

Dectin-2 Promotes House Dust Mite–Induced T Helper Type 2 and Type 17 Cell Differentiation and Allergic Airway Inflammation in Mice

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Abstract

The fact that sensitization against fungi is closely related to the severity of asthma suggests that immune systems recognizing fungi are involved in the pathogenesis of severe asthma. Recently, Dectin-2 (gene symbol, *Clec4n*), a C-type lectin receptor, has been shown to function as not only a major pattern-recognition receptor for fungi, but also a receptor for some components of house dust mite (HDM) extract, a major allergen for asthma. However, the roles of Dectin-2 in the induction of HDM-induced allergic airway inflammation remain largely unknown. Our objective was to determine the roles of Dectin-2 in HDM-induced allergic airway inflammation. We examined the roles of Dectin-2 in the induction of HDM-induced T helper (Th) 2 and Th17 cell differentiation and subsequent allergic airway inflammation by using *Clec4n*-deficient (*Clec4n*^{−/−}) mice. We also investigated Dectin-2-expressing cells in the lung and their roles in HDM-induced allergic airway inflammation. *Clec4n*^{−/−} mice showed significantly attenuated HDM-induced allergic airway inflammation and decreased Th2 and Th17 cell differentiation. Dectin-2 mRNA, together with Dectin-3 and Fc receptor-γ mRNAs, was expressed in CD11b⁺ dendritic cells (DCs),

but not in CD4⁺ T cells or epithelial cells in the lung. CD11b⁺ DCs isolated from *Clec4n*^{−/−} mice expressed lower amounts of proinflammatory cytokines and costimulatory molecules, which could lead to Th2 and Th17 cell differentiation than those from wild-type mice. HDM-pulsed *Clec4n*^{−/−} DCs were less efficient for the induction of allergic airway inflammation than HDM-pulsed wild-type DCs. In conclusion, Dectin-2 expressed on CD11b⁺ DCs promotes HDM-induced Th2 and Th17 cell differentiation and allergic airway inflammation.

Keywords: Dectin-2; house dust mite; CD11b⁺ dendritic cells; asthma

Clinical Relevance

We show here that Dectin-2 signaling enhances house dust mite–induced differentiation of T helper (Th) 2 cells and Th17 cells, and promotes the induction of allergic airway inflammation. These results suggest that Dectin-2 could be a novel therapeutic target for asthma.

Asthma is a complex human disease characterized by allergic airway inflammation and airway hyperresponsiveness (AHR). It is generally believed that these characteristics are mainly mediated by

antigen-specific T helper (Th) 2 cells, because elevated amounts of Th2 cytokines, such as IL-4, IL-5, and IL-13, and increased numbers of eosinophils are often observed in the airways of patients

with asthma (1, 2). In addition to eosinophils, it has been demonstrated that the number of neutrophils is increased in the airways in severe asthma (3–5), suggesting that neutrophilic inflammation

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is involved in the pathogenesis of severe asthma (5).

Neutrophilic inflammation is a hallmark of Th17-type immune response. Consistently, the amounts of IL-17A in bronchoalveolar lavage fluid (BALF) have been reported to be positively correlated with the severity of asthma (6, 7). In addition, we have shown that IL-23, one of Th17 cell-promoting cytokines, is expressed in the lung on antigen inhalation, and that IL-23 not only induces Th17 cell differentiation, but also enhances Th2 cell-dependent eosinophilic airway inflammation (8). Although these findings suggest that IL-23 and Th17 cells play important roles in the development of severe asthma, molecular mechanisms underlying the induction of IL-23 production and subsequent Th17 cell differentiation in the airways are still largely unknown.

A series of epidemiological and clinical studies suggest a link between fungal sensitization and severe asthma (9, 10). The link between immune responses against fungi and severe asthma was further proposed by the association of chronic fungal infection with asthma, and the improvement of asthma symptoms with antifungal treatment (11). In addition, recent studies have revealed that Dectin-2, a C-type lectin receptor expressed on a variety of myeloid cells, including dendritic cells (DCs), macrophages, and inflammatory monocytes (12), recognizes a fungal cell wall α -mannan and plays a major role in the induction of immune responses against fungi (13). Moreover, Dectin-2, but not Dectin-1, has been shown to be a major pattern-recognition receptor (PRR) for IL-23 induction and the development of Th17 cells (13–15). Importantly, it has been demonstrated that Dectin-2 recognizes an unidentified glycoprotein contained in house dust mite (HDM) allergen, and plays a key role in the development of HDM-induced allergic airway inflammation by activating cysteinyl leukotriene pathways (16–18). These results suggest that Dectin-2 could be a key molecule that causes severe asthma.

In this study, we analyzed the role of Dectin-2 in HDM-induced asthmatic responses by using *Clec4n*-deficient (*Clec4n*^{-/-}) mice, and found that *Clec4n*^{-/-} mice exhibited reduced amounts of HDM-induced Th2 and Th17 cell differentiation and airway inflammation.

We also found that Dectin-2 played an important role in HDM-induced activation of CD11b⁺ DCs, and that Dectin-2 expressed on DCs was crucial for HDM-induced Th2 and Th17 cell differentiation. Our results indicate the importance of Dectin-2 expressed on CD11b⁺ DCs in the induction of HDM-induced allergic airway inflammation.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories (Kanagawa, Japan). *Clec4n*^{-/-} mice on a C57BL/6 background were described previously (13). All mice were housed in microisolator cages under specific pathogen-free conditions, and all experiments were performed according to the guidelines of Chiba University (Chiba, Japan).

HDM-Induced Allergic Airway Inflammation in Mice

Mice were sensitized and challenged with intratracheal administration of HDM extracts (*Dermatophagoides pteronyssinus*, lot no. 189257; Greer Laboratories, Lenoir, NC) as described previously with minor modification (19) (see the online supplement for details).

Cytokine Assay

The amounts of IL-1 β , IL-5, IL-6, IL-13, IL-17, IL-23, and IFN- γ were measured by ELISA according to the manufacturer's instructions (see the online supplement for details).

Measurement of HDM-Specific Antibody

HDM-specific immunoglobulin G1 (IgG1) was measured as described elsewhere (20).

Cytokine Production of CD4⁺ T Cells

Single-cell suspensions were prepared from mediastinal lymph nodes (MLNs) of HDM-sensitized and -challenged *Clec4n*^{-/-} mice and wild-type (WT) C57BL/6 mice 48 hours after the last HDM challenge. Immediately after the preparation, these cells were stimulated with phorbol 12-myristate 13-acetate (20 ng/ml; Calbiochem, San Diego, CA) plus ionomycin (1 μ g/ml; Calbiochem) at 37°C for 4 hours in the presence of brefeldin A (10 μ mol/L; BD Bioscience, San Diego,

CA). Cytokine profiles (IFN- γ , IL-13, IL-17A, granulocyte/macrophage colony-stimulating factor [GM-CSF]) of CD4⁺ T cells were evaluated by flow cytometry, as described previously (21).

Histological Analysis

Lung sections (3 μ m thick) were stained with hematoxylin-eosin and periodic acid-Schiff (PAS) according to standard protocols. The number of goblet cells was counted on PAS-stained lung sections, as described elsewhere (22).

Measurement of AHR

Airway responsiveness to aerosolized acetylcholine was assessed by using a computer-controlled small-animal ventilator system (flexiVent; SCIREQ, Inc., Montreal, PQ, Canada), as described elsewhere (23).

FACS Analysis of Lung DCs

Lung cell suspensions were prepared and analyzed as described previously (24) (see the online supplement for details).

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was performed with a standard protocol on an ABI PRISM 7,300 instrument (Applied Biosystems, Foster City, CA; see the online supplement for details).

Preparation of Bone Marrow-Derived DCs

Bone marrow-derived DCs (BMDCs) were prepared as described previously (25) (see the online supplement for details).

Statistical Analysis

Data are summarized as means ($\pm$ SEM). The statistical analysis of the results was performed by the unpaired *t* test. *P* values less than 0.05 were considered significant.

Results

Dectin-2 Plays a Crucial Role in the Induction of HDM-Induced Allergic Airway Inflammation

Dectin-2, a C-type lectin receptor that plays an essential role in the development of immune responses against fungi (13), has been shown to recognize some unknown glycoprotein contained in mite allergen (16). In addition, recent studies using

lentivirus-mediated knockdown (17) and antibody-mediated neutralization (18) suggest that Dectin-2 is involved in the induction of HDM-induced allergic airway inflammation. To address the roles of Dectin-2 in the induction of allergic airway inflammation and its underlying mechanisms more precisely, we first examined HDM-induced allergic airway inflammation in *Clec4n*^{-/-} mice. *Clec4n*^{-/-} mice and WT mice were sensitized and challenged intratracheally with HDM or PBS (as controls) and 48 hours after the last HDM administration, inflammatory cell infiltration into BALF was evaluated. Consistent with a previous report (19), repeated administration of HDM into the airways induced lymphocyte and eosinophil infiltration into the BALF (Figure 1A). The numbers of lymphocytes and eosinophils in the BALF were significantly decreased in *Clec4n*^{-/-} mice as compared with those in WT mice (Figure 1A). Although the difference did not reach statistical significance, the number of neutrophils in the BALF tended to be decreased in *Clec4n*^{-/-} mice (Figure 1A). FACS analysis of BALF cells revealed that more than 70% of lymphocytes in the BALF were CD4⁺ T cells, and that HDM-induced CD4⁺ T cell recruitment in the BALF was significantly decreased in *Clec4n*^{-/-} mice (Figure 1A). Histological analysis of the lung showed that HDM-induced peribronchial and perivascular inflammation, which is mainly composed of eosinophils and lymphocytes, was significantly diminished in *Clec4n*^{-/-} mice (Figure 1B). PAS staining of the lung sections revealed that goblet cell hyperplasia was also significantly attenuated in *Clec4n*^{-/-} mice as compared with that in WT mice ($n = 4$, $P < 0.05$; Figure 1B, right panel). In addition, serum amounts of HDM-specific IgG1 antibody were significantly decreased in *Clec4n*^{-/-} mice as compared with those in WT mice (Figure 1C), whereas HDM-specific IgG2c and immunoglobulin E were below the detection limit in WT and *Clec4n*^{-/-} mice (data not shown). Consistent with the diminished airway inflammation in *Clec4n*^{-/-} mice, AHR to acetylcholine was significantly decreased in *Clec4n*^{-/-} mice (Figure 1D). These results indicate that Dectin-2 signaling enhances the induction of eosinophil and CD4⁺ T cell recruitment into the airways, goblet cell hyperplasia, IgG1 production, and AHR on HDM stimulation.

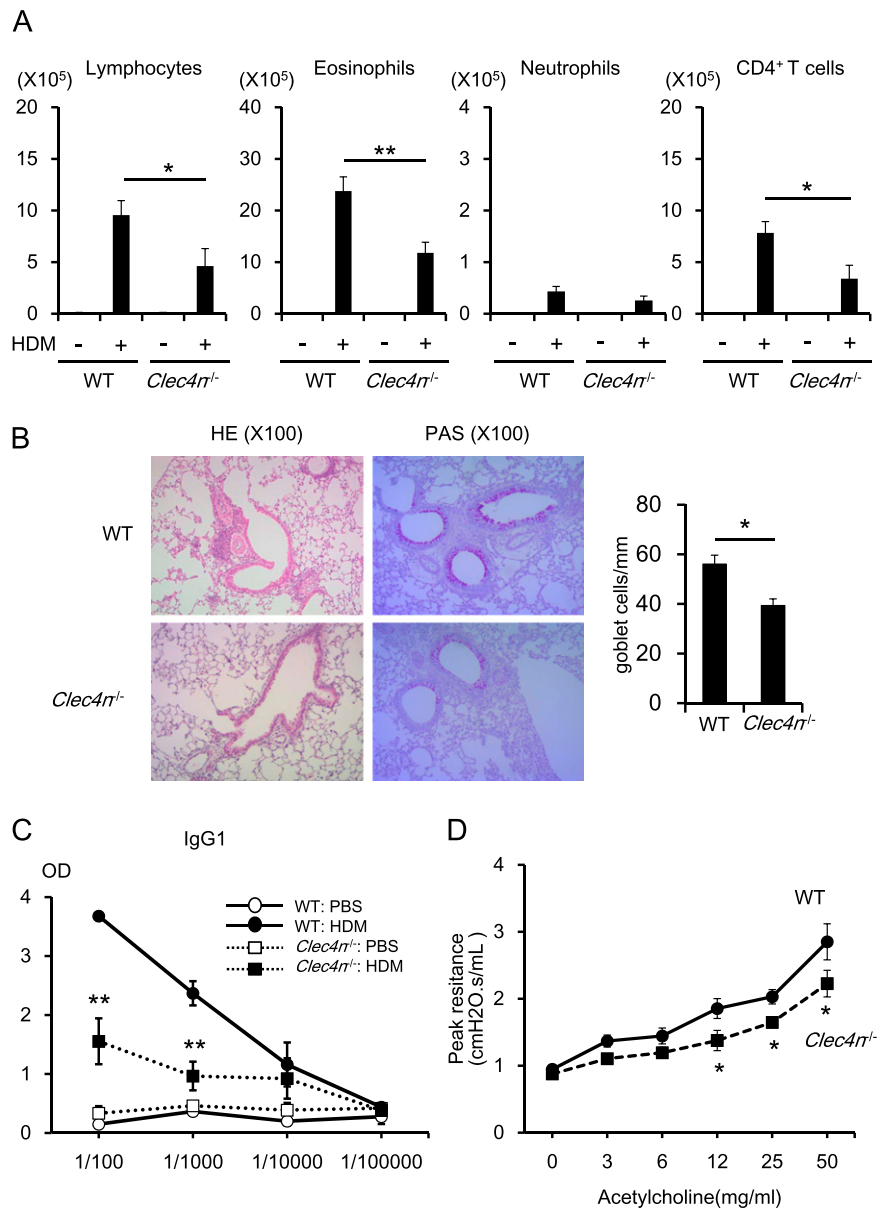


Figure 1. Dectin-2 plays a crucial role in the induction of house dust mite (HDM)-induced allergic airway inflammation. *Clec4n*-deficient (*Clec4n*^{-/-}) and wild-type (WT) mice were sensitized and challenged with HDM or PBS (as a control). (A) The numbers of lymphocytes, eosinophils, neutrophils, and CD4⁺ cells in the bronchoalveolar lavage fluid (BALF) were evaluated at 48 hours after the last HDM challenge. Data are means $\pm$ SEM for 10 mice in each group. * $P < 0.05$, ** $P < 0.01$. (B) Representative photomicrographs (hematoxylin-eosin [HE] and periodic acid-Schiff [PAS] staining) of lung sections and the numbers of goblet cells in the airways (left panels) at 48 hours after the last HDM challenge are shown. Data are means $\pm$ SEM for four mice in each group. * $P < 0.05$. (C) Serum amounts of HDM-specific immunoglobulin G1 (IgG1) are shown. Data are means $\pm$ SEM for five mice in each group. ** $P < 0.01$. (D) Airway resistance to acetylcholine was measured at 48 hours after the last HDM challenge. Data are means $\pm$ SEM for six mice in each group. * $P < 0.05$.

Dectin-2 Is Involved in Th2 and Th17 Cell Development during HDM-Induced Allergic Airway Inflammation

To examine the basis for Dectin-2-mediated enhancement of HDM-induced allergic

airway inflammation, we next examined cytokine profiles of MLN cells from HDM-sensitized and -challenged WT and *Clec4n*^{-/-} mice. Single-cell suspensions of MLN cells from HDM-sensitized

and -challenged mice were restimulated with HDM for 5 days, and cytokine amounts in the culture supernatants were evaluated by ELISA. As shown in Figure 2A, MLN cells from HDM-sensitized and -challenged *Clec4n*^{-/-} mice produced significantly less IL-5 and IL-13 than those from WT mice. MLN cells from *Clec4n*^{-/-} mice also produced significantly lower amounts of IL-17A than those from WT mice (Figure 2A). In contrast, although the differences did not reach statistical significance ($P = 0.057$), the amounts of IFN- γ tended to be higher in *Clec4n*^{-/-} mice (Figure 2A). Intracellular cytokine analysis showed that freshly isolated MLN CD4⁺ T cells from *Clec4n*^{-/-} mice produced lower amounts of IL-13 and higher amounts of IFN- γ than those from WT mice (Figure 2B). Furthermore, freshly isolated MLN CD4⁺ T cells from *Clec4n*^{-/-} mice produced less IL-17A and GM-CSF, recently identified as another effector cytokine produced from Th17 cells (26, 27). Absolute numbers of IL-13⁺ CD4⁺ T cells and IL-17A⁺ CD4⁺ T cells in MLNs were also significantly decreased in *Clec4n*^{-/-} mice (Figure 2C). These results suggest that Dectin-2 is involved in the development of Th17 and Th2 cells in HDM-induced allergic airway inflammation.

Dectin-2 Receptor Complex Is Expressed in CD11b⁺ DCs in HDM-Induced Allergic Airway Inflammation

To further address the mechanisms by which Dectin-2 enhances HDM-induced allergic airway inflammation, we next searched for Dectin-2-expressing cells in the lung. Because Dectin-2 needs Fc receptor- γ (FcR γ) for its surface expression and signal transduction (13, 14), and because Dectin-2 forms a heterodimeric complex with Dectin-3 for efficient ligand recognition (28), the expression of FcR γ and Dectin-3 was evaluated in parallel. Due to the lack of anti-Dectin-2 antibody applicable for FACS analysis, we examined mRNA expression of Dectin-2 in isolated cell populations from the lung in HDM-induced allergic airway inflammation. Because Dectin-2 has been identified as a DC-associated C-type lectin (29), we examined the expression of Dectin-2 in CD11b⁺ DCs and CD103⁺ DCs, two major DC subpopulations in the lung (32). Importantly, Dectin-2 mRNA was highly expressed in CD11b⁺ DCs as compared

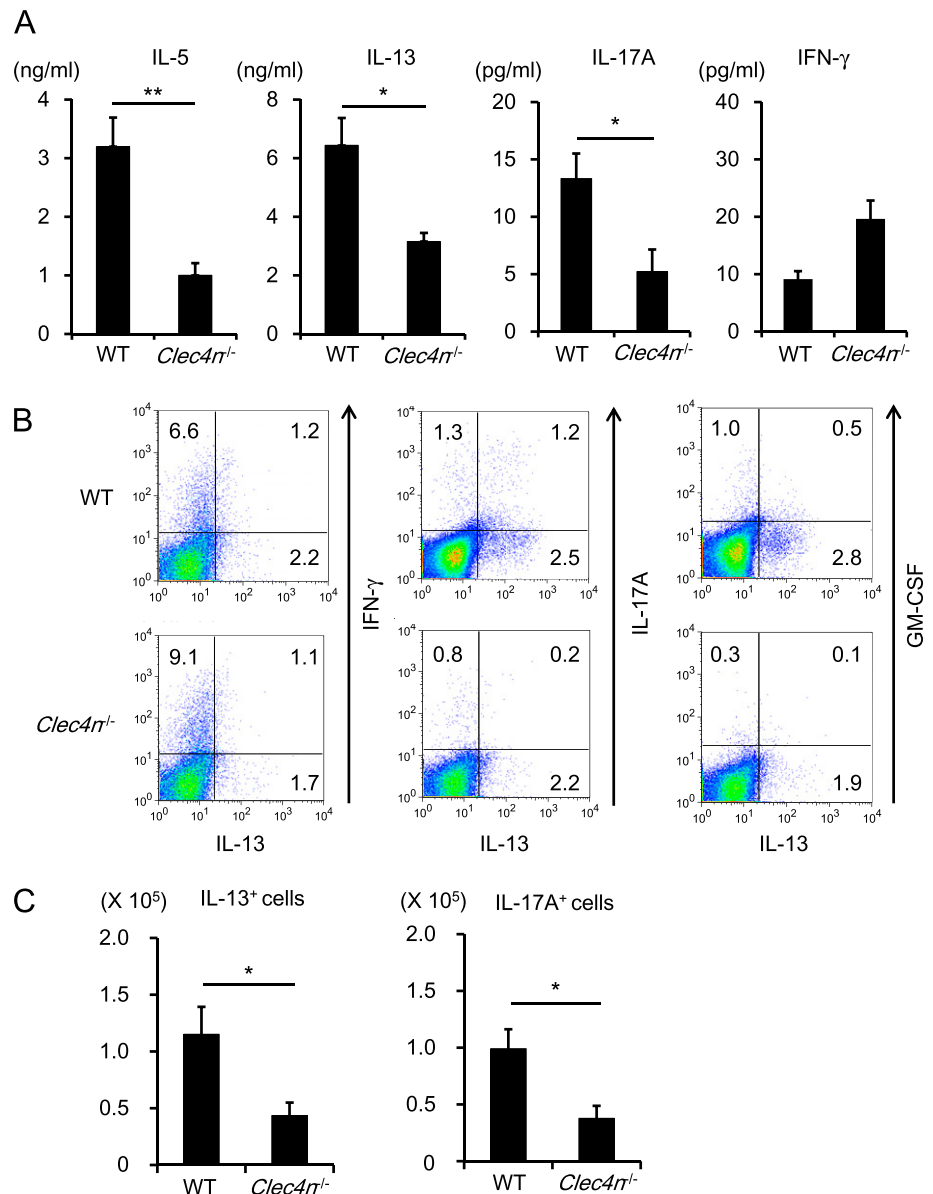


Figure 2. Dectin-2 is involved in T helper (Th) 2 and Th17 cell differentiation in HDM-induced allergic airway inflammation. (A) Mediastinal lymph node (MLN) cells were harvested from HDM-sensitized and -challenged *Clec4n*^{-/-} and WT mice, and were stimulated with HDM for 5 days. The amounts of IL-5, IL-13, IL-17A, and IFN- γ in the culture supernatants were examined. Data are means $\pm$ SEM for five mice in each group. * $P < 0.05$, ** $P < 0.01$. (B and C) Single-cell suspensions of MLN cells from HDM-sensitized and -challenged *Clec4n*^{-/-} and WT mice were stimulated with PMA plus ionomycin for 4 hours. Shown are representative FACS profiles of intracellular IL-13 together with IFN- γ , IL-17A, and granulocyte/macrophage colony-stimulating factor (GM-CSF) (B), and means $\pm$ SEM of absolute numbers of IL-13⁺ CD4⁺ cells and IL-17A⁺ CD4⁺ cells (C) ($n = 5$ mice in each group). * $P < 0.05$.

with that in CD103⁺ DCs (Figure 3). FcR γ and Dectin-3 were also highly expressed in CD11b⁺ DCs (Figure 3). Consistent with previous reports (12, 18), Dectin-2 was expressed in alveolar macrophages (Figure 3), but the expression levels of Dectin-3 in alveolar macrophages were lower than those in CD11b⁺ DCs

(Figure 3). On the other hand, Dectin-2, Dectin-3, and FcR γ mRNAs were not significantly expressed in CD4⁺ T cells or lung epithelial cells (Figure 3). These results suggest that Dectin-2 receptor complex is mainly expressed on CD11b⁺ DCs in HDM-induced allergic airway inflammation.

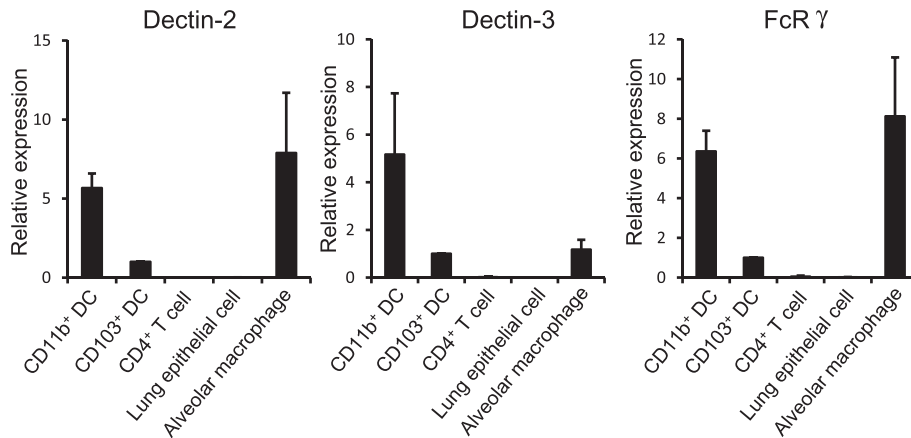


Figure 3. Dectin-2 receptor complex is expressed on lung CD11b⁺ dendritic cells (DCs) in HDM-induced allergic inflammation. CD11b⁺ DCs, CD103⁺ DCs, CD4⁺ T cells, lung epithelial cells, and alveolar macrophages were isolated from the lung of HDM-sensitized and -challenged WT mice at 48 hours after the last HDM challenge. The expression levels of Dectin-2, Dectin-3, and Fc receptor-γ (FcRγ) were analyzed by quantitative real-time PCR (qPCR). mRNA expression of each gene was normalized to that of β-actin and then expressed as the relative expression level to CD103⁺ DCs. Data are means ± SEM for three independent experiments.

Dectin-2 Is Crucial for the Activation of CD11b⁺ DCs in HDM-Induced Allergic Airway Inflammation

We next examined the roles of Dectin-2 in HDM-induced cytokine production from DCs. BMDCs from WT and *Clec4n*^{-/-} mice were activated with HDM, and the amounts of cytokines in the culture supernatants were evaluated. As shown in Figure 4A, HDM-induced production of inflammatory cytokines, such as IL-1β, IL-6, and IL-23, was significantly decreased in *Clec4n*^{-/-} BMDCs as compared with that in WT BMDCs.

We next examined cytokine expression in lung DCs in HDM-induced allergic inflammation. CD11b⁺ DCs and CD103⁺ DCs were isolated from the lung of HDM-sensitized and -challenged WT and *Clec4n*^{-/-} mice at 48 hours after the last HDM challenge, and then mRNA expression of cytokines was analyzed by quantitative real-time PCR. Consistent with a previous study (30), CD11b⁺ DCs expressed higher amounts of IL-6 and IL-23p19 mRNAs, but lower amounts of IL-12p40 than CD103⁺ DCs in WT mice (Figure 4B). Importantly, CD11b⁺ DCs from *Clec4n*^{-/-} mice expressed significantly lower amounts of IL-6 and IL-23p19 mRNAs than those from WT mice (Figure 4B). CD11b⁺ DCs from *Clec4n*^{-/-} mice also expressed significantly lower amounts of OX40L (Figures 4C and 4D), a representative costimulatory molecule that is essential for the development of

HDM-induced allergic airway inflammation (31). Moreover, the expression levels of other costimulatory molecules, such as CD40, CD80, and CD86, were also significantly attenuated in *Clec4n*^{-/-} CD11b⁺ DCs (Figures 4C and 4D). These results suggest that Dectin-2 is crucial for the production of inflammatory cytokines and the expression of costimulatory molecules in CD11b⁺ DCs in HDM-induced allergic airway inflammation.

Dectin-2 Expressed on DCs Is Crucial for the Induction of HDM-Induced Allergic Airway Inflammation

Finally, we evaluated the *in vivo* roles of Dectin-2 expressed on DCs in the induction of HDM-induced allergic airway inflammation by BMDC transfer experiments. HDM-pulsed BMDCs from WT or *Clec4n*^{-/-} mice were transferred to *Clec4n*^{-/-} or WT mice intratracheally and, 12 days after the transfer, the mice were challenged with HDM intratracheally, daily, for 3 consecutive days. As shown in Figure 5A, HDM-induced lymphocyte and eosinophil recruitment into the airways was significantly attenuated in *Clec4n*^{-/-} mice that received *Clec4n*^{-/-} BMDCs as compared with that in *Clec4n*^{-/-} mice that received WT BMDCs. Histological analysis of the lung showed that peribronchial and perivascular inflammation was diminished in *Clec4n*^{-/-} mice received *Clec4n*^{-/-} BMDCs (Figure 5B). FACS analysis

demonstrated that MLN CD4⁺ T cells from *Clec4n*^{-/-} mice that received *Clec4n*^{-/-} BMDCs produced less IL-13, IL-17A, and GM-CSF than those from *Clec4n*^{-/-} mice that received WT BMDCs (Figure 5C). MLN cells from *Clec4n*^{-/-} mice that received *Clec4n*^{-/-} BMDCs produced significantly less IL-5, IL-13, and IL-17A as compared with those from *Clec4n*^{-/-} mice that received WT BMDCs (Figure 5D). Similar, but less dramatic, results were obtained when WT mice were used as recipient mice (see Figure E1 in the online supplement), presumably due to the roles of Dectin-2 during the challenge phase of HDM-induced allergic airway inflammation. These results indicate that Dectin-2 expressed on DCs plays a critical role in the development of HDM-induced differentiation of Th2 and Th17 cells and subsequent allergic airway inflammation.

Discussion

Activation of PRRs on DCs is crucial for the induction of appropriate acquired immune responses. In this study, we show that Dectin-2 plays an important role in HDM-induced Th2 and Th17 cell differentiation and the induction of allergic airway inflammation (Figures 1 and 2). We also show that functional Dectin-2 receptor complex is expressed on CD11b⁺ DCs (Figure 3), an important DC subset for the induction of Th2 responses in the lung (32), and is involved in the induction of cytokines and costimulatory molecules (Figure 4), which lead to the development of Th2 and Th17 cells.

By using *Clec4n*^{-/-} mice, we demonstrate that Dectin-2 is crucial, not only for the development of Th2 cells, but also for the development of Th17 cells in HDM-induced asthma models (Figure 2). Previous studies with lentivirus-mediated knockdown of Dectin-2 (17) or antibody-mediated neutralization of Dectin-2 (18) have shown the importance of Dectin-2 in the development of Th2 cells, but not of Th17 cells, in HDM-induced asthma models. Our results with *Clec4n*^{-/-} mice clearly show that Dectin-2 has a broad range of function in the pathogenesis of asthma, as suggested by the previous studies (17, 18). The discrepancy between previous studies and ours is presumably due to the degree of Dectin-2 depletion in each experimental system, and suggests that

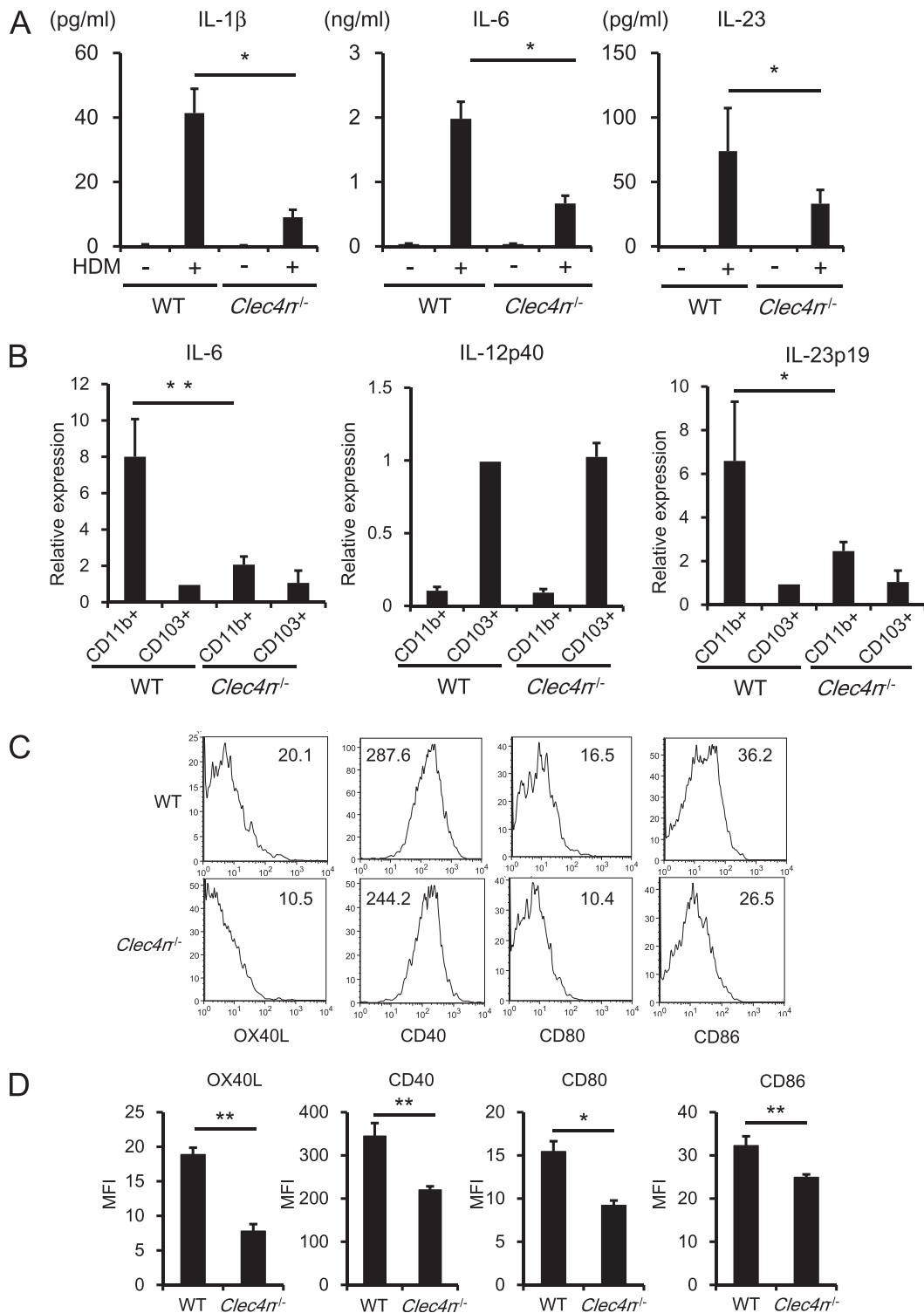


Figure 4. Dectin-2 is crucial for the activation of CD11b⁺ DCs in HDM-induced allergic airway inflammation. (A) Bone marrow–derived DCs (BMDCs) derived from *Clec4n*^{-/-} and WT mice were stimulated with HDM for 24 hours. The amounts of IL-1 β , IL-6, and IL-23 in the supernatants were evaluated by ELISA. Data are means $\pm$ SEM for four independent experiments. * P < 0.05. (B–D) *Clec4n*^{-/-} and WT mice were sensitized and challenged with HDM. (B) At 48 hours after the last HDM challenge, CD11b⁺ DCs and CD103⁺ DCs were isolated from the lung by FACS sorting, and mRNA expression of IL-6, IL-12p40, and IL-23p19 was evaluated by qPCR. mRNA expression of each gene was normalized to that of β -actin and then expressed as the relative expression level to WT CD103⁺ DCs. Data are means $\pm$ SEM for three independent experiments. * P < 0.05; ** P < 0.01. (C and D) At 48 hours after the last HDM challenge, the expression of OX40L, CD40, CD80, and CD86 on lung CD11b⁺ DCs was analyzed by FACS. Shown are representative histograms with mean fluorescence intensity (MFI) (C) and means $\pm$ SEM of MFI (D) from three independent experiments. * P < 0.05; ** P < 0.01.

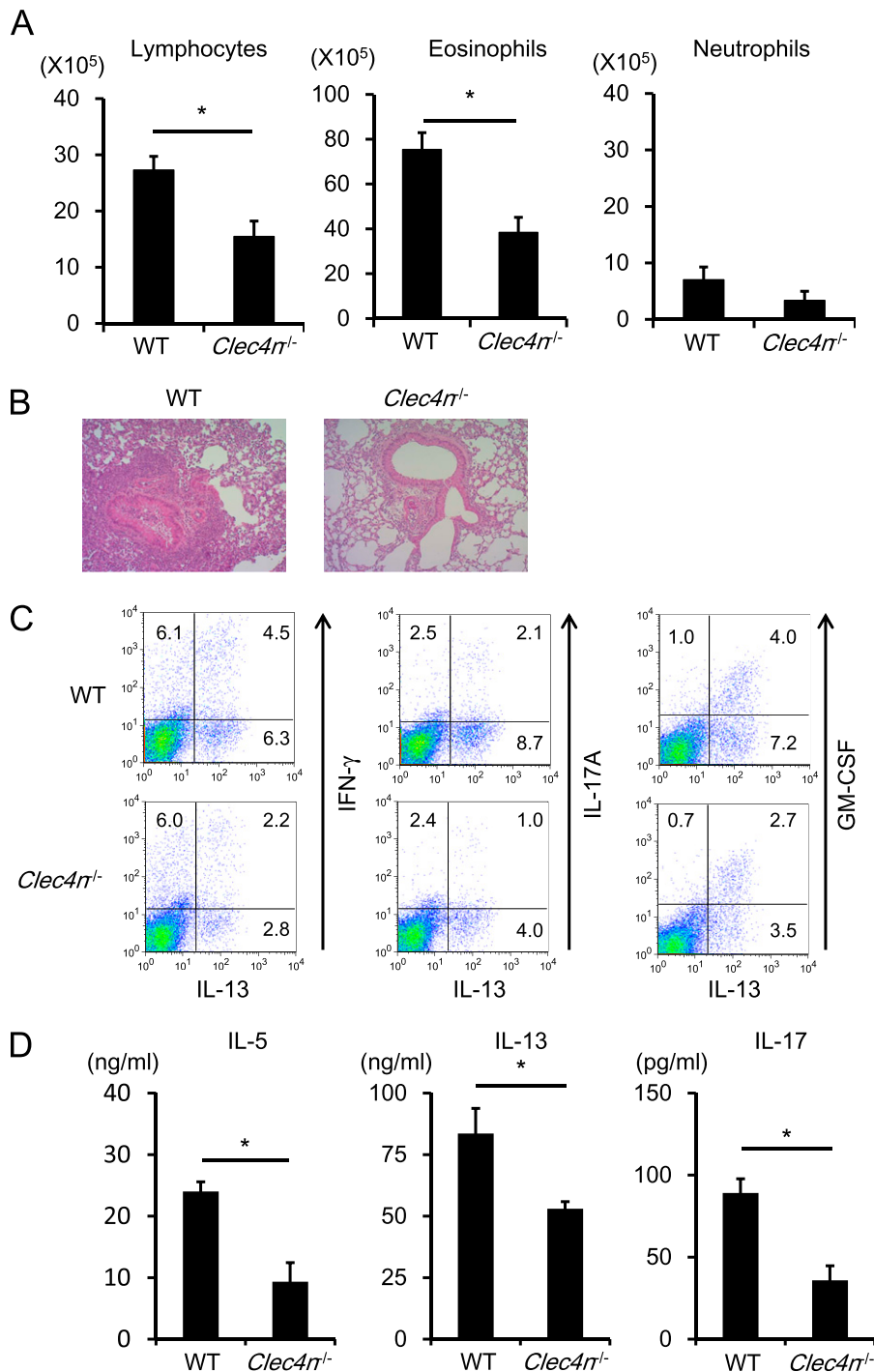


Figure 5. Dectin-2 expressed on DCs is crucial for the induction of HDM-induced allergic airway inflammation. (A–D) HDM-pulsed BMDCs prepared from WT and *Clec4n*^{-/-} mice were transferred to *Clec4n*^{-/-} mice intratracheally, and, 12 days after the transfer, the mice were challenged with HDM intratracheally daily for 3 consecutive days. (A) The numbers of lymphocytes, eosinophils, and neutrophils in the BALF were evaluated at 48 hours after the last HDM challenge. Data are means $\pm$ SEM for five mice in each group. * $P < 0.05$. (B) Representative photomicrographs (HE staining) of lung sections are shown. (C) At 48 hours after the last HDM challenge, CD4⁺ T cells from MLN were stimulated with PMA plus ionomycin and analyzed for intracellular IL-13 together with IFN- γ , IL-17A, and GM-CSF. Shown are representative FACS profiles from five mice. (D) At 48 hours after the last HDM challenge, MLN cells were stimulated with HDM and the as of IL-5, IL-13, and IL-17A in the supernatants were examined at 5 days after the stimulation. Data are means $\pm$ SEM for five mice in each group. * $P < 0.05$.

low amounts of Dectin-2 expression may be sufficient for HDM-induced development of Th17 cells. Because Th17 cells have been implicated in severe asthma or glucocorticoid resistance (33), our results suggest that Dectin-2 may be a key molecule in the development of such pathological conditions.

Regarding the mechanisms by which Dectin-2 enhances Th2 and Th17 cell differentiation *in vivo*, we found that Dectin-2 mRNA is preferentially expressed in CD11b⁺ DCs (Figure 3), which are essential DC subpopulations for the development of both Th2 and Th17 cells (30, 32). Furthermore, mRNAs for Dectin-3 and FcR γ , crucial components of Dectin-2 receptor complex for its efficient ligand recognition, surface expression, and signal transduction (13, 14, 28), were also expressed in CD11b⁺ DCs (Figure 3). In contrast, mRNA amounts for Dectin-2 and Dectin-3 in CD103⁺ DCs were significantly lower than those in CD11b⁺ DCs (Figure 3). Moreover, cell transfer experiments with HDM-pulsed BMDCs have shown that *Clec4n*^{-/-} BMDCs are less efficient for the induction of Th2 and Th17 cells and subsequent allergic airway inflammation than WT BMDCs (Figure 5). Taken together, these results suggest that Dectin-2 receptor complex expressed on CD11b⁺ DCs in the lung is involved in HDM-induced allergic airway inflammation through the induction of Th2 cells and Th17 cells.

Our results show that Dectin-2 is involved in HDM-induced IL-6 and IL-23 production from CD11b⁺ DCs (Figure 4B). Recently, it has been revealed that CD11b⁺ DCs are major producers of IL-6 and IL-23 in the lung (30). Importantly, previous studies have shown that IL-6 and IL-23 are involved not only in the differentiation of Th17 cells, but also in the differentiation of Th2 cells. We have previously shown that IL-23p19 neutralization during antigen challenge significantly inhibits Th2-mediated eosinophilic airway inflammation in murine asthma models (8). In addition, it has been reported that IL-23p19-deficient mice are defective in the induction of allergic inflammation and specific immunoglobulin E production (34). Moreover, IL-6 has been shown to enhance IL-4 production from CD4⁺ T cells (35). These findings suggest that the impaired IL-6 and/or IL-23 production of *Clec4n*^{-/-} CD11b⁺ DCs might be involved in the

reduced Th2 cell-mediated eosinophilic airway inflammation in *Clec4n*^{-/-} mice. On the other hand, previous studies have shown that Dectin-2 signaling primes Th2 cell differentiation via cysteinyl leukotriene production from DCs (17) or macrophages (18). Taken together, these results suggest that dectin-2 signaling enhances Th2 cell differentiation via multiple pathways.

It is well recognized that the allergy-promoting properties of HDM extract are associated not only with mite-derived allergens themselves, but also with mite-associated bacterial and fungal PRR ligands, such as LPS and β -glucans (36). We found that HDM-induced induction of Th2 cells was significantly reduced in *Clec4n*^{-/-} mice; however, HDM-induced induction of Th2 cells was still observed in *Clec4n*^{-/-} mice (Figure 2), indicating that Dectin-2-independent pathways are also involved in HDM-induced Th2 cell differentiation in the lung. Interestingly, it has been shown that Toll-like receptor 4-mediated activation of lung epithelial cells, but not of immune cells, plays a critical role in HDM-induced allergic airway inflammation (37). In addition, it has been demonstrated that HDM-induced CCL20 secretion from lung epithelial cells relies on β -glucan moieties within the HDM extract (38). These results suggest that several PRRs participate in the

development of HDM-induced allergic airway inflammation.

We also found that, although HDM-induced induction of Th17 cells was significantly reduced in *Clec4n*^{-/-} mice, Th17 cell differentiation was still observed in *Clec4n*^{-/-} mice (Figure 2), suggesting the existence of other signaling pathways for HDM-induced Th17 cell differentiation in the lung. In this regard, a recent study has shown that HDM-induced Th17 cell differentiation is attenuated in mice lacking MyD88 (39), suggesting that Toll-like receptor family and/or IL-1 receptor family members that use MyD88 as a signaling molecule are also involved in HDM-induced Th17 cell differentiation in asthma models. Furthermore, complement pathways have been shown to regulate the severity of allergic inflammation by modulating Th17 cell differentiation (40). Further studies that clarify the relative importance of these pathways in HDM-mediated Th17 cell differentiation are necessary for the development of efficient therapeutic approaches against severe neutrophilic asthma.

Although our results clearly demonstrate that Dectin-2 is involved in the activation of CD11b⁺ DCs in HDM-induced allergic airway inflammation, the corresponding DC subpopulation in humans is still largely unknown. Recently,

Schlitzer and colleagues (30) have reported that CD1c⁺ DCs isolated from lung express IL-23, and are capable of inducing Th17 cells *in vitro*. In addition, previous studies have shown that peripheral CD14⁺ monocytes and 6-sulfo N-acetyllactosamine (LacNAc)⁺ DCs could induce Th17 cells (41, 42). These findings suggest the existence of several cell types that lead to the development of Th17 cells in humans. Further studies are required to clarify the human DC subpopulation that induces the differentiation of Th17 cells and is involved in the pathogenesis of severe asthma.

In conclusion, our findings indicate that Dectin-2 expressed on CD11b⁺ DCs is crucial for HDM-induced Th2 and Th17 cell differentiation and subsequent allergic airway inflammation. Our results should add a new insight into the molecular mechanisms underlying HDM-induced allergic inflammation and raise the possibility that Dectin-2 could be a novel therapeutic target for asthma. ■

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References

- Locksley RM. Asthma and allergic inflammation. *Cell* 2010;140:777–783.
- Martinez FD, Vercelli D. Asthma. *Lancet* 2013;382:1360–1372.
- Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999;160:1532–1539.
- Louis R, Lau LC, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. *Am J Respir Crit Care Med* 2000;161:9–16.
- Hastie AT, Moore WC, Meyers DA, Vestal PL, Li H, Peters SP, Bleecker ER, National Heart, Lung, and Blood Institute Severe Asthma Research Program. Analyses of asthma severity phenotypes and inflammatory proteins in subjects stratified by sputum granulocytes. *J Allergy Clin Immunol* 2010;125:1028–1036.e1013.
- Chakir J, Shannon J, Molet S, Fukakusa M, Elias J, Laviolette M, Boulet LP, Hamid Q. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF- β , IL-11, IL-17, and type I and type III collagen expression. *J Allergy Clin Immunol* 2003;111:1293–1298.
- Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Page N, Olivenstein R, Elias J, Chakir J. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 2001;108:430–438.
- Wakashin H, Hirose K, Maezawa Y, Kagami S, Suto A, Watanabe N, Saito Y, Hatano M, Tokuhisa T, Iwakura Y, et al. IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice. *Am J Respir Crit Care Med* 2008;178:1023–1032.
- Black PN, Udy AA, Brodie SM. Sensitivity to fungal allergens is a risk factor for life-threatening asthma. *Allergy* 2000;55:501–504.
- Denning DW, O'Driscoll BR, Hogaboam CM, Bowyer P, Niven RM. The link between fungi and severe asthma: a summary of the evidence. *Eur Respir J* 2006;27:615–626.
- Ward GW Jr, Woodfolk JA, Hayden ML, Jackson S, Platts-Mills TA. Treatment of late-onset asthma with fluconazole. *J Allergy Clin Immunol* 1999;104:541–546.
- Taylor PR, Reid DM, Heinsbroek SE, Brown GD, Gordon S, Wong SY. Dectin-2 is predominantly myeloid restricted and exhibits unique activation-dependent expression on maturing inflammatory monocytes elicited *in vivo*. *Eur J Immunol* 2005;35:2163–2174.
- Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, Fujikado N, Kusaka T, Kubo S, Chung SH, et al. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against candida albicans. *Immunity* 2010;32:681–691.
- Robinson MJ, Osorio F, Rosas M, Freitas RP, Schweighoffer E, Gross O, Verbeek JS, Ruland J, Tybulewicz V, Brown GD, et al. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J Exp Med* 2009;206:2037–2051.
- Saijo S, Fujikado N, Furuta T, Chung SH, Kotaki H, Seki K, Sudo K, Akira S, Adachi Y, Ohno N, et al. Dectin-1 is required for host defense against *Pneumocystis carinii* but not against *Candida albicans*. *Nat Immunol* 2007;8:39–46.
- Barrett NA, Maekawa A, Rahman OM, Austen KF, Kanaoka Y. Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. *J Immunol* 2009;182:1119–1128.

17. Barrett NA, Rahman OM, Fernandez JM, Parsons MW, Xing W, Austen KF, Kanaoka Y. Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes. *J Exp Med* 2011;208:593–604.
18. Clarke DL, Davis NH, Campion CL, Foster ML, Heasman SC, Lewis AR, Anderson IK, Corkill DJ, Sleeman MA, May RD, *et al.* Dectin-2 sensing of house dust mite is critical for the initiation of airway inflammation. *Mucosal Immunol* (In press)
19. Hammad H, Plantinga M, Deswarte K, Pouliot P, Willart MA, Kool M, Muskens F, Lambrecht BN. Inflammatory dendritic cells—not basophils—are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med* 2010;207:2097–2111.
20. Cates EC, Fattouh R, Wattie J, Inman MD, Goncharova S, Coyle AJ, Gutierrez-Ramos JC, Jordana M. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J Immunol* 2004;173:6384–6392.
21. Suto A, Kashiwakuma D, Kagami S, Hirose K, Watanabe N, Yokote K, Saito Y, Nakayama T, Grusby MJ, Iwamoto I, *et al.* Development and characterization of IL-21–producing CD4⁺ T cells. *J Exp Med* 2008;205:1369–1379.
22. Tamachi T, Maezawa Y, Ikeda K, Kagami S, Hatano M, Seto Y, Suto A, Suzuki K, Watanabe N, Saito Y, *et al.* IL-25 enhances allergic airway inflammation by amplifying a Th2 cell-dependent pathway in mice. *J Allergy Clin Immunol* 2006;118:606–614.
23. Gavett SH, Madison SL, Chulada PC, Scarborough PE, Qu W, Boyle JE, Tiano HF, Lee CA, Langenbach R, Roggli VL, *et al.* Allergic lung responses are increased in prostaglandin H synthase-deficient mice. *J Clin Invest* 1999;104:721–732.
24. Iwata A, Kawashima S, Kobayashi M, Okubo A, Kawashima H, Suto A, Hirose K, Nakayama T, Nakajima H. Th2-type inflammation instructs inflammatory dendritic cells to induce airway hyperreactivity. *Int Immunol* 2014;26:103–114.
25. Fehervari Z, Sakaguchi S. Control of FOXP3⁺ CD25⁺ CD4⁺ regulatory cell activation and function by dendritic cells. *Int Immunol* 2004;16:1769–1780.
26. Codarri L, Gyulveszi G, Tosevski V, Hesske L, Fontana A, Magnenat L, Suter T, Becher B. ROR γ T drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. *Nat Immunol* 2011;12:560–567.
27. El-Behi M, Ciric B, Dai H, Yan Y, Cullimore M, Savafi F, Zhang GX, Dittel BN, Rostami A. The encephalitogenicity of T(h)17 cells is dependent on IL-1– and IL-23–induced production of the cytokine GM-CSF. *Nat Immunol* 2011;12:568–575.
28. Zhu LL, Zhao XQ, Jiang C, You Y, Chen XP, Jiang YY, Jia XM, Lin X. C-type lectin receptors dectin-3 and dectin-2 form a heterodimeric pattern-recognition receptor for host defense against fungal infection. *Immunity* 2013;39:324–334.
29. Arizumi K, Shen GL, Shikano S, Ritter R III, Zukas P, Edelbaum D, Morita A, Takashima A. Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its alternatively spliced isoforms. *J Biol Chem* 2000;275:11957–11963.
30. Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, Ho AW, See P, Shin A, Wasan PS, *et al.* IRF4 transcription factor–dependent CD11b⁺ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* 2013;38:970–983.
31. Seshasayee D, Lee WP, Zhou M, Shu J, Suto E, Zhang J, Diehl L, Austin CD, Meng YG, Tan M, *et al.* In vivo blockade of OX40 ligand inhibits thymic stromal lymphopoietin driven atopic inflammation. *J Clin Invest* 2007;117:3868–3878.
32. Plantinga M, Guillems M, Vanheerswynghe M, Deswarte K, Branco-Madeira F, Toussaint W, Vanhoutte L, Neyt K, Killeen N, Malissen B, *et al.* Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 2013;38:322–335.
33. Alcorn JF, Crowe CR, Kolls JK. Th17 cells in asthma and COPD. *Annu Rev Physiol* 2010;72:495–516.
34. Peng J, Yang XO, Chang SH, Yang J, Dong C. IL-23 signaling enhances Th2 polarization and regulates allergic airway inflammation. *Cell Res* 2010;20:62–71.
35. Rincon M, Anguita J, Nakamura T, Fikrig E, Flavell RA. Interleukin (IL)-6 directs the differentiation of IL-4–producing CD4⁺ T cells. *J Exp Med* 1997;185:461–469.
36. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol* 2011;32:402–411.
37. Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 2009;15:410–416.
38. Nathan AT, Peterson EA, Chakir J, Wills-Karp M. Innate immune responses of airway epithelium to house dust mite are mediated through beta-glucan-dependent pathways. *J Allergy Clin Immunol* 2009;123:612–618.
39. Phipps S, Lam CE, Kaiko GE, Foo SY, Collison A, Mattes J, Barry J, Davidson S, Oreo K, Smith L, *et al.* Toll/IL-1 signaling is critical for house dust mite-specific helper T cell type 2 and type 17 responses. *Am J Respir Crit Care Med* 2009;179:883–893.
40. Lajoie S, Lewkowich IP, Suzuki Y, Clark JR, Sproles AA, Dienger K, Budelsky AL, Wills-Karp M. Complement-mediated regulation of the IL-17a axis is a central genetic determinant of the severity of experimental allergic asthma. *Nat Immunol* 2010;11:928–935.
41. Rossol M, Kraus S, Pierer M, Baerwald C, Wagner U. The CD14(bright) CD16⁺ monocyte subset is expanded in rheumatoid arthritis and promotes expansion of the Th17 population. *Arthritis Rheum* 2012;64:671–677.
42. Hänsel A, Günther C, Ingwersen J, Starke J, Schmitz M, Bachmann M, Meurer M, Rieber EP, Schäkel K. Human slan (6-sulfo LacNAc) dendritic cells are inflammatory dermal dendritic cells in psoriasis and drive strong TH17/TH1 T-cell responses. *J Allergy Clin Immunol* 2011;127:787–795.

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