ERJ Express. Published on October 19, 2009 as doi: 10.1183/09031936.00022908

HDM induces direct airway inflammation in vivo: implications for future disease therapy?

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Short title: **Non-allergic inflammation by HDM**

Word count for the body of the manuscript: 4305

ABSTRACT

Background: House dust mite (HDM), the major source of allergen in house dust and strongly associated with the development of asthma, can evoke a direct, non-allergic, inflammatory reaction *in vitro*.

Objectives: To determine whether this apparent non-allergic inflammatory response can be observed in a more complex *in vivo* setting.

Methods: Vehicle, Alum^{TM} or HDM (*Der p* 5μg, i.p. with Alum) sensitised Brown Norway rats were challenged intratracheally with vehicle (saline), HDM (*Der p* 10μg) or heat-inactivated HDM on day 21. Lung function changes and the associated inflammatory response were evaluated.

Results: Tissue and BAL from Alum^{TM} sensitised *Der p* challenged animals exhibited strong eosinophilia and neutrophilia associated with an early release of pro-inflammatory cytokines (IL-13, IL-1β, eotaxin, TARC). This response was not attenuated by removal of HDM-associated protease activity. Interestingly the vehicle sensitised group (no Alum^{TM}) lacked this inflammatory response.

Conclusions: HDM allergen evokes non-allergic airways inflammation with an inflammatory profile similar to that of the asthmatic airway. This response, independent of the protease activity of the HDM extract, appeared to be linked to prior administration of the adjuvant Alum^{TM} and the subsequent increase in total IgE. This finding could have important implications in the development of future asthma therapies.

Key words: Allergy, Asthma, Cytokines, House dust mite, Inflammation, Rodent

Introduction

The exposure to HDM allergens is strongly associated with the development of allergic diseases, such as asthma, rhinitis or atopic dermatitis (1;2). Mites from the genus *Dermatophagoides,* such as *D. pteronyssinus* (*Der p*) and *D. farinae* (*Der f*) are thought to be the most important source of indoor allergens associated with human asthma (1;3). Over 30 proteins produced by *Dermatophagoides* mites induce IgE in allergic patients, but there are dominant antigens, especially the group 1 and 2 allergens, which can account for the bulk of allergenicity of HDM extracts (1). Among the group 1 allergens is *Der p 1* from *D. pteronyssinus*. *Der p 1* belongs to the papain-like cysteine protease family and exhibit cysteine protease activity (4). This proteolytic activity has been suggested to be involved in the pathogenesis of allergies by increasing the permeability of epithelial cells and allowing the passage of their own and other allergens across the epithelium (5-7), cleaving and/or interacting with cell surface molecules and intrinsic protease inhibitors (8-11) and modulating the function of a range of cells, such as basophils, mast cells, alveolar macrophages and airway epithelial cells (12-15). The effect on the airway epithelium is especially interesting, as it is likely to be the first cell type to interact with the allergen. Several authors have reported that *Der p 1* and other *Der p* antigens are able to directly induce the release of pro-inflammatory cytokines and chemokines from primary human bronchial epithelial cells and airway epithelial cell lines (14;16-20) by protease-dependent and independent mechanisms (14;16;19;21).

To our knowledge, however, this pro-inflammatory effect of HDM has not been shown in a more complex *in vivo* system. Therefore our objective was to investigate whether a commercial HDM extract from *D. pteronyssinus* was able to elicit a direct non-allergic pro-inflammatory effect in the Brown Norway (BN) rat. The BN rat was chosen because we, and others, have previously demonstrated that an allergic reaction to other antigens, i.e. ovalbumin, is achievable in this strain (22).

Methods

Animals

Male BN Rats (200-225g) were obtained from Charles River Laboratories, Inc (Lyon, France) and housed for 1 week before initiating experiments.

House Dust Mite extract

Purified HDM extract from *Dermatophagoides pteronyssinus* (*Der p*; GREER laboratories, USA) with a known content of *Der p 1* (39.77 μg/mg dry weight) was used in these experiments. The doses of HDM used in this manuscript refer to the amount of *Der p 1*.

Measurement of protease activity in the extract

Protease activity in the extract was assessed by a continuous rate (kinetic) assay using the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC (AMC, 7-amino-4-methylcoumarin; Boc, N-tert-butoxy-carbonyl) as previously described (23). Briefly, a known amount of HDM extract (final concentration Derp1 10nM) was added to a substrate standard curve (0-1mM), in 50mM sodium phosphate buffer, pH 7.0, containing 1mM EDTA and 1mM dithiothreitol (DTT) at 25ºC. Fluorescence associated with the hydrolysis of the substrate was monitored using a Synergy™ HT Multi-Mode Microplate Reader (Biotek Instruments Inc., USA) with λex=380nm and λem=460nm. Data were fitted to a Michaelis-Menten equation (y=V.x/(Km+x) and the kinetic constants Km and Vmax were calculated. HDM extract showed a positive protease activity with a Vmax of 76636 μmol/min and a Km of 304.9 μM. As a control, a similar amount of HDM extract was inactivated by heating to 65ºC for 30 minutes as previously described (19)

Dosing route selection

The majority of our previous work into allergic airway inflammation has involved aerosolised challenging with the antigen. Unfortunately we were unable to utilise this method because of the cost implications. Therefore our initial aim was to determine the optimum topical route of administration for the allergen by comparing intranasal and intratracheal. This was done by using LPS-induced TNFα release in the lung as a biomarker of appropriate delivery. Male BN rats (200-225g) were anaesthetised (4% halothane in oxygen for 3 minutes) and vehicle (saline) or lipopolysaccharide (LPS;

100µg/rat ie. the same amount in each case) were administered in various volumes either intranasally (5, 10, 20, 50 or 100µl in each nostril), using a pipette in a drop-wise fashion, or intratracheally (250µl).

Ninety minutes after blood, lavage and lung tissue samples were taken. TNFα was measured by ELISA (R&D systems Ltd) as described in Birrell *et al* (24).

House Dust Mite sensitising dose selection

Times for sensitisation challenge were extrapolated from our ovalbumin allergic inflammation model in the BN rat (24). An allergen dose-response study was performed to evaluate the optimum sensitising dose by measuring the levels of HDM specific IgE in plasma 14 days after i.p. dosing. Male BN rats (200-225g) were sensitised on day 0 with saline, Alum™ or HDM extract (Greer LT, *Der p 1*; 0.5-500 µg/rat, i.p.) administered with Alum™. At day 14 blood samples were collected and the plasma separated.

ELISA plates (Nunc MAXsorb) were coated with 50µl of 5µg/ml HDM protein in PBS overnight at room temperature (RT). Plates were then washed with wash buffer (Biosource) and blocked at least 30 minutes with 4% Bovine Serum Albumine (BSA; Sigma) in PBS. Plasma samples were then added to duplicate wells diluted 1:10 in PBS containing 4%BSA and 0.05% Tween 20. They were then incubated overnight at RT prior to adding biotinylated anti-IgE (1:10; Serotec) at 2µg/ml (in PBS containing 4% BSA and 0.05% Tween 20) and further incubated for 1 hr at RT. After washing, plates were incubated 30 minutes at RT with Horseradish peroxidase-Streptavidin (1:4000; Amersham). Following a last wash, TMB substrate (Sigma) was added to the plates and they were incubated at room temperature until colour developed. Reaction was stopped with 0.25M Sulphuric acid and plates were read at 450nM (Bistek Powerwave XS plate reader). Total IgE was measured using a commercial rat IgE quantitative ELISA kit (Bethyl Laboratories, USA) following the protocol of the manufacturer and expressed as µg/mg of lung tissue total protein.

House Dust Mite challenging dose selection

Following the determination of the optimal sensitising dose, we aimed to determine an appropriate HDM dose for challenging previously sensitised animals. Male BN rats (200-225g) were sensitised at day 0 and 14 with HDM (5 µg/rat, i.p.) administered

with Alum™. Rats were then challenged with intratracheal vehicle (saline) or HDM (0.1-100 µg/rat in 250µl saline) on day 21.

Forty-eight hours after HDM challenge, rats were lavaged (2 x 3 ml RPMI). Total cell counts recovered from the airway lumen were quantified using a Sysmex cell counter. Differential cell counts (eosinophils, neutrophils, lymphomononuclear cells expressed as absolute cell counts) of cells recovered from the airway lumen were made by light microscopy after Wright-Giemsa staining using standard morphological criteria.

Time course after HDM sensitisation /challenge on cell recruitment in the Brown Norway model.

To determine the effect of the acute exposure to HDM on asthma like end-points, male Brown-Norway rats (150-180g) were sensitised on day 0 and 14 with vehicle (saline) or HDM extract (*Der p 1*; 5 µg/rat, i.p.) administered with Alum™. Rats were challenged with intratracheal vehicle (saline) or HDM extract (*Der p 1*; 10µg i.t) on day 21.

Animals were lavaged 6, 24 or 48hrs later and differential white cell counts performed as above. Similarly white cell numbers and type in the lung tissue were determined as described before (27). The remaining lung was either snap-frozen or fixed in formalin for histological assessment. Inflammatory mRNA/protein expression was assessed by real–time PCR using fully validated primers and probes as detailed in McCluskie *et al* (25) or by specific ELISA (R&D Systems, USA), respectively. Cytokine location in the lung was visualised using immunohistochemistry.

Briefly, insufflated formalin fixed lung tissues were processed and wax embedded. 4µm sections of paraffin embedded tissue were dewaxed and rehydrated. Intrinsic peroxidase activity was blocked by incubating the sections with 3% hydrogen peroxide (IL-13; 25min) or 2% hydrogen peroxide (eotaxin and IL-1β; 20min). IL-13 and IL-1β slides were then subjected to antigen retrieval by pressure cooking in sodium citrate buffer (10mM; pH 6.0). Non-specific binding was reduced with 10% normal rabbit serum (Vector) in PBS containing 0.1% BSA and 0.025% Tween 80 for 20min for IL-13 IHC and 1% normal rabbit serum (Dako) in PBS containing 0.1% BSA and 0.025% Tween 80 for 20min for eotaxin and IL-1β IHC. Sections were then rinsed and the primary antibody (goat polyclonal anti-mouse IL-13 antibody; 1:250 dilution; Santa Cruz Biotechnology; goat polyclonal anti-mouse eotaxin/CCL11; 1:50

dilution; R&D Systems or goat polyclonal anti-rat IL-1β; 1:200 dilution; Santa Cruz Biotechnology) diluted in PBS containing 0.1% BSA and 0.025% Tween 80 was applied for 1 hour at room temperature. Sections were then incubated with a secondary biotinylated rabbit anti-goat IgG (IL-13 1:150 dilution for 30min; eotaxin 1:100 for 60min, IL-1β 1:120 for 60min; Vector Laboratories) followed by detection with a Vectastain Elite ABC kit for mouse IgG (Vector laboratories; PK6102). The staining was revealed using the diaminobenzidine (DAB; Sigma) procedure counterstaining with Mayer's hematoxylin. Tissues incubated with a blocking peptide instead of the primary were used as negative controls.

Images were captured using an Olympus BX-51 microscope (Olympus UK Ltd) fitted for both transmitted light and fluorescence imaging and a Qicam digital camera (Qimaging, Canada).

Non-invasive determination of the late bronchoconstrictor response to HDM challenge

After HDM challenge, conscious and unrestrained vehicle or HDM sensitised rats were placed in a whole body plethysmograph (WBP; Buxco Electronics, USA) and pressure changes were continuously registered by a Buxco XA-analyser (Buxco Electronics, USA). Enhanced pause (Penh) was recorded for up to 5 hours and mean values were taken in 10 minute intervals. A baseline value was obtained by recording the Penh for 10 minutes twenty-four hours before the challenge and baseline corrected values were used for analysis.

Invasive determination of changes in airway resistance in response to HDM challenge

Because of the controversy associated to using WBP (Penh) as a lung function measurement (28), we confirmed our results using conventional invasive lung mechanics. 48 hours after challenge vehicle or HDM sensitized male Brown Norway rats were anaesthetised (ketamine and xylazine, 144 and 10 mg/kg respectively, i.p.) and instrumented. Briefly, the trachea was cannulated and the animal artificially respired with a tidal volume of 2 ml/kg at a frequency of 90 breaths/minute. A water filled oesophageal cannula was inserted such that an estimate of transpulmonary pressure could be recorded. Resistance (R_L) ($cmH_2O/ml/s$) and compliance (C_{dyn})

(ml/cmH_2O) were continuously computed on a Buxco XA-analyser (Troy, New York, USA). Administration of aerosolised spasmogen was via a nebuliser (Buxco) connected in line with the ventilator. Airway responsiveness was assessed by nebulising increasing concentrations of methacholine (100µl of vehicle (saline), 2, 4, 8, 16 and 32 mg/ml). Maximal changes in airway resistance were monitored for 2 minutes after each administration of MCh.

Recall challenge of splenocytes from sensitised animals

Male Brown-Norway rats (150-180g) were "sensitised" on days 0 and 14 with saline (i.p.) or Alum^{TM} (20 mg/rat aluminium hydroxide and 20 mg/rat magnesium hydroxide, i.p.). In parallel, as a control for the recall challenge, animals were sensitised with ovalbumin as previously described (22). Spleens were removed on day 21 after an overdose of pentobarbitone (200 mg/kg, i.p.), and transferred to cold PBS. They were then flushed with phosphate buffered saline using a needle and syringe (without calcium and magnesium) (Sigma-Aldrich Co., Poole, UK), in order to obtain a splenocyte suspension. The pooled cell suspension was passed through a 70 µm cell sieve and centrifuged at 250 x *g* for 5 minutes at 4^{o}C, in a chill spin (Mistrall 3000i, MSE). The supernatant was discarded and the cell pellets were resuspended in PBS, then centrifuged. Splenocytes were then resuspended in RPMI 1640, supplemented with 10% FCS and 1% antibiotic and antimycotic solution (Penicillin/Streptomycin – Sigma-Aldrich Co., Poole, UK), counted and cell viability was determined using Trypan Blue. The splenocytes were diluted in RPMI 1640, supplemented with 10% FCS and 1% antibiotic and antimycotic solution, and 225µl of cells (8 x 10^5) were cultured in 24 well plates. 250µl of RPMI 1640, supplemented with 10% FCS and 1% antibiotic and antimycotic solution, with 0.1% DMSO v/v was added to each well. 25µl of varying concentration of HDM (*derp* 0.01-50 µg/ml**),** OVA (1 mg/ml**)** or saline were added to the corresponding wells. 200µl of cells were added to chamber slides. The plates and chamber slides were incubated for 72 hours at 37^{o}C in a humidified atmosphere (95% air, 5% (v/v) CO_2). Supernatants were collected and cytokine release measured by specific ELISA (R&D Systems, USA).

Data analysis

All the values in the figures and text are expressed as mean ± s. e. mean of n observations. Unpaired t-test was used when comparing two groups and a one-way ANOVA when comparing multiple groups with the appropriate post-test. When data were found not to be normally distributed Mann-Whitney was used when comparing two groups and a Kruskal-Wallis test when comparing multiple groups with the appropriate post-test. All treatments were compared to relevant vehicle control groups, differences were deemed significant when $P<0.05$.

Results

Dosing route selection

We found that in the rat, using LPS-induced TNFα as a marker of successful lung delivery, intranasal dosing gave variable results (data not shown). Intratracheal delivery of LPS, however, resulted in a larger and more robust signal in all animals (Figure 1). Similar data were obtained when we analysed the lung tissue (not shown) and interestingly we could not detect any increase in plasma TNFα, even though this dose given intraperitoneally would cause a massive increase in circulating TNFα (data not shown).

Due to the variability associated with intranasal delivery we opted for the intratracheal route to deliver the HDM.

HDM sensitising dose selection

Plasma levels of total IgE were significantly increased 14 days after administration of Alum™ compared to vehicle only (saline) but were not further increased by co-administration with HDM extract (Figure 2A). HDM extract co-administration did however cause a significant increase in HDM-specific IgE plasma levels compared to Alum™ alone (Figure 2B).

5 μg of *Derp 1* induced a robust increase in specific IgE and we adopted this dose for further studies.

HDM challenging dose selection

Intratracheal challenge with HDM caused significant BAL fluid eosinophilia at the top two doses tested (Figure 2C). The eosinophilia was accompanied by significant increase in neutrophil numbers at the highest dose (Figure 2D).

As 10 μg of *Derp 1* induced eosinophilia with less marked neutrophilia we adopted this dose to further our investigations.

Effect of HDM sensitisation /challenge on cell recruitment in BAL fluid and lung tissue

Six and forty-eight hours after challenge with HDM extract we could detect an increase in airway neutrophilia and eosinophilia, respectively, in both the BAL fluid and the lung tissue (Figure 3A-D). Interestingly we observed similar magnitudes of inflammation in the Alum^{TM}-only sensitised animals to that seen in the rats previously sensitised with HDM. Animals sensitised only with vehicle (saline) showed no sign of inflammation in either BAL or tissue (data not shown). This observation would suggest that Alum^{TM} is important in the direct inflammatory response to HDM.

Effect of HDM sensitisation/challenge on airway function measurements

There were no changes in airway function after challenge. Monitoring of LAR after HDM/vehicle challenge failed to show any significant differences between the groups studied (Figure 4A-left panel), whereas using our standard OVA driven model we can measure significant LAR in this strain of rats (4A-right panel)(24)**.** Similarly, there were no significant changes in the airway responsiveness to inhaled methacholine when measured 48 hours after the challenge (Figure 4B).

Effect of HDM sensitisation/challenge on cytokine/chemokines gene expression and release

To attempt to investigate the mechanism underlying the observed non-allergic inflammation we measured a range of asthma linked inflammatory cytokines at the mRNA and protein level. The results showed an increase in inflammatory mRNA expression: such as IL-13, IL-1β, eotaxin, Thymus and Activation-Regulated chemokine (TARC), Monocyte chemotactic protein-3 (MCP-3) and the rat equivalent of Growth Regulated Oncogene alpha (GROα) and Cytokine-Induced Neutrophil Chemoattractant-1 (CINC-1) six hours after challenge (Figure 5). We found parallel increases in protein expression of the mediators we could measure using available rat ELISAs (Figure 6). As with the increase in cellular inflammation, HDM induced increases in inflammatory cytokines both in the HDM sensitised and the Alum^{TM}-only sensitised rats. We could not detect any increase in IL-4 and IFNγ in these samples.

A histological assessment of the lung tissue from saline sensitised/saline challenge shows no inflammatory infiltration in the lung parenchyma at 48 hours (7A); whereas the time matched Alum-only sensitised/*Der p* challenged animals showed the existence of inflammatory infiltration 48 hours after challenge (7B).

Immunohistochemical staining demonstrated that the lungs of Alum-only sensitised /*Der p* challenged animals show positive staining for IL-13 (Figure 7C-D) in the airway epithelium and in inflammatory cells resembling alveolar macrophages 6 hours after challenge. In agreement with these findings, though IL-13 is generally associated to T-cells and sometimes to mast cells or eosinophils, it has been recently reported that it can be also be associated with airway macrophages (26) and airway epithelial cells (27;29). Eotaxin was also highly expressed but mainly in the epithelium of large and small airways (Figure 7E-F), while IL-1β staining was very low in the epithelium and it looked mainly associated to the inflammatory cell infiltrate (Figure 7G-H).

Effect of HDM protease activity on cell recruitment in BAL fluid and lung tissue

In order to determine whether the protease activity of the HDM extract was responsible for the apparent direct inflammatory response observed previously, animals were "sensitised" either with vehicle (saline) or Alum^{TM}-only and then challenged intratracheally with either proteolytically active or heat-inactivated HDM extract (demonstrated in Figure 8A). Saline sensitised animals, unlike those that were sensitised with Alum^{TM}-only, did not exhibit airway inflammation (Figure 8B). Heat inactivated HDM extract was also able to elicit an inflammatory response in the Alum^{TM} sensitised animals but not in the saline sensitised ones (Figure 8B).

Recall challenge of splenocytes from sensitised animals

To discount that the response observed in the Alum^{TM}-only sensitised animals could be due to an undefined protein, which was then delivered again during the intratracheal challenge, splenocytes from alum sensitised animals were challenged with HDM extract as described in the methods. This recall challenge method in splenocytes was previously validated by using splenocytes obtained from an ovalbumin model of allergic inflammation. Ovalbumin challenge in splenocytes from OVA sensitised animals caused a release of Th2 cytokines such us IL-4 (7±1.1 pg/ml vs. control saline challenged 1.2±0.3 pg/ml; $p<0.01$) or IL-13 (27.9±6.5 pg/ml vs. control saline challenged 3.5±2.4pg/ml; $p<0.01$). However HDM challenge, even at

the top concentration of 50μg/ml, failed to cause any significant release of Th2 cytokines from Alum™-only sensitised splenocytes. These results would support the non-allergic nature of the response observed in Alum™-only sensitised animals in vivo.

Discussion

In the current study we have shown that a HDM extract obtained from *D. Pteronyssinus* (*Der p*) is able to elicit a direct inflammatory response in the lungs of Brown Norway rats. The inflammation is characterised by neutrophilic and eosinophilic infiltration and associated with an early expression and release of pro-inflammatory cytokines such us IL-13, IL-1β, eotaxin, MCP-3, TARC and CINC-1. This study demonstrates for the first time that a direct pro-inflammatory effect of HDM, observed by others *in vitro* (14;16-20), can also be shown in a more complex biological *in vivo* setting in the BN rat. However, unlike in those studies, the inflammation did not appear to be associated with the protease activity of HDM but rather whether or not the animals had received Alum™ prior to challenge. This would suggest that the direct inflammatory response to inhaled HDM is dependent on a function of Alum™ such as increasing total IgE.

The study of the early immune events induced by allergens is currently under a great deal of scrutiny as it is likely that they have huge relevance to the pathogenesis of allergen-driven inflammatory diseases, such as asthma. These events have been poorly studied to date and they may be of great importance in the development of the allergic inflammation and our understanding of asthma pathology. It is currently believed that when HDM allergens interact with the bronchial epithelium, they induce direct damage through protease activity, disrupting the epithelial tight junctions and thereby increasing the bronchial epithelium permeability and facilitating trans-epithelial allergen delivery and interaction with antigen presenting cells (5-7). In the current study we have demonstrated that HDM allergens can interact with the lung, particularly the airway epithelium, and induce asthma-like inflammation i.e. eosinophilia and an increase in Th-2 type cytokine profile. What is very interesting is that HDM can elicit these responses in animals which have not been previously exposed to the same antigen. It has been recently reported that certain fungal allergens, with a proteinase activity, are able to induce eosinophilic airway inflammation in mice in the absence of adaptive immune cells. This direct inflammation was reported to be associated with the induction of chemokines such as MCP-3 (CCL7) and TARC. We observed a similar increase in these two cytokines which might have suggested a similar mechanism could be the cause of the early eosinophilia observed in this study. Furthermore, Heijink *et al* (20) has shown *in vitro* that this TARC expression, which may be critical for Th2 cell recruitment in

inflammation, can be directly induced by *Der p* in human bronchial epithelial cells. However, although this could have been a suitable explanation for our results, the fact that the inactivation of the protease activity in the extract does not prevent the development of the inflammatory response suggests a different mechanism. On the other hand, it has also been recently reported that non-proteolytic HDM allergens which could be present in the extract, such as *Der p* 2, are able to induce NF-κB and MAPK activation (30)

All animals that received Alum™ during sensitisation, with or without HDM, showed significant increases serum IgE. This phenomenon is not unique and other inorganic salts, such as $HgCl_2$, have been shown in the past to cause an elevation of total IgE in the BN rat (31). As expected only the rats that also received HDM during sensitisation showed significant levels of serum HDM-specific IgE. In the rats that did not receive Alum™, i.e. the ones with lower total IgE, we failed to observe the direct inflammatory response to inhaled HDM. The possible association of serum IgE levels and asthma, independent of specific allergic sensitisation has been previously reported (32;33). Non-atopic asthma accounts for between 10% and 33% of all asthma sufferers (34). At the cellular and molecular level, non-atopic asthma and atopic asthma are very similar, and the reason for their clinical difference remains to be fully understood (35). There is firm evidence that IgE mediated mechanisms are involved not only in the pathogenesis of atopic but also non-atopic asthma and the elevation of total serum IgE has been associated to asthma in non-allergic individuals (32;33). It has been suggested that in these individuals total IgE may represent just a consequence of asthma, a marker of allergic inflammation (32). However our results would suggest that there is an actual link, and we propose that the development of the inflammatory response seen in our model may be associated with the presence of high levels of total IgE in serum. If our data do translate to the situation in man it may explain why this antigen, which is so promiscuous, does not inflame everyone's airways. In fact, our assertion would be someone exposed to HDM would also need to have elevated IgE levels in order to evoke an inflammatory response in the lung. Indeed, a recent study suggests that individuals with IgE sensitisation to common allergens may develop an asthmatic response to a particular different allergen even though serum specific IgE levels suggest that they are not sensitised to it (36).

Interestingly, and despite showing an asthma-like inflammatory reaction and HDM specific IgE, none of the groups studied displayed any changes in airway

function after HDM challenge, either EAR or LAR. However as shown in previous studies (37;38) a single HDM challenge may not be enough to achieve reliable lung function changes in the rat and indeed it appears that in the mouse models multiple HDM challenges may be necessary (39-41).

One possible caveat to the suggestion that it is the HDM that is directly causing the inflammation is that something else within the HDM mixture is responsible i.e. the small amount of endotoxin. Furthermore, a recent study proposes that TLR-4 receptor activation could play a crucial part in HDM induced responses (41). Whilst endotoxin can cause some of the inflammation observed in the study we feel it is very unlikely to be responsible. The levels are very low (36 EU/ml as measured with a Pyrogen 5000 LAL endotoxin assay), the time of cellular influx is much later than one would expect after LPS, some of the mediators measured are not thought to be upregulated by endotoxin (i.e. TARC) and, probably most convincingly, there is no inflammation present in the HDM challenged but non-Alum^{TM} sensitised animals. Another possible explanation is that the Alum^{TM}-only sensitised animals had been exposed to an undefined protein, which was then delivered again during the intratracheal challenge, causing an allergic Th2 response. However, the negative results from the recall challenge experiment would seem to rule out this possibility. Nonetheless, we cannot completely discard that some of the inflammation observed is through a non-HDM mechanism.

In conclusion, these data demonstrate that HDM can, after exposure to an adjuvant which increases serum total IgE, evoke direct inflammation in the airways which in many ways is characteristic of the asthmatic airway. We feel this is a very interesting finding in that it might help unravel the complex association between high IgE levels, atopy and the development of the asthma phenotype. Indeed these data may significantly alter our understanding of the role of IgE in the pathogenesis of asthma.

Acknowledgements:

The authors would like to acknowledge GlaxoSmithKline for help with funding for the studies and Charles Dean for help with the preparation of this manuscript.

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FIGURE LEGENDS

Figure 1: Confirmation that intratracheal delivery is a robust route for lung delivery. Vehicle or LPS was dosed intratracheally to anaesthetised rats 90 minutes before BALF collection. TNFα levels were measured by ELISA.

Figure 1

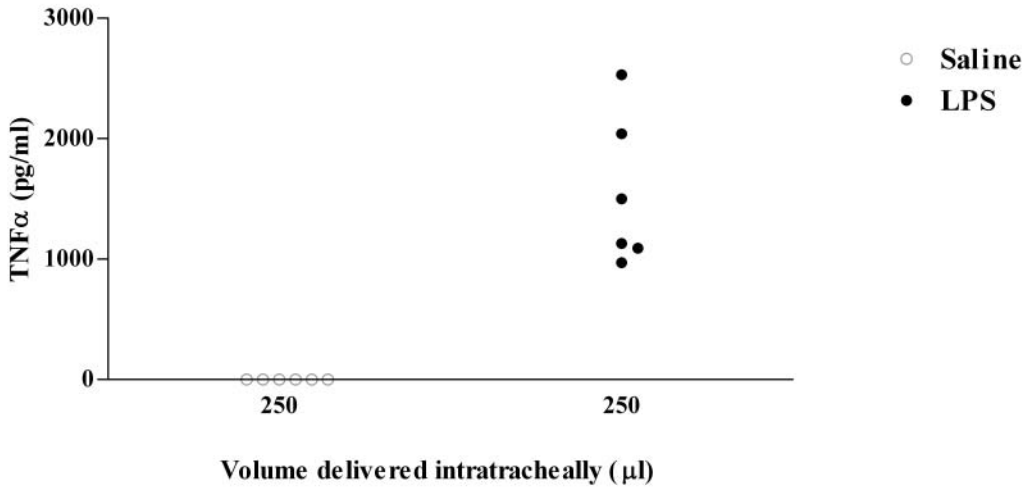

Figure 2: Determining the optimum HDM sensitising and challenging dose.
BN rats were sensitised with vehicle (with and without Alum™) plus increasing doses of HDM (i.p.) and plasma samples were collected 14 days after for measurement of total IgE (A) and HDM-specific IgE (B).
A second set of animals were sensitised with HDM (5ug, i.p.) with Alum™ on day 0 and 14. Seven days later they were challenged with vehicle or increasing doses of HDM. Two days after challenge the lungs were lavaged and BAL eosinophil (C) and neutrophil (D) numbers determined.

Data (n = 6-8) expressed as means ± s.e.mean and analysed by one-way ANOVA followed by a Dunnett's post-test. P value of less than 0.05 was deemed a significant difference.

Figure 2

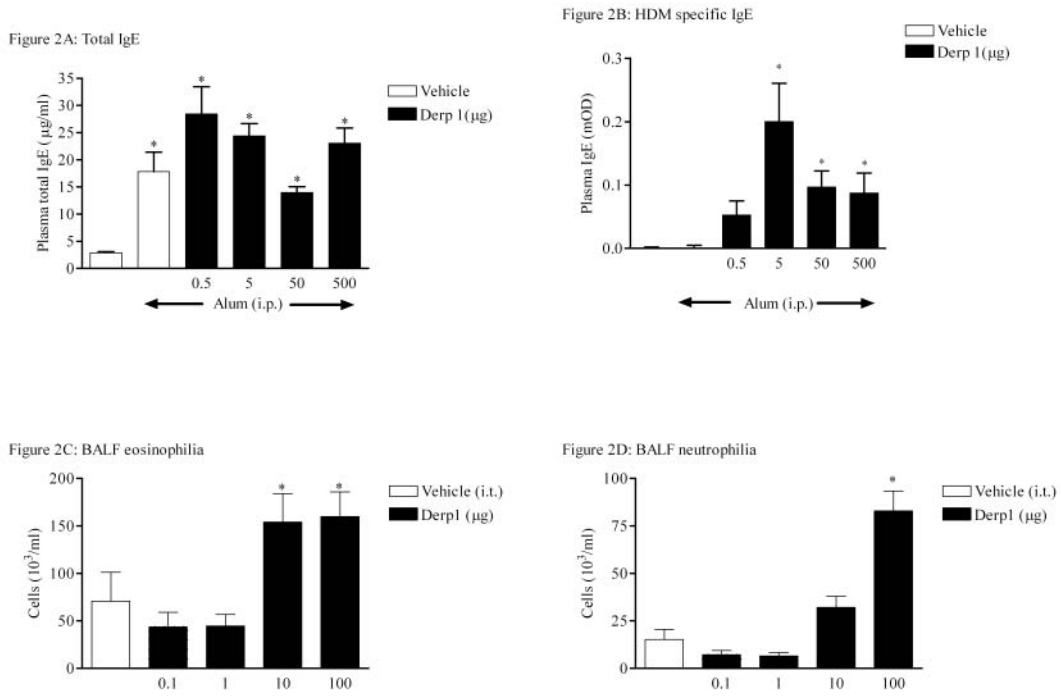

Figure 3: Effect of HDM challenge (*Der p*) on cell recruitment in the bronchoalveolar lavage and lung tissue – time course.

BN rats were sensitised with Alum™-only or HDM (5ug, i.p.) with Alum™ on day 0 and 14. Seven days later they were challenged with vehicle or HDM (10ug, i.t.). BAL fluid and lung tissue was collected 6, 24 and 48 hours later. Data shown is BAL fluid eosinophil (A) and neutrophil (C) numbers and lung tissue eosinophil (B) and neutrophil (D) numbers 48 hours after challenge.

Data (n = 4-8) expressed as means ± s.e.mean and analysed using Mann-Whitney with a P value of less than 0.05 deemed a significant difference.

Figure 3

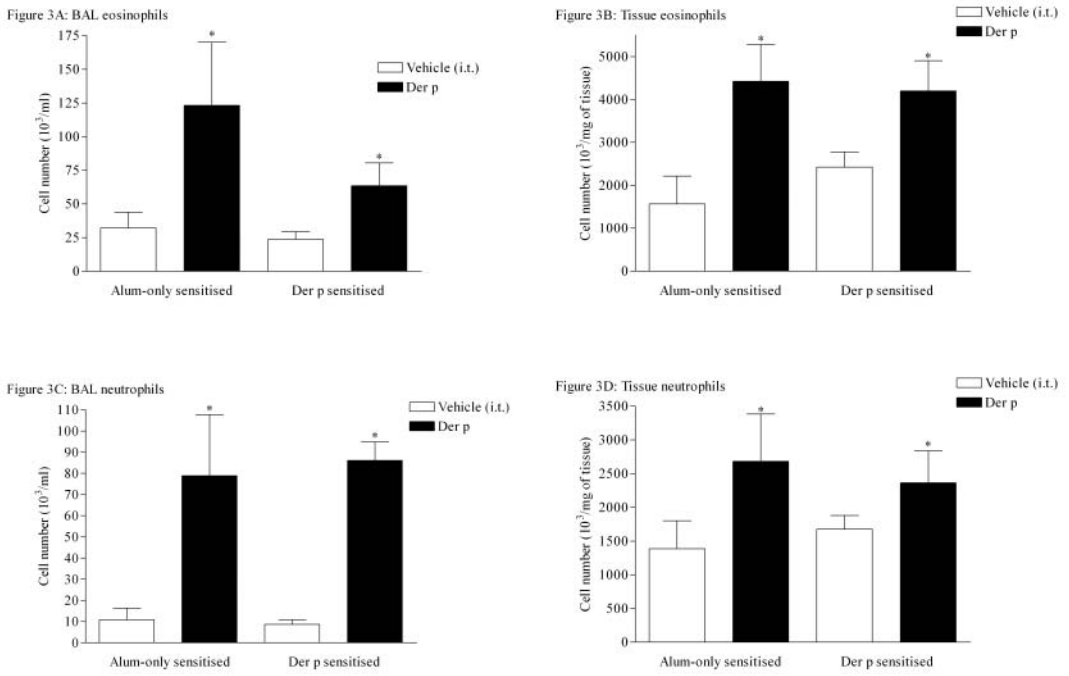

Figure 4: Effect of HDM challenge (*Der p*) on lung function.

A – Right panel demonstrates typical LAR after OVA challenge in OVA sensitised BN rats. The left panel depicts similar measurements made after HDM challenge in Alum^{TM}-only or HDM plus Alum^{TM}-sensitised Brown Norway rats. Sensitisation/Challenge regime: Alum-only/Vehicle (squares); HDM+Alum/vehicle (open triangles); Alum-only/ HDM (inverted triangle); HDM+Alum /HDM (circles). Mean Penh was for each 10 minute interval for 5 hours. Baseline corrected values were used for analysis. Each time point represents baseline corrected mean Penh ± s.e.mean. *n*=8.

(B) Changes in airway resistance in Alum-only or HDM+Alum sensitised Brown Norway rats following i.t. HDM exposure. Invasive airway resistance was recorded using a Buxco LS20 system. Average airway resistance (cmH_2O/ml/s) was recorded for 2 minutes before increasing concentration Methacholine challenge (2-32mg/ml). Baseline corrected data are presented as mean ± s.e.mean. *n*=8.

Figure 4

Figure 4A: Late asthmatic response after challenge in HDM model vs. OVA model

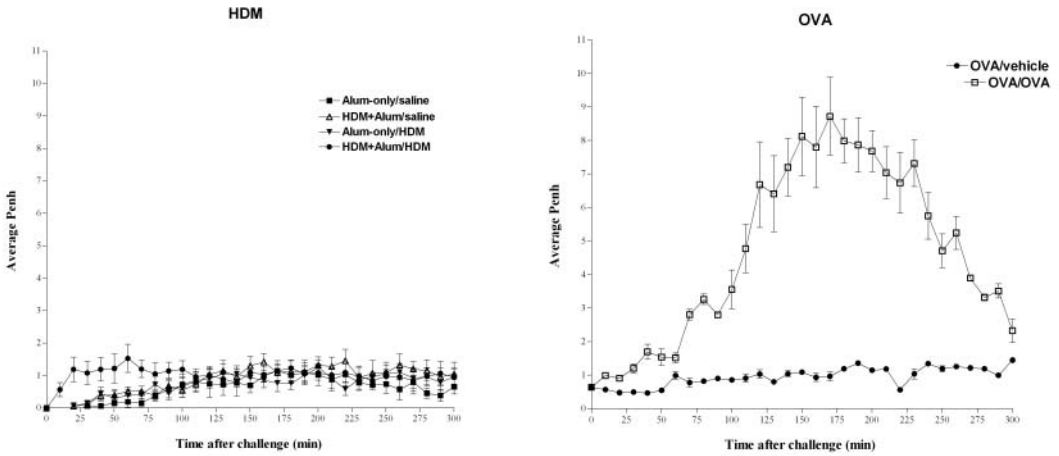

Figure 4B: Airway hyperrresponsiveness 48 hours after challenge

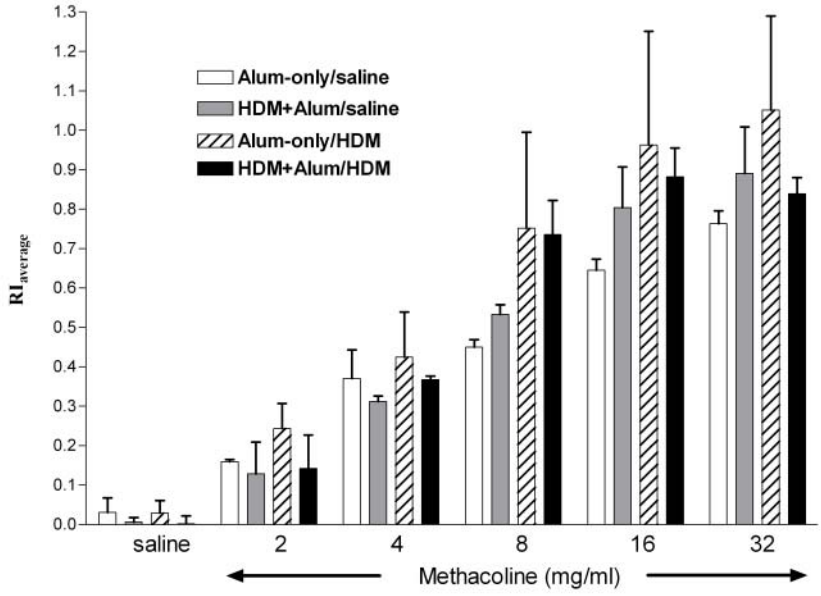

Figure 5: mRNA expression in lung tissue after HDM (*Der p*) challenge.

BN rats were sensitised with Alum only or HDM (5ug, i.p.) with Alum on day 0 and 14. Seven days later they were challenged with vehicle or HDM (10ug, i.t.). Lung tissue was collected 6, 24 and 48 hours later. Data shown is mRNA expression at 6

hours as measured by real-time RT-PCR: IL-13 (A), IL-1β (B), eotaxin (C) and CINC-1 (D), MCP-3 (E) and TARC (F).

Data (n = 4-8) expressed as means ± s.e.mean. Statistics were performed on the raw data using either t-test or Mann-Whitney depending on the distribution of the data. P value of less than 0.05 deemed a significant difference vs. the corresponding control.

Figure 5

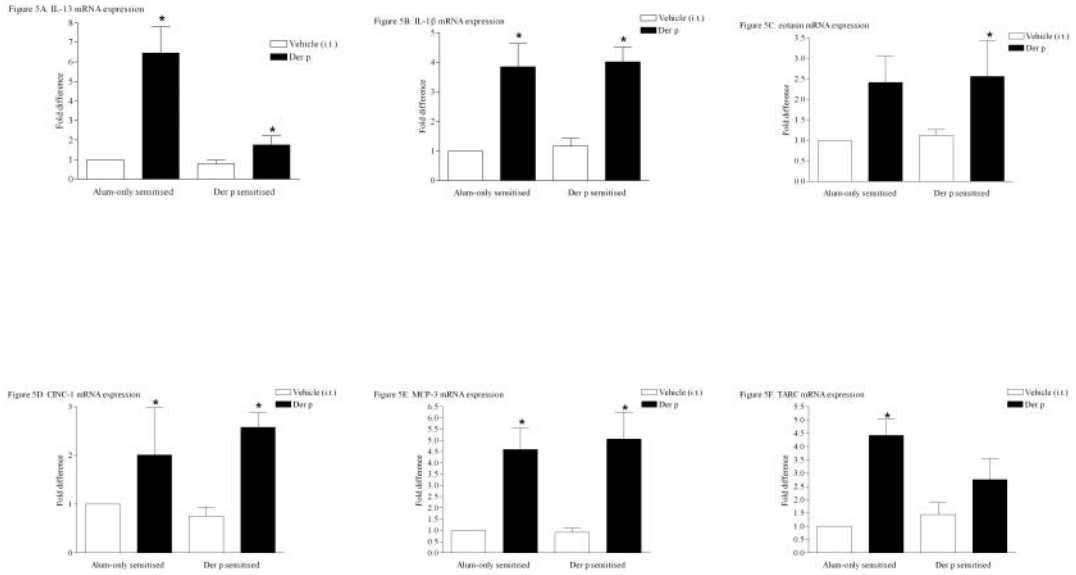

Figure 6: Protein expression in the BAL fluid after HDM (*Der p*) challenge.

BN rats were sensitised with Alum or HDM (5ug, i.p.) with Alum on day 0 and 14. Seven days later they were challenged with vehicle or HDM (10ug, i.t.). BAL fluid was collected 6, 24 and 48 hours later. Data shown is protein expression (pg/ml) at 6 hours as measured by ELISA: IL-13 (A), IL-1β (B), eotaxin (C) and CINC-1 (D). Data (n = 4-8) expressed as means ± s.e.mean and analysed using either t-test or Mann Whitney depending on the distribution of the data. P value of less than 0.05 deemed a significant difference vs. the corresponding control.

Figure 6

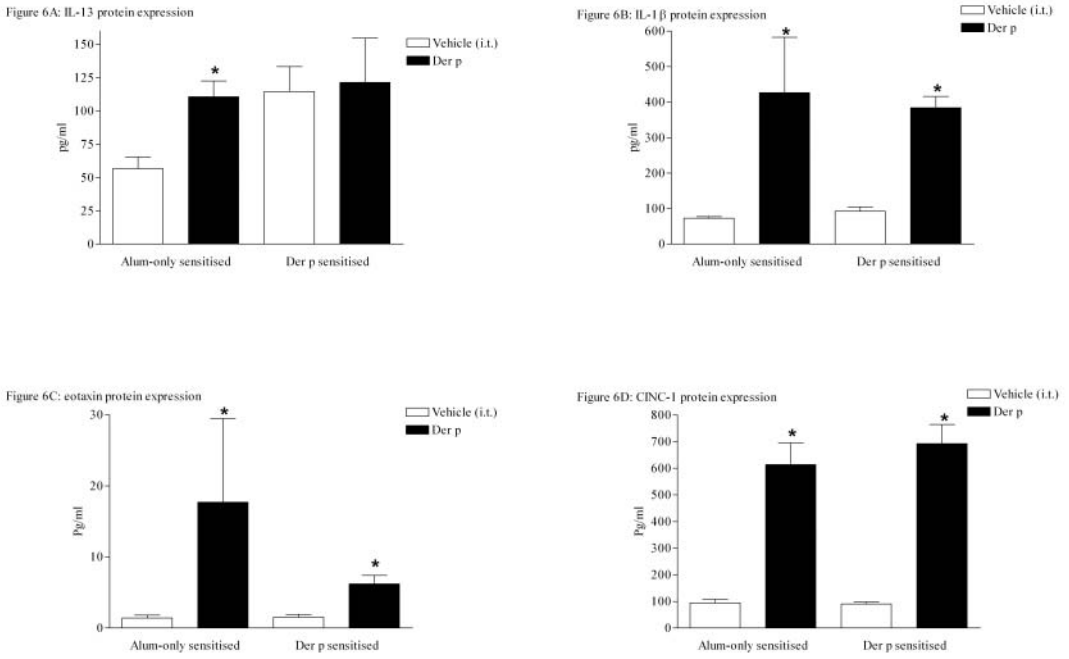

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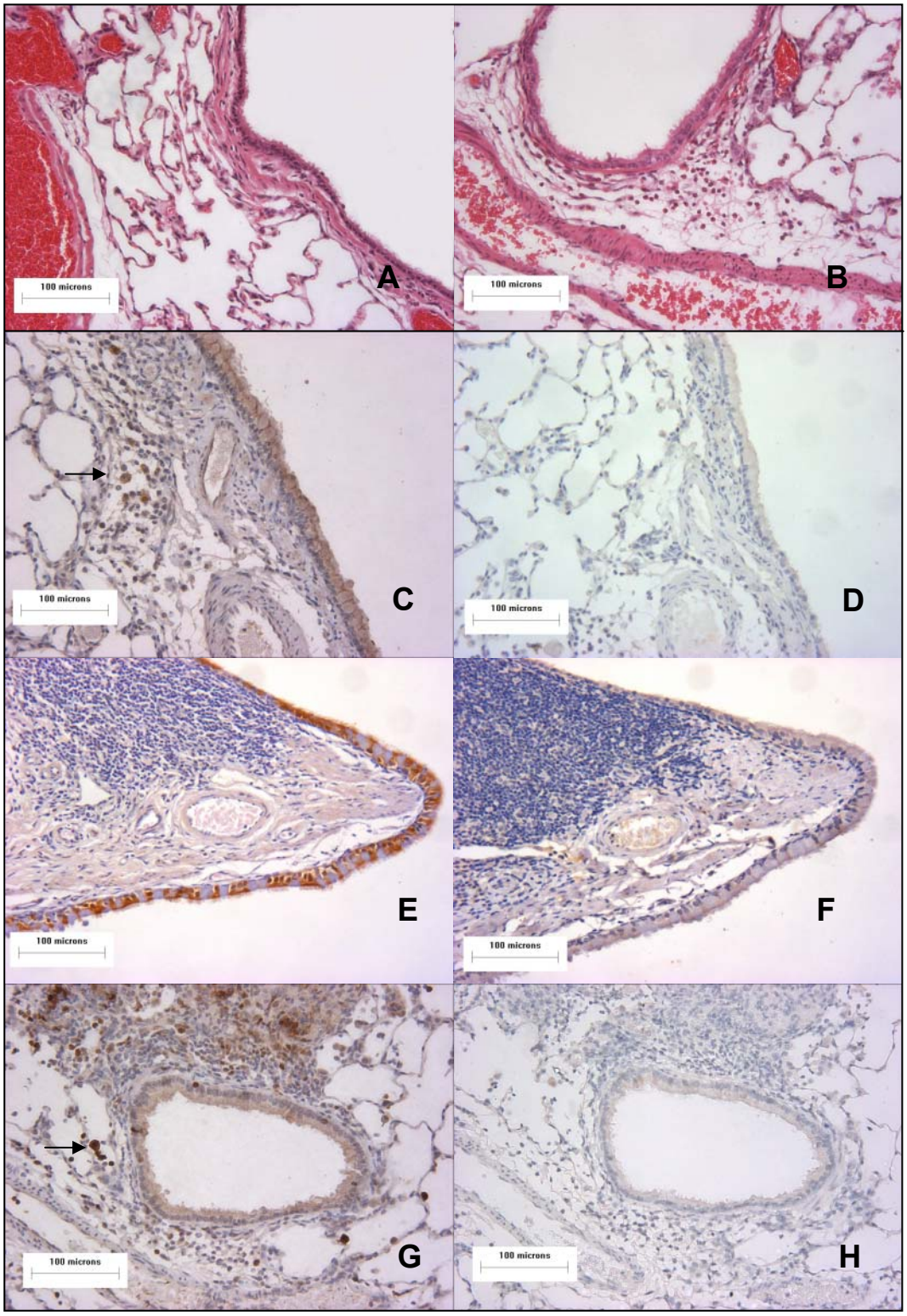
Figure 7: Histological examination of lung tissue sections after HDM challenge (x20).

BN rats were sensitised with Alum or HDM (5ug, i.p.) with Alum on day 0 and 14. Seven days later they were challenged with vehicle or HDM (10ug, i.t.). Lung tissue was collected 6 or 48 hours later.

Mayer's Haematoxylin-eosin staining of tissue from Alum-only sensitised/saline challenge shows no inflammatory infiltration in the lung parenchyma at 48 hours (A); whereas the time matched Alum-only sensitised/*Der p* challenged animals show significant inflammatory infiltration (B).

Lung tissue section from non-sensitised/*Der p* challenged (post 6 hours) animals shows positive staining for: IL-13(C) in the airway epithelium and in inflammatory cells resembling alveolar macrophages (arrow); panel D shows parallel slide with blocking peptide present. Panel E/G is stained for eotaxin/IL-1β respectively, F/H are parallel slides stained in the presences of the blocking peptides.

Figure 7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B
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Figure 8. Effect of HDM protease activity on cell recruitment in BAL fluid and lung tissue.

Protease activity in the extract was assessed by a continuous rate (kinetic) assay using the fluorogenic peptide substrate Boc-Gln-Ala-Arg-AMC. Data are fitted to a Michaelis-Menten equation (y=V.x/(Km+x). HDM extract showed a positive protease activity with a Vmax of 76636 μmol/min and a Km of 304.9 μM. Heat inactivated HDM extract showed no protease activity (A).

Tissue eosinophilia 48hrs after HDM challenge is shown in Figure 8B. Alum sensitised animals (black) showed significant inflammatory infiltration after HDM challenge when compared with saline sensitised animals (white). Heat inactivation of the HDM did not change this profile.

Figure 8

Figure 8A: Michaelis-Menten plot of HDM extract protease activity: [Substrate] vs. Velocity

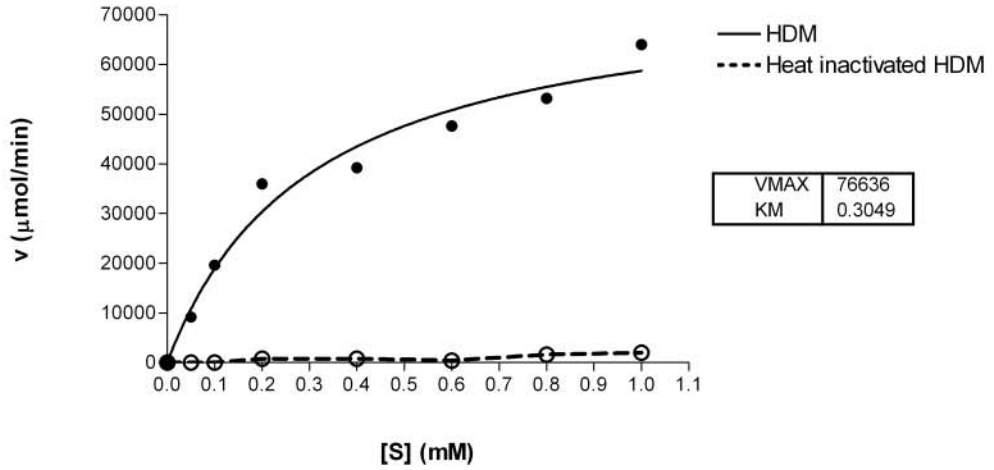

Figure 8B: Tissue eosinophils 48 hours after challenge