



## Regular Paper

## Anti-inflammatory modulation of chronic airway inflammation in the murine house dust mite model

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**Abstract**

Asthma affects 300 million people worldwide and continues to be a major cause of morbidity and mortality. Disease relevant animal models of asthma are required for benchmarking of novel therapeutic mechanisms in comparison to established clinical approaches. We demonstrate that chronic exposure of mice to house dust mite (HDM) extract results in allergic airway inflammation, that can be significantly attenuated by therapeutic intervention with phosphodiesterase 4 inhibition and corticosteroid treatment.

Female BALB/c mice were administered intranasally with HDM (*Dermatophagoides pteronyssinus*) extract daily for five weeks, and therapeutic intervention with anti-inflammatory treatment (dexamethasone 1 mg/kg subcutaneous once daily, prednisolone 10 mg/kg orally twice daily, fluticasone 3, 10 and 30 µg intranasally twice daily, roflumilast 10 mg/kg orally twice daily and intranasally 10 and 30 µg twice daily) was initiated after three weeks of exposure.

Chronic HDM extract exposure resulted in significant airway inflammation, demonstrated by bronchoalveolar lavage cell infiltration and lung tissue inflammatory gene expression by TaqMan low density array. Chronic steroid treatment significantly inhibited these parameters. In addition, roflumilast caused a significant reduction in airway inflammatory cell infiltration.

We have demonstrated that chronic HDM-induced allergic inflammation can be significantly ameliorated by steroid treatment, and that phosphodiesterase 4 inhibition modulates inflammatory cell infiltration. Therefore, the murine HDM model may be a useful tool for evaluating new targets for the treatment of asthma.

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**Keywords:** Asthma; Mouse; Phosphodiesterase 4; Corticosteroid; House dust mite

**1. Introduction**

Asthma is a chronic airway inflammatory disease, characterised by recurrent episodes of increased vascular permeability, airway eosinophilia, mucus hypersecretion and airway hyperresponsiveness. In atopic asthma, these inflammatory events are primarily driven by intermittent (seasonal) or persistent (perennial) exposure to allergens,

such as pollen and house dust mites (HDMs) [1], resulting in wheezing, breathlessness, chest tightness and cough [2].

Animal models that share the characteristics of the disease are essential to determine proof of concept for new therapeutic mechanisms, while allowing comparisons to existing treatments. However, no animal model available to date is capable of reproducing all of the known characteristics of asthma. Traditionally, rodent models of allergic inflammation have utilised the glycoprotein ovalbumin (OVA). While OVA-induced airway inflammation shares many features seen in asthmatics, it is not a naturally occurring allergen in man, and repeated exposure in the

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absence of systemic sensitisation leads to tolerance [3–5]. Structural changes to the airway wall have been demonstrated following chronic intranasal administration of OVA in mice [6,7], but this may be strain dependent as shown by Shinagawa et al. Chronic allergen exposure in asthmatics elicits a distinct set of immunological events, which ultimately leads to airway remodelling, believed to be an important factor leading to the clinical symptoms. Therefore, development of chronic animal models using common environmental allergens such as HDM [8] and ragweed [9] may lead to better understanding of the importance of airway remodelling, and may provide improved test systems for evaluation of novel therapeutic approaches. HDMs are a major source of indoor allergens, and are significantly linked to atopic asthma in man [10]. Furthermore, exposure of companion animals such as dogs to environmental HDMs is known to be associated with canine atopic dermatitis [11]. Proteolytic enzymes present in HDM fecal pellets cleave epithelial tight junctions, thus increasing epithelial permeability and aiding allergen delivery to the allergen presenting cells [12]. In addition, these proteases are potent inducers of pro-inflammatory cytokine release from the airway epithelium [13], and their proteolytic activity has been directly linked with increased IgE synthesis [14] and inflammatory cell infiltration in mice [15]. Johnson et al. have previously shown that prolonged exposure of mice to *Dermatophagoides pteronyssinus* extract resulted in persistent T helper (Th)2 cell-driven inflammation characterised by airway eosinophilia, Th2 cytokine production (interleukin (IL)-5 and IL-13) and airway hyperresponsiveness [8]. In addition, mice chronically exposed to HDM extract demonstrated several features typical of airway remodelling, including goblet cell hyperplasia, collagen deposition and peribronchial smooth muscle accumulation. Interestingly, while the airway inflammation resolved fully within 2 weeks of cessation of HDM exposure, remodelling and airway hyperresponsiveness persisted for up to 9 weeks, demonstrating the presence of a persistent underlying pathology distinct from the acute inflammatory response [8]. The marked HDM-induced allergic inflammation in the study by Johnson et al. was elicited following an intranasal dose of HDM of 25 µg/day. Direct scale up to a human equivalent dose would require ~0.8 g HDM, and therefore the HDM model is clearly not aligned with exposures comparable to man. It seems likely that the severity of the inflammatory response in the murine model is at least partly a result of a continued exposure to a high dose of allergen.

The mouse HDM model differs from acute inflammation models by incorporating persistent airway remodelling. We have investigated HDM-induced changes in lung tissue gene expression of a variety of pro-inflammatory cytokines, chemokines and remodelling markers. In addition, as the effect of anti-inflammatory treatment has not been investigated in this model, we evaluated the effects of three corticosteroids: dexamethasone, prednisolone, fluticasone;

and the phosphodiesterase (PDE) 4 inhibitor roflumilast on chronic HDM-induced allergic inflammation.

Some of the results of these studies have previously been reported in the form of an abstract [16].

## 2. Materials and methods

### 2.1. Animals

Female BALB/c mice (6–8 weeks, 16–22 g, Charles River, UK) were housed under specific pathogen-free conditions and a 12:12 h light dark cycle with access to food and water *ad libitum*. All experiments were carried out in compliance with UK legislation and subject to local ethical review.

### 2.2. HDM and compound administration

Animals were sensitised to HDM broadly as described by Johnson et al. [8]. In brief, mice were exposed to purified HDM extract (*D. pteronyssinus*; Greer Laboratories, Lenoir, NC) intranasally (25 µg in 10 µl of saline) while under transient isoflurane anaesthesia. Control animals received 10 µl saline intranasally. Exposures were carried out for 5 days/week for 5 weeks.

Anti-inflammatory treatment (dexamethasone 1 mg/kg; s/c; *qd* (Sigma, Poole, UK), prednisolone 0.1, 1 and 10 mg/kg; p.o.; *bid* (Sigma, UK), roflumilast 10 mg/kg; p.o., *bid* (Sequoia Research Products, Pangbourne, UK)) was initiated after three weeks of HDM exposure. Inhaled anti-inflammatory treatment (fluticasone 3, 10 and 30 µg; i.n.; *bid* (Sequoia Research Products, UK), roflumilast 10 and 30 µg; i.n.; *bid* (Sequoia Research Products, UK)) was initiated after 3 weeks of HDM exposure, and mice were dosed with 50 µl intranasally while under transient isoflurane anaesthesia. Daily exposure to HDM was continued throughout the period of therapeutic intervention.

Dexamethasone was dissolved in sterile saline, and prednisolone was prepared in sterile water, followed by sonication (heated ultrasonic bath, Fisher Scientific UK, Loughborough, UK) until dissolved. Roflumilast for oral administration was prepared in 0.5% methylcellulose (Methocel A4 M)/0.1% Tween 80 (polyoxyethylene sorbitan oleate, Sigma, UK), and a fine suspension was generated by brief homogenisation (IKA Ultra Turrax T8 homogeniser, Fisher Scientific, UK).

Fluticasone and roflumilast were prepared as fine milled suspensions in vehicle (0.5% Tween 80 in PBS (Sigma, UK)). In brief, the compounds were suspended in the vehicle at 10 × required concentration (fluticasone; 6 mg/ml, roflumilast, 6 mg/ml) and milled by stirring (1200 rpm on magnetic stirrer) for 24 h in the presence of lead-free glass beads (0.5–0.75 mm, GLENMILLS®, Clifton, NJ). Subsequently, the supernatant was removed, the glass beads were washed 3–6 times with the suspension vehicle, and the washes pooled. Lower concentration suspensions

of the compounds were prepared by diluting the stock with the suspension vehicle. Potency of the suspension was measured by sampling 150 µl of the suspensions and assaying by HPLC–UV.

### 2.3. Bronchoalveolar lavage (BAL) and flow cytometry

Seventy-two hours after the last HDM exposure, the mice were euthanised, BAL was performed and cells were stained for flow cytometric analysis on an LSRII flow cytometer (BD Biosciences Immunocytometry Systems, Oxford, UK). In brief, the trachea was exposed by blunt dissection, a 22-gauge cannula (3S Healthcare, London, UK) was inserted into the trachea, and the lungs were lavaged with 500 µl PBS (supplemented with 10 mM EDTA, Invitrogen, Paisley, UK) 4 times. Total BAL cell number was measured by an ACT 5 Diff Coulter Counter (BD Biosciences, Oxford, UK). The BAL fluid was centrifuged (700  $g_{av}$ , 5 min, 4 °C) to pellet the remaining cells. For each antibody combination,  $1 \times 10^6$  cells were blocked with FcBlock (rat anti-mouse CD16/CD32, BD Pharmingen, UK) for 15 min on ice, washed and incubated with monoclonal antibodies at 4 °C for 30 min. The cells were then washed, treated with Streptavidin-APC-Cy7 conjugate (BD Pharmingen, UK) for 30 min at 4 °C, washed and fixed in 1% paraformaldehyde (Sigma, UK) prior analysis on a LSRII flow cytometer (BD Biosciences Immunocytometry Systems, UK). The following antibodies were used: APC-Cy7-conjugated rat IgG<sub>2b</sub> anti-mouse CD11b (neutrophils, BD Pharmingen, UK), APC-conjugated hamster IgG1 anti-mouse CD11c (macrophages, BD Pharmingen, UK), FITC-conjugated rat IgG<sub>2a</sub> anti-mouse CD45/B220 (all leukocytes, BD Pharmingen, UK), PE-conjugated rat IgG<sub>2a</sub> anti-mouse CCR3 (eosinophils, R&D systems, Abingdon, UK), Biotin-conjugated rat IgG<sub>2a</sub> anti-mouse MHCII (BD Pharmingen, UK), PE-Cy7-conjugated hamster IgG1 anti-mouse CD3ε (T-cells, BD Pharmingen, UK), all appropriate isotype control antibodies. The antibodies were titrated to determine optimal concentration.

### 2.4. TaqMan low density array (TLDA)

Whole lungs were submerged in RNAlater (Ambion (Europe) Ltd., Huntingdon, Cambridgeshire, UK), stored at 4 °C for 24 h and frozen at –80 °C until further analysis. Samples were homogenised in RLT lysis buffer (supplemented with 1% β-mercaptoethanol, Qiagen; Crawley, UK) prior to extraction of total RNA using the RNeasy mini protocol (Qiagen) according to the manufacturer's recommendations. RNA quality and quantity was assessed by spectrophotometry (260/280 nm).

Fifty ng of RNA was converted to cDNA using the cDNA Archive Kit (Applied Biosystems, Warrington, UK) according to the manufacturer's recommendations, and was subsequently added to each sample port of a custom mouse Taqman Low Density Array (TLDA, Applied Biosystems, UK), containing 46 mouse gene expression

assays. Genes were chosen from their known involvement in asthma, and by their differential expression in microarray studies of several asthma models. Four to five samples were run for each group tested. Arbitrary copy numbers (ACN) were calculated from the cycle threshold ( $C_t$ ) values.

### 2.5. Statistical analysis

An assessment of the variation in initial BAL cell data was performed, and a sample size of 5 was found to be sufficient to detect at least a twofold difference as statistically significant. Data were graphed and analysed by Microsoft Excel and are expressed as mean ± SEM. Each figure represents data generated in a single experiment. Comparison of groups used analysis of variance (ANOVA) for multiple, unpaired, parametric groups with the data expressed on a logarithmic scale. The null hypothesis was rejected at  $p < 0.05$ .

## 3. Results

### 3.1. Chronic HDM administration in mice results in steroid-sensitive allergic inflammation

Intranasal administration of HDM over 5 weeks resulted in a significant ( $p < 0.01$ ) increase in total BAL leukocytes ( $286 \pm 28 \times 10^4$  cells compared to  $12 \pm 3 \times 10^4$  in saline controls) as measured by flow cytometry. In particular, a nearly 500-fold increase in BAL eosinophils (Fig. 1) was demonstrated ( $148 \pm 22 \times 10^4$  cells compared to  $0.3 \pm 0.04 \times 10^4$  cells in saline controls animals). Furthermore, significant ( $p < 0.01$ ) increases were also detected in macrophages ( $32 \pm 2 \times 10^4$  compared to  $7.9 \pm 1.8 \times 10^4$  in saline controls), neutrophils ( $49 \pm 6 \times 10^4$  compared to  $4 \pm 0.7 \times 10^4$  in saline controls), B cells ( $34 \pm 5 \times 10^4$  compared to  $0.13 \pm 0.03 \times 10^4$  in saline controls) and T cells ( $23 \pm 1.3 \times 10^4$  compared to  $0.36 \pm 0.08 \times 10^4$  in saline controls).

Daily treatment with dexamethasone initiated after 3 weeks of HDM administration resulted in a significant ( $p < 0.05$ ) reduction in all BAL cell types (eosinophils: 97% inhibition, macrophages: 53% inhibition, neutrophils: 90% inhibition, B cells: 59% inhibition and T cells: 91% inhibition) compared to control values (see above).

### 3.2. Chronic HDM administration results in increased expression of a variety of inflammatory genes

mRNA analysis of lung tissue from HDM-treated mice resulted in significantly ( $p < 0.05$ ) increased expression of 38 out of the total 46 genes studied by TLDA (Table 1). Significant ( $p < 0.01$ ) upregulation of various chemokines and receptors was demonstrated, including eotaxin-1 and CC chemokine receptor (CCR)3, which act primarily on eosinophils, and thymus- and activation-regulated chemokines (TARC), monocyte derived chemokine (MDC), macrophage inflammatory protein (MIP)-1α, MIP-3β and

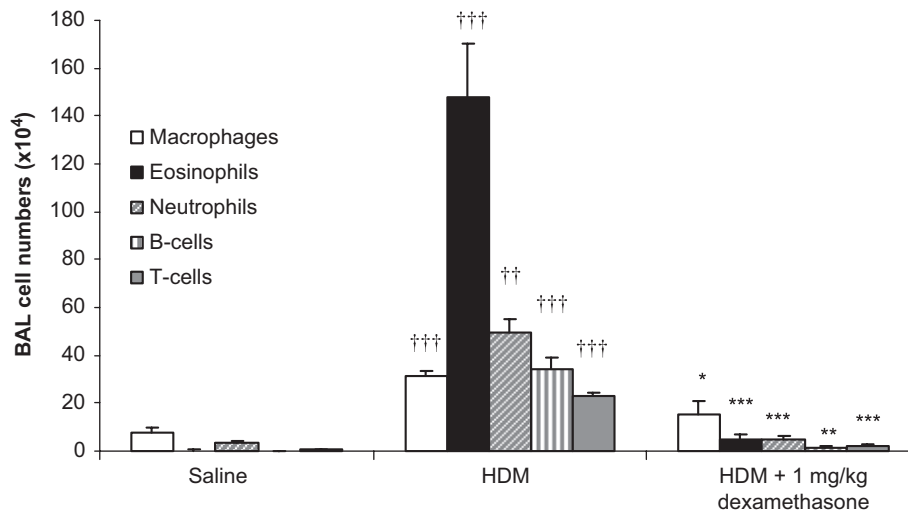


Fig. 1. Effects of therapeutic dexamethasone treatment on chronic HDM-induced BAL inflammatory cell infiltration. Cells were stained with monoclonal antibodies as described in Section 2 and analysed by four-colour flow cytometry. Data represent mean  $\pm$  SEM ( $n = 5$  per group).  $^{\dagger\dagger}p < 0.01$   $^{\dagger\dagger\dagger}p < 0.001$  compared to saline-treated animals.  $^*p < 0.05$ ,  $^{**}p < 0.01$   $^{***}p < 0.001$  compared to HDM-treated animals.

CCR4, which act on lymphocytes. In addition, significantly ( $p < 0.001$ ) increased monocyte chemoattractant protein (MCP)-1, MCP-3 and E selectin and P selectin expression demonstrate involvement of monocytes and endothelial cells in the HDM model. Involvement of interleukins in the HDM model was demonstrated by increased expression of IL-10, IL-6, IL-1 $\beta$  and IL-17 in addition to changes in IL-4, IL-5 and IL-13 demonstrated in Fig. 2(B). Furthermore, IL-18 was found to be significantly ( $p < 0.01$ ) downregulated following chronic HDM administration. Finally, chitinases, including acidic mammalian chitinase (AMCase), chitinase 3-like 1–3 (Chi3l1, Chi3l3 and Chi3l4), were all found to be significantly ( $p < 0.05$ ) upregulated as well as genes involved in mucus hypersecretion (MUC5AC, MUC5B and CLCA 1/3) and tissue remodelling (matrix metalloproteinase (MMP)12 and tissue inhibitors of matrix metalloproteinase (TIMP)-1).

### 3.3. Oral administration of prednisolone inhibits HDM-induced allergic inflammation in a dose-related manner

Treatment with 1 mg/kg prednisolone resulted in a significant ( $p < 0.05$ , Fig. 2(A) reduction in total BAL cells ( $95 \pm 10 \times 10^4$  compared to  $210 \pm 2 \times 10^4$  in HDM animals given vehicle; 55% inhibition), and eosinophils ( $40 \pm 9 \times 10^4$  compared to  $120 \pm 20 \times 10^4$  in HDM animals given vehicle; 67% inhibition), whereas no reduction was demonstrated in BAL neutrophils. This response was dose-related as treatment with 10 mg/kg prednisolone resulted in a significant reduction in BAL cells compared to animals receiving 1 mg/kg prednisolone (total cells: 86% inhibition and eosinophils: 97% inhibition compared to above HDM vehicle animals, neutrophils:  $2 \pm 0.6 \times 10^4$  compared to  $30 \pm 3 \times 10^4$  in HDM vehicle animals, 93% inhibition). Treatment with the lowest dose of prednisolone (0.1 mg/kg)

did not result in significant changes in BAL cell numbers. Similarly, no significant changes in BAL cells were observed after oral administration of vehicle.

Chronic HDM administration resulted in a significant ( $p < 0.05$ , Fig. 2(B) increase in expression of IL-4 ( $280 \pm 114$  ACN compared to  $25 \pm 11$  ACN in saline control animals), IL-5 ( $307 \pm 83$  ACN compared to  $57 \pm 12$  ACN in saline controls) and IL-13 ( $1347 \pm 647$  ACN compared to  $7.4 \pm 2.8$  ACN in saline controls). Prednisolone (10 mg/kg) treatment significantly ( $p < 0.05$ ) reduced mRNA expression of IL-4 ( $134 \pm 58$  ACN compared to  $280 \pm 114$  ACN in HDM control animals) and IL-13 in HDM-induced allergic inflammation. No significant changes were observed following treatment with 0.1 and 1 mg/kg prednisolone.

### 3.4. Oral prednisolone attenuates inflammatory gene expression in HDM-induced allergic inflammation in a dose-related manner

1 mg/kg prednisolone resulted in significantly ( $p < 0.05$ ) reduced expression of 17 out of the 38 genes shown to be upregulated by chronic HDM exposure (Table 2). Furthermore, 10 mg/kg prednisolone lead to significantly ( $p < 0.05$ ) reduced expression of 26 genes out of the total 38. Significant ( $p < 0.001$ ) down-regulation of various chemokines and receptors was demonstrated following treatment with 10 mg/kg prednisolone, including CCR3, TARC, MDC, MIP-1 $\alpha$ , MIP-3 $\beta$  and CCR4. Expression of IL-6, IL-1 $\beta$  and IL-17 was significantly ( $p < 0.05$ ) downregulated by 10 mg/kg prednisolone. Finally, Chi3l3 and Chi3l4 were found to be significantly ( $p < 0.05$ ) downregulated by 10 mg/kg prednisolone as well as MUC5AC and MMP12. Amongst 11 genes not affected by prednisolone treatment were MCP1 and 3, and endothelial cell mediators, E and P selectin.

Table 1  
mRNA expression profile by TLDA in lung tissue from animals exposed to HDM for 5 weeks

| Gene                         | Saline   |        | HDM       |         | Fold change | p-Value |
|------------------------------|----------|--------|-----------|---------|-------------|---------|
|                              | Avg.     | SEM    | Avg.      | SEM     |             |         |
| Arachidonate 15 lipoxygenase | 1209.7   | 158.6  | 16,212.2  | 2147.3  | 13.4        | 0.000   |
| Arginase 1                   | 1373.6   | 353.2  | 22,249.7  | 5386.1  | 16.2        | 0.011   |
| C3                           | 47,594.3 | 3565.9 | 180,468.9 | 13,433  | 3.8         | 0.000   |
| Eotaxin-1                    | 1189.6   | 82.9   | 24,922.6  | 3391.6  | 21.0        | 0.000   |
| TARC                         | 743.4    | 42.1   | 8622.2    | 414.1   | 11.6        | 0.000   |
| MIP-3B (CCL19)               | 856.8    | 147.4  | 3433.6    | 423.6   | 4.0         | 0.001   |
| MDC                          | 542.1    | 54.1   | 2411      | 115.7   | 4.4         | 0.000   |
| MCP1 (CCL2)                  | 504.4    | 26.8   | 1987.1    | 164     | 3.9         | 0.000   |
| MIP-1A (CCL3)                | 320      | 69.6   | 1927      | 198.4   | 6.0         | 0.000   |
| CCL6                         | 48,022.5 | 3751.6 | 263,470.7 | 15,779  | 5.5         | 0.000   |
| MCP-3                        | 224.3    | 25.1   | 1049.2    | 148.8   | 4.7         | 0.002   |
| CCL9                         | 4245.6   | 144.1  | 52,870.6  | 3170.2  | 12.5        | 0.000   |
| CCR1                         | 1169.9   | 107.1  | 52,65.7   | 130.9   | 4.5         | 0.000   |
| CCR2                         | 4300.7   | 757.3  | 11,917.6  | 607.1   | 2.8         | 0.000   |
| CCR3                         | 694.9    | 35     | 7365.5    | 629.4   | 10.6        | 0.000   |
| CCR4                         | 203.3    | 18.7   | 901.4     | 49.6    | 4.4         | 0.000   |
| Chitinase 3-like 1           | 104,727  | 6745.8 | 440,034.5 | 19,244  | 4.2         | 0.000   |
| Chitinase 3-like 3           | 23,006.2 | 2086.1 | 1,378,911 | 124,553 | 59.9        | 0.000   |
| Chitinase 3-like 4           | 2823.7   | 2791.1 | 98,602.4  | 8385.9  | 34.9        | 0.000   |
| AMCase                       | 20,430.4 | 1649.3 | 168,485   | 46,342  | 8.2         | 0.026   |
| CLCA1/3                      | 60.1     | 28     | 541,473.4 | 29,158  | 9012.8      | 0.000   |
| CSF2                         | 955.2    | 124.6  | 1442.7    | 134.5   | 1.5         | 0.036   |
| ICOS                         | 404      | 33.2   | 1733.1    | 121.1   | 4.3         | 0.000   |
| IL-10                        | 71       | 42.1   | 696.9     | 155.5   | 9.8         | 0.010   |
| IL-17                        | 0        | 0      | 1876.7    | 59.4    | 1876.0      | 0.000   |
| IL-18                        | 4002.8   | 427.7  | 1523.5    | 326.4   | 0.4         | 0.002   |
| IL-1B                        | 2228     | 69.8   | 4994.6    | 153.2   | 2.2         | 0.000   |
| T1/ST2                       | 493      | 23.3   | 2988.8    | 358.2   | 6.1         | 0.000   |
| IL-6                         | 82.9     | 14.1   | 338       | 47.5    | 4.1         | 0.002   |
| Intelectin                   | 4.3      | 2.6    | 452.7     | 48.1    | 104.6       | 0.000   |
| MMP-12                       | 1007.8   | 156.5  | 403,284.3 | 59,119  | 400.2       | 0.001   |
| Mucin 5AC                    | 218.1    | 39.7   | 13,742.7  | 1360.4  | 63.0        | 0.000   |
| Mucin 5B                     | 4056.6   | 523.3  | 19,805.8  | 1502.5  | 4.9         | 0.000   |
| E selectin                   | 108.5    | 24.7   | 464.8     | 131.4   | 4.3         | 0.050   |
| P selectin                   | 622.7    | 66.8   | 1448      | 148.3   | 2.3         | 0.002   |
| SYK                          | 6875.6   | 1185.5 | 19,209.3  | 1206.7  | 2.8         | 0.000   |
| TIMP-1                       | 832.9    | 102.9  | 3744.274  | 644     | 4.5         | 0.005   |
| TNF                          | 581.4    | 121.6  | 1108.6    | 94.9    | 1.9         | 0.010   |

Data expressed as mean ACN (arbitrary copy number)  $\pm$  SEM ( $n = 4$  per group).

### 3.5. Oral roflumilast partially inhibits HDM-induced allergic inflammation

The 10 mg/kg roflumilast resulted in a significant ( $p < 0.05$ ) reduction in total BAL cells ( $130 \pm 20 \times 10^4$  cells compared to  $250 \pm 30 \times 10^4$  cells in HDM animals receiving the vehicle only, 48% inhibition, Fig. 3). However, no significant reductions were observed in eosinophils and neutrophils, although a trend was demonstrated (eosinophils:  $67 \pm 10 \times 10^4$  cells compared to  $120 \pm 20 \times 10^4$  cells in vehicle control animals; neutrophils:  $12 \pm 3 \times 10^4$  cells compared to  $25 \pm 3 \times 10^4$  cells in vehicle controls). For comparison, dexamethasone significantly ( $p < 0.05$ ) inhibited total HDM-induced BAL inflammatory cell infiltration (61% inhibition) as well as

eosinophil (96% inhibition) and neutrophil infiltration (93% inhibition).

### 3.6. Inhaled fluticasone and roflumilast inhibit HDM-induced allergic inflammation in a dose-related manner

Inhalation of the lowest dose (3  $\mu$ g) of fluticasone resulted in a significant ( $p < 0.05$ , Fig. 4) reduction in total BAL cells ( $50 \pm 10 \times 10^4$  compared to  $190 \pm 10 \times 10^4$  in HDM animals given vehicle), eosinophils ( $10 \pm 4 \times 10^4$  compared to  $80 \pm 7 \times 10^4$  in HDM-vehicle animals), neutrophils ( $5 \pm 2 \times 10^4$  compared to  $20 \pm 6 \times 10^4$  in HDM-vehicle animals) and lymphocytes ( $10 \pm 2 \times 10^4$  compared to  $40 \pm 2 \times 10^4$  in HDM-vehicle animals). Further significant ( $p < 0.05$ ) efficacy was observed following the two higher doses of

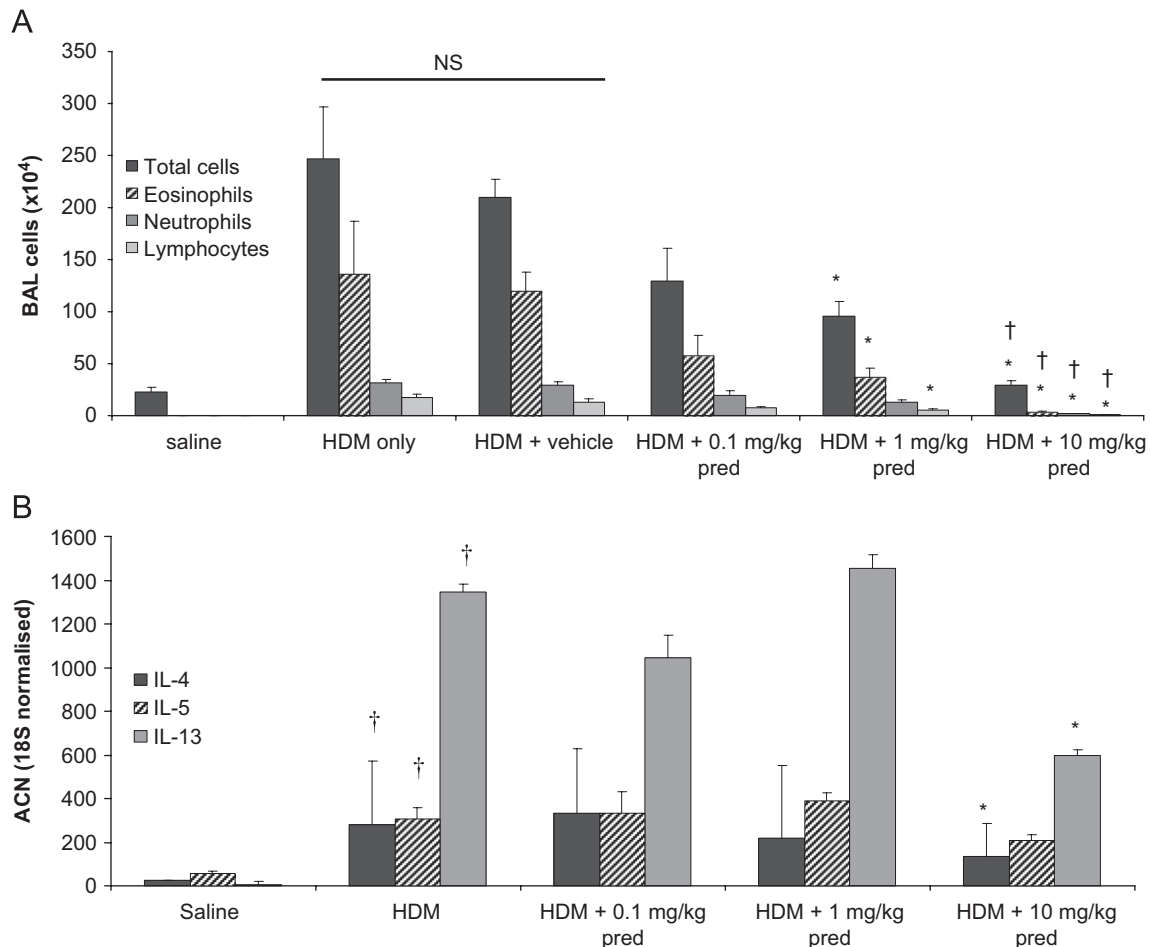


Fig. 2. Effects of oral prednisolone treatment on chronic HDM-induced BAL inflammatory cell infiltration (A). Cells were stained with monoclonal antibodies as described in Section 2 and analysed by four-colour flow cytometry. Data represent mean  $\pm$  SEM ( $n = 5$  per group). \* $p < 0.05$  compared to vehicle treated HDM animals. † $p < 0.05$  compared to HDM animals treated with 1 mg/kg prednisolone. Effects of continuous HDM exposure on Th2 cytokine mRNA expression profiles in whole lung tissue (B) Data expressed as mean ACN  $\pm$  SEM ( $n = 4$  per group). † $p < 0.05$  compared to animals receiving saline. \* $p < 0.05$  compared to animals receiving HDM alone.

fluticasone (10  $\mu$ g: total cells (79% inhibition), eosinophils (100% inhibition), neutrophils (75% inhibition), lymphocytes (85% inhibition), 30  $\mu$ g: total cells (84% inhibition), eosinophils (99% inhibition), neutrophils (97% inhibition), lymphocytes (93% inhibition)). This effect was dose-related as eosinophil, neutrophil and lymphocyte numbers were significantly ( $p < 0.05$ ) lower following 30  $\mu$ g fluticasone compared to 3  $\mu$ g fluticasone as well as neutrophils and lymphocytes being significantly ( $p < 0.05$ ) lower in the 30  $\mu$ g group compared to 10  $\mu$ g fluticasone.

Similarly, the lowest dose of roflumilast (10  $\mu$ g) significantly ( $p < 0.05$ ) inhibited BAL inflammatory cell infiltration (total cells: 53% inhibition, eosinophils: 63% inhibition, neutrophils: 55% inhibition, lymphocytes: 50% inhibition compared to HDM animals given vehicle as described above). The effect was further increased by treatment with 30  $\mu$ g roflumilast (total cells: 68% inhibition, eosinophils: 88% inhibition, neutrophils: 80% inhibition, lymphocytes: 70% inhibition compared to HDM animals given vehicle as described above). Again, the effect was demonstrated to be dose-related as

eosinophil and neutrophil numbers were significantly ( $p < 0.05$ ) lower following 30  $\mu$ g roflumilast compared to 10  $\mu$ g roflumilast.

Importantly, no significant changes in BAL cells were observed after chronic inhalation of the nanosuspension vehicle.

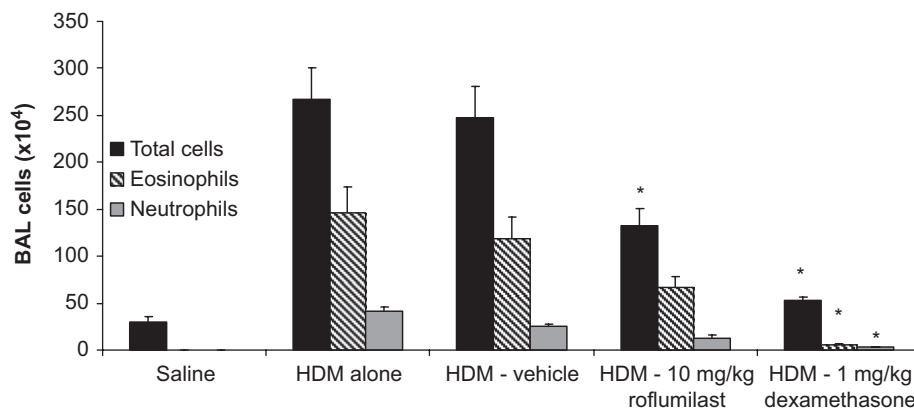
#### 4. Discussion

Asthma is a chronic inflammatory disease and thus animal models that reflect this are therefore potentially advantageous in the evaluation of the effect of novel anti-inflammatory mechanisms. Previous data from other investigators have shown that the mouse HDM model elicits significant chronic allergic inflammation and remodelling. Further characterisation of the mouse HDM model may lead to greater understanding of this model's relevance to human asthma, and increase the confidence in the outcome of studies evaluating novel anti-inflammatory treatments in this model.

Table 2

mRNA expression profile by TLDA in lung tissue from HDM-exposed animals treated with prednisolone (1 or 10 mg/kg p.o. *bid*)

| Gene                         | 1 mg/kg prednisolone |           |              |         | 10 mg/kg prednisolone |          |              |         |
|------------------------------|----------------------|-----------|--------------|---------|-----------------------|----------|--------------|---------|
|                              | Avg.                 | SEM       | % Inhibition | p-Value | Avg.                  | SEM      | % Inhibition | p-Value |
| Arachidonate 15 lipoxygenase | 10,260.2             | 1168.0    | 39.7         | 0.041   | 3362.6                | 551.6    | 85.7         | 0.000   |
| Arginase 1                   | 11,866.0             | 2538.7    | 49.7         | 0.119   | 5810.5                | 1181.0   | 78.7         | 0.018   |
| C3                           | 148,300.3            | 9467.3    | 24.2         | 0.086   | 105,282.3             | 13,590.3 | 56.6         | 0.004   |
| TARC                         | 7627.8               | 602.7     | 12.6         | 0.211   | 4442.7                | 596.2    | 53.0         | 0.000   |
| MIP-3B (CCL19)               | 2300.4               | 269.7     | 44.0         | 0.054   | 877.0                 | 140.1    | 99.2         | 0.000   |
| MDC                          | 1649.0               | 190.9     | 40.8         | 0.009   | 936.0                 | 149.8    | 78.9         | 0.000   |
| MIP-1A (CCL3)                | 888.9                | 121.1     | 64.6         | 0.002   | 597.7                 | 66.7     | 82.7         | 0.000   |
| CCL6                         | 193,860.2            | 6007.4    | 32.3         | 0.003   | 141,685.8             | 9438.1   | 56.5         | 0.000   |
| CCL9                         | 32,095.4             | 1219.9    | 42.7         | 0.000   | 17,674.0              | 1376.7   | 72.4         | 0.000   |
| CCR1                         | 4060.6               | 139.7     | 29.4         | 0.000   | 2879.1                | 201.1    | 58.3         | 0.000   |
| CCR2                         | 9271.1               | 312.4     | 34.7         | 0.005   | 6900.1                | 572.4    | 65.9         | 0.000   |
| CCR3                         | 5830.7               | 469.3     | 23.0         | 0.086   | 1823.4                | 354.5    | 83.1         | 0.000   |
| CCR4                         | 581.6                | 28.2      | 45.8         | 0.001   | 404.1                 | 62.1     | 71.2         | 0.000   |
| Chitinase 3-like 3           | 809,245.0            | 134,290.2 | 42.0         | 0.014   | 281,071.1             | 58,811.5 | 81.0         | 0.000   |
| Chitinase 3-like 4           | 97,270.2             | 14,621.4  | 1.4          | 0.939   | 50,309.8              | 14,871.1 | 50.4         | 0.022   |
| ICOS                         | 1017.2               | 19.1      | 53.9         | 0.000   | 736.6                 | 144.6    | 75.0         | 0.001   |
| IL-17                        | 1240.2               | 127.3     | 33.9         | 0.002   | 530.9                 | 216.6    | 71.7         | 0.000   |
| IL-18                        | 1873.0               | 326.5     | 14.1         | 0.471   | 3246.0                | 524.3    | 69.5         | 0.024   |
| IL-1B                        | 3774.8               | 218.7     | 44.1         | 0.002   | 3790.9                | 297.4    | 43.5         | 0.007   |
| T1/ST2                       | 2281.6               | 181.2     | 28.3         | 0.116   | 1611.4                | 234.3    | 55.2         | 0.012   |
| IL-6                         | 194.7                | 19.9      | 56.2         | 0.024   | 172.1                 | 17.5     | 65.0         | 0.011   |
| Interlectin                  | 336.7                | 28.0      | 25.9         | 0.071   | 131.7                 | 32.9     | 71.6         | 0.001   |
| MMP-12                       | 146,861.7            | 34,682.7  | 63.7         | 0.006   | 21,548.1              | 6114.5   | 94.9         | 0.000   |
| Mucin 5AC                    | 9711.8               | 910.5     | 29.8         | 0.039   | 5045.6                | 1098.1   | 64.3         | 0.001   |
| SYK                          | 13,283.4             | 1125.1    | 48.0         | 0.007   | 8567.7                | 583.6    | 86.3         | 0.000   |
| TNF                          | 803.8                | 68.2      | 57.8         | 0.031   | 647.4                 | 83.7     | 87.5         | 0.007   |

Data expressed as mean ACN  $\pm$  SEM ( $n = 4$  per group), and statistical comparisons were made to animals receiving HDM alone (see Table 1).Fig. 3. Effects of oral roflumilast treatment on chronic HDM-induced BAL inflammatory cell infiltration. Cells were stained with monoclonal antibodies as described in Section 2 and analysed by four-colour flow cytometry. Data represent mean  $\pm$  SEM ( $n = 5$  per group). \* $p < 0.05$  compared to vehicle treated HDM animals.

#### 4.1. Chronic exposure to HDM results in Th2-driven allergic inflammation

Our data confirm and extend the findings of Johnson et al. [8] by demonstrating significant chronic airway inflammation, consisting of primarily BAL eosinophils, but also involving a significant degree of neutrophil and lymphocyte infiltration. Significant T cell activation and T helper (Th)2 cell involvement was also demonstrated by

increased ICOS and T1/ST2 gene expression. We have also confirmed the pathological findings made by Johnson et al. [8] by demonstrating significant goblet cell hyperplasia, collagen deposition, and peri-bronchial accumulation of contractile tissue following chronic HDM exposure (data not shown). Th2 cytokines, such as IL-4, -5 and -13 are known to be important in asthma. Both IL-4 and IL-13 levels are raised in BAL fluid derived from asthmatics [17,18], and these cytokines have been shown to be

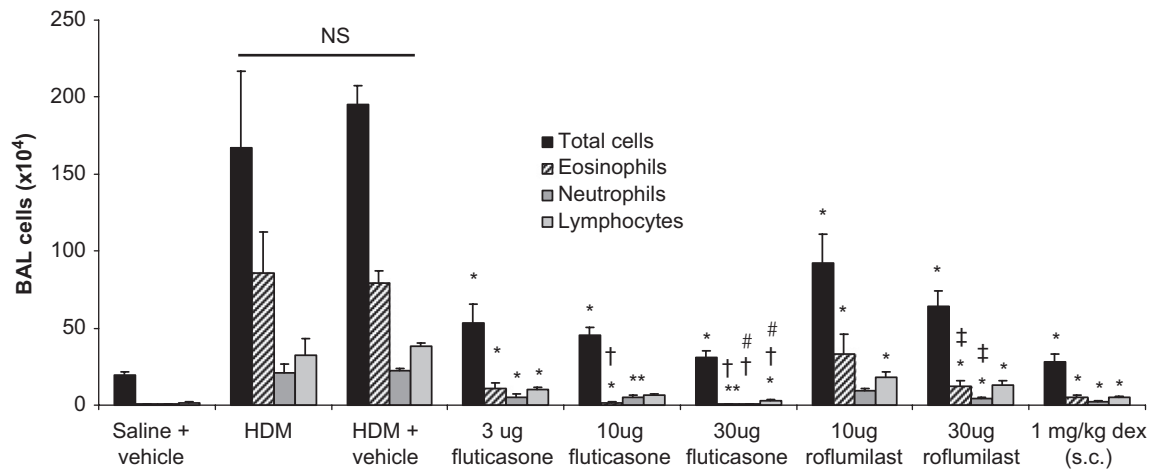


Fig. 4. Effects of inhaled fluticasone and roflumilast treatment on chronic HDM-induced BAL inflammatory cell infiltration. Dexamethasone (1 mg/kg s.c.) was included as a positive control. Stained BAL cells were analysed by FACSarray flow cytometry as described in Section 2. Data represent mean  $\pm$  SEM ( $n = 5$  per group). \* $p < 0.05$  compared to vehicle treated HDM animals. † $p < 0.05$  compared to 3  $\mu$ g fluticasone. # $p < 0.5$  compared to 10  $\mu$ g fluticasone. ‡ $p < 0.05$  compared to 10  $\mu$ g roflumilast.

important in Th2 cell function, eosinophil recruitment, airway hyperresponsiveness and mucus secretion [19–21]. IL-5 is responsible for terminal differentiation of eosinophils within the bone marrow and levels are raised in serum and bronchial samples from asthmatics [22]. In our studies, we were unable to detect IL-4, -5 and -13 proteins in the BAL fluid by ELISA following chronic HDM administration. However, by profiling gene expression in the lung tissue using TLDA, we observed significant increases in the expression of these cytokines in HDM exposed animals.

#### 4.2. Chronic HDM exposure induces changes in lung cytokine gene expression

We demonstrated that gene expression of a number of other cytokines were significantly upregulated following chronic HDM exposure, namely IL-1 $\beta$ , -6, -10, -17 and tumour necrosis factor (TNF)- $\alpha$ . While asthma is primarily a Th2-driven eosinophilic disorder, some Th1 characteristics become evident as the disease becomes more severe, including neutrophil infiltration and tissue destruction. IL-6 and IL-17 are involved in recruitment and activation of neutrophils, which are believed to be important in acute exacerbations of asthma [23,24]. Furthermore, severe asthma is often accompanied by marked increases in TNF- $\alpha$  production [25]. IL-1 $\beta$  is found in BAL fluid from asthmatics, and has been shown to increase the response of airway smooth muscle to a variety of contractile agonists *in vitro* and *in vivo* including bradykinin, methacholine and 5-hydroxytryptamine (5-HT) [26,27]. Importantly, the expression of IL-18, a classical Th1 cytokine, was demonstrated to be significantly downregulated, indicating that while some Th1 characteristics are present in the HDM model, others are downregulated. Interestingly, the expression of IL-10, a known anti-inflammatory cytokine, was found to be significantly upregulated in the HDM model. Granulocyte-macrophage colony stimulating factor

(GM-CSF or CSF2) production is elevated in BAL and sputum from asthmatics, and is believed to promote and maintain a Th2 driven immune response [28]. The importance of GM-CSF in the murine HDM model has previously been demonstrated by the ability of anti-GM-CSF-neutralising antibodies to significantly reduce inflammatory responses in the lung [29].

#### 4.3. Chronic HDM exposure causes increased lung chemokine and chemokine receptor gene expression

We found increased expression of a range of chemokines and their receptors following chronic HDM administration. Eotaxin binds to CCR3 receptors expressed on eosinophils, Th2 cells, mast cells and the airway epithelium causing cell activation and migration. CCR3 expression is increased in asthmatic airways, and has been linked to airway hyperresponsiveness [30]. Similarly, expression of TARC, a Th2 cell chemotactic factor secreted by epithelial [31] and smooth muscle cells [32] was found to be upregulated in the HDM model, as was MDC and MIP-1 $\alpha$  which both affect eosinophil function [33]. Other chemokines and receptors found upregulated in the HDM model included MIP-3 $\beta$ , MCP-1, MCP-3, CCR1, CCR2, CCR4, CCL6 and CCL9, which are involved in leukocyte recruitment and airway smooth muscle hyperplasia [34–37].

#### 4.4. Other HDM-induced lung gene expression changes

The expression of E and P selectins, present on activated vascular endothelium to mediate the adhesion of circulating leukocytes [38], was significantly increased following chronic HDM exposure. We also found significant increases in AMCase, chitinase 3-like 1, 3 and 4 expression, and while the role of these enzymes has not been clearly elucidated, increased AMCase levels have been demonstrated in

asthmatic lung tissue [39]. Similarly, HDM-induced upregulation of arachidonate 15-lipoxygenase is noteworthy, as metabolites of this pathway have been found in BAL fluid from asthmatics, and may be linked to pro-fibrotic effects of Th2 mediators [40]. Increased C3 expression in the HDM model indicates upregulation of the complement pathway, which confirms findings by other investigators [41]. Upregulation of spleen tyrosine kinase (Syk), as demonstrated in the HDM model is likely to increase mast cell activation [42], and thereby the release of pro-inflammatory mediators such as prostaglandins and leukotrienes. In addition, some evidence of altered nitric oxide (NO) homeostasis in the HDM model was demonstrated by increased expression of arginase 1. Arginase 1 is believed to be involved in the deficiency in cNOS-derived NO by competition with cNOS for the common substrate, L-arginine, resulting in reduced airway relaxation [43]. Finally, several genes linked to airway remodelling were shown to be upregulated in the HDM model, including CLCA1/3, MUC 5AC, MUC 5B, TIMP-1 and MMP-12, which are involved in mucus hypersecretion [44] and extracellular matrix degradation [45].

#### 4.5. Chronic HDM-induced inflammation is highly steroid sensitive

Current asthma treatment strategies primarily involve inhaled bronchodilators and inhaled steroids, which provide significant control for mild-to-moderate asthmatics.  $\beta_2$ -agonist bronchodilators are used either on an “as needed” basis (short-acting) or as maintenance medication (long-acting), and inhaled corticosteroids (e.g. fluticasone) aim to control the airway inflammation in mild-to-moderate asthma. Oral corticosteroids (e.g. prednisolone) are limited to severe or difficult-to-control asthma due to the risk of side effects from chronic systemic exposure [46]. In order to understand the relevance of animal asthma models, benchmarking of agents available in the clinic is important. In mouse models of OVA-induced allergic asthma, oral prednisolone has been shown to significantly inhibit BAL inflammatory cell infiltration, IL-4, -5 and -13 levels and airway hyperresponsiveness to methacholine [47–49], while resulting in some loss of bodyweight over time. We have confirmed a dose-related effect of oral prednisolone on BAL inflammatory cell infiltration in the HDM model, where the highest dose of prednisolone (10 mg/kg) resulted in near complete inhibition of the HDM-induced cell response. Importantly, in these studies treatment was not initiated until after three weeks of HDM exposure. Thus, the therapeutic effect of the corticosteroids was demonstrated against pre-existing HDM-induced inflammation, as opposed to the pre-challenge administration applied in many OVA models. In addition, we found that the inhibitory effect of prednisolone when given in a therapeutic manner was of the same order of magnitude as demonstrated by others using pre-challenge administration [48]. Prednisolone also

significantly inhibited the expression of a wide range of pro-inflammatory genes in a dose-related manner. In addition to downregulation of the classical Th2 cytokines, such as IL-4, -5 and -13, prednisolone also inhibited the expression of several other cytokines, chemokines and receptors. This effect was most clearly demonstrated following 10 mg/kg prednisolone, where expression levels of 26 out of 38 genes were inhibited between 43% and 99% compared to HDM animals not receiving prednisolone.

We also investigated whether inhaled delivery of the corticosteroid fluticasone could attenuate HDM-induced allergic inflammation, and have demonstrated a dose-related effect on BAL inflammatory cell infiltration. The highest dose of fluticasone (30  $\mu$ g) completely inhibited BAL cell infiltration, while the lowest dose (3  $\mu$ g) resulted in approximately 80% inhibition of the HDM-induced response, demonstrating that repeated administration of anti-inflammatory compounds via the intranasal route is well tolerated in this model. A comparable dose of inhaled fluticasone has previously been shown to reduce BAL inflammatory cell infiltration and remodelling of airway smooth muscle in a chronic OVA mouse model [50], although others have failed to demonstrate a therapeutic effect on OVA-induced BAL inflammatory eosinophil infiltration [51].

We demonstrated that the HDM model incorporated some degree of neutrophilic inflammation, which in turn was shown to be sensitive to steroid treatment. This finding is in contrast to clinical evidence which largely indicates a poor neutrophilic response to corticosteroids in both severe asthma, but also other neutrophilic inflammatory lung diseases such as chronic obstructive pulmonary disease (COPD) and acute respiratory distress syndrome (ARDS) [52]. It is possible that the neutrophilic inflammation in the HDM model occurs through a different mechanism to that observed in patients, perhaps arising from the presence of endotoxin in the HDM extract, or that the steroid doses administered in this model are higher than what would be given in man.

#### 4.6. PDE-4 inhibition attenuates chronic HDM-induced inflammation

Phosphodiesterase (PDE) 4-type enzymes control the hydrolytic degradation of cyclic 3', 5' adenosine monophosphate (cAMP), and selective inhibition of enzymes of the PDE4 family, inhibits the function of a variety of inflammatory and immunomodulatory cells including eosinophils, mast cells, neutrophils, macrophages, T cells and epithelial cells. Thus, PDE4 inhibitors may be useful as alternatives or adjuncts to corticosteroids in the treatment of asthma, as they have been shown to exhibit significant anti-inflammatory activity in animal models of allergic inflammation [53]. Oral administration of roflumilast, a selective PDE4 inhibitor, has been demonstrated to reduce BAL eosinophil infiltration, collagen accumulation and thickening of the airway epithelium in a chronic mouse

model of OVA-induced allergic inflammation [54]. Another PDE4 inhibitor, ciclamilast, similarly reduced OVA-induced BAL inflammatory cells, mucus secretion and airway hyperresponsiveness [55]. We demonstrated a small but significant effect on HDM-induced total BAL inflammatory cell infiltration following oral roflumilast administration, but were unable to show significant effects on BAL eosinophils and neutrophils. However, inhaled administration of a milled roflumilast suspension resulted in a significant dose-related reduction in BAL inflammatory cells in HDM animals, with the highest dose of roflumilast attenuating HDM-induced cell infiltration between 69% and 85%. Clinical studies using roflumilast have demonstrated some efficacy in both asthma and chronic obstructive pulmonary disease (COPD). In asthma, roflumilast suppressed the development of bronchial hyperresponsiveness after antigen challenge and improved lung function [53]. However, one of the major hurdles for this mechanism is overcoming the tolerability profile with respect to nausea and emesis induced by PDE4 inhibition, an issue that could be addressed by changing delivery route to inhalation.

In summary, we have further characterised the mouse HDM model by evaluating the lung gene expression profiles following chronic HDM exposure, and demonstrated upregulation of a variety of important cytokines, chemokines and receptors. In addition, several genes involved in remodelling were also found to be upregulated. While the model is primarily characterised by Th2 driven inflammation, a certain degree of neutrophilic inflammation was also evident, which may have parallels to severe asthma in man. In addition, we have shown that HDM-induced chronic inflammation were highly sensitive to corticosteroid treatment, and that PDE4 inhibition resulted in a significant anti-inflammatory effect. This model appears to recapitulate a wider range of features than many other models, better enables evaluation of the effect of genetic manipulations, and demonstrates responsiveness to clinically used agents, in particular when used in a therapeutic mode. Thus, the HDM model offers considerable promise as a test system of potentially high clinical relevance.

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