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Suppression of adrenomedullin contributes to vascular leakage and altered epithelial repair during asthma

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Abstract

Background: The anti-inflammatory peptide, adrenomedullin (AM), and its cognate receptor are expressed in lung tissue, but its pathophysiological significance in airway inflammation is unknown.
Objectives: This study investigated whether allergen-induced airway inflammation involves an impaired local AM response.
Methods: Airway AM expression was measured in acute and chronically sensitized mice following allergen inhalation and in airway epithelial cells of asthmatic and nonasthmatic patients. The effects of AM on experimental allergen-induced airway inflammation and of AM on lung epithelial repair *in vitro* were investigated.
Results: Adrenomedullin mRNA levels were significantly ($P < 0.05$) reduced in acute ovalbumin (OVA)-sensitized mice after OVA challenge, by over 60% at 24 h and for up to 6 days. Similarly, reduced AM expression was observed in two models of chronic allergen-induced inflammation, OVA- and house dust mite –sensitized mice. The reduced AM expression was restricted to airway epithelial and endothelial cells, while AM expression in alveolar macrophages was unaltered. Intranasal AM completely attenuated the OVA-induced airway hyperresponsiveness and mucosal plasma leakage but had no effect on inflammatory cells or cytokines. The effects of inhaled AM were reversed by pre-inhalation of the putative AM receptor antagonist, $AM_{(22-52)}$. AM mRNA levels were significantly ($P < 0.05$) lower in human asthmatic airway epithelial samples than in nonasthmatic controls. *In vitro*, AM dose-dependently (10^{-11}–10^{-7} M) accelerated experimental wound healing in human and mouse lung epithelial cell monolayers and stimulated epithelial cell migration.
Conclusion: Adrenomedullin suppression in T_H2-related inflammation is of pathophysiological significance and represents loss of a factor that maintains tissue integrity during inflammation.

Abbreviations
AHR, airway hyperresponsiveness; AM1 and AM2 receptors, adrenomedullin receptors; AM, adrenomedullin; BAL, Bronchoalveolar lavage; CGRP1Rs, calcitonin gene–related peptide-1 receptors; CLR, calcitonin-like receptor; HDM, house dust mite; OVA, ovalbumin; RAMPS, receptor-activity-modifying proteins.

Allergic asthma is a chronic inflammatory disease of the airways characterized by eosinophilic inflammation, T_H2 cell-associated cytokine production, and airway hyperresponsiveness (AHR). Dysregulation of interactions between the airway epithelium and mucosal immune system is considered to be a major promoter of the disease (1, 2).

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Adrenomedullin (AM) is a 50/52 amino acid peptide (mouse/human) (3) that is expressed in bronchio-alveolar epithelial cells, alveolar macrophages, endothelium, and smooth muscle cells (4–6). In other mucosal tissues, AM is induced in response to bacterial exposure (7) and appears to act locally to dampen inflammation and support tissue homeostasis, by suppressing pro-inflammatory cytokine synthesis and promoting wound healing (8, 9). AM may be a normal part of the inflammatory response as it is induced in response to inflammatory injury in the lung, heart, liver, and kidney and during experimentally induced sepsis (10). Mice overexpressing AM are resistant to septic shock (11).

Adrenomedullin exerts its biological actions through calcitonin gene–related peptide-1 receptors (CGRP1Rs) and specific AM receptors (AM_1 and AM_2 receptors), which are pharmacologically unique, but share a common molecular structure known as the calcitonin-like receptor (CLR). The function and ligand specificity of CLR depends on the coexpression of the so-called receptor-activity-modifying proteins (12). Coexpression of CLR with RAMP-1 generates a CGRP receptor, while coexpression with RAMP-2 generates an AM receptor. AM activation of CLR-RAMP2 can be competitively antagonized by $AM_{(22\text{-}52)}$.

Although the anti-inflammatory actions of AM are well described and the peptide and its cognate receptor are known to be expressed in lung tissue, little is known concerning the possible role of AM in airway inflammation. This study aimed to test the hypothesis that the pathophysiology of allergic airway inflammation involves impaired AM response within the airways.

Material and methods

Methods are described in detail in Data S1.

Patients and sampling procedure

See Data S1.

Animals

See Data S1.

Mouse models of allergic airway inflammation

Acute ovalbumin (OVA)-(13), chronic OVA-, and House dust mite–induced allergic airway inflammation model were performed as described and shown in Data S1 and Fig. S1.

Determination of AHR

Airway hyperresonsiveness was determined as described in Data S1.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed, as previously described (13).

Measurement of immunoglobulins

Blood samples were taken from the axillary vessels, 48 h after the last challenge, and serum levels of anti-OVA IgE were measured by ELISA, as previously described (13, 14).

Measurement of cytokines

Cell-free supernatants from BAL fluids were analyzed for IL-5 and IL-13 as described previously (13, 15). The ELISA detection limit of each ELISA was 10 pg/ml.

Measurement of albumin

Albumin was measured in cell-free supernatants from BAL fluids using an ELISA as described by the manufacturer (Bethyl Laboratories delivered by Biomol, Hamburg, Germany).

Isolation of primary tracheal epithelial cells (MTECs), primary alveolar type II epithelial cells (AECII), primary alveolar macrophages, and lung endothelial cells

Isolation of primary cells is described in Data S1.

Gene expression analysis

Human primary cells, human bronchial epithelial (HBE) cells, primary mouse cells, LA4-cells, and mouse lung tissues were processed for gene expression analysis, as described in the Data S1. For primer sequences, see Table S1 in this article's Data S1.

Laser-assisted microdissection

See Data S1.

Cell culture

Human bronchial epithelial- and LA4- epithelial cells were cultivated as described in Data S1.

Wound-healing assay and *in vitro* migration assay

Wound-healing assay and *in vitro* migration assay were performed, as described in Online Repository.

Statistical analysis

Data analyses were performed using the PRISM 4 Software package (GraphPad Software, San Diego, CA, USA). Where appropriate, a two-tailed Student's *t*-test was used for the analysis. For multiple group comparisons, one-way ANOVA followed by Tukey's post-test was applied. Each set of data was expressed with the standard error of the mean (SEM), and P values of ≤ 0.05 were regarded as statistically significant.

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Results

Adrenomedullin mRNA and peptide levels were reduced in allergen-induced airway inflammation

During acute airway inflammation in a well-established adjuvant-free mouse model of OVA allergy-induced airway inflammation, whole lung levels of AM mRNA were significantly ($P < 0.05$) reduced, 48 h after allergen challenge of OVA-sensitized mice, when compared to sham-sensitized mice (see Fig. 1A). There was a parallel significant decrease in AM peptide content of BAL ($P < 0.001$) (Fig. 1B) and of systemic plasma ($P < 0.05$) (Fig. 1C), suggesting a down-regulation of AM biosynthesis. The kinetics of this apparent reduced AM expression indicated a nadir for mRNA levels at days 1 and 2 ($P < 0.001$) after allergen challenge with a slow return to baseline levels by day 6 (Fig. 1D).

Allergen-induced reduction in airway AM expression appears restricted to epithelial and endothelial cells, with no change in macrophage expression of AM

To identify the cellular location of the reduced AM expression observed in the whole lung of allergen-challenged OVA-sensitized mice, AM mRNA levels were quantified in different cell types (airway epithelial cells, type II pneumocytes, endothelial cells, and alveolar macrophages) isolated from the lungs of mice. Compared to sham-sensitized control mice, allergen-challenged OVA-sensitized mice had significantly reduced levels of AM mRNA in structural cells of the airway, namely, epithelial ($P < 0.05$) (see Fig. 2A,B) and endothelial cells ($P < 0.001$) (Fig. 2C), but unchanged levels in alveolar macrophages (Fig. 2D). These findings were confirmed in parallel analysis of cells isolated from bronchi and septa using laser-assisted microdissection. A significant ($P < 0.05$) (Fig. 2E) reduced level of AM mRNA was detected in microdissected airways. A lower level of AM mRNA was also observed in the microdissected septa of allergen-challenged OVA-sensitized mice, but this did not reach statistical significance (Fig. 2F).

Allergen-induced reduction in AM expression was also demonstrated in two additional mouse models of allergen-induced airway inflammation

To elucidate whether the reduced AM expression in the acute OVA model of allergic airway inflammation represents only a phenomenon during the acute phase, these studies were extended to the chronic OVA model. Compared to sham-sensitized mice,

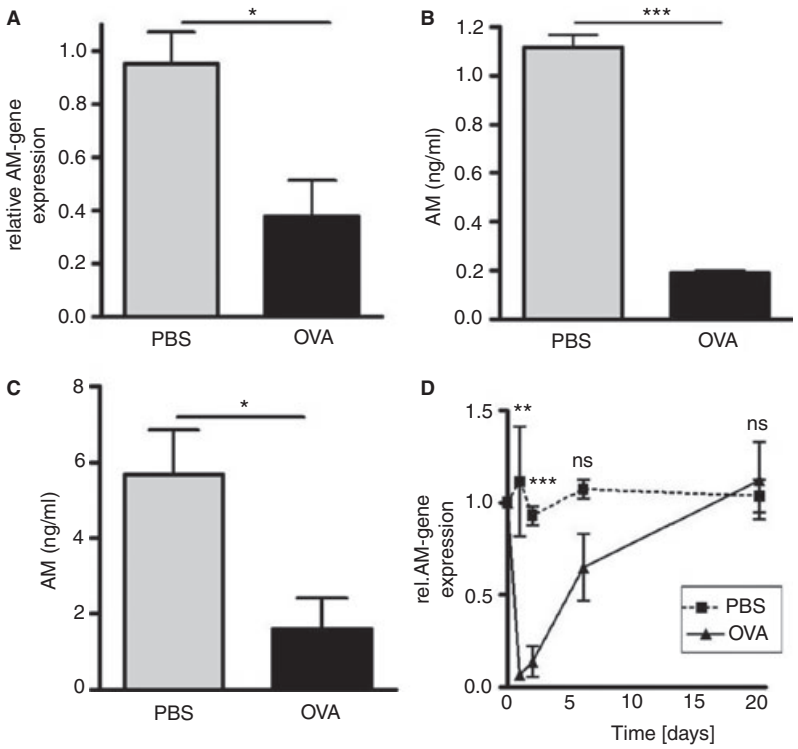


Figure 1 Adrenomedullin (AM) airway expression after allergen challenge of ovalbumin (OVA)-sensitized Balb/c mice. Mice were subcutaneously sensitized with OVA followed by an OVA aerosol challenge on three consecutive days. The control mice were sham-sensitized with PBS and then challenged with OVA aerosol (Fig. S1A). (A) AM mRNA levels were determined in whole lung homogenates by real-time PCR. (B,C) AM protein levels in bronchoalveolar lavage and plasma were determined by AM-EIA – 48 h after the last challenge. (D) kinetic of AM expression in lung homogenates by real-time PCR. Data are shown as mean ± SEM of values of eight mice per group and represent one of three independently performed experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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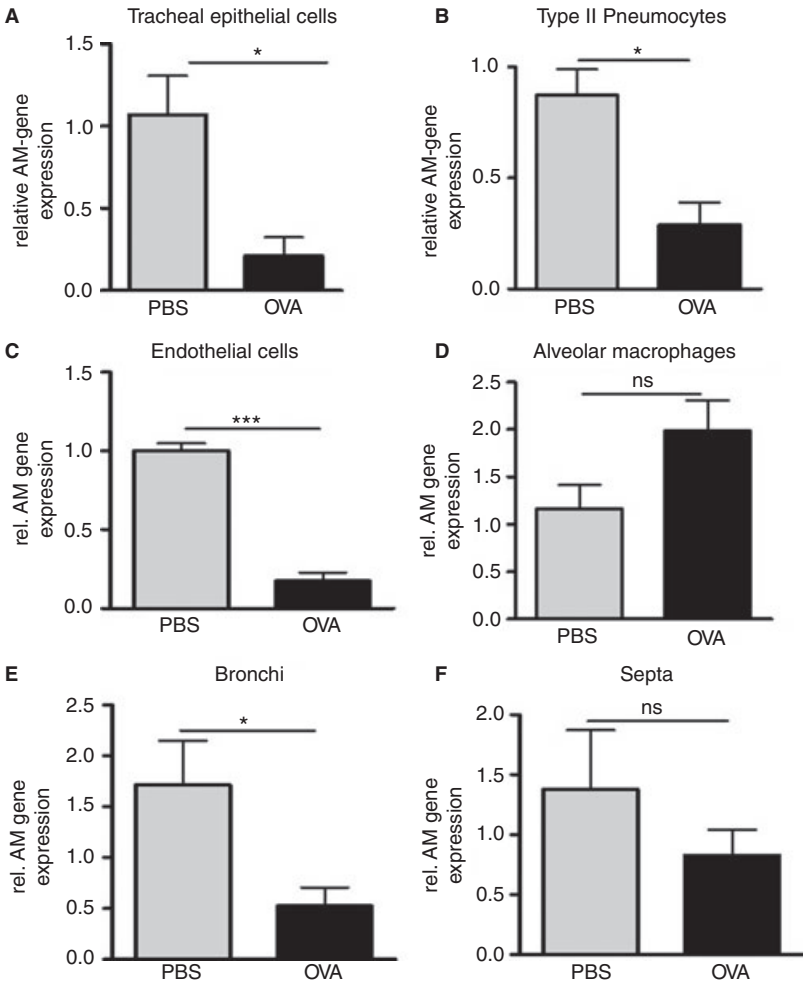


Figure 2 Adrenomedullin (AM) expression in different cell types of the lung after allergen challenge of ovalbumin (OVA)-sensitized Balb/c mice. Quantitative real-time polymerase chain reaction analysis for AM mRNA of (A) tracheal epithelial cells, (B) type II pneumocytes), (C) endothelial cells, (D) alveolar macrophages and (E,F) laser microdissected bronchi and septa, of OVA-sensitized/challenged and control mice (treatment of the mice Fig. S1A). Cells were isolated from OVA-sensitized/challenged mice and control mice as described in material and methods. Data are shown as mean ± SEM of values of six animals per group and represent one of two independently performed experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

chronically challenged, OVA-sensitized mice had significantly ($P < 0.001$) reduced whole lung AM mRNA levels after chronic allergen challenge (48 h after last challenge). AM mRNA levels had began to return to baseline after 4 weeks of no further allergen challenge (=resolution) and, after 8 weeks, had returned to baseline levels (Fig. 3A). A similar significant ($P < 0.05$) reduction in AM mRNA levels was seen after chronic allergen challenge of house dust mite–sensitized mice (Fig. 3B), which represents a clinically relevant inhaled allergen.

Intranasal administration of AM attenuates allergen-induced AHR and plasma leakage without any effect on pro-inflammatory cells and cytokines

Next, in pharmacological experiments, we tested whether inhaled AM in allergen-challenged, OVA-sensitized mice may provide an anti-inflammatory effect that compensates for the down-regulation of airway AM. The data indicate that the significant ($P < 0.001$) increased responsiveness of airways (AHR) of allergen-challenged, OVA-sensitized mice was significantly ($P < 0.01$) attenuated by inhaled AM (Fig. 4A). This effect of inhaled AM was significantly ($P < 0.05$) blocked by pretreatment with the AM receptor antagonist $AM_{(22\text{-}52)}$, indicating that AM acted via the specific AM receptor.

Further investigations with this pharmacological model investigated the effect of inhaled AM on systemic and local inflammatory parameters. The allergen-challenged, OVA-sensitized mice exhibited the characteristic increase in OVA-specific IgE levels, BAL cell content of primarily eosinophils and lymphocytes, and Th2-cytokine (IL-5 and IL-13) levels. These were not affected by inhaled AM (Fig. 4B

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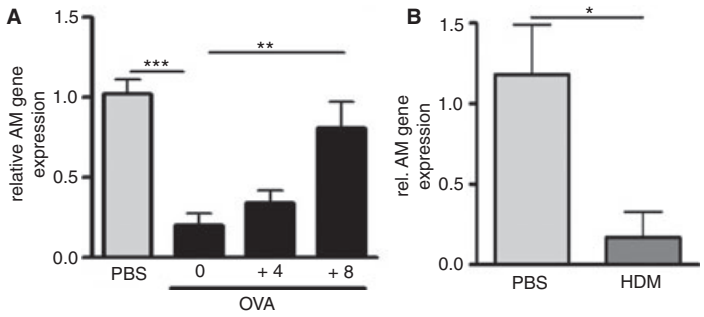
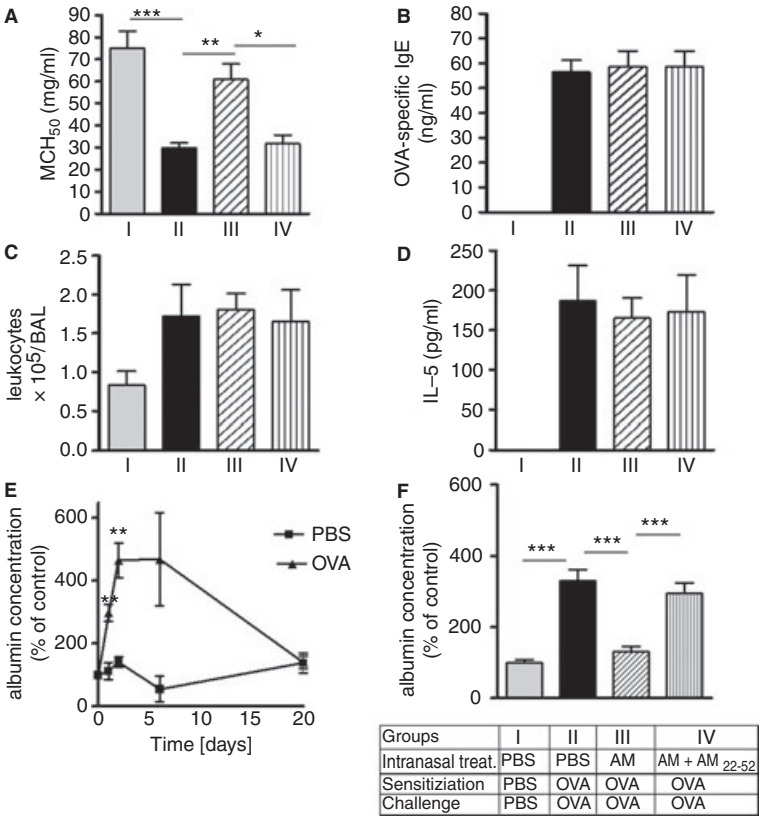


Figure 3 Confirmation of reduced adrenomedullin (AM) expression in chronic ovalbumin (OVA) and house dust mite (HDM) induced model of allergic airway inflammation. Allergic airway inflammation in Balb/c was induced as described in the material and methods section (A) chronic OVA model and (B) HDM model. Forty-eight hour after the last allergen challenge, animals were killed and AM mRNA levels were determined in lung homogenates by real-time PCR. Data are shown as mean ± SEM of values of eight mice per group and represent one of two independently performed experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.



Groups	I	II	III	IV
Intranasal treat.	PBS	PBS	AM	AM + AM $_{22-52}$
Sensitiziation	PBS	OVA	OVA	OVA
Challenge	PBS	OVA	OVA	OVA

Figure 4 Effect of intranasal application of adrenomedullin (AM) on asthmatic phenotype. (A) Administration of intranasally applied AM led to improvement of airway hyperresponsiveness. This effect was antagonized by a 15-min prior treatment with antagonist $AM_{(22-52)}$. Lung function was analyzed by head-out body plethysmography. Treatment with AM did not influence (B) ovalbumin (OVA)-specific IgE levels in serum; (C) numbers of leukocytes in bronchoalveolar lavage (BAL); (D) IL-5 concentrations in BAL; (E) kinetic of plasma leakage, revealed by increasing albumin concentrations in BAL of OVA-sensitized and challenged mice in comparison with control mice, (F) reduction in BAL albumin levels in AM-treated OVA-sensitized and challenged mice. Group I, PBS-sensitized mice challenged with OVA; Group II, OVA-sensitized mice challenged with OVA – both treated intranasally with PBS; OVA-sensitized and challenged mice treated intranasally with AM (group III) or AM and $AM_{(22-52)}$ antagonist (group IV) as described in material and methods. Data are shown as mean ± SEM of values of eight mice per group and represent one of two independently performed experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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–D) (data for eosinophils, lymphocytes, and IL-13 are not shown).

We next investigated the effect of inhaled AM on the plasma extravasation of the airway mucosa that accompanies allergen-induced airway inflammation. Increased BAL albumin levels were taken as a measure of mucosal plasma extravasation (16) and there was a significant ($P < 0.01$) increase at 72 h after allergen challenge in OVA-sensitized mice (Fig. 4E). Inhaled AM significantly attenuated ($P < 0.001$) the allergen-induced elevation (at 48 h) of BAL albumin by about 60%. This effect of inhaled AM was, in turn, significantly ($P < 0.001$) inhibited by pretreatment with $AM_{(22\text{-}52)}$ (Fig. 4F).

Reduced airway AM expression was observed in human asthmatic airway epithelial samples

Using human airway tissue, we made a preliminary step to ascertain whether our findings in murine allergy models are of relevance to human asthma. We investigated whether expression of AM is reduced in the human asthmatic airway. Levels of AM mRNA were quantified in samples of total RNA, obtained from airway epithelial cells isolated from nonatopic healthy ($n = 21$) and atopic asthmatic children ($n = 14$). The clinical and demographic characteristics of the children and procedure for extracting total mRNA have been previously published (17) and are summarized, along with the method for quantifying AM mRNA in these samples, in the Data S1. As shown in Fig. 5A, epithelial cells of asthmatic children had significantly ($P < 0.05$) reduced AM gene expression, as compared to nonatopic healthy children. Lower AM gene expression in airway epithelial cells of asthmatics children was corroborated by lower protein expression observed. Results showed a marked reduction in the intensity of AM-specific staining, from epithelial cells derived from the same asthma patients, as compared with their healthy control group counterparts (Fig. 5B).

AM enhanced human and murine epithelial wound repair *in vitro*

Because of our finding of reduced AM expression in experimental and clinical allergen-induced airway inflammation, we next investigated the possible role of AM in the airway epithelium. AM was applied to an *in vitro* model of epithelial wound healing. In separate experiments with monolayers of different airway epithelial cells (HBE and LA4 cells), we initially confirmed the expression of the AM receptor system (CLR/RAMP2) as well as AM itself within these cell lines (Fig. 6A,B). The rate of closure of a 'wound' in the cell monolayer, by a single incision, was measured in the presence and absence of AM after 24-, 48-, and 72-h incubation (after 72 h the monolayer was confluent). This effect of AM was reversed in the presence of the AM receptor antagonist $AM_{(22\text{-}52)}$, which indicated that the observed AM -stimulated wound closure was a specific receptor-mediated effect

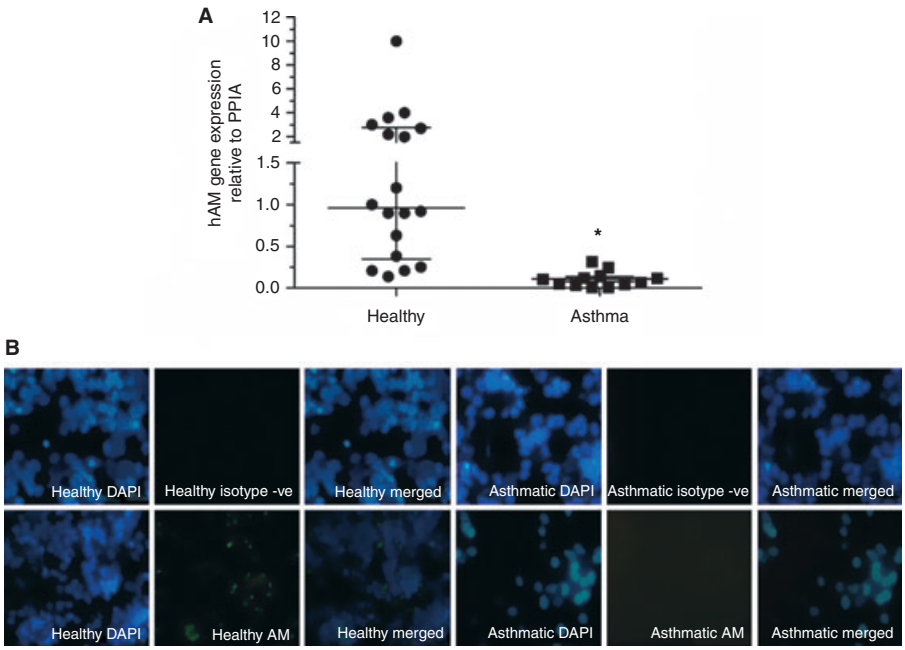


Figure 5 Adrenomedullin (AM) gene and protein expression in healthy and asthmatic epithelium. (A)Total RNA extracted from healthy nonatopic airway epithelial cells (Healthy) and atopic asthmatic airway epithelial cells (Asthma) was assessed for AM gene expression using quantitative polymerase chain reaction. In asthmatic, expression of AM was approximately 20-fold lower than that in healthy airway epithelial cells. *$P < 0.05$. (B) Immunocytochemical staining for AM protein expression results with a marked reduction in AM-specific staining intensity in asthmatic epithelial cells compared to their healthy counterparts. Note: magnification 600×.

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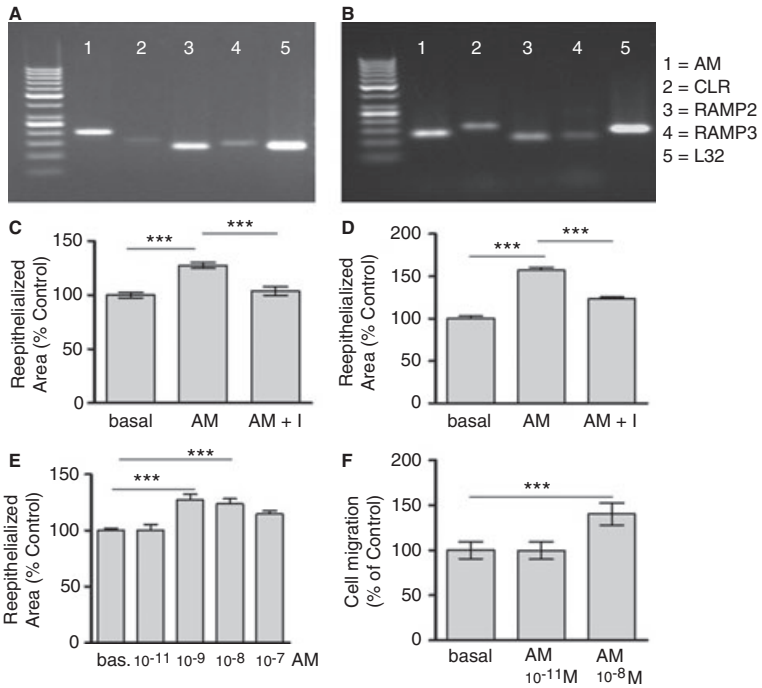


Figure 6 Adrenomedullin (AM) promotes airway epithelial wound repair and cell migration *in vitro* Evidence of expression of AM and its receptor system (CLR/RAMP2/RAMP3) in (A) human bronchial epithelial (HBE) and (B) LA4 cells. Confluent monolayers of (C) HBE and (D) LA4 cells were scratched, and the kinetics of wound closure was monitored in monolayers grown in medium only (basal) or in medium with 10^{-8} M adrenomedullin (AM), or with 10^{-8} M AM and $AM_{(22\text{-}52)}$ (inhibitor) at 24 h. Cells were pre-incubated for 30 min with the inhibitor before applying AM;(E) Dose-dependent effect of AM on epithelial regeneration (wound healing) in HBE cells; (F) Effect of AM on HBE cell migration in a Transwell migration assay in comparison with cells incubated with medium alone (basal). Data are shown as mean ± SEM of three independently performed experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

(Fig. 6C,D). A potent and dose-dependent (10^{-11}–10^{-7} M) increase in the rate of wound closure was observed with AM (Fig. 6E). (Similar results were observed for A549 cells, data not shown). As wound repair involves movement or spreading of epithelial cells and/or cell proliferation, we investigated whether AM might affect these processes. As shown in Fig. 6F, AM at a concentration of 10^{-8} M significantly increased the degree of cell migration. However, different concentrations of AM (10^{-7} M–10^{-11} M) had no effect on the proliferation of the tested epithelial cell lines (data not shown).

Discussion

Although AM is known to have potent anti-inflammatory pharmacological effects and to be present in the lung, its role in airway inflammatory disease, such as asthma, has not been investigated in detail. As AM is considered to act as a local autocrine/paracrine regulator of tissue homeostasis (8), we hypothesized that impairment of airway expression of AM may contribute to dysregulation of epithelial and mucosal function in allergen-induced airway inflammatory diseases such as asthma.

One major novel finding of this study is the decreased expression of lung AM during allergen-induced airway inflammation. This is notable, especially as it contrasts with increased AM expression that is reported in typical T_H1-related inflammation such as sepsis (18, 19). Our consistent findings, in different mouse models of allergic airway inflammation, suggest that down-regulation of the anti-inflammatory AM represents a pathophysiological characteristic of allergic inflammation. That AM down-regulation is a key pathophysiological process in the development of allergen-induced airway inflammation is suggested by (i) the rapidity of AM down-regulation in response to exposure to allergen; and (ii) the sustained AM down-regulation in response to persistent allergen exposure. Interestingly, Fujikura and Okubo recently reported that nasal AM levels are reduced in rhinitis patients (20).

Furthermore, we provide evidence that, in allergic airway inflammation in the mouse, AM expression is specifically down-regulated within the airway epithelium and mucosal vascular endothelium, which are sites of pathophysiological changes in the asthmatic airway (16, 21).

On the basis of these findings, we applied exogenous AM, via inhalation, directly to the airway to test whether AM

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replacement may attenuate the phenotype of allergic airway inflammation. We confirmed in mice the earlier finding in guinea pig (22), that inhaled AM inhibits allergen-induced AHR and airway mucosal extravasation, while additionally showing that this is a specific AM receptor-mediated effect. We also showed that inhaled AM had no simultaneous effect on the allergen-induced increase in BAL content of leukocytes and T_H2/T_H1 cytokines. These results suggest that AM exerts vascular effects without directly affecting the immunological components of airway inflammation. However, this requires further investigation including the application of higher doses of AM. Consistent with our findings, OVA-sensitized and challenged AM heterozygous mice (23) did not influence airway allergen inflammatory responses but enhanced AHR to allergen. The therapeutic potential of AM as a targeted treatment of AHR is underscored by the recent finding that AHR, and not airway inflammation, may be the primary factor of asthma-induced airway remodeling (24).

The inhibition by AM of allergen-induced mucosal plasma extravasation is consistent with the known vascular stabilizing effects of AM (11, 25, 26) and may be of possible clinical significance. It is well established that AM inhibits plasma extravasation (27) with the postcapillary endothelium being a particular AM target (28). AM also reduced lung permeability in an acute lung injury model of rats (29). Although plasma leakage in the asthmatic airway mucosa has long been considered (30) a hallmark of the disease, its pathophysiological significance has remained unclear. Recently, Khor et al. (16) demonstrated a clear association between the occurrence of plasma protein extravasation in bronchial tissues and a worsening of clinical asthma.

Epithelial dysfunction is now recognized as an important feature of airway inflammatory disease with disruption of epithelial tight junctions and increased permeability, impaired innate immunity, reduced local antioxidant protection, and defective epithelial repair processes (1, 21, 31, 32). In contrast to the well-known vascular effects of AM, the role of AM in the epithelium is unclear and was investigated here in regard to airway inflammation. Revealing the suppression of AM expression in the mouse airway epithelium, in a model of allergic airway inflammation, we were also able to demonstrate this in atopic pediatric asthmatic patients, underscoring the relevance of our findings to humans. AM has been shown previously to promote lung growth and repair in neonatal mice, effects that were considered to be due to the vascular stabilizing effects of AM (33). We have shown before that human asthmatic airway epithelial cells have a slower than normal proliferation rate and a considerably impaired wound-healing property (33). Here, we provide evidence that, *in vitro*, AM may directly promote human and mouse airway epithelial repair, by stimulating cell migration but without affecting cell proliferation. These *in vitro* findings are the first evidence that AM may act to support and restore airway epithelial barrier function, as has been demonstrated in experimental intestinal inflammation (34).

Taken together, our findings strongly suggest that decreased AM expression in airway T_H2-related inflammation may be a pathophysiological factor in airway inflammatory diseases, such as asthma, as it represents the loss of a factor that acts to maintain tissue integrity in the presence of inflammation. Of note, our data indicate that, in the airway mucosa, AM may act primarily to promote tissue repair and maintain microvascular function without any direct effect on the processes that initiate and drive inflammation. Further studies are required (i) to determine whether direct effects of AM on the airway epithelium and mucosal microvasculature underlie the attenuation of AHR observed after AM inhalation; and (ii) to assess AM and its cognate receptor as potential therapeutic targets for asthma therapy.

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Authors contribution

S.H. conceived the study, delineated the hypothesis, designed the study, acquired the data and wrote the article. H.W. and A.K. acquired the data and involved in its revision prior to submission. M.A., L.M.M., E.N.S., K-M.L., S.M.S., B.M., and N.W acquired the data. H.R. conceived the study, revised and approved the final version the article.

Conflict of interest

The authors declare that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Figure S1. Mouse models of allergic airway inflammation.

Table S1. Patient demographics.

Table S2. Primers for PCR.

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Online Supplemental Information

Suppression of adrenomedullin contributes to vascular leakage and altered epithelial repair during asthma

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Methods

Mouse models of allergic airway inflammation

Female Balb/c mice (6-8 weeks old) were housed in a pathogen-free facility with individual ventilated cages and received an OVA-free diet. All animal experiments were approved by the local authorities (Regierungspräsidium Gießen). At least 6-8 animals were used per group.

Acute OVA model (subcutan): Mice were sensitized by three subcutaneous injections of 10 µg of OVA diluted in 200 µl of PBS, or sham injections of PBS on days 0, 7 and 14. The sensitization phase was followed, on days 26, 27 and 28, by 20 minute aerosol application of 1% OVA or sham PBS, as described previously [(1)]. Adrenomedullin (5 µg/kg), adrenomedullin receptor antagonist (AM_{22-52}) (15 µg/kg) were administered intranasally, starting one day before challenge. On day 29, lung function was assessed and on day 30, all other analyses were performed. The kinetic analyses involved measurements at the selected time intervals of days 1, 2, 6 and 20 after the final OVA-challenge (s. Fig S.1A).

Chronic OVA model: Mice were sensitised to OVA by three intraperitoneal (i.p.) injections of 10 µg OVA adsorbed to 1, 5 mg $Al(OH)_3$ diluted in 200 µl PBS. Mice were challenged with 1% OVA aerosol via the airways twice a week on 2 consecutive days over a period of up to 12 weeks. In some mice OVA was replaced by PBS for further 4 or 8 weeks. 24h after the last allergen treatment lung tissues were processed for AM gene expression (s. Fig S.1B).

House dust mite (HDM) induced allergic airway inflammation model:

Female Balb/C mice (6-8 weeks) were treated intranasally with 100µg Dermatophagoides pteronyssinus extract (Greer Laboratories, Lenoir,USA) dissolved in 50 µl PBS on day 0, day 7, day 14 and 21, control animals received 50 µl PBS.

24h after the last allergen treatment lung tissues were processed for AM gene expression (s. Fig S.1C).

Assessment of airway reactivity

Lung function measurements were made 24 h after the last challenge, in conscious animals using non-invasive head-out body plethysmography. The mid-expiratory airflow (EF_{50}) of bronchial responsiveness to varying doses of ß-methacholine (MCh) was measured as described previously (2). Our laboratory has established that values measured by head-out body plethysmography correlated well with values obtained by invasive measurement (Flexivent, Scirec).

Bronchoalveolar lavage (BAL) and differential cell counts

Balb/c mice were sacrificed 48 h after the last challenge (day 30). Using a tracheal cannula, BAL was performed one time using 1 ml PBS containing 1x protease inhibitor cocktail (Roche). Total leukocytes were counted with an automated cell counter (Casy TT; Schaerfe Systems), and cell-free supernatant was stored at –20°C for cytokine measurement. Cytospin preparations were stained with Diff-Quick (Merz & Dade AG). Macrophages, lymphocytes, eosinophils and neutrophils were identified by standard morphologic criteria and 100 cells were counted per cytospin.

AM-EIA

After incubation with the different stimuli, culture supernatants were harvested and stored at -20°C until assayed for AM by enzyme immunoassay according to the manufacturer’s instructions (Phoenix Pharmaceuticals).

Isolation of primary murine tracheal epithelial cells (MTECs)

Primary murine tracheal epithelial cells (MTECs) were isolated from female Balb/c mice as described previously (3), with the small modification that the trachea tissues were transferred into dissociation media and incubated at 4°C for 18h before the enzymatic dissociation was stopped by addition of FCS.

Isolation of primary alveolar type II epithelial cells (AECII)

AECIIs were isolated from female Balb/c mice using a previously described protocol (4).

Isolation of primary alveolar macrophages and lung endothelial cells

The lungs of female Balb/c mice were perfused with 20 mL of sterile PBS until free of blood, as judged by visual inspection, then removed and transferred into Petri dishes containing 0.7 mg/ml collagenase A and 30 µg/mL DNAse I in RPMI-Medium. Lungs were minced, cut into small pieces and agitated on a shaker (1h, 37°C) in a humified atmosphere containing 5% CO_2. Cell aggregates were dispersed by passage through a GentleMACs and filtered through a 30 µm cell strainer (BD Biosciences) in order to obtain single cell suspension. After washing with a solution consisting of PBS, 2mM EDTA and 0.5% FCS, cells were stained with magnetic bead-conjugated anti-CD45 antibodies separated into CD45-negative and CD45-positive cells, using magnetic separation according to the manufacturer's instructions (Miltenyi Biotec). Cell numbers were assessed using an automated Casy TT cell counter (Schaerfe Systems). Subsequently, the CD45 positive cell population was stained with $F480^+$ Alexa Fluor and $CD11c^+$ PerCP/Cy5.5 conjugated antibodies (BD Pharmingen), the CD45 negative cell population was stained with CD31 APC and CD45 FITC conjugated antibodies. Staining was performed at 4°C in the dark for 20 min. After

staining, cells were washed twice in the PBS/2mM EDTA/0.5 % FCS solution. Cell sorting was performed with a MoFlow^{TM} flow cytometer. Purity of sorted cell was ≥ 98% as determined by flow cytometry and differential cell counts stained with DiffQuick. After sorting, cells were frozen at -80 C in RLT lysis buffer (Qiagen) with 1% mercaptoethanol (Sigma).

Laser-assisted microdissection

Cryo sections (10 µm) from lung tissues of Balb/c mice were mounted on glass slides. After hemalaun staining for 45 s, the sections were subsequently immersed in 70 % and 96% ethanol and stored in 100 % ethanol until use. Thirty bronchi and 20 septa per animal were selected and microdissected under optical control using the Laser Microdissection System (LMD 6000, Leica, Nussloch, Germany). They were then directly transferred into a reaction tube containing 200 µl RNA lysis buffer (Qiagen).and immediately frozen at -80 C.

Patients & Sampling procedure

This study was approved by the Princess Margaret Hospital for Children's Human Ethics Committee. Bronchial brushings were obtained from individuals undergoing elective surgery for non-respiratory related conditions (table S.1). Children with an existing bacterial or viral chest infection were excluded from recruitment. Asthma was defined as physician-diagnosed based upon physician documented wheezing episodes in the 12-months preceding their recruitment and confirmed by positive responses on the International Study of Asthma and Allergies in Children (ISAAC) and American Thoracic Society (ATS) respiratory questionnaires (5; 6) All asthmatic children had mild disease, such that none were receiving glucocorticosteroids (inhaled or oral) and less than 10% were taking β-agonists. Atopy status of all

subjects were determined by a positive radioallergosorbent test (RAST) to a panel of common allergens including; grass pollens, milk, mould, peanut, egg white and animal hair. Airway epithelial cells were harvested via trans-laryngeal, non-bronchoscopic brushing of the tracheal mucosa of children through an endotracheal tube as previously described (7; 8) or via a portable 'bronchoscope directed' sampling methodology (9). A minimum of 3 x 10^6 viable cells were collected for RNA extraction (7; 10; 11).

Human gene expression

Total RNA was extracted from samples using RNeasy extraction kit (QIAGEN) and human adrenomedullin gene expression determined by two-step RT-PCR as previously described (8; 10; 12). Expression of adrenomedullin was quantified relative to the expression of PPIA. The primers are listed in table S.2.

Statistics

Statistical significance regarding human gene analysis was assessed using Mann Whitney non parametric analysis. All values presented are means ± SD. All p values less than 0.05 were considered significant.

Mouse gene expression

Total RNA from homogenized lung tissues, primary cells and cell-lines was extracted in accordance with manufacturer's instructions (Qiagen, Hilden, Germany) and stored at -80°C. A total of 500 ng of total RNA preparation was reverse transcribed (Omniscript RT Kit, Qiagen, Hilden, Germany) using oligo (dT) primers.
Real-Time PCR was performed using the QuantiTect SYBR® Green PCR Kit (Qiagen, Hilden, Germany). Cycling conditions were 95°C for 15 min, followed by 40

cycles of 94°C for 10s, 60°C for 15 s, 72°C for 20s. Relative mRNA expression was calculated using the △△CT method. Individual data were normalized against the housekeeping gene (L32). It was also confirmed that L32 primers were not affected by treatment versus control. Results were expressed as fold change in expression over control.The primers are listed in table S.2.

Immunocytochemistry

Epithelial cells were cytospun onto glass slides and then fixed using neutral buffered formalin for 20 minutes at RT. Cells were then washed three times in PBS and stained for the human Adrenomedullin (Abcam, Cambridge, UK). Briefly, autofluorescence of the cells was blocked by incubating the slides in a solution of 0.5% (w/v) Sudan Black B in 70% (v/v) ethanol for 10 minutes. Cells were then washed in PBS and antigen retrieval was performed by incubating cells with proteinase K (35µg/ml) for 30 minutes at 37°C which were then cooled to RT for 10 minutes followed by 3 washes in PBS containing (1% v/v) saponin. Cells were then blocked for 1 hour at RT in 5% BSA (w/v), 10%FBS (v/v), 0.1% Triton X-100 (v/v) in 1x PBS/saponin solution. Cells were again washed and incubated with human adrenomedullin primary antibody (1:100) for 24 hours at 4°C. After this period, cells were washed in PBS/saponin and incubated with the secondary antibody (anti-rabbit FITC conjugate (1:100 Sigma, MO, USA). for 24 hours at 4°C. Fluorescent antibody complexes were then visualized using a fluorescent microscope (Nikon, Corp, Tokyo, Japan).

Cell culture

Human immortalized bronchial epithelial cells (HBECs) were kindly provided by Dr. G. John; A549 cells (a human alveolar epithelial cell carcinoma) and LA4 murine

bronchial epithelial cells were purchased from the American Type Culture Collection, Rockville, USA), respectively. HBE- and A549 cells were cultured in RPMI supplemented with 10% FCS, 1% L-Glutamine and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin), LA4 cells were incubated in F12-Kaigingh's medium supplemented with 15 % FCS, 1% L-Glutamine and antibiotics all at 37 °C in a humidified atmosphere.

Wound healing assay

HBE/LA4 cells were grown to over-confluence in six-well plates, after which the monolayer was scratched in a straight line from top to bottom with the tip of a 200 µl pipette tip. The cells were rinsed with PBS and replaced with either medium or medium containing AM (10^{-7}M -10^{-11}M); medium was replaced every 24 hours. The area of the denuded surface was measured in μm^2 by light microscopy immediately after wounding and after 24, 48 and 72 hours later (Olympus, Hamburg, Germany) using the Cell^F imaging software program (Soft Imaging System GmbH, Münster, Germany). The assay was based on independent measurements made by two experimenters who were blinded to the treatment conditions.

In vitro migration assay

Cell migration in response to AM was quantified using a 24-well modified Boyden chamber (Falcon; BD) with trans-well membranes (8 µm pore size). The upper chamber contained 10^5 HBE/LA4 cells in 500 µL medium, the lower chamber 500 µL of cell-free medium containing AM (10^{-8}M). Cells were incubated for 6h at 37°C with 5% CO_2, after which non-migrated cells were removed from the membranes with a cotton swab. The migrated cells on the lower surface were counterstained with Diff-Quick (Dade, Behring, Germany) and counted.

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Fig. S1: Mouse models of allergic airway inflammation

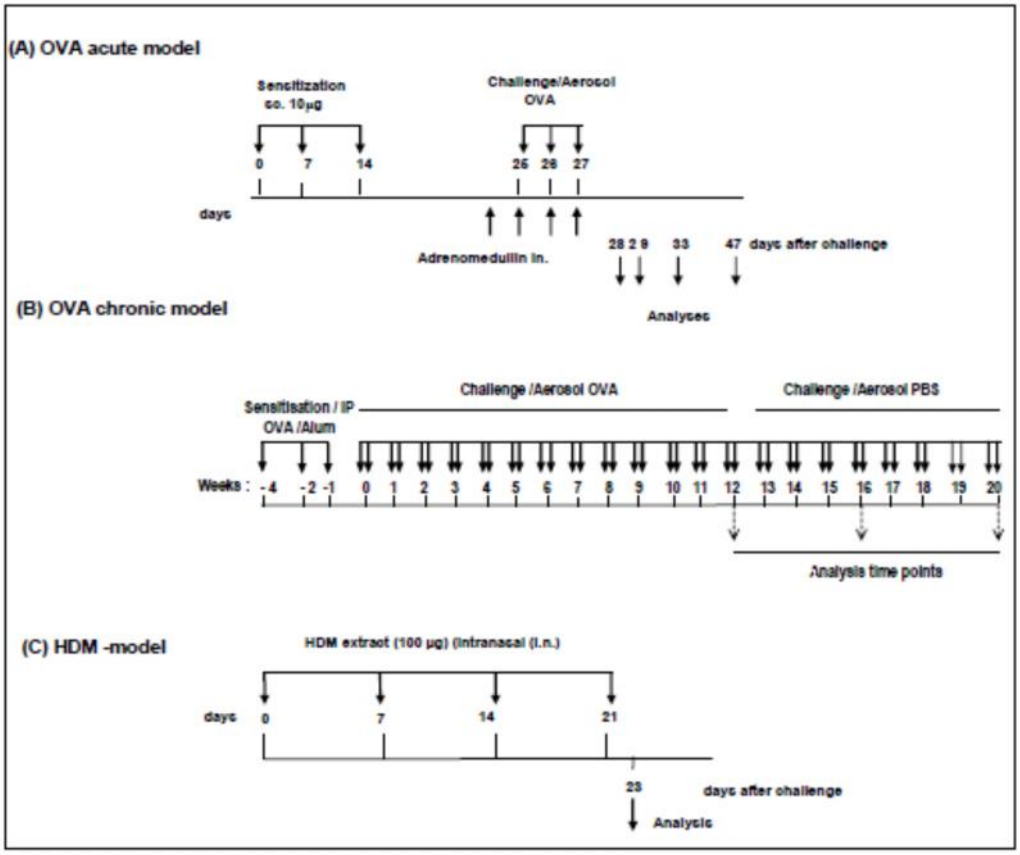


Table S.1. : Patient demographics

Phenotype	Sex	Average Age (yr)	Age Range (yr)	Number	Total (n)	Average IgE (kU/L)	Hay Fever	Eczema
Healthy	M	5.9	1.7-12.3	12				
	F	6.3	1.9-10.5	9	21	26	0	2
Asthmatic	M	7.4	5.3-13.5	11				
	F	12.3	11.9-13.1	3	14	220	7	9

Table S.2: Primers for PCR

Gene	Species	Sequence
	Human	
AM		
Forward:		5’-CTCTGAGTCGTGGGAAGAGG-3’
Reverse:		5’-CCCTGGAAGTTGTTCATGCT-3’
CLR		
Forward:		5‘-AACAGAACCTGGGATGGATG-3’
Reverse:		5‘-TCTGTTGCTTGCTGGATGTC-3’
RAMP1		
Forward:		5’-CTGCCAGGAGGCTAACTACG-3’
Reverse:		5’-GCTCCCTGTAGCTCCTGATG-3’
RAMP2		
Forward:		5’-GCCATGATTAGCAGGCCTTA-3’
Reverse:		5’-GTTGGCAAAGTGGATCTGGT-3’
RAMP3		
Forward:		5’-CTCATCCCGCTGATCGTTAT-3’
Reverse:		5’-AACTTTCTTCCAGCTTGCCA-3’
PPIA		
Forward:		5’-AGGAAAGAGAGGTCACGAGT-3’

Reverse:	5’-CCATTATGGCGTGTAAAGTCA-3’

Mouse

AM	
Forward primer	5’-ACAACGCACCCCTTTATCAG-3’
Reverse primer	5’-CGGAACTGCGAAGGAGTATC-3’
CLR	
Forward primer	5’-TAAGTTGCCAACGGATCACA-3’
Reverse primer	5’-GCCCATCAGGTAGAGATGGA-3’
RAMP1	
Forward primer	5’-GGACTCTCATCCAGGAGCTG-3’
Reverse primer	5’-GGTGGACAGCGATGAAGAAT-3’
RAMP2	
Forward primer	5’-CTGAGGACAGCCTTGTGTCA-3’
Reverse primer	5’-GTCCAGTTGCACCAGTCCTT-3’
RAMP3	
Forward primer	5’-TGCATCTTAGTTGGCCATGA-3’
Reverse primer	5’-CTCTCCAGCATCCCTGTCTC-3’
L32	
Forward:	5’-GCAAGTTCCTGGTCCACAAT-3’
Reverse:	5’-GGGATTGGTGACTCTGATGG-3’