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House dust mite allergic airway inflammation facilitates neosensitization to inhaled allergen in mice

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Abstract

Background: The mechanism by which many monosensitized allergic individuals progress to polysensitization over time remains to be elucidated. Mouse models have contributed greatly to the understanding of sensitization to inhaled allergens in healthy airways but hardly any studies have addressed sensitization during established allergy. We hypothesized that an allergic inflammatory milieu might facilitate sensitization to inhaled allergens by the presence of mature dendritic cells (DCs) and IL-4.

Methods: Mice with house dust mite (HDM)-induced allergic airway inflammation received a single intratracheal dose of ovalbumin (OVA), 2 days after the last HDM exposure. Ten days later, sensitization was assessed by rechallenger with OVA. We evaluated the following factors for their importance in neosensitization: (1) maturation and recruitment of DCs to the airways, (2) dependency on DCs using CD11cDTR conditional knockout mice, (3) presence of ongoing airway inflammation by comparing sensitization at day 2 and day 14 after the last HDM exposure and (4) dependency on IL-4 by treatment with blocking antibodies.

Results: House dust mite -induced inflammation facilitated neosensitization to OVA. HDM-induced inflammation increased the number of airway DCs with a mature phenotype but a DC reduction of 93% did not inhibit sensitization. Neosensitization to OVA was dependent on ongoing inflammation and in particular on IL-4.

Conclusions: These findings show that HDM-induced allergic airway inflammation facilitates neosensitization to a second inhaled allergen in an IL-4-dependent manner and provide insight into the underlying mechanism of the frequently observed progression to polysensitization in HDM-monosensitized individuals.

Abbreviations

BAL, bronchoalveolar lavage fluid; DC, dendritic cell; DTR, diphtheria toxin receptor; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence activated cell sorter; HDM, house dust mite; i.n., intranasally; i.t., intratracheally; IFN γ , interferon gamma; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; MHCII, major histocompatibility complex class 2; OVA, ovalbumin; PBS, phosphate-buffered saline; TCR, T-cell receptor; Tg, transgenic; Th2, T helper 2; TLR4, toll-like receptor 4.

It is eminent that a number of allergic individuals develop a second or third allergy over time (1–3). Several human and animal studies support the hypothesis that one allergy favours sensitization to another allergen, referred to as T helper 2 (Th2) spread (1–6). In different cohorts of monosensitized children, a majority of children developed one or more subsequent sensitizations within 6 years (1–3). This suggests that inhalational tolerance might be broken more readily in allergic airways than in healthy airways. The underlying mechanism resulting in successive sensitizations is still poorly understood. Eisenbarth et al. (4) demonstrated in an adoptive transfer

model in mice, using T-cell receptor (TCR) transgenic Th2 cells, that the presence of Th2 cells to one allergen can act as an adjuvant for sensitization to a second inhaled allergen. This phenomenon was described as 'collateral priming'. In this model, sensitization to a second allergen was dependent on the production of IL-4 by the transferred T cells. We described recently that neosensitization in stably remodelled but inflammation-free lungs is dependent on dendritic cells (DCs). We showed that airway DCs retained their activated phenotype after resolution of the inflammation and that they were instrumental in facilitating neosensitization to a newly encountered allergen (7). The aim of the present study was to investigate whether the milieu of ongoing allergic airway inflammation induced by a relevant allergen source, that is, house dust mite (HDM), is sufficient to facilitate neosensitization to a second allergen. Besides the induction of typical Th2 cytokines and the recruitment of inflammatory cells to the airways, also the recently described induction of innate cytokine production by the airway epithelium, that is, of GM-CSF, TSLP, IL-25 and/or IL-33, may potentially contribute to breaking tolerance to newly inhaled aeroallergens (8,9). In this study, we investigated whether mice with an established eosinophilic airway inflammation without airway remodelling, induced by repeated airway exposure to HDM, were prone to develop neosensitization to a second allergen upon a single adjuvant- and HDM-free exposure via the same route, and which role DCs and inflammatory cytokines have in the neosensitization to a secondary allergen.

Materials and methods

Mice

Female BALB/c mice (Harlan, Horst, the Netherlands) and female CD11cDTR (10) (backcrossed on BALB/c background) mice were housed under specific pathogen-free conditions at the animal facility of the AMC. All experiments were approved by the animal ethics committee of the AMC, the Netherlands.

OVA sensitization during HDM-induced airway inflammation

Mice were anaesthetized with isoflurane, and 100 µg HDM extract (Greer Laboratories, equivalent to 1 µg Der p 1; Lenoir, NC, USA) in 30 µl PBS was administered i.n. at days 0, 7, 14 and at days 21, 22, 23. To induce sensitization to OVA, 100 µg OVA (Sigma, grade III; Zwijndrecht, The Netherlands) or as a control, PBS was administered at day 25 intratracheal administration (i.t.). Ten days later, OVA sensitization was read out by challenging these mice for 30 min on three consecutive days with OVA aerosol [10 mg OVA (Sigma)/ml PBS]. One day after the last aerosol challenge, mice were killed.

CD11cDTR tg mice underwent the same experimental protocol but CD11c⁺ DCs (and CD11c⁺ alveolar macrophages) were depleted at day 25 before OVA exposure by 50 ng Diphtheria Toxin (Calbiochem; Merck Chemical Ltd, Nottingham, UK) i.t. as described earlier (11). OVA was administered 8 h

later, which was shown to be sufficient to abrogate DC function (10). Depletion of CD11c⁺ cells was confirmed 24 h after DT administration by flow cytometric analysis on digested lung cells.

For IL-4-blocking experiments, BALB/c mice were treated twice with 500 µg anti-IL-4 (clone 11B11) or isotype control GL113 mAb (Bioceros B.V., Utrecht, the Netherlands) i.p. 24 h after the last HDM exposure (day 24) and 30 min before OVA sensitization (day 25).

Collection and analysis of tissues

Bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid inflammatory cells were obtained by airway lumen lavage with 3 × 1 ml PBS containing 0.1 mM EDTA. Cell differentiation was made by flow cytometric analysis as described earlier (12).

Ex vivo restimulation of lung-draining lymph nodes

Lung-draining lymph node cell suspensions were restimulated for 4 days with 10 µg/ml OVA (Worthington, Lakewood, NJ, USA) or 100 µg/ml HDM extract. Supernatants were analysed for IL-4, IL-5, IL-13 and IFNγ production by ELISA (Ready-set-go!, eBioscience Inc, San Diego, CA, USA).

Serum OVA-specific immunoglobulins

Serum was analysed for the level of OVA-specific IgE by ELISA (Opteia; BD, San Diego, CA, USA). A standard curve of murine IgE was used as a quantitative reference.

Airway histology

Frozen sections (6 µm) were stained with periodic acid Schiff's reagent (Sigma-Aldrich). Degree of inflammation and mucus-producing goblet cells were semi-quantified as described earlier (7).

DC phenotype in lung

Lungs of HDM-sensitized and challenged mice and PBS control mice were digested as described earlier (7). DCs were identified as CD11c^{hi}, MHCII^{hi}, CD11b^{hi}, CD19/CD3/B220/CCR3^{neg}, and the expression of CD80, CD86 and CD40 was determined by flow cytometric analysis as described earlier (7).

Statistical analysis

For statistical analysis, Kruskal–Wallis one-way ANOVA and the Mann–Whitney *U*-test were performed using PASW statistics. All experiments were performed 2–5 times with 5–7 animals per group. Differences were considered to be significant at a *P* value of <0.05.

Results

HDM-induced airway inflammation facilitates neosensitization to OVA

To study the effect of ongoing Th2-mediated airway inflammation on the adaptive immune response to a second inhaled

allergen, mice were first sensitized and challenged with HDM i.n. to induce the main hallmarks of allergic asthma (Fig. S1 A–D). Mice with or without HDM-induced airway inflammation were exposed to a single dose of OVA i.t. (resp. HDM/OVA_OVA and PBS/OVA_OVA). As a control for OVA sensitization, one group of HDM-allergic mice received PBS instead of OVA (HDM/PBS_OVA). Ten days later, sensitization was read out by challenging all mice with three OVA aerosols (Fig. 1A). As expected, OVA sensitization and challenge did not recruit eosinophils to the airways of control mice (PBS/OVA_OVA). However, in mice that had a HDM-mediated airway inflammation prior to sensitization with OVA (HDM/OVA_OVA), significantly more eosinophils were recruited to the airways (Fig. 1B). Mice that were not sensitized with OVA but challenged with OVA aerosols (HDM/PBS_OVA) did not develop airway inflammation. Prior HDM-induced inflammation also increased production of Th2 cytokines IL-4, IL-5 and IL-13 compared with the two control groups. In HDM/PBS_OVA, some IL-5 and IL-13 was produced, while PBS/OVA_OVA mice did not produce any Th2 cytokines. IFN γ levels were not significantly different between groups (Fig. 1C). Histological analysis revealed significantly more mucus-producing goblet cells and peribronchial infiltrates compared with the two control groups (Fig. 1D,E). In concordance with the other inflammatory parameters, only HDM/OVA_OVA mice showed a significant increase in OVA-specific IgE and not the HDM/PBS_OVA or PBS/OVA_OVA mice (Fig. 1F).

HDM-induced airway inflammation induces maturation in airway DCs

We and others have shown that airway DCs have an activated phenotype during ongoing inflammation and are crucial for sensitization to inhaled allergens (7,11,13–15). We hypothesized that HDM-induced inflammation would induce the activation of airway DCs facilitating sensitization to a newly encountered allergen. We killed HDM- and PBS-exposed mice right before OVA sensitization (day 25). HDM-induced airway inflammation increased the number of MHCII⁺CD11c⁺ DCs present in the airways and significantly enhanced the expression of CD86 and CD40 compared with noninflamed airways, while for CD80 a trend was observed that did not reach statistical significance (Fig. 2A,B).

Strong reduction of airway CD11c⁺ cells does not inhibit neosensitization to OVA

To investigate whether airway DCs are crucial for neosensitization to OVA during HDM-induced inflammation, we used CD11cDTR tg mice to deplete DCs during sensitization. Three groups of CD11cDTR tg mice were exposed to HDM. Two days after the last HDM exposure, one group was treated with DT to deplete DCs (DC[−]) and one group with PBS (DC⁺) (Fig. 3A). In line with earlier reports (16), CD11c⁺ DCs and alveolar macrophages were successfully depleted by 93% and 94%, respectively. After DT or PBS treatment,

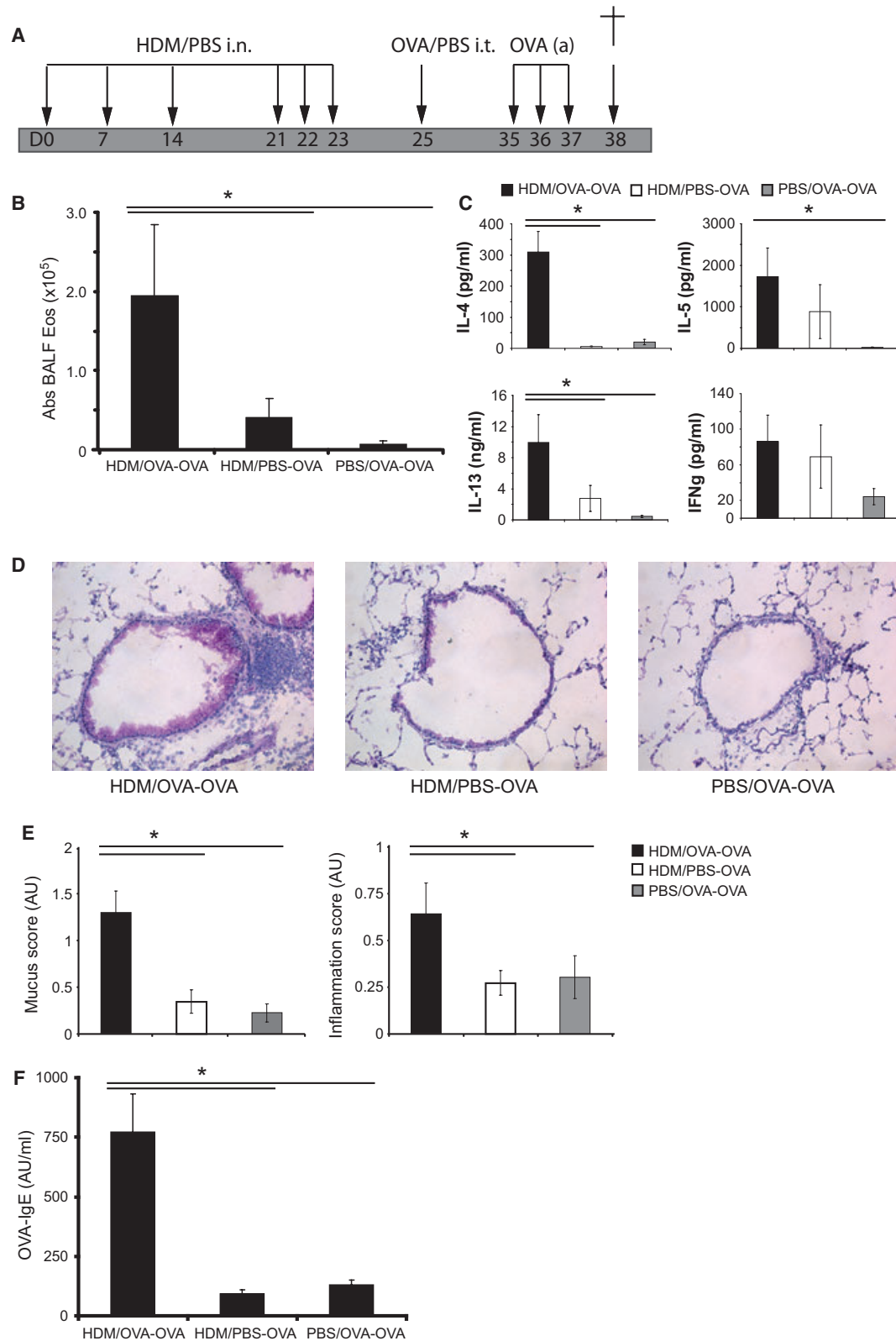
mice were sensitized intratracheally to OVA (resp. DC[−]/OVA_OVA and DC⁺/OVA_OVA) or as a control to PBS (DC⁺/PBS_OVA). As expected, DC⁺/OVA_OVA mice developed an eosinophilic airway inflammation after challenge with OVA aerosols. Surprisingly, DC depletion during sensitization did not inhibit eosinophil recruitment but instead even showed a trend towards an increase (Fig. 3B). As in the BALB/c, OVA aerosols without a prior OVA sensitization did not recruit eosinophils to the airways in DC-sufficient CD11cDTR tg mice (DC⁺/PBS_OVA). DC depletion during OVA sensitization also did not result in a lower Th2 cytokine production in response to the OVA aerosols but instead increased IL-4 and IL-13 production (Fig. 3C). Histological analysis of the airways revealed that DC depletion did not dampen the formation of peribronchial inflammatory infiltrates (Fig. 3D). DC-sufficient, OVA-sensitized mice had a significantly higher level of OVA-IgE compared with PBS sham-sensitized mice. Mice depleted of DCs during sensitization had a 10-fold higher level of OVA-IgE in serum compared with DC-sufficient mice (Fig. 3E). Overall, these results indicate that CD11c⁺ cell depletion enhanced neosensitization and associated Th2 inflammation.

Neosensitization to OVA during HDM-induced airway inflammation is dependent on ongoing inflammation

Although HDM-allergic individuals are usually perennially exposed to HDM allergens, we investigated whether inhalational tolerance could be restored after resolution of HDM allergen-mediated eosinophilic airway inflammation. The airway eosinophilia observed at day 2 was completely resolved at day 14 after challenge. Mice were intratracheally instilled with OVA 2 or 14 days after the last HDM challenge. Ten days later, mice were challenged with three OVA aerosols. Exposure to OVA 14 days after the last HDM challenge did not induce any sensitization, that is, there was no sign of airway eosinophilia (Fig. 4A), Th2 cytokine production (Fig. 4B), mucus production or peribronchial inflammatory infiltrates (Fig. 4C) or OVA-specific IgE (Fig. 4D) in contrast to mice sensitized 2 days after the last HDM exposure.

Neosensitization during HDM-induced airway inflammation is dependent on IL-4

Next, we explored whether IL-4 is crucial for neosensitization in the inflammatory lung environment after HDM-induced airway inflammation. IL-4 was blocked at the time of sensitization to OVA, and 10 days later, mice were challenged with OVA (Fig. 5A). Mice were compared with mice that were either not sensitized to OVA (HDM/PBS_OVA) or that did not have a HDM allergy prior to OVA sensitization (PBS/OVA_OVA). Anti-IL-4 treatment abrogated the recruitment of eosinophils and reduced IL-4, IL-5 and IL-13 production significantly compared with the group treated with the isotype control. The response was inhibited to the level seen in the two control groups (Fig. 5B,C). Production of IFN γ was not affected (Fig. 5C). Anti-IL-4 treatment did not inhibit



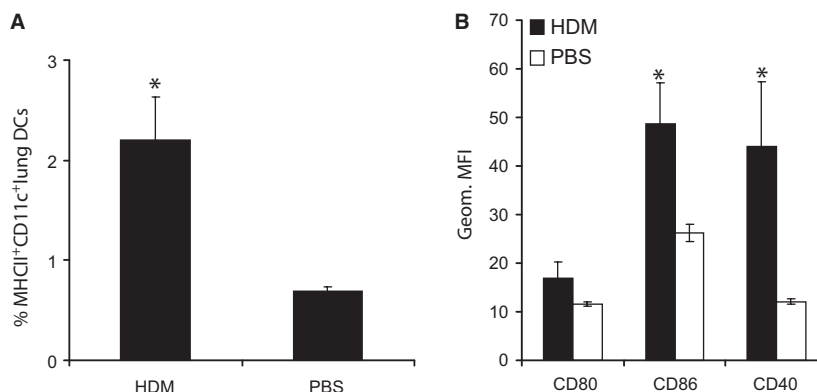


Figure 2 House dust mite (HDM)-induced allergic airway inflammation induces maturation and recruitment of airway dendritic cells. BALB/c mice were intranasally exposed to HDM and were killed (day 25) immediately before OVA administration. The number of

recruited airway MHCII^{hi}CD11c^{hi}CD3/CD19[−] DCs was analysed in HDM allergic airways (HDM) or in control (phosphate-buffered saline) airways (A) and analysed for the expression of CD80, CD86 and CD40 (B). Data are presented as means ± SEM **P* < 0.05.

mucus production and inflammatory infiltrates (Fig. 5D). Anti-IL-4 treatment was capable of inhibiting sensitization at the humoral level as well; serum OVA-IgE levels in the anti-IL-4-treated group did not exceed the levels found in the control groups and were significantly lower than in the isotype-treated group (Fig. 5E).

Discussion

There is an increasing recognition that sensitization to one allergen can favour sensitization to unrelated allergens (1–3), while immunotherapy can successfully suppress progression to polysensitization. The aim of this study was to investigate the immunological mechanism underlying progression to polysensitization. In a murine model for HDM-induced allergic airway inflammation, we showed that during ongoing inflammation, inhalational tolerance to inhaled allergen was broken in an IL-4-dependent manner. Although increased numbers of DCs with a more mature phenotype were recruited to the airways, an almost complete depletion of DCs did not inhibit sensitization to an inhaled secondary allergen.

Over the last decades, tremendous progress has been made in the elucidation of the underlying mechanism of sensitization in naïve airways, but surprisingly only a few papers have addressed sensitization during ongoing allergic airway inflammation. This is clinically relevant because patients with a perennial allergy such as that to HDM are constantly

exposed to other potential allergens. It is likely that the inflammatory milieu alters the requirements for sensitization. DCs are recognized for their unique capacity to prime naïve T cells. HDM-induced inflammation increased the maturation of lung DCs, but surprisingly, an almost complete depletion of lung CD11c⁺ DCs did not impede neosensitization. This suggests that during ongoing inflammation, non-DC inflammatory cells like eosinophils, basophils, B cells or inflammatory macrophages can substitute for DCs as antigen-presenting cells and promote neosensitization to inhaled allergens (17–19), such as OVA in the present study. Interestingly, we have shown previously in a chronic model of asthma that in remodelled but inflammation-free lungs, DCs retained their mature phenotype and were crucial for the induction of neosensitization (7). However, the present study provides evidence that ongoing inflammation can contribute to neosensitization in a DC-independent manner.

Commercially available OVA has been reported to have significant contamination with LPS (20), which has been described to induce a reactivation of a Th2 response in post-asthmatic allergen-sensitized mice (21). In our study, this artefact could be excluded because the HDM/PBS_OVA group did not develop airway inflammation. In addition, the induction of OVA-specific IgE in the HDM/OVA_OVA, but not in HDM/PBS_OVA, confirmed that neosensitization indeed occurred and was accompanied by airway inflammation upon exposure to the neoallergen.

Figure 1 House dust mite (HDM)-induced allergic airway inflammation facilitates ovalbumin (OVA) sensitization. BALB/c mice were intranasally exposed to HDM or phosphate-buffered saline (PBS) as a control. OVA (or phosphate-buffered saline as a control) was administered 2 days after the last HDM exposure, and all mice were exposed 10 days later to three daily OVA aerosols (A). Eosinophil recruitment to the airways was examined 24 h after the last OVA aerosol (B). Production of Th2 cytokines IL-4,

IL-5, IL-13 and IFN γ was determined in supernatants of *ex vivo*-restimulated lung-draining lymph node cells with ovalbumin (OVA) (C). Lung slides were stained with PAS to determine peribronchial inflammatory infiltrates and mucus production (D). Peribronchial infiltrates were quantified (right graph). Mucus production was quantified (left graph) (E). OVA-IgE levels were determined in serum by ELISA (F). Data are presented as means ± SEM **P* < 0.05.

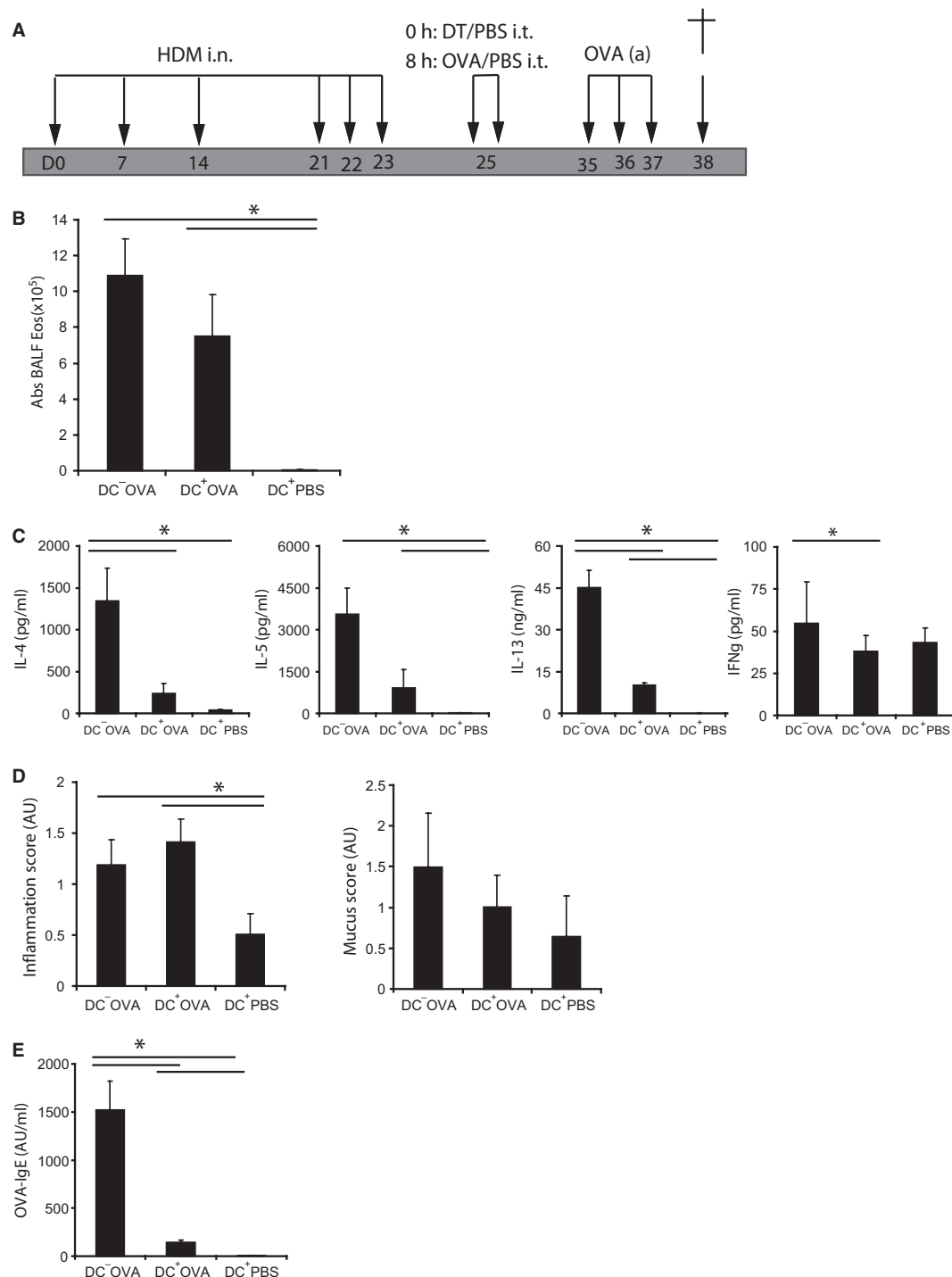


Figure 3 Partial depletion of airway dendritic cells does not affect neosensitization to ovalbumin (OVA). House dust mite (HDM)-induced allergic airway inflammation was induced in CD11cDTR transgenic mice (on BALB/c background). Before intratracheal administration of OVA, airway DCs were depleted by an intratracheal instillation with DT or as a control with phosphate-buffered saline (PBS). One control group of HDM-allergic CD11cDTR tg mice were sham-sensitized with PBS i.n. and challenged with OVA aerosols (A). Eosinophil recruitment was determined in

BALF by flow cytometric cell differentiation (B); production of the Th2 cytokines IL-4, IL-5, IL-13 and IFN γ was determined in supernatants of *ex vivo*-restimulated lung-draining lymph node cells (C). Lung slides were stained with PAS to determine peribronchial inflammatory infiltrates and mucus production. Peribronchial infiltrates were quantified (D: left graph). Mucus production was quantified (D: right graph). OVA-IgE levels were determined in serum by ELISA (E). Data are presented as means $\pm$ SEM * $P < 0.05$.

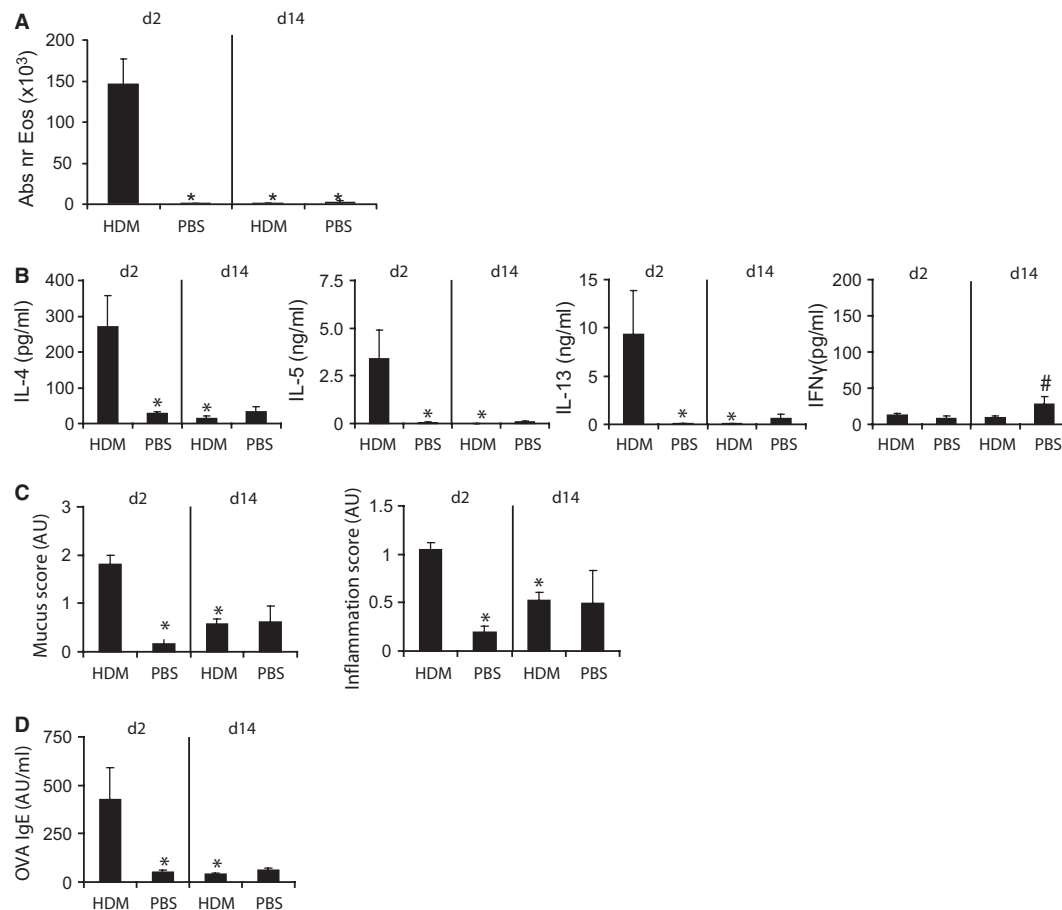


Figure 4 House dust mite (HDM)-induced inflammation facilitated ovalbumin (OVA) sensitization which is dependent on acute inflammation. BALB/c mice were intranasally exposed to HDM to induce allergic airway inflammation or phosphate-buffered saline (PBS) as a control. OVA (or PBS as a control) was administered 2 or 14 days after the last HDM exposure, and all mice were exposed 10 days later to three daily OVA aerosols. Eosinophil recruitment was determined by flow cytometric cell differentiation of BALF

cells (A). Th2 cytokine production was measured in the supernatant of *ex vivo*-restimulated lung-draining lymph nodes with OVA (B). Lung slides were stained with PAS for quantitative determination of mucus production and peribronchial inflammatory infiltrates (C). OVA-IgE levels were determined in serum by ELISA (D). Data are presented as means $\pm$ SEM * P < 0.05 vs HDM day 2 (mice sensitized with OVA 2 days after last HDM exposure) # P < 0.05 vs HDM day 14

The process of neosensitization was shown to be IL-4 dependent by the administration of anti-IL-4 antibodies during OVA exposure. Because specific immunotherapy has been reported to decrease IL-4 cytokine production (22–24), our results could explain the clinical observation that early immunotherapy protected HDM-monosensitized children for progression to polysensitization (2). Our results further suggested that the window of opportunity for neosensitization is extended from a simultaneous encounter with HDM allergens (4,25) to the duration of the active immune response which is accompanied by IL-4 production. Perennial exposure to HDM will probably fuel ongoing inflammation in HDM-allergic individuals constantly. Whether the production of other cytokines such as TSLP, IL-25, GM-CSF and IL-33 by airway epithelium exposed to HDM could facilitate sensitization as demonstrated here for IL-4 is an interesting hypothe-

sis (8,9). We measured these cytokines in BAL at the time of encounter with the new allergen, but levels detected were close to the detection limit and did certainly not reveal any significant increases (data not shown). In contrast, expression of these cytokines after HDM exposure has been described by others (8,9) and could have been detected in lung tissue or at earlier time points in our study as well. However, our study indicates that the probable presence of these cytokines was not sufficient to induce sensitization when IL-4 was inhibited: identifying IL-4 as a key cytokine.

In conclusion, we have shown that HDM-induced airway inflammation can facilitate sensitization following a single mucosal exposure to the innocuous protein OVA, without the need of extensive simultaneous exposure to HDM as described in earlier reports (4,25). Neosensitization was dependent on IL-4 and the presence of inflammatory cells in

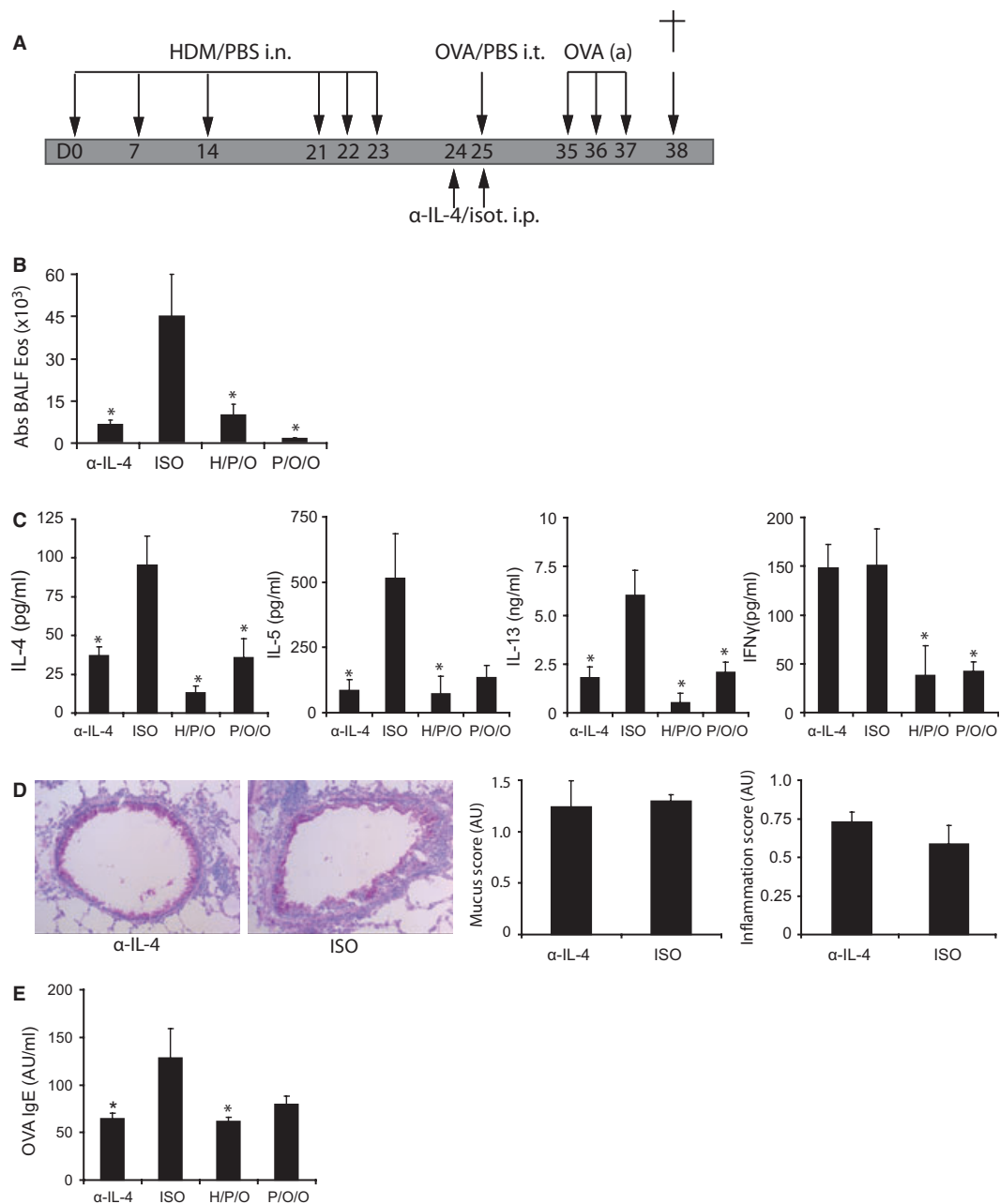


Figure 5 Sensitization to OVA during HDM allergic airway inflammation is dependent on IL-4. BALB/c mice were intranasally exposed to HDM before anti-IL-4 or an isotype (ISO) control antibody was administered 24 h before and 30 min before OVA sensitization. Ten days later, mice were re-exposed to the same allergen, OVA, by three daily aerosols. One HDM-allergic control group was sham-sensitized with phosphate-buffered saline (PBS) and challenged with OVA aerosols (H/P/O). Another control group received PBS instead of HDM and was sensitized and challenged

with OVA(P/O/O) (A). Eosinophil recruitment was determined in BALF by flow cytometric cell differentiation (B). Production of the Th2 cytokines IL-4, IL-5, IL-13 and IFN γ was determined in the supernatants of *ex vivo*-restimulated lung-draining lymph node cells with OVA (C). Lung slides were stained with PAS for quantitative determination of peribronchial inflammatory infiltrates and mucus production (D). OVA-IgE levels were determined in serum by ELISA (E) * $P < 0.05$ compared with the HDM/OVA/OVA treated with the isotype control.

the airways. Although these cells include activated DCs, surprisingly they were not crucial for neosensitization. The clinical implications of our findings relate to how a substan-

tial percentage of HDM-mono-sensitized allergic individuals progress to one or more additional allergies against unrelated allergens and contributes to an explanation how early immu-

notherapy in HDM-mono-sensitized children might prevent progression to polysensitization.

Conflict of interest

DC is granted by EU Marie Curie Initial Training Network 'INFLAME'. There are no potential conflicts of interest for any authors.

Authors contribution

LvR, LB and RvR involved in the conception and design of the study. LvR, AL, LU and DC involved in the acquisition of data.

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Supporting Information

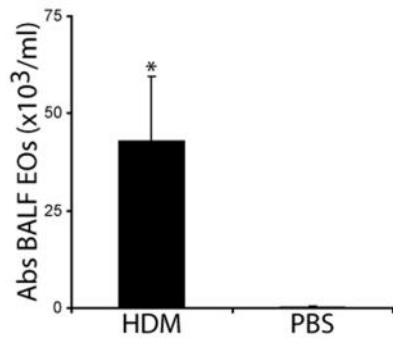
Additional Supporting Information may be found in the online version of this article:

Figure S1. Intranasal exposure to HDM induced the main characteristics of asthma.

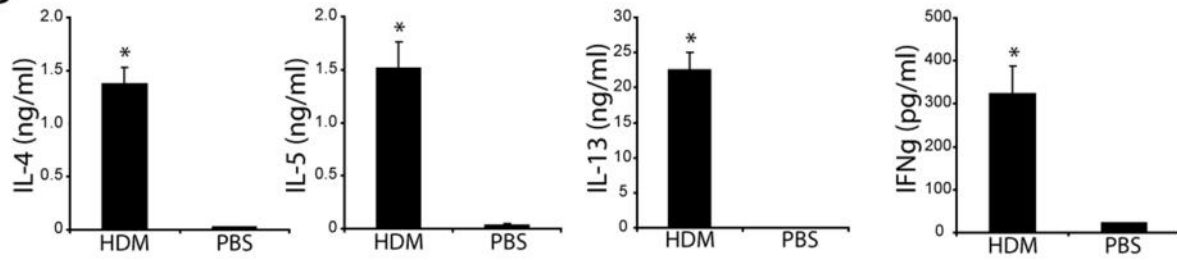
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Supplementary figure 1

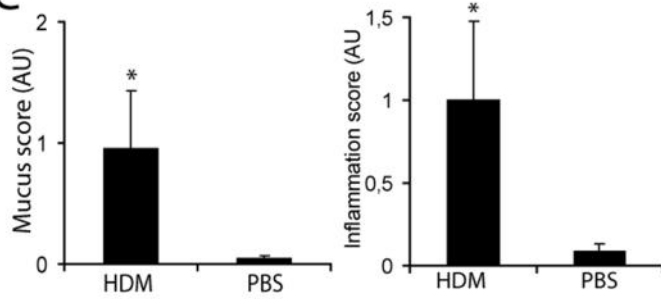
A



B



C



D

