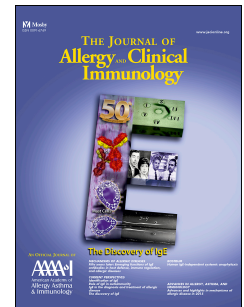


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Epicutaneous sensitization to house dust mite allergen requires IRF4-dependent dermal dendritic cells

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27

Abstract

Background

Exposure to allergens like house dust mite (HDM) via the skin often precedes allergic inflammation in the lung. It was proposed that Th2 sensitization via the skin occurs when skin barrier function is disrupted for example by genetic predisposition, mechanical damage or enzymatic activity of allergens.

Objective

To study how HDM applied to unmanipulated skin leads to Th2 sensitization and to study which antigen presenting cells mediate this process

Methods

HDM was applied epicutaneously by painting HDM on unmanipulated ear skin, or under an occlusive tape. HDM challenge was via the nose. Mouse strains lacking different dendritic cell (DC) populations were used, and 1-DER T cells carrying a transgenic TCR reactive to Der p 1 allergen used as readout for antigen presentation. The Th2-inducing capacity of sorted skin-derived DC subsets was determined by adoptive transfer to naïve mice.

Results

Epicutaneous HDM application led to Th2 sensitization and eosinophilic airway inflammation upon intranasal HDM challenge. Skin sensitization did not require prior skin damage or enzymatic activity within HDM extract, yet was facilitated by applying the allergen under an occlusive tape. Primary proliferation of 1-DER T cells occurred only in the regional skin draining lymph nodes. Epicutaneous sensitization was found to be driven by two variants of IRF4-dependent dermal conventional dendritic cell (cDC2) subsets, and not by epidermal Langerhans cells.

Conclusion

These findings identify skin cDC2 as crucial players in Th2 sensitization to common inhaled allergens that enter the body through the skin, and can provoke features of allergic asthma.

Key messages

- Epicutaneous sensitization to mite allergen can occur through an intact skin, yet is facilitated by an occlusive tape
- Epicutaneous sensitization to mite allergen does not require the enzymatic activity of the allergen
- Epicutaneous sensitization to mite allergen is driven by 2 variants of IRF4-dependent dendritic cells, and not by Langerhans cells

Capsule summary

Our findings identify skin IRF4-dependent cDC2 and not Langerhans cells as crucial players in Th2 sensitization to HDM. How alterations in skin-expressed proteins or cytokines in patients at risk for developing asthma could interfere with this basic mechanism of DC-driven type 2 immunity will need to be addressed.

Key words

Epicutaneous sensitization

House Dust Mite

Allergy

Airway inflammation

Skin Dendritic Cells

Abbreviations used: HDM, house dust mite; DC, dendritic cells; IRF, Interferon Regulatory

Factor; BAL, bronchoalveolar lavage; LC, Langerhans cells; DTR, diphtheria toxin receptor;

SCLN, superficial cervical lymph node; BLN, brachial LN; ALN, axillary LN; ILN, inguinal

LN; MesLN, mesenteric LN; MLN, mediastinal LN.

Introduction

Although most clinically relevant allergens like house dust mite (HDM) fecal pellets, pollen, animal dander and fungal spores are aeroallergens that are easily inhaled, the precise route by which individuals mount an adaptive immune response to them has been poorly defined. It is well known that airborne particles can trigger an immune response in the respiratory epithelium that leads to systemic sensitization¹⁻³. It is however equally possible that aeroallergens enter the body via other routes. As the dust mite *Dermatophagoides pteronyssinus* feeds on human skin, HDM allergens are abundant in mattress covers and blankets and could be recognized by the structural and immune cells of the skin barrier^{4, 5}. The idea that sensitization can occur via the skin is also supported by the fact that children who later develop allergic asthma or rhinitis often had eczema in the first year of life⁶. It has been hypothesized that some children might have an intrinsically altered skin barrier integrity, which could lead to increased penetration of allergens to the cells of the immune system. These children indeed show single nucleotide polymorphisms (SNPs) in genes encoding for skin barrier proteins, like filaggrin⁷⁻⁹. However, these SNPs are only present in 20% of asthmatic patients¹⁰⁻¹², suggesting that other mechanisms might be responsible for Th2 sensitization through the skin. Intrinsic barrier defects in the skin are not a prerequisite for allergy development via that route. Many allergens including the HDM allergens Der p 1 and Der p 9 are proteolytic enzymes that have the capacity to cleave tight junctions that are abundantly present in skin and mucosal barriers, and thus get access to immune cells across intact skin. Other risk allele SNPs that are associated with allergy and asthma are found in genes encoding regulators of the process of antigen presentation (e.g. certain major histocompatibility complex (MHC) haplotypes), pattern recognition receptors, as well as multiple cytokine (receptor) genes involved in the innate and adaptive immune system. Indeed, allergic sensitization occurs when allergens are recognized by pattern recognition receptors on antigen-presenting dendritic cells (DCs) that degrade the antigen into small peptides for presentation on MHC molecules to the T cell receptor^{1, 2, 13}. In the lungs and nose, it has been shown that subsets of conventional (c)DCs, especially those expressing the

integrin CD11b and SIRP α $\square\square\square\square\square\square\square\square\square\square$, and now referred to as cDC2, are crucial for the recognition of aeroallergens and for subsequent development of Th2 immunity to allergens like HDM, and development of asthma or allergic rhinitis^{1, 2, 14-22}. Other DC subsets of the lung (cDC1s expressing XCR1 and the integrin CD103 or plasmacytoid DCs) induce tolerance to inhaled allergens²³⁻²⁵.

If the skin is a portal for allergic sensitization through an intact skin barrier, then DCs are likely to play an equally important role. Also in the skin, different subsets of DCs with different anatomical localizations and functions have been described²⁶⁻²⁹. Langerhans cells (expressing CD11c, langerin (a.k.a CD207), and CD11b) are located in the epidermis and were considered for a long time as the main antigen-presenting cells (APCs) in the skin. In the dermis, studies have reported the presence of several cDC populations like cDC1s and cDC2s which respectively rely on the transcription factors IRF8 and IRF4 for their development and function^{22, 30-33}. During inflammation, monocyte-derived DCs (expressing CD11b and intermediate levels of the macrophage markers CD64 and MERTK) also get recruited to the skin³². IRF4-dependent cDC2s were recently reported to control Th2 responses in the skin³⁴⁻³⁶, and this property was mostly restricted to Klf4-dependent Mgl2⁺CD11b⁻CD24⁻ “double negative” (DN) cDC2s³⁶. However, in most studies using sensitization via the skin, the pro-Th2 antigens (papain, alum, ovalbumin) have been injected through the skin or applied to the skin after tape stripping, causing either a direct breach of skin barrier integrity or irritation of the barrier by the process of tape stripping^{34, 35, 37}, and no study has studied the role of DC subsets in the process of epicutaneous allergic sensitization.

In this paper, we have addressed whether Th2 immunity would occur in response to an aqueous extract of the ubiquitous environmental allergen HDM, when applied epicutaneously on intact mouse ear skin. We demonstrate clear signs of systemic Th2 sensitization that lead to allergic airway inflammation following airway allergen challenge. We found that HDM sensitization via this route did not require skin barrier disruption by tape stripping or the proteolytic activity of HDM, yet was facilitated by applying the allergen under an occlusive

144 tape that caused mild barrier impairment. Epicutaneous sensitization was mediated by two
145 subsets of IRF4-expressing dermal cDC2s, whereas Langerhans cells were not required.
146 These findings identify skin cDC2 as crucial players in Th2 sensitization to common inhaled
147 allergens that enter the body through an intact skin and can provoke features of allergic
148 asthma.
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150

151 **Methods**

152

153 **Mice**

154 C57Bl/6 were obtained from Harlan or generated in house and were maintained in the animal
 155 facility of the Inflammation Research Center (IRC) of Ghent under specific-pathogen free
 156 conditions. *Langerin*-DTR transgenic mice were obtained from Dr. Bernard Malissen (Centre
 157 d'Immunologie de Marseille-Luminy, France)³⁸. *Irf4*^{fl/fl} mice were obtained from Dr. William
 158 Agace (University of Lund, Sweden)³⁹. 1-DER TCR transgenic mice in which CD4 T cells
 159 react to an immunodominant epitope in Der p 1 have been described in detail⁴⁰. Experiments
 160 were approved by the animal ethical committees of the University of Ghent and the
 161 Inflammation Research Center.

162 **Flow cytometry**

163 To determine the cellular composition of BAL fluids, cells were stained with moAbs directed
 164 against MHCII (M5/114.15.2), CD11c (N418), CD19 (1D3) and CD3 (145-2C11)
 165 (eBioscience), Siglec F (E50-2440), Ly-6G (1A8) (BD). For detection and phenotyping of DC
 166 subsets in cell suspensions of skin, or skin draining LN, cells were stained with MHCII
 167 (M5/114;Biolegend), CD11c (N418), CD103 (2E7), CD3 (145-2C11), CD19 (1D3), F4/80
 168 (BM8), CD172a (P84) (eBioscience), XCR1 (ZET, Biolegend), CD11b (M1/70), CD24
 169 (M1/69), CD64 (X54-5/7.1), CD26 (H194-112) and SAV (BD). For intracellular IRF4, IRF8
 170 or CD207 staining, cells were fixed using a fixation/permeabilization kit (eBioscience) before
 171 they were stained with labeled CD207 (929F3.01, Dendritics) and/or IRF8 (C19, eBioscience)
 172 or unlabeled IRF4 (M17, Santa Cruz Biotechnology). Then, unlabeled IRF4 was bound to
 173 labeled donkey anti-goat Ab (Invitrogen). To check purity and proliferation of CD4⁺ '1der' T-
 174 cells following Ab were used: CD4 (RM4-5), CD3 (17A2), CD44 (IM7) (eBioscience),
 175 CD45.1 (A20), CD45.2 (104), TCR V β 4 (KT4) and CD69 (H1,2F3) (BD). Dead cells were
 176 always excluded from the analysis by using fixable viability dye eFluor506 or eFluor780
 177 (eBioscience). Acquisition of multi-color samples was done on a LSR Fortessa flow
 178 cytometer (BD Bioscience). Final analysis and graphical output were performed using FlowJo
 179 software (Tree Star Inc.).

180 **HDM labeling and uptake by dendritic cell subsets**

181 HDM extract (Greer Laboratories; Derp1 content: 227.64 μ g/vial; Endotoxin content: 1397
 182 EU/vial; Protein content: 5.59 mg/vial) was labeled with AF647 succinimidyl ester (Life
 183 Technologies) by the protein service facility (PSF) from the IRC of Ghent. 100 μ g HDM

were applied on the ear skin and analysis of uptake was performed in the skin or in SCLN using flow cytometry at different time points.

Epicutaneous sensitization and development of allergic airway inflammation

Sensitization was induced on day 0 by applying 100 µg HDM in 20 µl PBS (*Dermatophagoides pteronyssinus* extracts, Greer Laboratories) on the dorsal ear skin or on the shaved flank, which was then covered with a 5mm x 5mm gauze under an acrylic adhesive waterproof film (Leukoflex). The tape was gently removed using scissors taking care to avoid skin stripping or abrasion. To induce allergic airway inflammation, mice were challenged intranasally (i.n) with 10 µg HDM on days 7–11. On day 14, serum, BAL and mediastinal lymph nodes were collected and analyzed.

In experiments where Langerin-DTR mice were used, the animals were injected intraperitoneally with 500 ng diphtheria toxin on the day before HDM epicutaneous sensitization.

Collection of serum, BAL, MLN cells.

Blood was collected from the iliac vein for serum preparation. Bronchoalveolar lavage (BAL) was obtained by injecting 3 x 1 ml of PBS containing EDTA in the cannulated trachea. MLN cells (2×10^6 cells/ml) were restimulated for 3 days *ex vivo* with 15 µg/mL HDM extracts in 96 round-bottomed plates. Cytokine production was determined in supernatants by specific ELISA (eBioscience).

Immunofluorescence staining of lung tissue sections

7µm-thick lung slices were incubated with rat anti-Siglec F (BD Bioscience) or with mouse anti-Muc5ac antibodies (Abcam). For Muc5ac staining, slides were treated with the Mouse on Mouse staining kit according to the manufacturer's instructions. Slides were then incubated with Cy3-labeled secondary antibodies and nuclei were counterstained with DAPI before analysis on a LSM710 microscope (Zeiss) equipped with a tunable 2-photon Mai Tai and a 561 nm laser. Analysis of the acquired images was done using Imaris software (Bitplane).

Lymph nodes and skin preparations for dendritic cell sorting

Lymph nodes were manually cut in small pieces and these were enzymatically digested with 20 µg/mL LiberaseTM research grade and 10 U DNase (both from Roche) in RPMI medium for 30' at 37°C. Skin samples were incubated overnight at 4°C with 200 µg/mL Dispase II (from *Bacillus polymerase grade 2*, Roche) to facilitate manual cutting and isolation of cells. Small skin pieces were further digested with 1,5 mg/mL Collagenase Type 4 (Worthington) and 10 U DNase (Roche) in RPMI medium buffered with Hepes and supplemented with 2%

Fetal Calf Serum (FCS). The suspension was each 30' resuspended and provided with fresh digestion buffer for a total of 90 minutes at 37°C. After digestion, debris and dead cells were removed using a density gradient (Percoll).

CFSE labeling of HDM-specific T cells

LNs and spleen from CD45.1 congenic '1-DER' mice were isolated and homogenized in PBS through a 70 µM cell strainer (Falcon). After red blood cell lysis, CD4⁺ T-cells were purified using the MagniSort™ Mouse CD4 T-cell enrichment kit according to the manufacturer's protocol. The purity of the cells was assessed by flow cytometry and was higher than 98%. Isolated CD4⁺ T-cells were incubated for 7 minutes at 37°C with 5 µM CellTrace™ CFSE (ThermoFisher Scientific) in FCS-free medium. Cells were washed with RPMI containing 10% FCS.

In coculture experiments, 1-DER T cells were placed with sorted DC subsets for 3 days at a ratio of 5:1.

Dendritic cell and 1-DER T cell adoptive transfer

In experiments where DCs were transferred, naïve C57Bl/6 mice were applied PBS or HDM epicutaneously. Thirty-six hours later, SCLN were collected and cell suspensions were prepared. Cells were stained for the presence of different DC subsets. After washing, the different DC subsets were sorted on an Aria III (BD). Twenty thousand cells were injected intradermally into naïve recipient mice on day 0. On days 7-11, mice were challenged intranasally with 10µg HDM. On day 14, BAL and lung-draining mediastinal lymph nodes were collected and analyzed for the presence of eosinophils and Th2 cytokine production, respectively.

In 1-DER adoptive transfer experiments, 3 million CD4⁺ CFSE-labeled 1-DER T cells were injected into mice via the tail vein. T cell proliferation was assessed 4 and 7 days later by flow cytometry.

HDM-specific ELISA

96 well plates were coated with 100 µg/mL HDM extracts overnight at 4°C. After washing, serum was incubated on the plates for 3h. Biotin-coupled anti-IgE detection antibodies and avidin-HRP reagent were then added for 1h. TMB substrate was added after extensive washing and chemiluminescence was determined by spectrophotometry (absorbance at 450 nm).

Transepidermal water loss (TEWL) measurement

The belly of the mice was shaved in the morning, and mice were applied an occlusive tape (Leukoflex) on the shaved area in the evening. As a control, some mice did not receive a tape. One group of shaved mice were tape stripped 10 times before TEWL measurement. One day later, TEWL was measured with a Tewameter® TM300 (Courage + Khazaka electronic).

Statistics

For all experiments, the difference between 2 groups was calculated with the Mann-Whitney U test for unpaired data. Differences were considered significant when $p < 0.05$ (*).

Results

Epicutaneous sensitization to HDM does not require skin damage

In several models of epicutaneous sensitization, antigens are applied on the skin after disruption of the stratum corneum using different mechanical methods like tape stripping^{37, 41, 42} or after applying irritating depilatory creams⁴³. To be able to study epicutaneous sensitization to the relevant HDM allergen while minimally altering skin barrier, we applied HDM extract to the backside of a mouse ear on a sterile gauze under an occlusive flexible dressing tape (Leukoflex) devoid of latex extracts. We gently removed the tape after 16h taking care not to disturb epithelial cell integrity, by cutting away, rather than stripping the tape off. For comparison, we also included a group where we applied the allergen under a similar occlusive tape on shaved flank skin. All mice were then challenged with HDM intranasally (i.n) 7 days later for 5 days, and the degree of airway eosinophilic inflammation was read out 3 days after the last challenge (see Figure 1A for experimental setup). After challenge, all mice sensitized epicutaneously with HDM on the ears (HDM/HDM group) developed airway eosinophilia (Figure 1B), had HDM-specific IgE in the serum (Figure 1C), and showed strong IL-5 and IL-13 production when mediastinal lymph node cells (MLN) were restimulated *ex vivo* with HDM (Figure 1D), compared to mice applied with PBS epicutaneously (PBS/HDM group). However, when HDM was applied on shaved flank skin, the severity of all features of allergic inflammation was significantly higher than in mice in which the allergen was applied on the ears (Figure 1B-D). We also characterized lung changes more carefully. There was increased peribronchial and perivascular infiltration of SiglecF⁺ eosinophils in the HDM/HDM group, compared with the PBS/HDM group (Figure 1E). Moreover, mice in the HDM/HDM group showed increased staining intensity for Muc5ac, a gel forming protein present in mucus lining the airways particularly when there is goblet cell metaplasia (GCM), compared to control PBS/HDM mice (Figure 1E). These data indicate that allergens applied epicutaneously on the skin can induce systemic Th2 sensitization to allergen and cause features of allergic airway inflammation upon repeated exposure to the lung when the same allergen is subsequently inhaled.

Although we took great care in avoiding manual barrier disruption by cutting away, rather than stripping off the occlusive tape after 16h, we still wanted to study the impact of the occlusive dressing in our model. A good measure of epidermal barrier function is measurement of transepidermal water loss (TEWL). Unfortunately, due to the size of the probe, we could not measure this on mouse ear skin. We therefore measured the effect of the occlusive tape removal on shaved back skin. For comparison, we also performed tape stripping by manually stripping off adhesive tape 10 times prior to TEWL measurement. As shown in Figure 1F, overnight application of occlusive tape, followed by our gentle removal

procedure did modify the skin barrier integrity, as reflected by the increased TEWL compared with mice that did not get a tape applied. However, the degree of barrier defect induced was much smaller compared with the tape stripping procedure commonly used in allergen skin application models. This increased TEWL induced by the occlusive tape was associated with a very mild inflammatory response in the skin, as assessed by the recruitment of innate cells Ly-6G^{hi} neutrophils and MHCII^{hi} CD11c⁺ dendritic cells (DCs) to the draining node 24 hours after the tape application (Figure 1G). Whether sensitization can still be achieved by the application of allergens on an intact and unmanipulated skin, or whether it always requires some degree of inflammation in the skin is still debated⁴⁴. To address the impact of the occlusive tape on the strength of Th2 responses to HDM, we applied an occlusive tape on the ears overnight. As a control for the tape effect, some mice did not receive a tape application. The next day, the occlusive tape was removed. All mice were then anaesthetized and 100 µg HDM extract, or PBS as a control, was applied for 1 hour by painting the HDM solution on the ear skin. After i.n HDM challenges, even mice sensitized epicutaneously to HDM in the absence of prior tape application (No tape-HDM 1h e.c) developed airway eosinophilia (Figure 1H). However, removal of the overnight tape just prior to HDM sensitization (Tape ON-HDM 1h e.c) further increased the strength of the model, as reflected by the increased BAL fluid eosinophilia (Figure 1H). The group of mice where the tape was removed prior to HDM (Tape ON-HDM 1h e.c) in fact showed similar intensity of inflammation as a group of mice in which the HDM was applied under the tape overnight (HDM under tape ON; Figure 1H). These data point to the fact that Th2 sensitization to HDM can be achieved even through an intact unmanipulated ear skin, but that sensitization is further enhanced if an occlusive tape is used.

Epicutaneous sensitization to HDM does not require proteolytic activity of HDM

We next wanted to understand how Th2 sensitization to HDM could be achieved through the skin barrier, and investigated whether the disruption of tight junctions by proteolytically active allergens contained in our HDM extracts (like Der p 1) could be involved in this process. For this, mice were sensitized e.c either with HDM or with heat-inactivated HDM under an occlusive dressing tape on mouse ears, and were challenged with HDM i.n. We verified that heat inactivation of HDM for 45 min on 95 °C inactivated the proteolytic activity of HDM (data not shown). Epicutaneous sensitization with HDM or with heat-inactivated HDM (HI-HDM) induced similar numbers of BAL eosinophils and lymphocytes (Figure 1I), and of cytokine production by MLN cells restimulated *ex vivo* with HDM (Figure 1J), indicating that the proteolytic activity of HDM was not required to induce epicutaneous Th2 sensitization. Similar data were obtained when we used the highly selective cysteine protease inhibitor E64 (Figure I-J), in which case HDM sensitization was also intact.

T cell activation after epicutaneous sensitization occurs in skin-draining lymph node

We next wanted to study where sensitization to HDM occurred when applied on the skin. For this, we made use of the recently generated '1-DER' mice, which express an MHCII-restricted TCR specific for a Der p 1₃₃₋₄₅ peptide (peptide sequence SCWAFSGVAATES), a major allergen of HDM. To test the importance of single versus dual TCR reactivity, these mice were generated on a *Rag*^{-/-} C57Bl/6 background and a C57Bl/6 background, respectively. Four days after e.c HDM application, proliferation of WT CD4⁺ '1-DER' T-cells only occurred in the superficial cervical lymph node (SCLN) that drain the ear skin on the side of allergen application (Figure 2A-C). No primary T cell divisions were detectable in the contralateral SCLN, or in other non-draining lymph nodes like the inguinal lymph node (ILN; Figure 2B-C) or spleen (Figure 2A). A low percentage of divided 1-DER T cells could be recovered from the spleen, but these had a CFSE^{lo}CD44^{hi} phenotype, suggesting they were recirculating T cells (Figure 2A). When *Rag*^{-/-} 1-DER T cells were injected into mice exposed to HDM e.c, these cells showed higher proliferation and activation capacities in the draining SCLN 4 days after HDM application (Figures 2E-F) than WT 1-DER T cells (Figures 2B-C). The number of *Rag*^{-/-} CFSE^{lo}CD44^{hi} recirculating T cells was significantly increased in the ILN of mice exposed to HDM compared to PBS applied animals (Figure 2F).

Four and 7 days after HDM e.c. application, ear draining SCLN cells were obtained from mice that had received WT 1-DER T cells and were restimulated with HDM for 3 days *ex vivo* (Figure 2D). The supernatants contained high amounts of IFN γ , IL-10 and IL-13 cytokines when cells were derived from mice injected with WT 1-DER T cells and exposed to HDM e.c for 4 days (Figure 2D). In mice exposed to HDM for 7 days, IL-17 and IL-13 production were very high compared to mice exposed to PBS (Figure 2D). Similar cytokine profiles were observed in SCLN cells of mice injected with *Rag*^{-/-} 1-DER T cells (Figure 2G). These data show that HDM e.c application induces primary T cell divisions only in the SCLN draining the ear to which the allergen was applied, subsequently leading to Th2 and Th17 cytokine production, as well as mild IFN γ induction. Similar responses were observed with WT 1-DER T cells bearing dual TCR reactivity and *Rag*^{-/-} 1-DER cells bearing single TCR reactivity and lacking naturally-occurring Tregs, indicating that the response induced by the application of HDM extracts is specific for Der p 1. The proliferative response of 1-DER T cells observed following HDM e.c application in WT mice was absent when the allergen was applied on the ears of *Ccr7*^{-/-} mice (Suppl. Figure 1), showing that the activation of HDM-specific T cells requires a CCR7-dependent active transport of the allergen by DCs to the draining node.

DCs capture epicutaneous HDM in the skin and transport it to the draining lymph nodes

Priming of naïve T cells is a function performed by DCs after they have captured antigens in the periphery and migrated to the nodes in a CCR7-dependent manner. The skin and skin-draining LN contain five major DC subsets^{22, 29, 32} which share high expression of CD11c and MHCII, but can be discriminated based on the surface expression of CD26, CD64, CD24, CD207 (langerin), CD103, XCR1, CD172a and CD11b; and by intracellular expression of lineage defining transcription factors IRF4 and IRF8 (see Figure 3 for detailed gating process)²². The function of skin DCs has mainly been studied after painting irritative haptens to the skin, or injection of antigens subcutaneously or intradermally. Exposure of the ear skin to e.c. HDM induced a rapid increase in the number of DCs in SCLN. As early as 1 day after allergen application, we observed an increase in the number of CD11b⁺ cDC2 and CD11b⁻ CD24⁻ double negative (DN) cDC2 in SCLN (Figure 4A). The number of DN cDC2 peaked at day 2 post e.c HDM and returned to baseline by day 4, whereas the number of CD11b⁺ cDC2 kept increasing until day 4 post e.c HDM application. CD24⁺ cDC1 and Langerhans cells (LCs) were recruited to the SCLN only after 2 days post HDM e.c application. We did not find any increase in the number of CD11c⁺ MHCII⁺ monocyte-derived cells (MCs or moDCs) in the SCLN (Figure 4A). We next went on to identify the specific subsets of skin DCs, which were able to transport e.c. applied HDM to SCLN. For this, ears were exposed to fluorescently labelled (Alexa Fluor 647) HDM. Four hours after the application, fluorescent HDM could be detected in CD11c^{lo} cells of the skin (Figure 4B). The application of fluorescent HDM did not induce any changes in the number of skin macrophages and DC subsets at this time point compared to PBS application (Figure 4C). The fluorescent allergen was contained mostly in skin macrophages, in monocyte-derived cells (MCs), Langerhans cells (LCs) and CD11b⁺ cDC2 (Figure 4D). Almost no DN cDC2 and cDC1 were found to carry HDM in the skin (Figure 4D). When SCLN were analysed 48h after e.c application, the fluorescent HDM was detected mostly in very few CD11c^{hi} cells (Figure 4E). Contrary to the skin, HDM e.c application induced a significant increase in the number of all DC subsets analysed compared to mice exposed to PBS (Figure 4F). In SCLN, the fluorescent HDM was mostly contained in cDC2 (CD11b⁺ and DN subset) (Figure 4G). Strikingly, very few cDC1, MCs and LCs were found to carry HDM in SCLN (Figure 4G).

Epicutaneous sensitization to HDM is induced by IRF4-dependent cDC2

Since several skin-derived DC subsets can capture allergen applied on the skin and transport it to the draining SCLN, we next wanted to know which subset would be responsible for Th2 sensitization to e.c HDM. To address the contribution of MCs and LCs in this process, we made use of Flt3L-deficient (*Flt3l*^{-/-}) mice that still have LCs and MCs, but in which all

subsets of cDCs are lacking (Figure 5A). As expected, WT mice exposed to HDM e.c and challenged i.n with HDM developed BAL lymphocytosis and eosinophilia (Figure 5B). These mice also showed a strong IL-5 and IL-13 production by MLN cells after *ex vivo* restimulation with HDM compared to mice sensitized with PBS e.c (Figure 5C). BAL eosinophilia was reduced by 50% and Th2 cytokine production was almost completely abolished in *Flt3l*^{-/-} mice sensitized and challenged with HDM compared to control WT mice (Figure 5B-C), suggesting that cDCs are involved in Th2 sensitization to e.c. applied HDM. However, LCs and MCs were sufficient to induce some degree of BAL eosinophilia. To test the contribution of LCs and CD103⁺ cDC1s, we made use of Langerin-DTR (*Lang*-DTR) mice lacking all Langerin⁺ cells (LCs and one subset of (CD103⁺) cDC1s) after DT injection (Figure 5D). To study the contribution of cDC2s, we made use of *Cd11c*Cre-*Irf4*^{fl/fl} mice that lack both subsets of cDC2s in the skin draining lymph nodes due to a migration defect of these cells (Figure 5G). When *Lang*-DTR mice injected with DT were subjected to the e.c sensitization protocol when both LCs and CD103⁺ cDC1 were absent, they showed similar degrees of airway eosinophilia than their WT counterparts (Figures 5E-F). Surprisingly, Th2 cytokine production by MLN cells was strongly increased compared to WT littermates. Similar results were obtained if HDM was applied e.c at a time when only LCs were absent (Supplementary Figure 2), suggesting that LCs and/or CD103⁺ cDC1s might play a role in inducing T cell tolerance to e.c. applied allergen. When *CD11c*-Cre^{Tg} *Irf4*^{fl/fl} mice were exposed to the same protocol, they failed to develop features of allergic airway inflammation compared to the littermate controls (Figure 5H-I). Altogether, these results suggest that mainly cDC2, and not LCs or Langerin⁺ CD103⁺ cDC1, are necessary to induce Th2 sensitization to e.c. applied HDM.

IRF4-dependent skin-derived cDC2 are sufficient to induce allergic airway inflammation to HDM

We next wanted to know which subsets of skin DCs were sufficient to induce Th2 sensitization to HDM. We first investigated which DC subsets would be able to stimulate the proliferation of CD4⁺ 1-DER naïve T cells *ex vivo*. For this, different subsets of DCs (CD11b⁺ cDC2s, DN cDC2s, CD103⁺ cDC1s, and LCs) were sorted from SCLN 36 hours after e.c. applied HDM or control PBS (Figure 6A), and were cocultured for 3 days with CFSE-labelled HDM-specific naïve T-cells. As shown in Figure 6B, only HDM-exposed CD11b⁺ cDC2s and DN cDC2s induced the proliferation of 1-DER T-cells, while CD103⁺ cDC1s and LCs did not (Figure 6B). The degree of proliferation induced by cDC2 was, however, very low compared to the positive control where exogenous HDM was added to the co-culture (Figure 6B). Since cDC2 were the only subsets able to stimulate naïve T cells to proliferate, we wanted to know whether they would be able to induce Th2 sensitization when adoptively transferred into

naïve mice. Different DC subsets were sorted from SCLN of mice exposed epicutaneously to HDM or to control PBS, and were injected intradermally (i.d.) in naïve C57Bl/6 mice on day 0. On days 7-11, mice were challenged i.n with HDM. Only mice injected with HDM-exposed CD11b⁺ cDC2s or with HDM-exposed DN cDC2s developed features of allergic airway inflammation, as evidenced by the presence of BAL eosinophilia (Figure 6C) and by the production of the Th2 cytokines IL-5 and IL-13 by MLN cells restimulated with HDM *ex vivo* (Figure 6D). Mice injected with CD103⁺ cDC1 or with LC failed to mount Th2 immunity to HDM. Together, these data show that IRF-4-dependent CD11b⁺ cDC2 and DN cDC2s are necessary and sufficient to induce type 2 immunity to epicutaneously applied HDM allergen.

DISCUSSION

The first barrier to protect against sensitization to allergens is made up by the epithelial layer, a physical, chemical and immunological barrier found in skin and the mucosa covering the gastrointestinal, genitourinary and respiratory tract⁴⁵. Allergic inflammatory diseases are accompanied by increased permeability of various epithelia including the skin and respiratory epithelium caused for example by disruption of tight-junction (TJ) function or genetic or metabolic defects in filaggrin or hornerin^{11, 46, 47}. As a consequence of subtle defects in epithelial barrier function, it is thought that underlying structural cells and immune cells become more susceptible for environmental triggers, like HDM, pollutants and viruses that all contain pathogen associated molecular pattern (PAMPS)⁴⁸. Various epithelial cells, including skin keratinocytes, express Toll-like receptors (TLR) and protease-activated receptors (PARs) to interact with allergens and other environmental factors, leading to production of cytokines that promote Th2 immunity by activating DCs and other innate immune cells⁴⁹ (reviewed in²).

It has long been established that eczema constitutes a risk factor for development of sensitization to aeroallergens and to (severe) asthma later in life^{5, 50, 51}. Also, many children who have signs of altered and defective epithelial barrier function (measured by transepidermal water loss) are at increased risk of developing sensitizations to aeroallergens⁵². Although this has led to the assumption that the skin might be a portal of entry for allergens, the actual route and mechanism of sensitization to aeroallergens has not been studied in great detail, but has however been modelled in the mouse skin, where epicutaneous application of model and real life allergens does lead to Th2 immunity^{37, 40, 44, 53, 54}. One caveat to many of the previously published models of skin sensitization is the fact that the skin was altered by shaving or tape stripping, or by the use of irritants such as DMSO or depilatory creams^{37, 42, 43}. The ensuing epithelial leakiness, damage and inflammation caused by these actions might act as an adjuvant, overestimating the real potential of allergens to cause sensitization when applied on an intact skin⁵⁵⁻⁵⁷. We have therefore developed a model of epicutaneous application of allergens on the intact ears of mice, and compared it to a model where allergens were applied on shaved skin. We essentially found that sensitization did not require shaving of the mice or the application of an occlusive dressing tape, but instead, it could be induced by the simple painting of HDM extracts on the ear skin of an anesthetized mouse. However, both shaving on the flank skin and the use of an occlusive dressing on the ears did enhance most features of allergic inflammation that were induced. It is therefore very likely that these methods induce some degree of keratinocyte damage⁵⁸ associated with a stronger innate response. This damage was reflected by a mild increase in TEWL that was however not near the degree of TEWL induced by the tape stripping procedure.

In this model of epicutaneous sensitization under an occlusive dressing overnight, the penetration of the skin could occur as a result of the proteolytic activity of some allergens like Der p 1 or Der p 9 capable of cleaving epithelial tight junction proteins and, in this way, gaining access to immune cells^{53, 59-62}. Although this is an elegant hypothesis, the requirement for the proteolytic activity of allergens in the process of sensitization is still debated. As such, some studies show that the induction of Th2 responses via the skin by the cysteine protease papain is mediated by its enzymatic activity^{53, 54, 60, 63}, whereas others have shown that this is not the case⁶⁰. The reason for such discrepancy might come from the differences in application of the allergen at times of first allergen application, with one study employing hair removal and the other one using tape stripping, raising the issue as to whether proteolytically active allergens are responsible for Th2 sensitization after application on intact skin. We have carefully compared the development of Th2 immunity between mice applied with crude or heat-inactivated HDM extracts on intact ear skin, and found that both types of extracts induced sensitization to a similar extent. Our data show that proteases like Der p 1 and Der p 9, which are present in our allergen extracts, are not required to induce proper sensitization leading to development of allergic airway inflammation. This was also confirmed by using an irreversible cysteine proteinase inhibitor which was previously used to also block the activity of Der p 1 and the cysteine proteinase papain in a model of epicutaneous sensitization⁶³.

Which allergens are responsible for HDM sensitization via the skin is currently unknown. Recent studies have shown the possibility to induce Th2 immunity to HDM allergens such as Der p1 and Der p2, which are contained in fecal pellets of the mites^{5, 53, 59}, but this was studied in the context of altered skin barrier function. Although many allergens are found in the fecal pellets of the HDM, it was also shown previously that sensitization via the skin in humans with atopic dermatitis leads to IgE reactivity to mite body allergens, not contained in the fecal pellets⁵. As the allergen used was whole HDM extract (including fecal pellets) it is possible that epicutaneous sensitization in mice also favours sensitization to mite body allergens, although a clear response to Der p 1 antigen was detected using TCR transgenic 1-*DER* T cells.

Underneath the skin barrier, DCs are lined up to scan for incoming allergens. The skin and skin-draining LN contain five major DC subsets^{29, 32}, whose function has mainly been studied after painting of irritative haptens to the skin, or injection antigens subcutaneously or intradermally. Through extension of dendritic processes, DCs, and especially Langerhans cells (LCs) can penetrate the epithelium until just below the stratum corneum without breaching the barrier function⁶⁴. In this way, LCs were shown to gain access to and capture antigens deposited on the skin. It was therefore very surprising that fluorescently labelled HDM applied on the skin was not found in LCs or in the different dermal cDC subsets

analyzed, but rather in macrophages localized in the dermis. In our study, a high dose of allergen was used and it is possible that such dose is able to reach cells in the dermis without the need for LCs. Another explanation might be that most LCs and dermal cDCs that would have taken up the allergen emigrated from the skin to reach the draining SCLN. However, 24 hours after HDM epicutaneous application, the major cell types that contained allergens in the ipsilateral SCLN were 2 subsets of cDC2 (CD11b⁺ and double negative DN cDC2) and almost no allergen-bearing LCs were present, which is consistent with a previous report showing that skin-derived cDCs migrate faster to the LNs than LCs^{38, 65, 66}. Whether cDC2 have captured the allergen in the skin or whether the allergen would have been transferred by other DC subsets and/or macrophages remains to be addressed, but in any case the CCR7 chemokine receptor was involved, implying a direct transport by DCs. Also, after skin administration of proteolytic HDM allergen extract, divisions were only seen in the ipsilateral skin draining lymph node, suggesting that there was no leakiness of skin applied antigen to the systemic circulation, in which case the spleen would show T cell divisions. This is in clear contrast to the lung, where the same dose of HDM extract does reach the systemic circulation and induce primary divisions in HDM-reactive splenocytes (data not shown). In the skin, the migration of DCs to the draining LNs is controlled by CCR7 responsiveness⁶⁷ and by the transcription factor IRF4, which is mainly expressed by cDC2s⁶⁸. Not surprisingly, the amount of cDC2s was strongly reduced in the SCLN of *Cd11cCre Irf4^{fl/fl}* mice that received HDM allergen. The underrepresentation of cDC1 in the SCLN 24-48h after allergen e.c exposure is best explained by the fact that these cells have a poor chemotactic response to CCR7 ligands⁶⁶.

A consequence of DC migration to draining LN is the activation of antigen-specific T cells. Our data clearly show that both cDC2 subsets sorted from SCLN 24h after HDM exposure were the only DCs capable of inducing Der p 1-specific T cell proliferation to some extent (Figure 5). The few LCs or cDC1 present in the SCLN at this time point were unable to do so. It was surprising to us that LCs failed to induce HDM-specific T cell proliferation, because in a model where the protein ovalbumin was applied on the skin of mice, T cell proliferation was blunted when LCs were depleted⁶⁹. A major difference with our model is that the authors shaved the skin of the mice before the application of the antigen, which might have facilitated the uptake and migration of LCs to the draining LN, through induction of the cytokine TSLP. As a consequence, Nakajima et al also found that LCs were responsible for Th2 epicutaneous sensitization to the antigen⁶⁹. In our model of epicutaneous sensitization to HDM, depletion all langerin⁺ cells (LCs and langerin⁺ dermal cDC1), at the time of HDM application, did not lead to a significant reduction in sensitization to aeroallergens, as read out by the similar degree of airway inflammation upon challenge of the lung with HDM extracts. However, we noticed that the amount of Th2 cytokines produced by draining mediastinal lymph node T

cells were increased in mice lacking langerin⁺ DCs, suggesting that in response to allergens, langerin⁺ DCs might have some regulatory role and keep immune responses in check as already shown in CHS models^{30, 70-73}. In our study, we have not fully excluded the involvement of langerin⁺ cDC1 in Th2 skin sensitization to HDM, as only langerin⁺ cDC1 were targeted in langerin-DTR mice. However, Batf3-dependent cDC1 have been reported to be a major source of IL-12, favouring Th1 immune responses, and in this way, they might prevent Th2 immunity from developing^{74, 75}. We have also previously demonstrated that langerin⁺ CD103⁺ cDC1 can drive the generation of regulatory T cells through the production of retinoic acid²⁸. It is therefore possible that the increased production of Th2 cytokines observed in langerin⁺ DC-depleted animals might be attributed to a lack of these regulatory cells (Figure 4).

We recently described that CD11b⁺ cDC2s are the most important subset inducing sensitization after application of HDM extracts in the lungs of adult and neonatal mice, whereas CD103⁺ cDC1s were not necessary^{17, 76}. When our epicutaneous HDM sensitization model was performed in *Flt3l*^{-/-} mice lacking cDCs, there was a strong reduction in the degree of BAL eosinophilia and Th2 cytokine production by SCLN cells, pointing to the fact that cDCs are crucial in the induction of Th2 responses to HDM applied on skin. One difference with the lung however, is that the same 100 µg of HDM applied to the lung, also caused sensitization in *Flt3l*^{-/-} mice, whereas a low dose of antigen could not cause sensitization in *Flt3l*^{-/-} mice. The explanation that we found in the lung experiments, was that the dose of antigen stimulating the lung epithelium was decisive in causing monocyte-derived cells (MCs) to also present antigen in the mediastinal nodes¹⁷. In the current epicutaneous model, we therefore believe that there is much less antigen penetration into the skin barrier with 100 µg of HDM extract, a finding supported by the idea that very little HDM-positive cells could be traced in the SCLN. Experiments performed in *CD11cCre Irf4*^{fl/fl} identified IRF4-dependent cDC2 as the main inducers of skin sensitization to HDM. Our data are in agreements with other studies showing that IRF4-expressing DCs could control Th2 responses to allergens in the lung and to papain injected into the skin^{19, 34, 36} because IRF4 controls cDC2 migration to SCLN⁶⁸, the permeability and fibrosis of afferent lymphatics⁷⁷ and the expression of costimulatory molecules and MHCII on this subset of DCs⁷⁸. Within the IRF4-dependent skin cDC2 comprising CD11b⁺ cDC2 and DN (double negative) cDC2, the pro-Th2 potential was recently attributed solely to the Klf4-dependent DN subset³⁶. However, when we sorted both cDC2 subsets from SCLN of HDM e.c.-exposed mice and adoptively transferred them intradermally into naïve recipients, we found that both subsets of cDC2 could promote Th2 sensitization to HDM. Although the idea of a specialized subset of Klf4⁺ cDC2 restricted to the induction of Th2 responses is appealing, our data suggest that the

type of antigen encountered together with environmental cues may give some plasticity to Klf4⁺ cDCs to also drive Th2 responses. Within the population of IRF4-dependent DCs, there is also a subset of cells expressing the C-type lectin Mgl2, which was also shown to promote Th2 responses to the model allergen papain. Recently, this subset was also shown to suppress humoral immune responses and formation of T follicular helper (T_{FH}) cells to epicutaneous HDM allergen⁷⁹. Although this would suggest a tolerogenic role for Mgl2⁺ cDC2s, it is also possible that induction of T_{FH} cells and effector or resident memory Th2 cells are mutually exclusive fates of naïve allergen reactive T cells, and that suppression of T_{FH} formation inevitably leads to increased Th2 immunity. Such a model was recently proposed by different groups studying HDM-reactive T cell differentiation in B cell deficient and Bcl6-deficient mice that also lack T_{FH} formation^{80, 81}.

The fact that murine DN cDC2 can induce Th2 responses to allergens in the skin brings up the question of translation of these findings in humans, since no counterparts for this murine subset has been identified in human skin^{22, 29}. In our hands however, IRF4-dependent CD11b⁺ cDC2 can also induce Th2 sensitization to HDM via the skin. In humans, CD1c⁺ DCs are the counterpart of this mouse population²⁹ and have been shown to activate Th2 responses to inhaled allergens ex vivo⁸². Whether this human subset is involved in the induction of Th2 responses in the skin remains to be addressed.

In conclusion, we have shown that sensitization to relevant inhaled allergens like HDM can occur through intact skin, and does not require the protease activity of the allergen. This type of epicutaneous sensitization is driven by two variants of IRF4-dependent cDC2 subsets, not by Langerhans cells. Future studies will have to reveal how genetic or inflammation induced alterations in skin barrier proteins (like the well known filaggrin polymorphism) or cytokines (like the *TSLP* gene polymorphism) in patients at risk for developing asthma could interfere with this basic mechanism of DC driven type 2 immunity.

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Figure legends

Figure 1. HDM sensitization via the skin does not depend on barrier disruption or proteolytic activity of the allergen

(A) Protocol of allergic airway inflammation induced by epicutaneous sensitization to HDM. (B) Differential cell counts in BAL fluids. (C) Serum levels of HDM-specific IgE. (D) Cytokine production by MLN cells. (E) Siglec F (left) and Muc5ac (right) confocal staining in lungs. DAPI, nucleus counterstaining. (F) Transepidermal water loss after overnight tape occlusion (tape ON) or acute tape stripping. (G) Frequency of neutrophils and DCs in SCLN after overnight tape occlusion. (H) Differential cells counts in BAL fluids after e.c HDM application in the absence of tape occlusion. (I) Effect of the enzymatic activity of HDM on cell counts in BAL fluids. (J) Cytokine production by MLN cells. Data represent means $\pm$ SE, n = 5-10 mice/group, *p< 0.05. O.D; optical density.

Figure 2. Sensitization via the skin occurs only in the skin-draining lymph node and this is der p 1 specific

(A) Proliferation (CFSE dilution) and activation (CD44 expression) of 1-Der T-cells in draining (SCLN) and non-draining LNs, 4 days after e.c. HDM application. (B) Percentage of cells in division and of (C) CD44⁺ cells in draining SCLN and non-draining LN (ILN) 4 or 7 days after e.c. application of HDM or PBS. (D) Cytokine production by SCLN cells. (E-G) The same protocol as in Figure 2A-D was repeated using Rag^{-/-} '1der' CD4⁺ T-cells. Data are representative of four independent experiments, means $\pm$ SE, n = 3-6, *p< 0.05.

Figure 3. Gating strategy of DC subsets in the skin and skin-draining lymph node

(A) Gating strategy of DC subsets in the skin of naïve C57Bl/6 mice. (B) Gating strategy of DC subsets in the skin-draining LN (Superficial Cervical LN, SCLN) 24 hours after e.c. treatment with HDM. (C) Expression of different markers in skin DC subsets. (D) Expression of different markers in migratory DC subsets from SCLN.

Figure 4. Epicutaneous application of HDM induces the migration of DCs to the draining LN

(A) Kinetics of DC migration in SCLN after e.c. application of HDM. (B) Uptake of fluorescent HDM by skin CD45⁺ cells and (C) Number of total skin macrophages (MFs) and DC subsets 4 hours after PBS (white bars) and HDM (grey bars) e.c application. (D) Number of HDM-AF647⁺ cells in the skin 4 hours after e.c. application of HDM-AF647. (E) Uptake of fluorescent HDM by CD45⁺ SCLN cells and (F) Number of total DC subsets in the SCLN 48 hours after e.c. application of PBS or HDM-AF647. (G) Number of HDM-AF647⁺ cells in

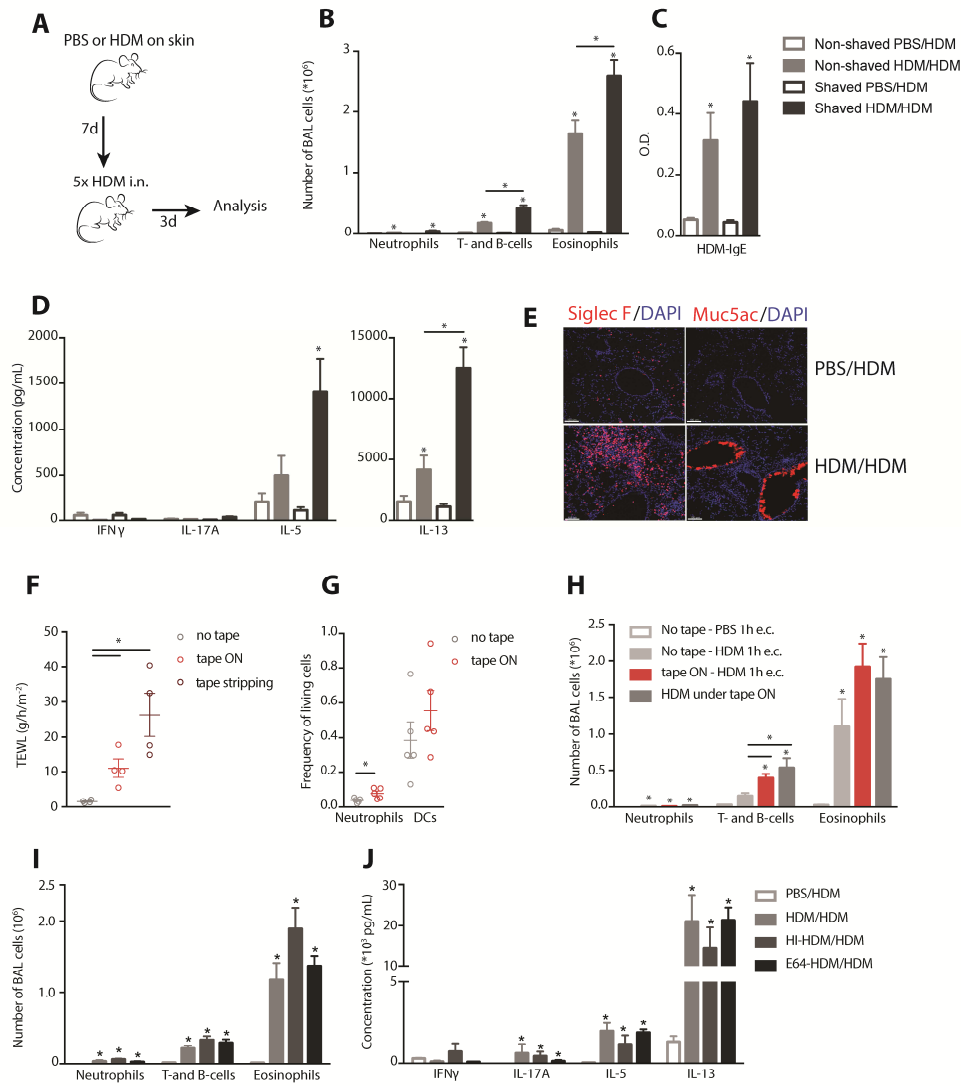
the SCLN 2 days after e.c. application of PBS or HDM-AF647. Data are representative of at least two independent experiments, means $\pm$ SE, n = 6, *p < 0.05

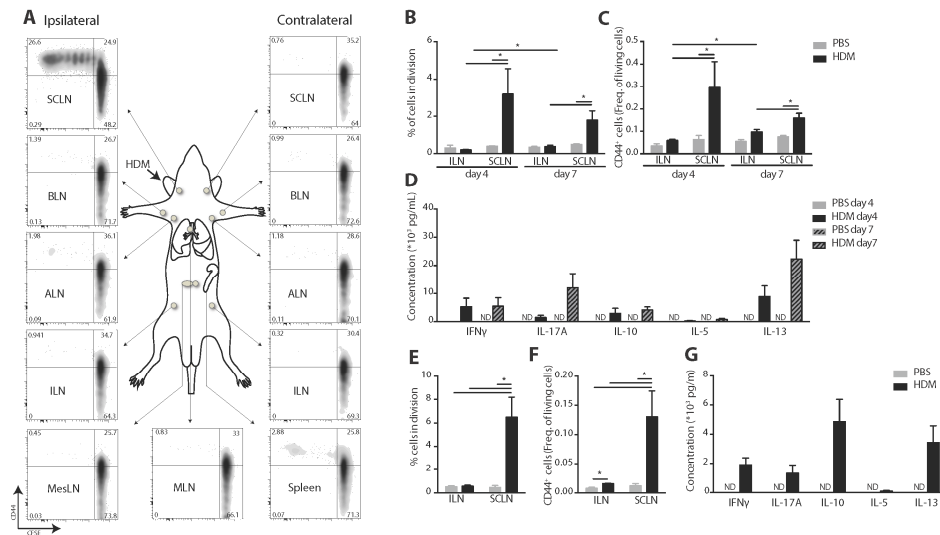
Figure 5. Allergic airway inflammation induced by skin sensitization depends on cDC2s.

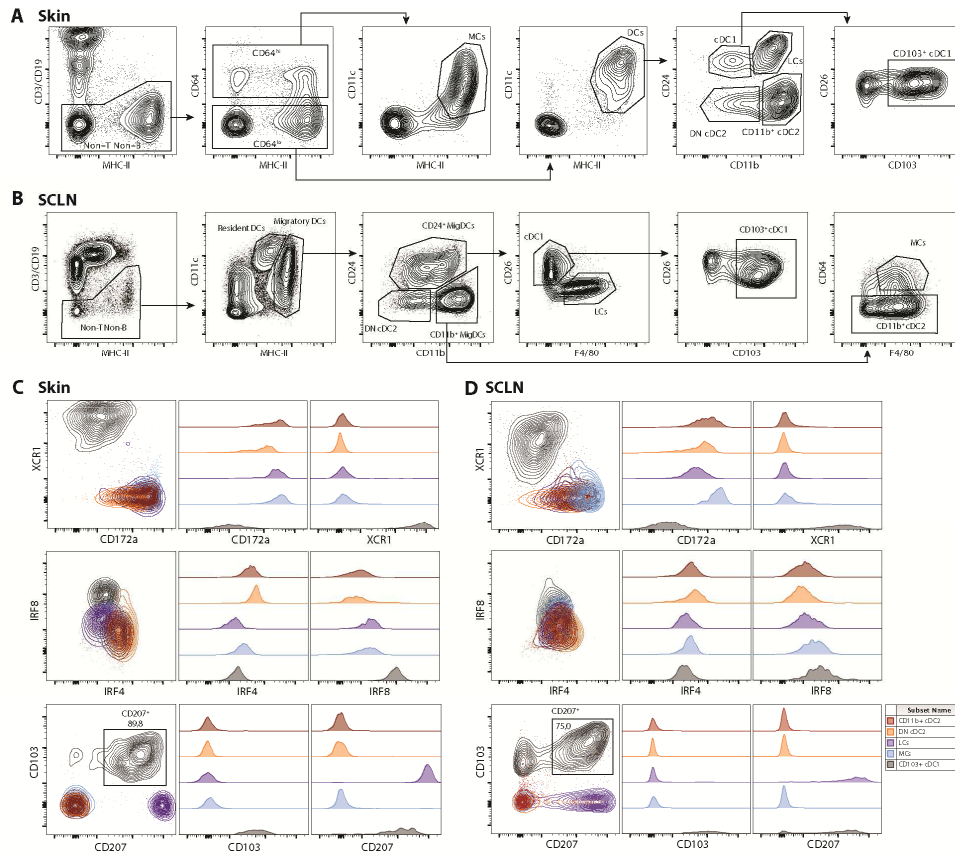
(A, D and G) Number of migratory DC subsets in the SCLN of *Flt3*^{-/-}, *Langerin*-DTR and *Irf4* ^{Δ/Δ} *Cd11c*-Cre mice, respectively, in steady state conditions. (B, E and H) Differential cell counts in BAL fluids and (C, F and I) cytokine production of MLN cells. Data are representative of at least two independent experiments, means $\pm$ SE, n = 5-6, *p < 0.05

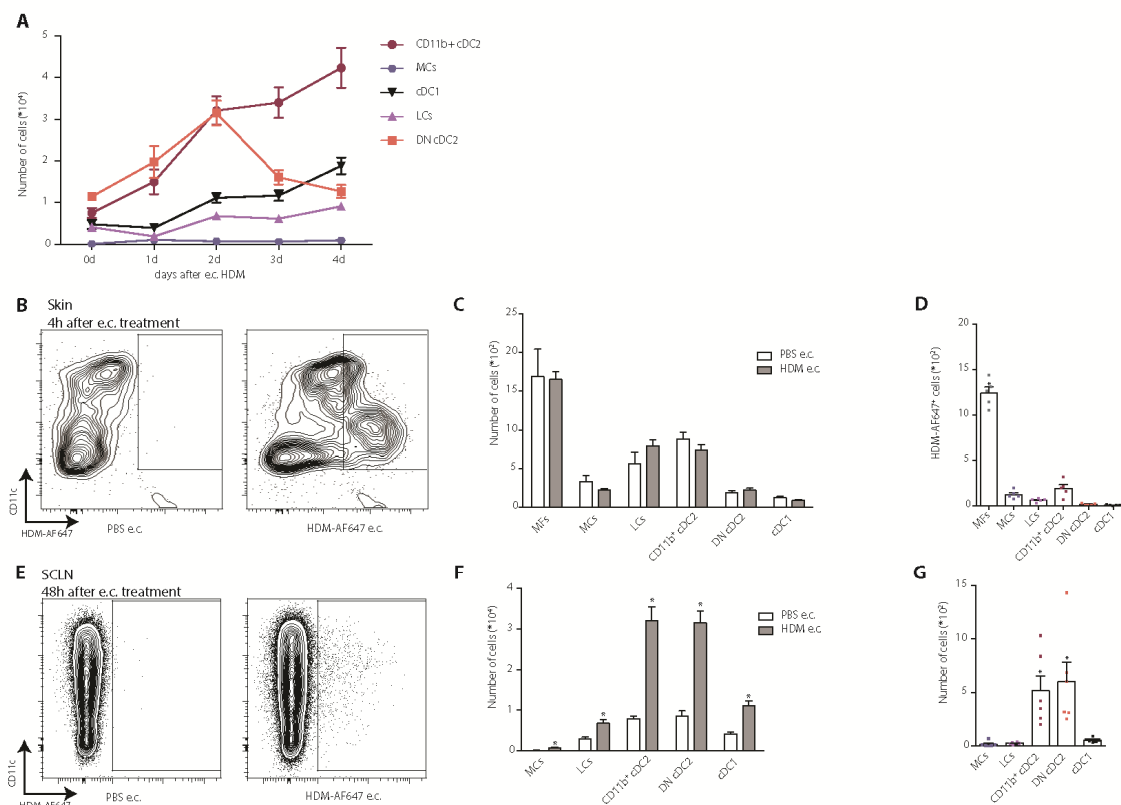
Figure 6. IRF4-dependent cDC2 are sufficient to induce allergic airway inflammation to HDM

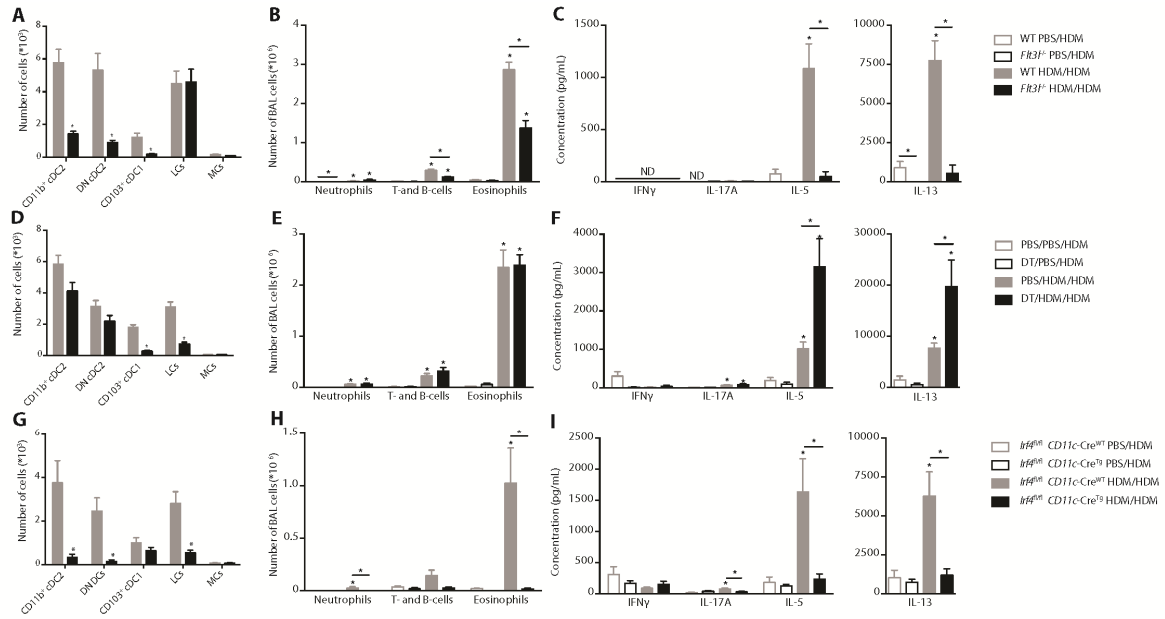
(A) Experimental setup. (B) Proliferation and CD44 expression of '1-DER' CD4⁺ T-cells cocultured *ex vivo* with sorted HDM⁺ DC subsets. (C) Differential cell counts in BAL and (D) cytokine production of MLN cells from mice injected intradermally with HDM⁺ DC subsets. (B) Data are representative of at least four independent experiments. (C-D) Data from two independent experiments have been pooled, means $\pm$ SE, n = 4-12, *p < 0.05

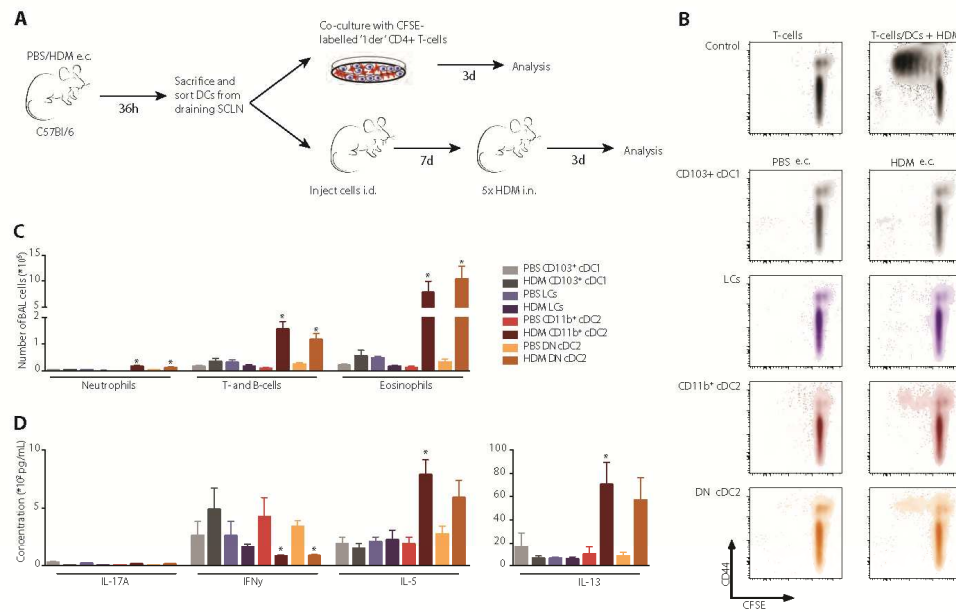


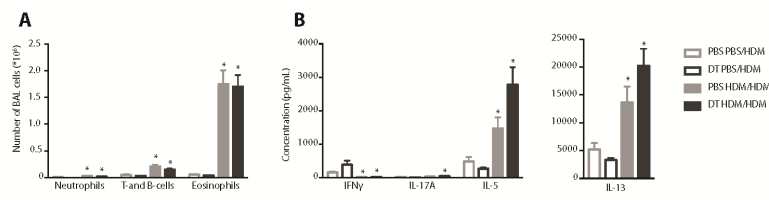


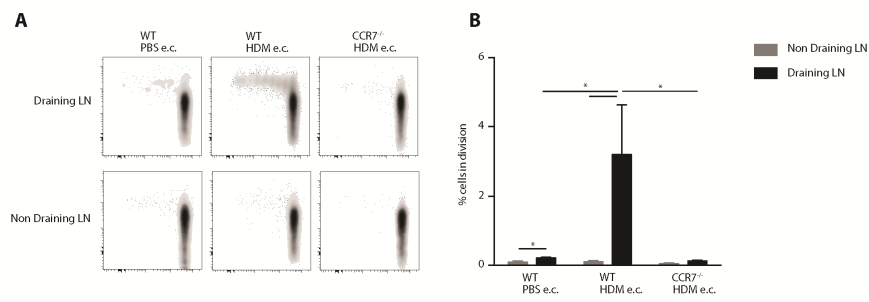












Supplementary Figures**Supplementary Figure 1. Sensitization via the skin requires CCR7-dependent active transport of HDM to the draining lymph node**

CFSE-labeled WT '1der' CD4⁺ T cells were injected intravenously in C57Bl/6 mice applied with HDM or PBS on the ear skin. (A) Proliferation of injected 1-Der T cells in draining (ipsilateral SCLN) and non-draining LNs (contralateral ILN), 4 days after e.c. application of HDM extract or PBS control. (B) Percentage of cells in division. Data are representative of two independent experiments, means $\pm$ SE, n = 6-7, *p < 0.05.

Supplementary Figure 2: Langerhans cells are not involved in Th2 sensitization to epicutaneously-applied HDM.

Langerin-DTR mice and WT counterparts were injected i.p with 500 ng diphtheria toxin (DT) or PBS on day 0. On day 7, mice were sensitized with HDM or PBS e.c, and were challenged with HDM intranasally on days 14-18. (A) Differential cell counts in BAL fluids and (B) cytokine production of MLN cells restimulated with HDM for 3 days *ex vivo*. Mean $\pm$ SE, n = 5-6, *p < 0.05