

Longitudinal characterization of a model of chronic allergic lung inflammation in mice using imaging, functional and immunological methods

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Abstract

The present study investigated the role that imaging could have for assessing lung inflammation in a mouse model of HDM (house dust mite)-provoked allergic inflammation. Inflammation is usually assessed using terminal procedures such as BAL (bronchoalveolar lavage) and histopathology; however, MRI (magnetic resonance imaging) and CT (computed tomography) methods have the potential to allow longitudinal, repeated study of individual animals. Female BALB/c mice were administered daily either saline, or a solution of mixed HDM proteins sufficient to deliver a dose of 12 or 25 μg total HDM protein $\pm$ budesonide (1 mg/kg of body weight, during weeks 5–7) for 7 weeks. AHR (airway hyper-responsiveness) and IgE measurements were taken on weeks 3, 5 and 7. Following imaging sessions at weeks 3, 5 and 7 lungs were prepared for histology. BAL samples were taken at week 7 and lungs prepared for histology. MRI showed a gradual weekly increase in LTI (lung tissue intensity) in animals treated with HDM compared with control. The 25 μg HDM group showed a continual significant increase in LTI between weeks 3 and 7, the 12 μg HDM-treated group showed a similar rate of increase, and plateaued by week 5. A corresponding increase in AHR, cell counts and IgE were observed. CT showed significant increases in lung tissue density from week 1 of HDM exposure and this was maintained throughout the 7 weeks. Budesonide treatment reversed the increase in tissue density. MRI and CT therefore provide non-invasive sensitive methods for longitudinally assessing lung inflammation. Lung tissue changes could be compared directly with the classical functional and inflammatory readouts, allowing more accurate assessments to be made within each animal and providing a clinically translatable approach.

Key words: asthma, computed tomography (CT), inflammation, lung, longitudinal, magnetic resonance imaging (MRI)

INTRODUCTION

Allergic mouse models are widely used to model aspects of human disease in asthma research [1,2]. The allergic ovalbumin model [3] has been extensively used but its acute nature produces little lung remodelling and chronic exposure often leads to the development of immune tolerance. The HDM (house dust mite) model [4] was developed to overcome these limitations and its use has increased substantially in recent years. Repeated episodes of HDM (without adjuvant) to the lung via intranasal ad-

ministration produce a severe and persistent airway inflammation that is characterized by airway eosinophilia, airway remodelling and a Th2-biased immune response [4–6]. Functional measurements of the lung during inflammation and remodelling suggest increased airway resistance and bronchoconstrictor response to methacholine, but this can vary between strains of mice studied [7]. The inflammatory response associated with HDM exposure has been shown to be reduced by corticosteroids and phosphodiesterase 4 inhibitors [8–10]. However, one major drawback with the HDM model is the length of time that it takes to produce a

Abbreviations: ABPAS, Alcian Blue Periodic Acid Schiff; AHR, airway hyper-responsiveness; BAL, bronchoalveolar lavage; CT, computed tomography; H&E, haematoxylin and eosin; HDM, house dust mite; HU, Hounsfield unit; LTI, lung tissue intensity; MRI, magnetic resonance imaging; MSB, Martius Scarlet Blue.

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robust and reproducible response. Generally mice are intranasally dosed with HDM for up to 7 weeks, after which terminal readouts such as BAL (bronchoalveolar lavage) lavage for cells and cytokines, histopathology and lung function mechanics may be performed. From a drug discovery perspective, the length of time of the model, dictated by the current endpoints, can be limiting if it is to be used as a routine model to assess efficacy of anti-inflammatory therapies.

In recent years, there has been a marked increase in the use of three-dimensional imaging modalities, particularly MRI (magnetic resonance imaging) and CT (computed tomography), to evaluate structural and functional changes in rodent models of lung disease. CT and MRI are non-invasive and this allows repeated evaluations in the same animal over time. Proton MRI has been used to measure inflammation in LPS (lipopolysaccharide)-treated [11] and allergic rodents [12]. CT has been used to characterize allergic inflammation in rats and mice [13–15]. Several studies have used hyperpolarized ^3He MRI to assess regional ventilation in rodent lungs [16,17]. However, there are no studies using imaging in a longitudinal fashion to evaluate lung pathology in mice with lung inflammation induced by HDM. The present study characterizes the lung inflammation as measured by MRI and CT (as well as the conventional measurements) on a weekly basis to understand how imaging biomarkers may play a role in studying this chronic model. In addition, the studies described have been the result of cross company collaboration between GlaxoSmithKline and AstraZeneca as part of an effort to share best practice in model development at pre-competitive stages of development. The same animal models were set up at the respective companies with conventional measures being made simultaneously, but with the MRI methods being conducted at GlaxoSmithKline and the CT at AstraZeneca.

MATERIALS AND METHODS

Ethics

All animal studies carried out at GlaxoSmithKline were ethically reviewed and carried out in accordance with European Directive 86/609/EEC and the GlaxoSmithKline Policy on the Care, Welfare and Treatment of Animals. All animal studies performed at AstraZeneca were carried out under licences issued under the Animals (Scientific Procedures) Act 1986 following local ethical committee review.

HDM model methods

Female BALB/c mice, aged 8–12 weeks ($n = 6$ per group), were purchased from Charles River UK. The animals were housed in specific pathogen-free conditions in plastic cages with absorbent bedding material and maintained on a 12 h daylight cycle. Food and water were provided *ad libitum*. The animals were acclimatized for a minimum of 7 days before commencement of studies. Mice were exposed to a solution of HDM proteins according to an established chronic allergen exposure protocol. Under sterile conditions, extract of whole body purified lyophilized HDM, *Dermatophagoides pteronyssinus* (Greer Laboratories), was re-

suspended in sterile saline at a solution concentration of either 240 or 500 $\mu\text{g}/\text{ml}$ total HDM protein, so that animals received either 12 or 25 μg of HDM protein on each occasion. The dose solution also contained bacterial endotoxin at a concentration of 13 endotoxin units/ml. The same batch (no. 124632) of HDM was used for studies at AstraZeneca and GlaxoSmithKline. HDM aliquots were maintained at $+4^\circ\text{C}$ until use. A single aliquot was used each day and any remaining solution was discarded once the aliquot had been opened. All mice receiving HDM were exposed to the allergen through a single daily 50 μl intranasal dose given under light inhalation anaesthesia, on 5 days each week, for up to 7 weeks. Control animals received a vehicle exposure of 50 μl of sterile saline over the same time period using the same method of administration. One group of HDM exposed animals was dosed orally with budesonide (1 mg/kg of body weight) twice daily during weeks 6 and 7, and a parallel control group was dosed with the vehicle solution (water with hydroxypropyl methylcellulose and Tween).

MRI methodology

Animals were imaged prior to the start of HDM exposure and thereafter were imaged 2 h after the final HDM dose of each treatment week. Free-breathing mice anaesthetized with 2% isoflurane were placed into the bore of a 4.7 T MRI Scanner (Bruker Instruments) while the scanner animals were remotely monitored for respiration rates via a pneumatic pressure pad placed under the animals (Small Animal Monitoring System; SA Instruments). Initial pilot scans were carried out in each of the three planes: sagittal, coronal and axial. A multi-slice gradient echo (flow compensated) was set up using the pilot scans to provide ten coronal slices through the chest cavity (scanning parameters: TE 3.0 ms, TR 125 ms, 15° pulse angle, 16 averages, slice thickness 1.0 mm, slice separation 1.5 mm, field of view 3.5×3.5 cm and matrix of 128×128 , non-respiratory gated).

MRI image analysis

Lung volume and bright signal within the lung were quantified using Analyze 7.0 software (Mayo Clinic). A contour encompassing the lung was drawn and applied to each of the seven slices of the scan (the trachea and main bronchi were not included within the contour). The total volume of this lung contour was calculated. An intensity threshold value applied to the contour enabled the quantification of absolute bright signal (mm^3) for each scan. The thresholds (mean ± 2 S.D.) for determining the intensity of voxels associated with the lung tissue were computed by placing six ROIs (regions of interest) of about 1.2 mm radius in homogeneous areas within the subtracted image. The signal intensity was assessed for all voxels within the previously determined lung region (within the contour). Volumetric quantitative data were calculated as the number of voxels below the calculated threshold (normal lung tissue) and above the threshold (oedematous tissue), resulting in lung and oedematous volumes (as well as mean $\pm$ S.D. intensity of oedematous voxels).

CT imaging methodology

CT images were obtained once a week from the week prior to initial HDM exposure and 48 h following the last HDM exposure

in weeks 1, 2, 3, 4, 5, 6 and 7. CT-imaged mice were all dosed with 25 μ g of HDM or vehicle. All imaging was performed on spontaneously breathing animals, anaesthetized with isoflurane, on a nanoSPECT/CT small animal imager (Bioscan). CT acquisition was made at 45 kVp over 360 continuous projections with an exposure time of 500 ms per projection. The resultant image matrix was $352 \times 352 \times 436$ voxels (isometric) at 200 μ m resolution. Respiratory gating was not used. Calibration of images for HU (Hounsfield unit) scaling was performed using a water-filled phantom once per day of imaging.

CT image analysis

CT images were processed with Amira 4.1 software (Visage Imaging). The three-dimensional images were low-pass filtered (Gaussian) and the lungs were selected using a computer-assisted volume segmentation from -1000 to -250 HU, which was visually inspected and altered as necessary to ensure inclusion of all lung areas and exclusion of any non-lung areas.

Density histograms were produced with 150 bins describing a range from -1000 to 0 HU (7 HU/bin). The modal lung density of each animal was used for further analysis.

Invasive lung function

Animals were anaesthetized, tracheotomized and mechanically ventilated at 150 beats/min and 0.16 ml tidal volume with 2–3 cmH₂O positive end expiratory pressure, on a Flexivent system (SCIREQ). Baseline respiratory mechanics were measured using an 8-s broadband oscillatory waveform and interpreted using the constant phase model [18]. AHR (airway hyper-responsiveness) was then assessed by measuring the change in respiratory resistance and compliance (measured using monofrequency oscillation) with increasing intravenous doses of methacholine, as previously described [19]. Following lung function measurements the animals were killed and BAL fluid was collected.

BAL and cell counting

At various times during the HDM exposure period, animals were humanely killed by an intra-peritoneal overdose of sodium pentobarbitone. The trachea was cannulated, and the lungs were lavaged with isotonic electrolyte solution. Total and differential cell counts were measured on a Sysmex XT-2000i automated analyser (Sysmex) or on a BD FACSCanto II flow cytometer (BD Biosciences).

Measurement by ELISA of total IgE and HDM-specific IgE

To detect the total IgE in serum samples, flat bottomed 96-well MaxiSorb immunoplates (Nunc) were coated overnight at room temperature (19–21 °C) with 50 μ l of purified anti-mouse IgE (BD Pharmingen) diluted 1:250 in PBS. Plates were washed with 0.05% Tween 20/10% (w/v) PBS, and the coated wells were blocked with 100 μ l of 4% (w/v) BSA in PBS for between 1 and 5 h at room temperature. ELISA was performed using dilutions of serum samples prepared using reagent diluent (4% BSA/0.05% Tween 20 in PBS) incubated at room temperature overnight. Serial dilutions of the serum samples were 1:100, 1:500 and 1:2000 and the standard range of the assay was 100–1.6 ng/ml

IgE. After incubation and washing, 50 μ l of biotinylated rat anti-mouse IgE (2 μ g/ml; BD Pharmingen) was added to each well and incubated for 1 h. Streptavidin HRP (horseradish peroxidase) complex (Amersham Bioscience) was used as the second step reagent for detection of the biotin-labelled antibodies with a 30 min incubation.

The HDM-specific IgE ELISA was carried out exactly as for the total IgE assay with the exception that flat bottomed plates were initially incubated overnight with sterile 50 μ l aliquots of 1 mg/ml HDM protein stock solution diluted 1:200 in PBS. The standard range was prepared using 0.2% standard mouse serum in 4% (w/v) BSA/0.05% Tween 20 in PBS together with HDM-pooled serum (in-house preparation) which was diluted 1:2 to give a standard range from 1 to 0.015 arbitrary standard units/ml.

Histological preparation of lungs

At necropsy, the thoracic cavity was opened, the trachea clamped and cannulated and the lungs instilled with fixative. As CT and MRI were performed by two different organizations on different sites, there were some minor differences in fixation, tinctorial staining, and digital imaging at the two locations. After CT imaging, the lungs were instilled with 10% neutral buffered formalin at a constant pressure of 25 cmH₂O. Once the lungs were fully distended, the trachea was tied off, the lungs removed from the thoracic cavity and post-fixed in 10% neutral buffered formalin for 48 h. Individual lobes were then trimmed, processed and embedded in paraffin wax. The lungs assessed by MRI were instilled with 4% neutral buffered paraformaldehyde using slow gentle pressure on a 1 ml syringe without any standardization of inflation pressure. Once inflated, the lungs were removed from the thoracic cavity and held by the trachea for about 30 s before the tracheal clamp was removed and the lungs post-fixed without the use of any ligatures in 4% neutral buffered paraformaldehyde for a minimum of 24 h.

Following fixation and embedding, the lungs analysed by CT were sectioned (4 μ m) and stained with Gills II Haematoxylin (Pioneer Research Chemicals) and Eosin Y (Acros Organics; Fisher Scientific). Sections were also stained with MSB (Martius Scarlet Blue) trichrome [19a] to highlight the peribronchiolar smooth muscle and collagen. After paraffin processing, the lungs from each mouse analysed by MRI were embedded whole, dorsal surface down, with the trachea facing the cassette label to provide tissue sections in the same plane as MRI scanning. In order to provide good correlation with the MRI, sectioning of the lungs was delayed until it was confirmed that the hyper-intense MRI signal was present throughout the full depth of the lung tissue, at which point a set of serial 4 μ m coronal sections were cut through all lobes in the plane of the main airways. Sections were stained with Harris's H&E [haematoxylin and 1% (w/v) aqueous eosin], to demonstrate general lung morphology and inflammation, and ABPAS (Alcian Blue Periodic Acid Schiff) to demonstrate neutral and acid mucins in the airway goblet cells. Periodic Acid Schiff was supplied by Sigma Aldrich; all other staining reagents were supplied by Pioneer Research Chemicals.

Prior to histopathological assessment, stained slides were digitally imaged using either an Aperio scanner (CT mice) or a

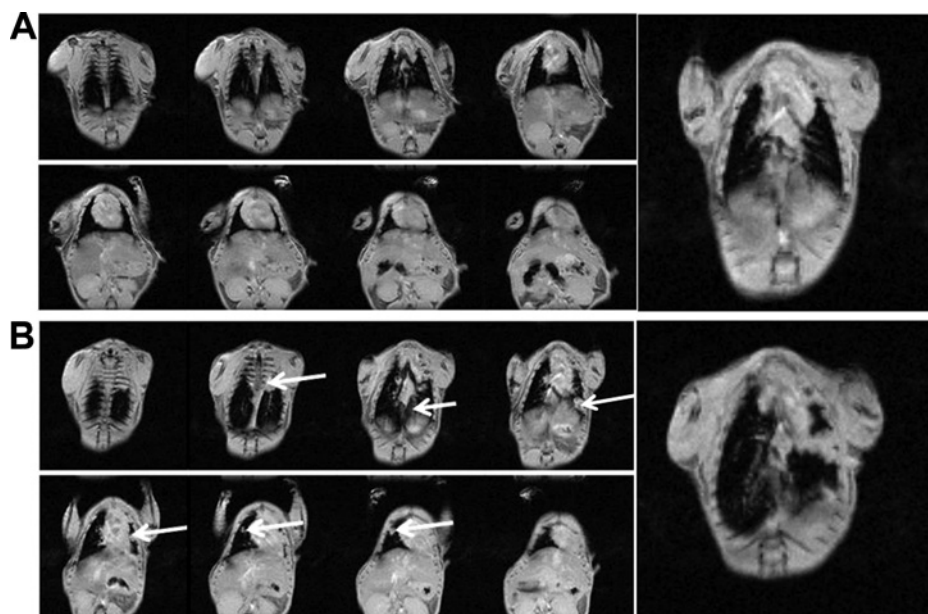


Figure 1 Sequential MRI coronal slices through the lung cavity of a mouse exposed to saline (A) and a 25 μ g HDM-treated animal (B)

(A) Sequential MRI coronal slices through the lung cavity of a mouse exposed to saline. No increase in signal intensity was observed throughout the cavity suggesting absorption of the saline dose. (B) Similar slices through the lung from a 25 μ g HDM-treated animal. Clear areas of hyperintense regions indicated by the arrows are associated with localized inflammation. Large images represent a single slice through the lung cavity of a mouse pre and post 25 μ g HDM challenge.

Hamamatsu Nanozoomer (MRI mice). Histopathological assessment of features such as the number of eosinophils present in the tissue was by semi-quantitative scoring. All scoring was performed blind with regard to treatment groups (results not shown) by a single pathologist at each site.

Statistical analysis of data

Experimental power: pilot experiments demonstrated that sample sizes of $n = 6$ were sufficient to detect at least a 2-fold difference as statistically significant.

For MRI data, repeated measures ANOVA was applied to the data. For the lung volume data only, the baseline values were used as a covariate. Comparisons between group means were made in the form of differences and were tested to 95% confidence intervals.

For CT data, a two-way ANOVA was applied to the volume and density data with main effect of treatment (HDM or vehicle) and week of dosing, using PROC MIXED in SAS v9.2. At each time point, pairwise comparisons across treatment were performed for density data and a one-way ANOVA for volume data. Results are expressed as means $\pm$ S.E.M. and were deemed significant if $P < 0.05$.

Lung mechanics data were compared between treatment groups using one-way ANOVA and between treatment groups and across time using two-way ANOVA. In both cases, *post-hoc* comparisons were made with Bonferroni's test.

Results are expressed as means $\pm$ S.E.M. Differences between groups were considered significant if $P < 0.05$.

RESULTS

MRI results

Typical MRI images of lung are predominantly dark in appearance because of the low signal associated with lung parenchyma. Any cell recruitment and/or accumulation of oedema within the lung produces an increase in MRI signal. Figure 1(A) shows the effect of topical administration of saline resulting in lung cavities that have a predominantly dark appearance and hence low lung tissue signal intensity. In contrast, animals challenged with 12 and 25 μ g of HDM showed a global increase in lung tissue signal intensity as well as discrete areas where an extensive increase in signal intensity was observed (Figure 1B). Over the 7 week HDM exposure period there was no change in lung tissue signal intensity in any of the control animals treated with saline (Figure 2). Animals treated with both 12 and 25 μ g of HDM showed equivalent increases in lung signal intensity between weeks 2 and 4 after which 12 μ g HDM-dosed animals remained at a level of approximately 20 mm³. The 25 μ g dose continued to show a steady linear increase from weeks 4 to 7, resulting in a total high intensity volume of 80 mm³ ($P < 0.01$ compared with 12 μ g at weeks 6 and 7 and $P < 0.05$ at week 5). Significant increases in lung intensity measurements at both dose groups were seen as early as week 3. Figure 3 illustrates the changes associated with lung volume over the same period where the two HDM dose groups showed equivalent increases up until week 3 after which the 25 μ g HDM group continued on a steeper increase in volume. There was a slight increase in lung volume (approximately 30 mm³ which equated to a 11% increase from baseline

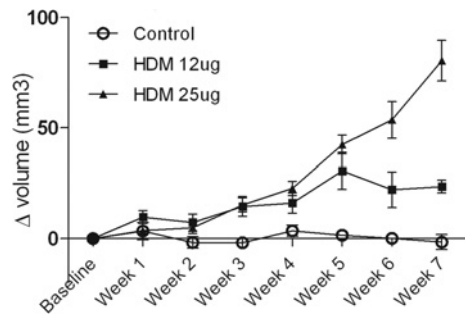


Figure 2 Total volume increase of the hyper-intense MRI signal over the 7-week period in mice challenged with saline, 12 μ g HDM and 25 μ g HDM

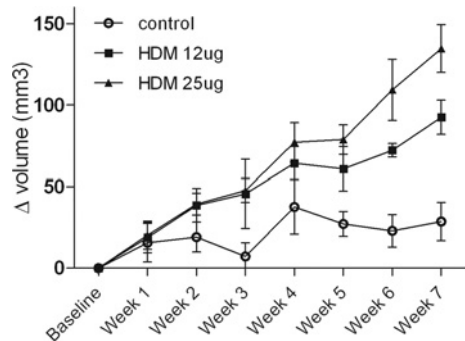


Figure 3 Total lung volume increase over the 7 week period in mice challenged with saline, 12 and 25 μ g HDM

values) for the saline treated animals, which may have reflected overall animal weight gain. However, lung volume increases of 90 and 130 mm³ were seen in the 12 and 25 μ g HDM-dosed animals (see large images within Figures 1A and 1B that represent an equivalent slice from a mouse at baseline and following 7 weeks of 25 μ g HDM challenge) equating to percentage increases of 27 and 58%, respectively (Figure 3).

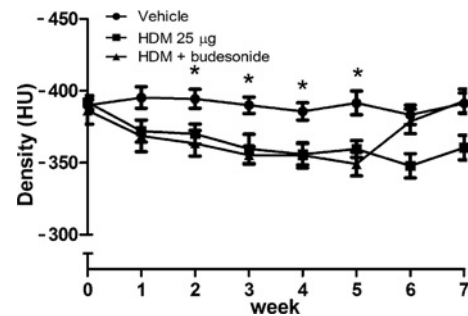


Figure 5 CT lung density in HU over time

Values are means $\pm$ S.E.M. * P < 0.05 compared with the 25 μ g HDM group.

CT results

Figure 4 shows representative thoracic transverse sections of HDM and saline-treated animals. There was an increase in lung density with a peribronchial pattern in the HDM-treated animals. Figure 5 shows the mean modal lung density in HU. There was a significant increase in lung density in the HDM-dosed groups compared with the saline-dosed groups, and this difference was abolished by budesonide treatment. Lung volumes did not change significantly over time with either HDM or saline dosing (results not shown).

Respiratory mechanics and airway responsiveness

Airway resistance, tissue damping and tissue elastance all increased significantly in the HDM-dosed group compared with vehicle (Figure 6), with differences apparent after 3 weeks for resistance and elastance and 5 weeks for tissue damping. These changes produced a marked increase in the sensitivity of the airways to the bronchoconstrictor effects of intravenous methacholine at 7 weeks (Figure 7). In the HDM-dosed groups, measurements were only made at low doses of methacholine

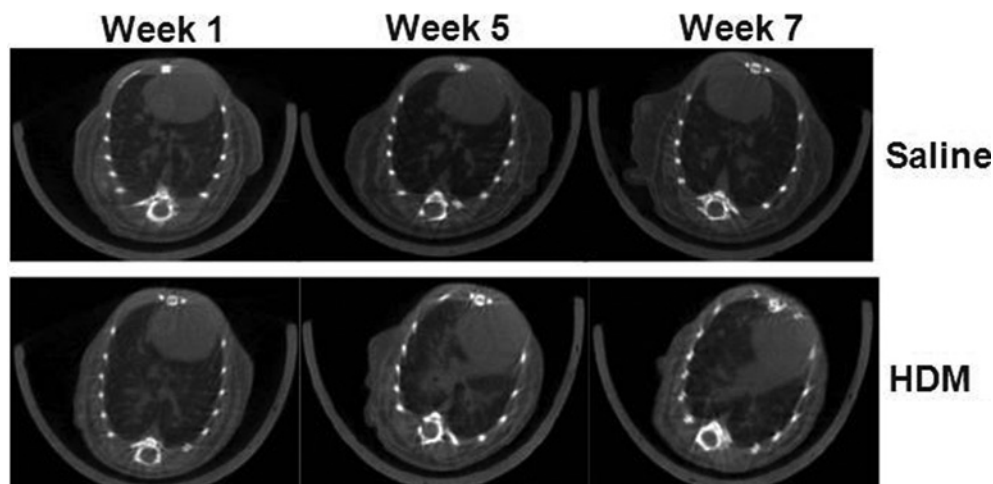


Figure 4 Transverse thoracic CT slice of saline (upper row) and HDM 25 μ g (lower row) dosed mice after 1, 5 and 7 weeks of dosing

HDM-exposed mice show an increased CT density with a peribronchial pattern.

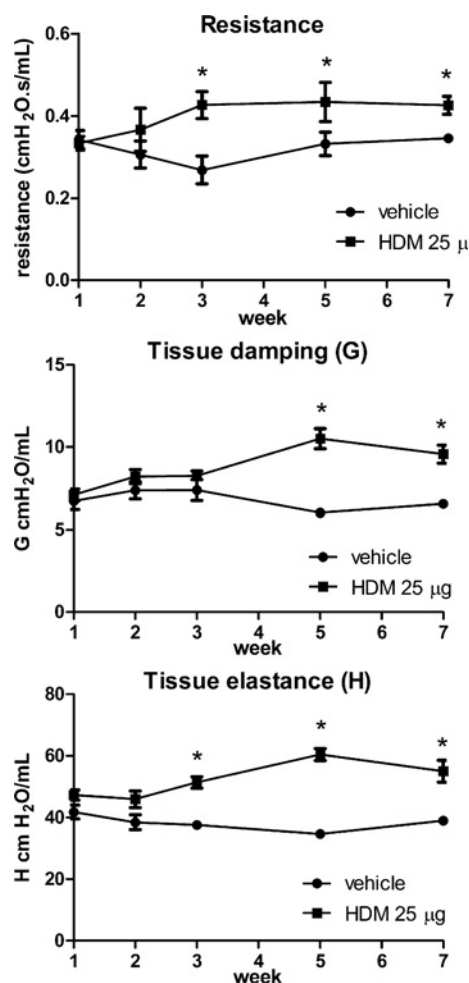


Figure 6 Lung mechanics measured by oscillometry over time in HDM-dosed mice compared with vehicle-dosed animals
Values are means $\pm$ S.E.M. * $P < 0.05$ compared with HDM group.

because the marked bronchoconstriction made it difficult to ventilate the lungs at higher doses.

Histopathology of the HDM model

Repeated daily intranasal challenge with HDM extract resulted in prominent perivascular, peribronchiolar and alveolar inflammation (alveolitis and pneumonitis, Figures 8A and 8B), consisting primarily of mononuclear cells (T-cells, B-cells and macrophages) and eosinophils, with smaller numbers of neutrophils. At the highest doses and longest dosing regimens, there was evidence of pneumonia and consolidation of the peripheral airspaces with large activated macrophages and multinucleate giant cells. The overall response was dependent on the daily dose level and the duration of dosing. The most consistent and prominent effects were observed with 7 weeks at the 25 μ g HDM dose. The dose–response relationship demonstrated by the inflammatory load in BAL and in tissue sections after the different dosing regimens was mirrored by tissue eosinophils. There was little difference observable between the 5 and 7 week dosed animals but the extent and severity of the alveolitis/pneumonitis was re-

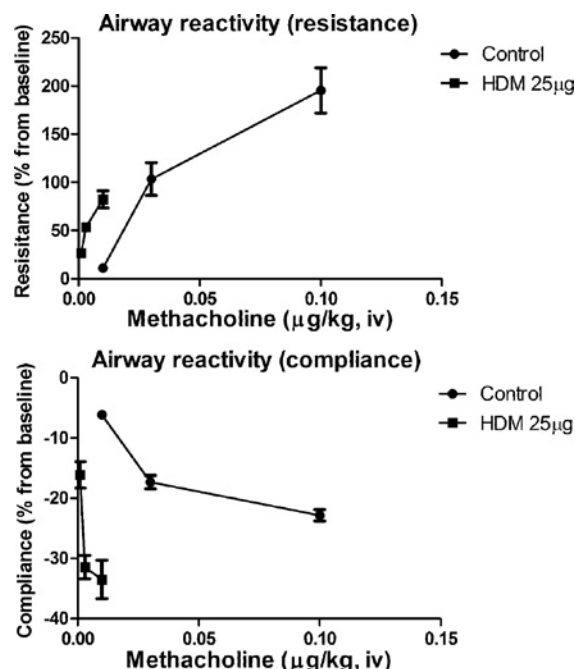


Figure 7 Airway responsiveness at week 7, expressed as the percentage increase in resistance and fall in compliance with increasing dose of intravenous methacholine
Values are means $\pm$ S.E.M.

duced at week 5 and with the lower HDM dose. Administration of HDM resulted in a marked goblet cell metaplasia of the airway epithelium that extended down the tracheobronchial tree to the terminal bronchioles. Very occasionally, mucus plugs were observed. In addition to the synthesis of mucus, HDM-treated animals also demonstrated a marked accumulation of eosinophilic material within the epithelial cells. This material was shown to be ABPAS-negative, but appeared to be present within airway goblet cells. As it increased in quantity and prevalence with prolonged duration of HDM dosing, this material appeared to have a negative impact on the amount of mucus within the goblet cells of these animals. Concomitant with the presence of eosinophilic material within the epithelium, increasing duration of HDM dosing also resulted in the accumulation of eosinophilic crystalline material within the alveolar macrophages (Figures 8C and 8D).

Although not of direct relevance to the use of the model for inflammation research, increasing cumulative dose of HDM also resulted in a marked vascular remodelling, characterized by increased smooth muscle and collagen leading to intimal thickening and partial occlusion of some vessels, particularly the smaller arteries.

Although not formally determined, peribronchiolar smooth muscle in the animals dosed at 3, 5 and 7 weeks appeared to be greater than in corresponding controls. MSB (Martius Scarlet Blue) trichrome staining to demonstrate collagen and muscle indicated that the apparent increase in muscle mass was because of an increase in collagen deposition around the airways (Figures 8E and 8F) as well as smooth muscle myocyte hypertrophy.

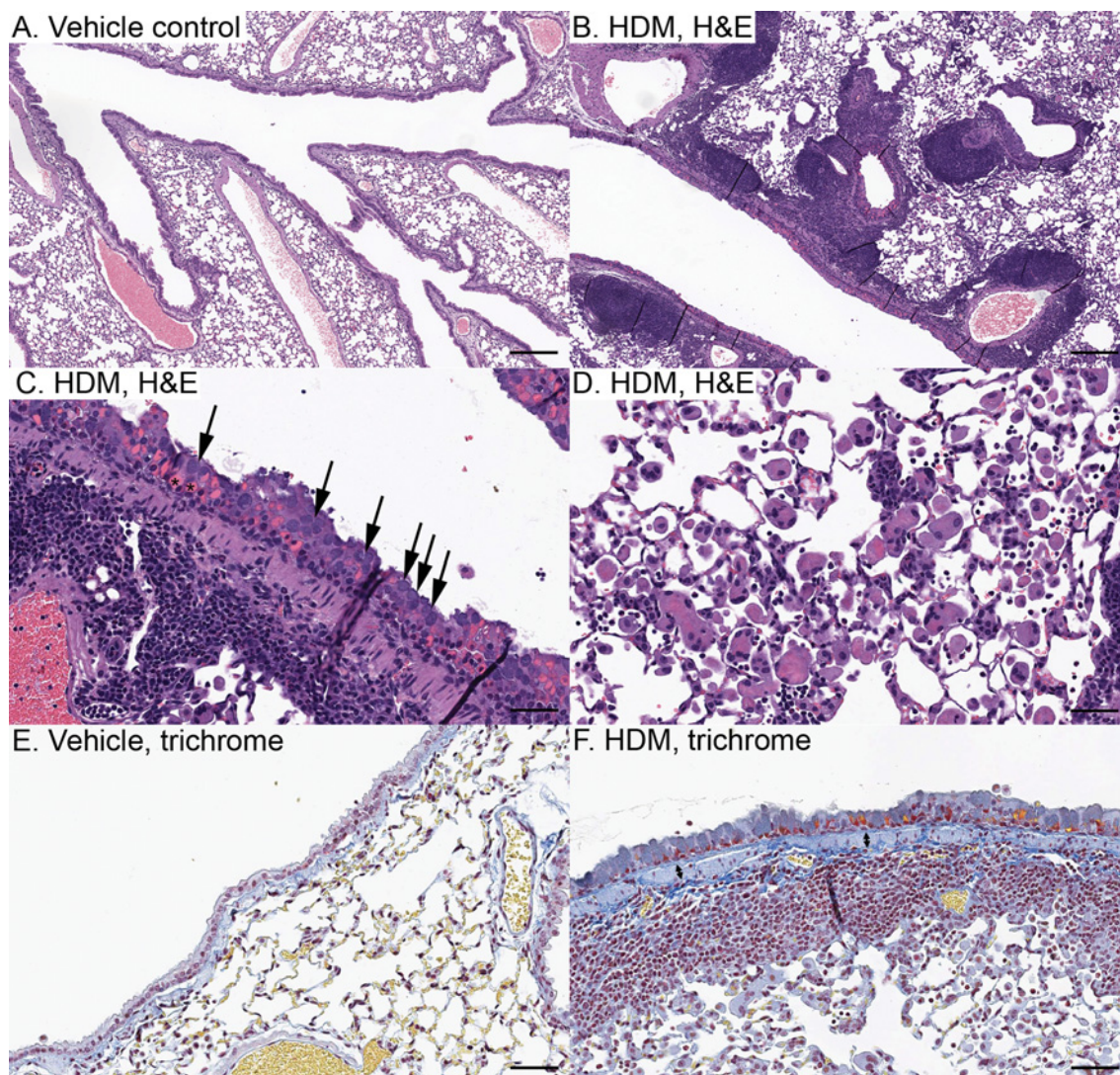


Figure 8 Histopathology of HDM-challenged rat lungs

Mouse lungs were insufflated in formalin, processed into wax and stained with either H&E (A–D) or MSB trichrome (E, F). (A, E). Vehicle control mice did not show any findings of pathological significance. (B) Mice treated with HDM for 7 weeks showed marked perivascular and peribronchiolar cuffing consisting of mixed inflammatory cells. There was also an inflammatory infiltrate into the alveolar bed and alveolar airspaces. (C) There was a marked epithelial reaction including increased numbers of mucus producing goblet cells (arrows) that extended down the tracheobronchial tree. The epithelial cells also showed prominent eosinophilic inclusions (asterisks). (D) Within the alveolar bed there was a marked alveolitis, including granulocytes and multinucleate giant cells within the airspaces. (F) There was an apparent thickening of the peribronchiolar smooth muscle layer (arrows) along with increased deposition of collagen (bright blue stain) in the animals dosed with HDM compared with those dosed with vehicle (E). Scale bars in (A, B), 250 μ m; and (C–F), 50 μ m.

DISCUSSION

The results of the present study showed that mice chronically treated with HDM extract developed progressive increases in MRI signal intensity and CT lung density, demonstrating an increase in lung water and/or tissue density. These changes are most likely to reflect increased mucus and oedema in the periphery of the lungs of the mice.

This increase was associated with lung inflammation, increased airway responsiveness, altered respiratory mechanics,

and histopathological changes in the lung. The use of non-invasive imaging allowed serial measurements of the lungs in the same mouse over time which considerably reduced the number of animals required for a longitudinal study. There are no previous full reports of MRI or CT changes in HDM exposed mice. Nouis et al. [20] presented preliminary data using proton and hyperpolarized ^3He MRI to image mouse lungs treated with HDM for 2 weeks. The proton MRI data were consistent with this study, showing fluid-filled regions in the lungs of HDM-exposed mice that were unventilated on ^3He MRI. Intravenous

methacholine resulted in bronchoconstriction and ventilation defects. Jobse et al. [13] used CT to examine Brown Norway rats exposed to HDM and analysed the images using two different methods; their method 2 corresponded to the method used on CT images in this study. They showed an increase in lung density in the high dose group after 7 days of treatment. Our study in mice similarly showed an increase in lung density in the HDM treated animals that started after 2 weeks of dosing and persisted for the duration of the study. The increase in CT density was rapidly reversed by treatment with budesonide, suggesting that the change was because of inflammation and/or fluid accumulation rather than structural modification of the lung. The MRI data showed an increase in bright signal (proton density) in both a focal and diffuse pattern. There was also a significant increase in lung volume, which was not seen in the CT data but remains consistent with clinical findings from asthma patients [21,22]. This may be because the MRI segmentation method was based on a manually drawn region of interest (similar to method 1 of Jobse et al. [13]) rather than automated segmentation based on density together with the greater contrast within the MRI image to better delineate boundaries. With automated methods areas of hyperintense signal within the lung cavity but adjacent to the lung cavity wall may not be included in the overall lung volume measurement. This could also underestimate the total amount of inflammation within the lung cavity itself which probably reflects the differences in magnitude of the volume changes seen by the two modalities. Previous work looking at other chronic lung conditions such as bleomycin induced fibrosis have also shown similar increases in overall lung cavity volume (results not shown). An acute study using ovalbumin in the mouse also showed no differences in lung volume by manually segmented CT analysis but this may be because of the acute nature of the study (Riesenfeld et al. [14]).

Histopathological examination of the lungs showed prominent perivascular, peribronchiolar and alveolar inflammation (alveolitis and pneumonitis), consisting primarily of mononuclear cells and eosinophils, with smaller numbers of neutrophils. There was also evidence of remodelling with increased collagen deposition around the airways and airway smooth muscle hypertrophy. There was a marked goblet cell metaplasia of the airway epithelium that extended down the tracheobronchial tree to the terminal bronchioles. Similarly Johnson et al. [4] described increased goblet cell mucin, sub-epithelial collagen deposition and smooth muscle hypertrophy in HDM-exposed mice. Other markers of allergic inflammation measured in this study were in line with published studies. Total and specific IgE increased (cf. Johnson et al. [4]; Kelada et al. [7]) and there was an increase in all cell types in the BAL fluid (Supplementary Results section at <http://www.clinsci.org/cs/125/cs1250555add.htm>). Oral budesonide decreased BAL lymphocytes and eosinophils but not neutrophils. Interestingly, the same heterogeneous cellular response was seen by Ulrich et al. [10] using oral prednisolone at 1 mg/kg of body weight. In the same study, prednisolone at 10 mg/kg of body weight decreased BAL neutrophils as well, suggesting a relative rather than absolute steroid resistance of the neutrophilic response to HDM extract.

Changes in respiratory mechanics caused by chronic HDM exposure were measurable after 3–5 weeks. Airway resistance

(R), tissue damping (G) and tissue elastance (H) were all increased compared with control mice. Changes in airway resistance are caused by airway narrowing and/or peripheral airway closure, but although the airway epithelial pathology suggests that airway narrowing could be the cause of the increase in R, the tissue parameters G and H indicate otherwise. G and H are both considerably increased in the HDM-exposed mice, reflecting changes in the peripheral lung. The ratio G/H was unchanged in this study (results not shown) and thus the changes were due to de-recruitment rather than peripheral lung inhomogeneity [23]. Hence, the changes in baseline respiratory mechanics are due to loss of peripheral lung units via closure of small airways and this is likely to be due to mucus plugging given the increased mucin production seen on pathology and the increased water content of the lung seen on MRI and CT.

Administration of the bronchoconstrictor methacholine to anaesthetized mice at the end of 7 weeks of treatment showed that the HDM-exposed mice had a much greater rise in respiratory resistance and fall in respiratory compliance compared with control animals (Figure 7). In these studies, the methacholine was given intravenously and hence the changes were not due to any alterations in epithelial permeability to methacholine [24]. Instead, the abnormal mechanical response was probably because of an increased tendency towards peripheral airway closure [23]. Given the evidence for increased mucin in this model, a unifying hypothesis that explains the alterations in both the baseline mechanics and the response to methacholine, and is consistent with the imaging and pathology data, is an increased production of mucin leading to peripheral airway plugging and an increased tendency towards critical airway closure during bronchoconstriction. This is consistent with the emerging importance of peripheral airway closure as the major cause of physiological dysfunction in human asthma and inflammatory animal models of asthma [21].

The chronic HDM model has been developed as a model of allergic asthma and shares many of the features of the human disease [1,4,10]. Lung volumes increase in asthma due to peripheral airway closure [21,22] and the increased MRI lung volume seen in this study is probably because of a similar mechanism. The mean X-ray density of the lung tends to decrease in human asthma and is correlated with severity [25,26] because air trapping, which reduces density, has a greater effect than the increase in density because of airway inflammation. The opposite effect was observed in the HDM-exposed mice (in spite of an increased MRI lung volume) suggesting that inflammation is the dominant effect in mice. The imaging results from this study suggest that, despite the chronic nature of the HDM mouse model, significant increases in lung inflammation can be seen using 25 µg from as early as the second week of challenge. By the third week of dosing a good window can be observed potentially allowing therapeutic intervention to the same animal. With different animals presenting with slightly different levels of lung inflammation at any one specific timepoint, each animal can act as its own control to produce a relative change rather than absolute measure, generating greater statistical power to the data. In addition, there was a significant reduction in the total numbers of animals required and the overall time taken to complete these types of studies. Another feature of this type of approach was the fact that

at baseline, prior to any intranasal challenge, a couple of animals presented with significant levels of lung pathology as measured by MRI. These animals were removed from the study reducing the overall variability of the final dataset.

Conclusions

The present study gives a comprehensive evaluation of a model of chronically induced allergic lung disease in mice using a clinically relevant allergen. The use of non-invasive MRI and CT imaging allowed longitudinal measurements in the same animal which markedly reduced the number of mice needed to achieve a similar degree of characterization using animals assessed only on a single occasion. This collaboration has allowed two pharmaceutical companies to jointly undertake research that may benefit the wider research community under the UBIOPRED Innovative Medicines Initiative (<http://www.ubiopred.european-lung-foundation.org/>), a joint venture between the European Union and EFPIA (European Federation of Pharmaceutical Industries and Associations). The present paper has explored how imaging may provide an important non-invasive imaging modality that can pick up subtle changes of disease at a very early time point. In addition, by allowing longitudinal assessments to be made for individual animals, variability sometimes associated with traditional techniques becomes less of an issue due to a change from baseline being measured. This then allows screening of animals that may not have responded well to the initial HDM challenge, as well as allowing therapeutic intervention to the same cohort of animals. Imaging can have a major impact on reducing total animal numbers required for any particular study through longitudinal assessment as illustrated in this characterization of the model.

From a translational viewpoint, CT is the most widely used imaging modality in asthma [27] in spite of the radiation burden. MRI has much to offer but requires the uptake of short echo time sequences into clinical practice to obtain useful signal from the lung parenchyma.

CLINICAL PERSPECTIVES

- There are several animal models of asthma where the inflammatory component of the disease is assessed using terminal procedures such as bronchoalveolar lavage and histopathology.
- The present study has used MRI and CT imaging techniques to longitudinally assess the degree of lung inflammation. These techniques have shown good correlation with traditional end points yet can be performed within the same animal on several occasions. A profile of inflammation over time can be measured which provides an opportunity to monitor therapeutic intervention.
- The imaging readouts are sensitive and accurate and could be applied clinically, but further work is needed to understand how the increased signal intensity and lung density seen in mice relates to the decreased density and signal in humans.

This will probably be resolved by sub-segmenting the mouse lung into airway and parenchymal compartments and comparing them to the human equivalents.

AUTHOR CONTRIBUTION

Kumar Changani helped in the MRI set up, design, execution and preparation of the paper; Catherine Pereira helped in the CT imaging; Simon Young assisted in study design, data analysis and preparation of the paper. Robert Shaw helped in functional lung measurements, cell population and preparation of the paper; Simon Campbell helped in MRI data analysis. Kashmira Pindoria assisted in MRI in life organization and scanning; Steve Jordan performed the lung function measurements. Katherine Wiley was involved in the animal model development and characterization; Sarah Bolton helped in histopathological analysis; Tony Nials helped in study design and preparation of the paper. Michael Haase helped with the *in vivo* dosing and scanning. Mike Pedrick assisted in the histopathological analysis, histological and preparation of the paper. Richard Knowles worked as U-BIOPRED work-package co-ordinator, study design and preparation of the paper.

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SUPPLEMENTARY ONLINE DATA

Longitudinal characterization of a model of chronic allergic lung inflammation in mice using imaging, functional and immunological methods

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RESULTS

Cellular and immunological response to HDM

MRI-scanned animals

Repeated intranasal delivery of HDM extract to mice for 7 weeks resulted in a robust increase in inflammatory cells in BAL fluid. Lymphocytes, neutrophils and eosinophils were all significantly increased after 7 weeks of HDM dosing compared with saline controls, with the increase in eosinophils being particularly marked. In contrast, macrophages remained relatively unchanged after HDM treatment (Supplementary Figure S1).

CT-scanned animals

Seven weeks of HDM treatment produced increases in inflammatory BAL cells compared with vehicle-treated animals that were similar to the MRI-scanned group (Supplementary Figure S2). Dosing with budesonide from weeks 5 to 7 reduced the numbers of lymphocytes and eosinophils to near control levels but did not significantly affect neutrophil and monocyte numbers (Supplementary Figure S2).

HDM-specific IgE levels rose slowly and were significantly increased after 7 weeks of 25 μ g HDM exposure (Supplementary Figure S3).

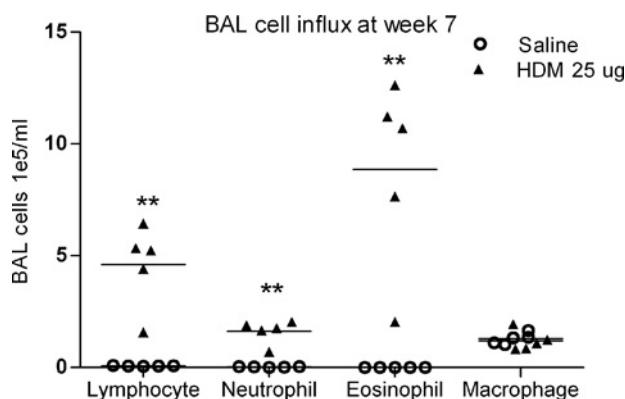


Figure S1 Change in BAL cell populations after 7 weeks of HDM or saline vehicle dosing

Numbers of lymphocytes, neutrophils and eosinophils significantly differed between the two treatment groups $**P < 0.01$ compared with the HDM group.

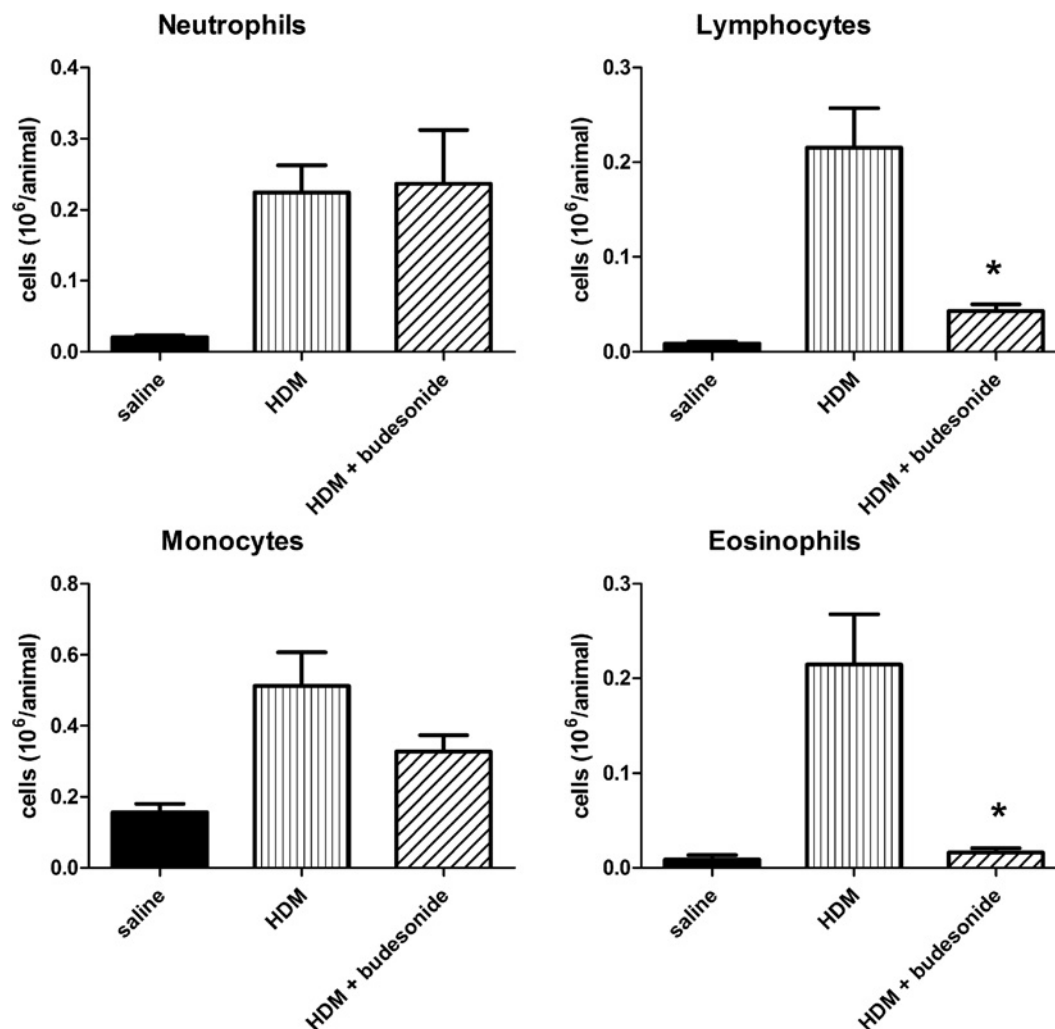


Figure S2 Effect of budesonide treatment on BAL cells at 7 weeks

Values are means $\pm$ S.E.M. All cell types were significantly increased in the HDM dosed group compared with the saline-dosed group. * $P < 0.05$ compared with the HDM + budesonide group.

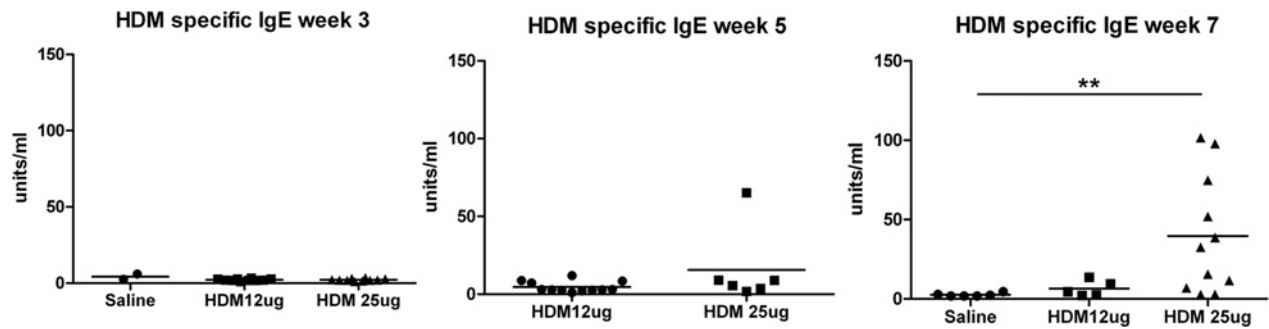


Figure S3 Generation of HDM-specific IgE following repeated administration of HDM over 7 weeks

Samples were taken 48 h after the final preceding HDM or saline dose of that week. Significant increases in the specific IgE were seen at week 7. $**P < 0.01$ compared with the vehicle group.

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