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Dectin-2 Regulates the Effector Phase of House Dust Mite–Elicited Pulmonary Inflammation Independently from Its Role in Sensitization

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The myeloid C-type lectin receptor Dectin-2 directs the generation of Th2 and Th17 immune responses to the house dust mite *Dermatophagoides farinae* through the generation of cysteinyl leukotrienes and proinflammatory cytokines, respectively, but a role for Dectin-2 in effector phase responses has not been described. In this study, we demonstrate that administration of the Dectin-2 mAb solely at the time of *D. farinae* challenge abrogated eosinophilic and neutrophilic inflammation in the bronchoalveolar lavage fluid and Th1, Th2, and Th17 inflammation in the lung of previously sensitized mice. Furthermore, Dectin-2 null mice (*Clec4n*^{−/−}) sensitized with the adoptive transfer of *D. farinae*–pulsed wild-type (WT) bone marrow–derived dendritic cells (DCs) also had less *D. farinae*–elicited pulmonary inflammation, supporting an effector function for Dectin-2. The protection from pulmonary inflammation seen with the Dectin-2 mAb or in *Clec4n*^{−/−} mice was associated with little or no reduction in lung-draining lymph node cells or their cytokine production and with no reduction in serum IgE. WT and *Clec4n*^{−/−} mice recipients, sensitized with *D. farinae*–pulsed WT bone marrow–derived DCs, had comparable levels of *D. farinae*–elicited IL-6, IL-23, TNF- α , and cysteinyl leukotrienes in the lung. By contrast, *D. farinae*–elicited CCL4 and CCL8 production from pulmonary CD11c⁺CD11b⁺Ly6C⁺ and CD11c⁺CD11b⁺Ly6C[−]CD64⁺ monocyte–derived DCs was reduced in *Clec4n*^{−/−} recipients. Addition of CCL8 at the time of *D. farinae* challenge abrogated the protection from eosinophilic, neutrophilic, and Th2 pulmonary inflammation seen in *Clec4n*^{−/−} recipients. Taken together, these results reveal that Dectin-2 regulates monocyte–derived DC function in the pulmonary microenvironment at *D. farinae* challenge to promote the local inflammatory response. *The Journal of Immunology*, 2014, 192: 000–000.

House dust mite (HDM) is the most common allergen worldwide, and sensitization to HDM is present in almost half of U.S. asthmatics (1). Although HDM exposure increases the risk of developing asthma in a dose-dependent fashion (2, 3), suggesting a causal role in asthma pathogenesis, the mechanism(s) by which HDM is so immunogenic is only recently being uncovered. In mouse models, HDM can promote Th2-dependent

pulmonary inflammation through the specific activation of pattern recognition receptors (4–8). The HDM species *Dermatophagoides pteronyssinus* can activate lung stromal cell TLR4 signaling to drive LPS-dependent pulmonary eosinophilia and serum IgE (4). This activation is, in part, due to structural homology between the *D. pteronyssinus*–derived protein, Der p 2, and MD-2, the LPS-binding portion of the TLR signaling complex that is critical to facilitate signaling in epithelial cells (5). TLR5, a receptor for the bacterial protein flagellin, also promotes eosinophilic pulmonary inflammation and impairment of airflow to HDM in mice, with contributions from both the hematopoietic and structural compartments (7).

Carbohydrate structures in HDM also can trigger dendritic cell (DC)–mediated Th2 immune responses through interactions with members of the C-type lectin receptor (CLR) family. The mannose receptor binds to the immunodominant proteins Der p 1 and Der p 2, and inhibition of its signaling by small interfering RNA in human monocyte–derived DCs (MDDCs) reduces allergen uptake and inhibits Th2 cell development in DC–T cell cocultures (9). Moreover, mannose receptor binding and endocytosis of Der p 1 is enhanced in allergic individuals, suggesting a role in disease pathogenesis (10). Der p 1 and Der p 2 bind to DC-specific ICAM-3–grabbing nonintegrin, but inhibition of DC-specific ICAM-3–grabbing nonintegrin in human MDDCs has variably led to enhancement or inhibition of Th2 cell development (11, 12), indicating that HDM-dependent Th2 immunity might be finely regulated by multiple carbohydrate receptors.

The CLR Dectin-2 recognizes high-mannose ligands found in diverse microbes, including *Candida albicans* (13), *Malassezia*

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Abbreviations used in this article: BAL, bronchoalveolar lavage; BMDC, bone marrow–derived dendritic cell; CAE, chloroacetate esterase; CLR, C-type lectin receptor; cys-LT, cysteinyl leukotriene; DC, dendritic cell; *Df/Df* Dectin-2 Ab, *D. farinae*–sensitized and –challenged WT mice treated with the Dectin-2 mAb; *Df/Df* Isotype Ab, *D. farinae*–sensitized and –challenged mice treated with isotype control; HDM, house dust mite; LT, leukotriene; LTC₄, 5(S)-hydroxy-6(R)-S-glutathionyl-7,9-trans-11,14-cis-eicosatetraenoic acid; LTD₄, 5 (S)-hydroxy-6(R)-S-cysteinylglycyl-7,9-trans-11,14-cis-eicosatetraenoic acid; LTE₄, 5S-hydroxy-6R-(S-cysteinyl)-7,9-trans-11,14-cis-eicosatetraenoic acid; MDDC, monocyte–derived dendritic cell; PAS, periodic acid–Schiff; Sal/Df Isotype Ab, *D. farinae*–challenged mice sensitized with saline and treated with isotype control; WT, wild-type.

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furfur (14), and *Schistosoma mansoni* (15), and we have previously demonstrated that Dectin-2 also recognizes HDM (16). HDM-induced activation of Dectin-2 in DCs triggers the generation of cysteinyl leukotrienes (cys-LTs), proinflammatory lipid mediators, through an $\text{Fc}\gamma\text{R}$ -, Syk-, and LTC_4 synthase-dependent pathway (16). Activation of Dectin-2 by HDM and by other ligands additionally triggers Syk- and CARD9-dependent activation of NF- κB and production of proinflammatory cytokines IL-1 β , IL-6, IL-23, and TNF- α (13, 17–19). Dectin-2 signaling in DCs instructs Th2 and Th17 immunity. Dectin-2 null mice (*Clec4n*^{−/−}) or mice treated with a Dectin-2 blocking Ab have impaired Th17 immunity to *C. albicans* (13, 17), and knockdown of Dectin-2 in human DCs similarly impairs in vitro Th17 development in response to *C. albicans* (20). Dectin-2 knockdown in murine bone marrow-derived DCs (BMDCs) attenuates their capacity to sensitize wild-type (WT) recipients for the generation of Th2 pulmonary inflammation and lymph node expansion after *Dermatophagoides farinae* challenge (6). Moreover, administration of the Dectin-2 blocking Ab solely during sensitization similarly attenuates eosinophilic pulmonary inflammation and airways hyperresponsiveness induced by HDM (21). Although Dectin-2 has an established role in sensitization, the influence of this receptor in regulating systemic immunity and tissue inflammation in effector phase responses is unknown. Because Dectin-2 mediates DC production of many proinflammatory mediators, we sought to determine whether Dectin-2 signaling could promote inflammation in the effector phase of HDM-elicited pulmonary immune responses in addition to its established role in sensitization.

In this study, we show that Ab-mediated blockade of Dectin-2 in the challenge phase reduced *D. farinae*-elicited bronchoalveolar lavage (BAL) inflammation and T cell recruitment to the lungs of actively sensitized mice. Furthermore, *D. farinae*-elicited BAL inflammation and T cell recruitment to the lung was similarly reduced in *Clec4n*^{−/−} mice sensitized by the adoptive transfer of WT *D. farinae*-pulsed DCs. The attenuated pulmonary inflammation in *Clec4n*^{−/−} mice was associated with a reduction in MDDC generation of CCL4 and CCL8 in the lung and was reversed with addition of CCL8 to the airway at the time of *D. farinae* challenge. In contrast, cell numbers and Th2 cell cytokine production from the lung-draining lymph nodes showed little or no reduction, and the serum levels of IgE and specific IgG1 were unchanged in *Clec4n*^{−/−} mice. These findings indicate that Dectin-2 exerts a dominant effect on pulmonary MDDCs at challenge that is independent from its role in sensitization. Furthermore, they suggest that innate inflammatory pathways induced simultaneously with classical Th2 immunity can contribute significantly to allergic pulmonary inflammation elicited by native allergens.

Materials and Methods

Clec4n^{−/−} mice

Clec4n^{−/−} mice (13) were backcrossed to C57BL/6 for eight generations and to the C57BL/6 substrain (Charles River Laboratories) for an additional three generations. *Clec4n*^{−/−}, WT C57BL/6, and WT BALB/c mice were used at 12–16 wk age. All animal studies were approved by the Animal Care and Use Committee of the Dana-Farber Cancer Institute.

D. farinae sensitization and challenge protocols

D. farinae extract (Greer Laboratories, Lenoir, NC) contained 0.2 mg Der f1 and 6136 EU endotoxin per mg protein weight. For direct sensitization and challenge experiments, WT BALB/c mice received saline or 1 μg *D. farinae* on days 0 and 1 and 1 μg *D. farinae* on days 14 and 15 by intranasal injection. Two days after the last injection, mice were killed by i.p. injection of pentobarbital. For blocking experiments, 200 μg Dectin-2 Ab (clone D2.11E4; Thermo Scientific Pierce, Rockford, IL) or a rat IgG2a control (BioLegend, San Diego, CA) was injected i.p. on days 13 and 15,

12 and 6 h prior to each *D. farinae* challenge, respectively. For sensitization by adoptive transfer of *D. farinae*-pulsed BMDCs into naive mice, the protocol was carried out as described previously (6). In brief, bone marrow cells from C57BL/6 mice were harvested and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine, 50 μM 2-ME, and recombinant mouse GM-CSF, as described previously (22). Day 7 BMDCs were pulsed with either PBS or 50 $\mu\text{g}/\text{ml}$ *D. farinae* at a concentration of 1×10^6 cells/ml in a 35-mm culture dish (Sumilon Celltight X; Sumitomo Bakelite, Tokyo, Japan) for 24 h. The next day, the BMDCs were washed twice with PBS and resuspended in PBS. Cells (1×10^4) in 20 μl were transferred intranasally to C57BL/6 *Clec4n*^{−/−} and WT littermate recipients. The cells were routinely >85% CD11c⁺. At days 12 and 14 after DC transfer, recipient mice were challenged with 1 μg *D. farinae* intranasally. Two days after the last challenge, mice were killed by i.p. injection of pentobarbital. For CCL8 reconstitution experiments, 5 μg CCL8 (BioLegend) was given by intranasal injection on days 12 and 14, 30 min prior to *D. farinae*.

BAL fluid cell analysis

Two days after the last intranasal injection, the trachea was cannulated, and BAL fluid was obtained by three repeated lavages with 0.75 ml Ca^{2+} - and Mg^{2+} -free PBS with 1 mM EDTA. The BAL fluid was centrifuged at $500 \times g$ for 5 min. Cells were resuspended in 0.2 ml PBS with 1% FCS, and the total cells were counted manually with a hemocytometer. For the differential cell counts of macrophages, neutrophils, eosinophils, and lymphocytes, the cells were cytospun onto a glass slide and stained with Diff-Quik, and cell types in a total of 200 cells were identified by morphologic criteria.

Intracellular cytokine staining of pulmonary mononuclear cells

For pulmonary mononuclear cell assessment, the lungs were homogenized with sterile scalpels and digested for 30 min with 500 U/ml type IV collagenase (Worthington, Lakewood, NJ) and 20 $\mu\text{g}/\text{ml}$ DNase I at 37°C. Mononuclear cells were separated on a Nycoprep gradient (Cosmo Bio, Carlsbad, CA) at $600 \times g$ for 20 min at 4°C; resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.1 mM nonessential amino acids, 43 μM 2-ME, 2 mM L-glutamine, 25 mM HEPES buffer, and 1 mM sodium pyruvate; plated at 8×10^6 cells/ml in 24-well plate; and stimulated with 50 ng/ml PMA and 1 μM ionomycin for 2 h at 37°C. A total of 2.5 μM monensin was added, and the cells were incubated for an additional 4 h. Cells were then treated with DNase I at 60 $\mu\text{g}/\text{ml}$ for 15 min at 37°C; fixed and permeabilized according to the manufacturer's protocol (eBioscience, San Diego, CA); blocked with 1% mouse IgG (Sigma-Aldrich, St. Louis, MO) and 1% anti-mouse CD16/CD32 (BD Biosciences, San Jose, CA) for 20 min; and then stained with anti-mouse CD4-allophycocyanin (clone RM4-5, BD Biosciences), anti-mouse CD8 α -allophycocyanin-Cy7 (clone 53-6.7; BD Biosciences), anti-mouse IL-4-PE (clone 11B11; BD Biosciences), anti-mouse IL-5-PE (clone TRFK5; BD Biosciences), anti-mouse IL-17A-PE (clone TC11-18H10; BD Biosciences), anti-mouse IFN- γ -PE (clone XMGI.2; eBioscience), or rat IgG1-PE isotype control Ab (BD Biosciences) at a concentration of 0.2 $\mu\text{g}/0.1$ ml/ 1×10^6 cells. Analyses were performed on a FACSCanto flow cytometer, and data were analyzed with the FlowJo 7.5.

Cytokine production by peribronchial lymph node cells after ex vivo *D. farinae* restimulation

Two days after the last intranasal injection, three peribronchial lymph nodes were excised from each mouse and homogenized. The cell suspensions were filtered through a 70- μm cell strainer, centrifuged at $300 \times g$ for 5 min at room temperature, and resuspended in RPMI 1640 medium containing heat-inactivated 10% FCS. After the total number of cells was counted for each mouse, cells were cultured at 4×10^6 cells/ml in the presence of 5 $\mu\text{g}/\text{ml}$ *D. farinae* (BALB/c) or 20 $\mu\text{g}/\text{ml}$ *D. farinae* (C57BL/6) for 72 h (23–25). The concentrations of IL-4, IL-5, IL-13, IL-17A, and IFN- γ in the supernatants were measured with ELISA kits (eBioscience).

Measurement of total IgE and *D. farinae*-specific IgG1

Sera were collected by cardiac puncture 2 d after the last intranasal injection. Total IgE was determined with an ELISA kit (BD Biosciences). *D. farinae*-specific IgG1 was measured as described previously (26). Briefly, 96-well plates were coated with a 5 $\mu\text{g}/\text{ml}$ solution of *D. farinae* and incubated with diluted serum followed by alkaline phosphatase-conjugated anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) and *p*-nitrophenyl phosphate substrate (Sigma-Aldrich).

Histology

The lung tissues were excised, fixed, and stained as described previously (27). For general morphology, tissue sections were stained by chloroacetate esterase (CAE) reaction and counterstained with hematoxylin. Congo red staining was used to identify eosinophils, and periodic acid–Schiff (PAS) staining was used to assess mucus and goblet cells. The slides were analyzed with a Leica DM LB2 microscope (Leica Microsystems, Wetzlar, Germany). The pictures were taken with a Nikon digital camera DXM 1200 with Nikon ACT-1 (version 2.70) image acquisition software.

Flow cytometry, sorting, and culture of Dectin-2-expressing populations

To assess Dectin-2 expression on lung cells, the lungs were perfused with PBS plus 1 mM EDTA, homogenized with sterile scalpels, and digested in a shaker for 40 min with 650 U/ml type IV collagenase and 0.02 mg/ml DNase I at 37°C. Single-cell suspensions were blocked with 10% donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and serially stained with rat anti-Dectin-2 (Abcam, Cambridge, MA) or rat IgG2a isotype control Ab (BD Biosciences) at 1 µg/0.2 ml/10⁶ cells, donkey anti-rat IgG-allophycocyanin (Jackson ImmunoResearch Laboratories) at 1:100 dilution, and then a tertiary Ab. Combinations of CD11c-UV (clone N418), CD11b-PE-Cy7 (clone M1-70), CD103-PE (clone 2E7), Ly6C-allophycocyanin-Cy7 (clone HK1.4), and CD64-PE (clone X54-5/7.1) were used (BioLegend). Propidium iodide or Fixable Viability Dye eFluor780 (eBioscience) was used to exclude dead cells. Analyses were performed on a FACSCanto II flow cytometer (BD Biosciences), and data were analyzed with FlowJo 7.5. Lung cells were sorted on a FACSARIA IIu (BD Biosciences) and cultured for 24 h with 5 ng/ml GM-CSF, CCL-4 and

CCL-8 in culture supernatants were measured by ELISA (R&D Systems, Minneapolis, MN).

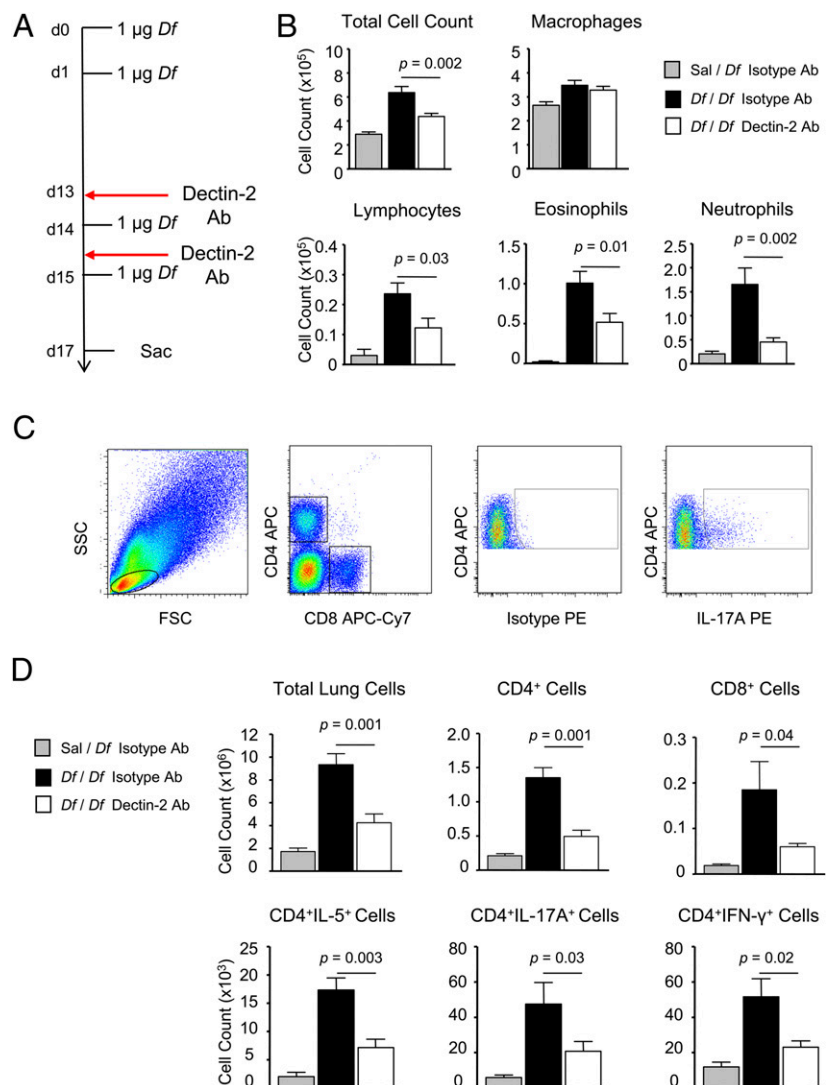
Measurement of cytokine mRNA and protein expression in the lung

Total RNA was isolated from the right lungs with TRIzol reagent, and cDNA was generated using the first-strand kit (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Quantities of IL-6, IL-10, IL-23, TNF-α, CCL2, CCL4, CCL8, CCL12, CCL17, CCL22, CXCL1, and CXCL2 were measured relative to GAPDH using the Mx3005P Real-Time PCR System (Agilent Technologies, Santa Clara, CA) with gene-specific primers (SA Biosciences, Valencia, CA). Protein levels of chemokines were assessed by ELISA (R&D Systems) on lung homogenates.

BAL fluid cys-LT measurement

Twenty-four hours after the last intranasal injection, the trachea was cannulated, and BAL fluid was obtained by three repeated lavages with 0.75 ml Ca²⁺- and Mg²⁺-free PBS with 1% BSA. The BAL fluid was centrifuged at 500 × g for 5 min, and the supernatant was mixed with 5 volumes of methanol on ice for 30 min and spun at 3000 × g. The methanolic supernatant was dried on a rotovac, resuspended in a 2:1 mixture of PGB₂/methanol (400 ng/ml) and HEPES buffer (50 mM), and reversed-phase HPLC for cys-LT separation was performed as described previously (28). Briefly, samples were applied to a C18 Ultrasphere reversed-phase column (Restek, Bellefonte, PA) equilibrated with a solvent of methanol/acetonitrile/water/acetic acid (10:15:100:0.2, v/v; pH 5.6) (solvent A). After injection of the sample, the column was eluted at a flow rate of 1 ml/min with a programmed concave gradient to 55% of the equilibrated solvent A and 45%

FIGURE 1. Dectin-2 blocking Ab inhibits *D. farinae*-elicited pulmonary inflammation in sensitized WT mice. WT mice were sensitized with 1 µg intranasal *D. farinae* or saline control on days 0 and 1, challenged with 1 µg intranasal *D. farinae* on days 14 and 15, treated with 200 µg Dectin-2 Ab or a rat IgG2a control i.p. on days 13 and 15, and killed on day 17. (A) Experimental diagram. (B) Cells from the BAL fluid were counted, cytospin preparations were stained, and 200 cells/slide were counted for specific cell types. Results are means ± SEM (*n* = 10–12 mice/group) combined from three independent experiments. (C and D) Pulmonary mononuclear cells were isolated, stimulated with 50 ng/ml PMA and 1 µM ionomycin for 6 h in the presence of 2.5 µM monensin, stained for cell surface expression of CD4 and CD8 and for intracellular expression of IL-4, IL-5, IL-17A, and IFN-γ, and analyzed by flow cytometry. (C) Representative dot plots gating on cell size, CD4 and CD8 expression, and IL-17A are shown. (D) The total numbers of cells and subsets recruited to the lung are shown. Data are means ± SEM (*n* = 6–8 mice/group) combined from two independent experiments. FSC, Forward light scatter; SSC, side scatter (of light).



of solvent B (100% methanol) over 2.5 min. After 5 min, solvent B was increased linearly to 75% over 15 min. The retention times for PGB₂, 5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTC₄), 5(*S*)-hydroxy-6(*R*)-*S*-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTD₄), and 5(*S*)-hydroxy-6(*R*)-*S*-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTE₄) were 20.8, 21.7, 23.5, and 24.2 min, respectively. Individual HPLC fractions containing LTC₄, LTD₄, and LTE₄ were collected at 54, 162, and 204 s after the peak of PGB₂, respectively, dried, and reconstituted in assay buffer. Cys-LT quantification was performed by ELISA, per the manufacturer's protocol (GE Healthcare Life Sciences, Pittsburgh, PA).

Statistical analysis

Results were expressed as means $\pm$ SEM. Student unpaired two-tailed *t* test was used for the statistical analysis. A *p* value < 0.05 was considered significant.

Results

Dectin-2 Ab inhibits *D. farinae*-elicited pulmonary inflammation in sensitized mice

To assess the role of Dectin-2 in the effector phase of allergic pulmonary inflammation, we administered Dectin-2 mAb or rat IgG2a isotype control i.p. preceding each *D. farinae* challenge of previously sensitized mice (Fig. 1A). *D. farinae*-sensitized and -challenged mice treated with isotype control (*Df/Df* Isotype Ab) developed a significant influx of BAL fluid inflammatory cells, as compared with *D. farinae*-challenged mice sensitized with saline and treated with isotype control (Sal/*Df* Isotype Ab) (Fig. 1B). This influx included recruitment of lymphocytes, eosinophils, and neutrophils to the BAL fluid. *D. farinae*-sensitized and -challenged WT mice treated with the Dectin-2 mAb (*Df/Df* Dectin-2 Ab) had significant reductions in BAL fluid total cells (31.3%), lymphocytes (48.0%), eosinophils (48.5%), and neutrophils (72.9%), as compared with *Df/Df* Isotype Ab-treated mice.

To determine whether the reductions in BAL fluid cells were associated with reductions in T cell infiltration in the lung, pulmonary mononuclear cells were isolated from sensitized and challenged mice, stimulated with PMA and ionomycin and assessed for the numbers of CD4⁺ and CD8⁺ cells, and for IL-5, IL-17A, and IFN- γ -positive subsets. Fig. 1C shows representative staining, including the lymphocyte gate (*far left*), CD4 and CD8 staining (*left*), and intracellular cytokine staining on CD4⁺ lymphocytes using anti-IL-17A PE (*far right*) or isotype control (*right*). As compared with saline controls, *Df/Df* Isotype Ab-treated mice had dramatic increases in pulmonary mononuclear cells, including CD4⁺ cells, CD8⁺ cells, and CD4⁺IL-5⁺, CD4⁺IL-17A⁺, and CD4⁺IFN- γ ⁺ subpopulations (Fig. 1D). In contrast, *Df/Df* Dectin-2 mAb-treated mice had significant reductions in total mononuclear cells (54.6%) and in each subpopulation at 67.6% (CD4⁺), 63.4% (CD8⁺), 58.9% (CD4⁺IL-5⁺), 56.2% (CD4⁺IL-17A⁺), and 55.0% (CD4⁺IFN- γ ⁺), as compared with *Df/Df* Isotype Ab-treated controls. The number of CD4⁺IL-5⁺, CD4⁺IL-17A⁺, and CD4⁺IFN- γ ⁺ cells from *Df/Df* Dectin-2 Ab-treated mice was 2-fold above that of Sal/*Df* Isotype Ab-treated controls and paralleled the finding that the total CD4⁺ T cell count remained 2-fold above baseline.

We next sought to determine whether the effects of systemic Dectin-2 blockade extended to the lung-draining lymph nodes. Upon ex vivo restimulation with 5 μ g/ml *D. farinae*, lymph node cultures from Sal/*Df* Isotype Ab-treated mice produced little IL-4, IL-5, or IL-17A (Fig. 2A). IFN- γ was produced in these cultures, reflecting an innate response to *D. farinae*. A small amount of IL-13 was also produced that was not statistically significant. In cultures from *Df/Df* Isotype Ab-treated mice, *D. farinae* restim-

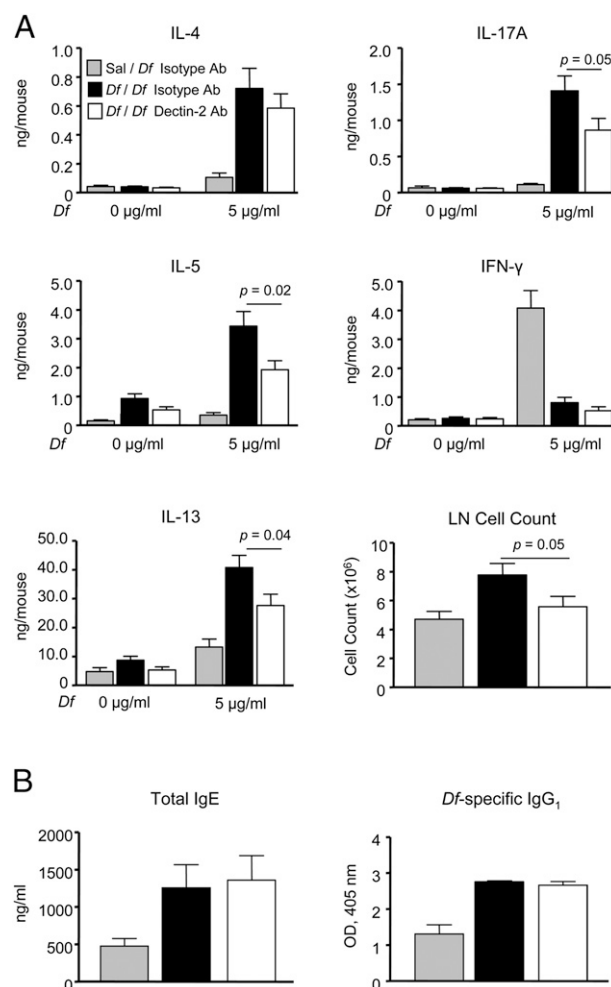


FIGURE 2. Dectin-2 blocking Ab reduces lymph node cytokine production in sensitized WT mice and has no effect on serum Igs. WT mice were sensitized, challenged, and treated with Ab as in Fig. 1. (A) Peribronchial lymph node cells were isolated, counted, and restimulated for 72 h with 0 or 5 μ g/ml *D. farinae*, and cytokines in the supernatant were measured by ELISA. Data are means $\pm$ SEM ($n = 10$ –12 mice/group) combined from three independent experiments. (B) Serum IgE and serum *D. farinae*-specific IgG₁ were measured by ELISA. Data are means $\pm$ SEM ($n = 10$ –12 mice/group) combined from three independent experiments.

ulation generated IL-4, IL-5, IL-17A, and IL-13. There were modest but statistically significant reductions in IL-5 (43.9%), IL-13 (32.1%), and IL-17A (38.6%) in cultures from *Df/Df* Dectin-2 Ab-treated mice, as compared with *Df/Df* Isotype Ab-treated controls, which resulted from a 28.4% reduction in total lymph node cell count, and not from significant differences in cytokine generation on a per cell basis. The levels of IL-5 and IL-17A in cultures from *Df/Df* Dectin-2 Ab-treated mice remained 4- to 5-fold above that of Sal/*Df* Isotype Ab-treated controls.

Total serum IgE and *D. farinae*-specific IgG₁ were also measured in each treatment group (Fig. 2B). After sensitization and challenge, *Df/Df* Isotype Ab-treated mice had significant increases in serum total IgE and *D. farinae*-specific IgG₁, as compared with Sal/*Df* Isotype Ab-treated controls. However, serum IgE and *D. farinae*-specific IgG₁ titers were not reduced in *Df/Df* Dectin-2 Ab-treated mice, suggesting a dominant role for Dectin-2 in the pulmonary microenvironment during the effector phase.

Dectin-2-deficient recipients sensitized with WT DCs have impaired *D. farinae*-elicited pulmonary inflammation

To assess Dectin-2 function in the effector phase by an alternate approach, we generated WT C57BL/6 BMDCs, pulsed them with PBS (PBS-DCs) or *D. farinae* (Df-DCs), and adoptively transferred 1×10^4 BMDCs intranasally to sensitize WT or *Clec4n*^{-/-} (Dectin-2 null) recipients (Fig. 3A) (13). WT mice sensitized with *D. farinae*-pulsed BMDCs and challenged with *D. farinae* had a robust recruitment of inflammatory cells to the BAL fluid including macrophages, lymphocytes, eosinophils, and neutrophils, as compared with WT mice sensitized with PBS-pulsed BMDCs and challenged with *D. farinae* (Fig. 3B). In contrast, *Clec4n*^{-/-} mice sensitized with WT *D. farinae*-pulsed BMDCs had a significantly lower number of *D. farinae*-elicited total BAL fluid cells (56%), as compared with WT recipients. This included significant reductions of macrophages (49.3%), lymphocytes (76.3%), eosinophils (57.3%), and neutrophils (63.2%). Histologic analysis of the lung showed a cellular infiltrate around the bronchovascular bundles of WT recipients that was reduced in *Clec4n*^{-/-} recipients (Fig. 3C, left panel). Goblet cell metaplasia and mucous production, as determined by PAS staining, also was present in WT and largely absent in *Clec4n*^{-/-} recipients (Fig. 3C, middle panel). Congo red staining identified abundant tissue eosinophilia

in WT that was reduced in *Clec4n*^{-/-} recipients (Fig. 3C, right panel).

Pulmonary mononuclear cells from each treatment group were isolated, stimulated with PMA and ionomycin, and assessed for the numbers of CD4⁺ and CD8⁺ cells and for IL-5, IL-17A, and IFN- γ -positive subsets (Fig. 3D). WT mice sensitized with *D. farinae*-pulsed BMDCs and challenged with *D. farinae* recruited more CD4⁺ cells, CD8⁺ cells, and CD4⁺IL-5⁺, CD4⁺IL-17⁺, and CD4⁺IFN- γ ⁺ subpopulations, as compared with mice sensitized with PBS-pulsed BMDCs. *Clec4n*^{-/-} recipients had a significant reduction in total mononuclear cells (42.7%) and in each subpopulation at 58.3% (CD4⁺), 47.9% (CD8⁺), 59.8% (CD4⁺IL-5⁺), 48.6% (CD4⁺IL-17A), and 62% (CD4⁺IFN- γ ⁺), as compared with WT recipients.

D. farinae-induced cytokine generation from the lung-draining lymph nodes of each treatment group also was assessed (Fig. 4A). Upon ex vivo restimulation with 20 μ g/ml *D. farinae*, lymph node cultures from WT mice sensitized with *D. farinae*-pulsed BMDCs and challenged with *D. farinae* produced greater quantities of IL-5, IL-13, IL-17A, and IFN- γ than WT mice sensitized with PBS-pulsed BMDCs. Lymph node cultures from *Clec4n*^{-/-} recipients sensitized with *D. farinae*-pulsed BMDCs and challenged with *D. farinae* had similar numbers of lung-draining lymph node cells

FIGURE 3. Sensitized *Clec4n*^{-/-} mice have impaired *D. farinae*-elicited pulmonary inflammation. WT or *Clec4n*^{-/-} mice were sensitized with 1×10^4 WT BMDC pulsed with PBS (PBS-DC) or with *D. farinae* (Df-DC) and challenged with 1 μ g *D. farinae* on days 12 and 14. (A) Experimental diagram. (B) On day 16, cytopins from BAL fluid were stained and counted. Data are means $\pm$ SEM ($n = 8$ –11/Df-DC; $n = 3$ /PBS-DC) combined from three independent experiments. (C) Histologic analyses of the lung. Lung tissues were fixed with paraformaldehyde and stained with the CAE reaction and hematoxylin, PAS, or Congo red. Eosinophils are indicated by white arrows in Congo red staining, and mucus is stained in purple in PAS. Images were acquired at $\times 20$ (CAE and PAS) and $\times 40$ (Congo red) magnifications. (D) On day 16, pulmonary mononuclear cells were isolated, stimulated, fixed, and stained for CD4, CD8, and intracellular cytokines and analyzed by flow cytometry, as in Fig. 1C and 1D. Data are means $\pm$ SEM ($n = 13$ –15/Df-DC; $n = 5$ /PBS-DC) combined from three independent experiments.

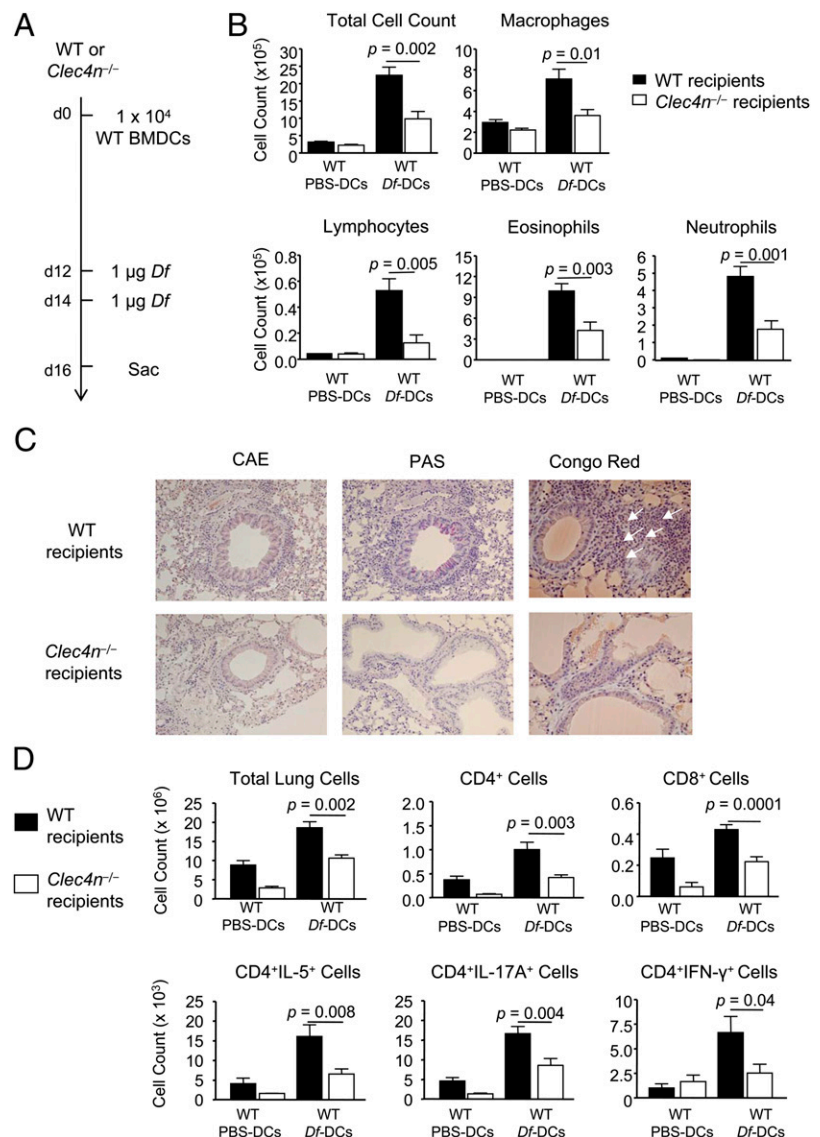
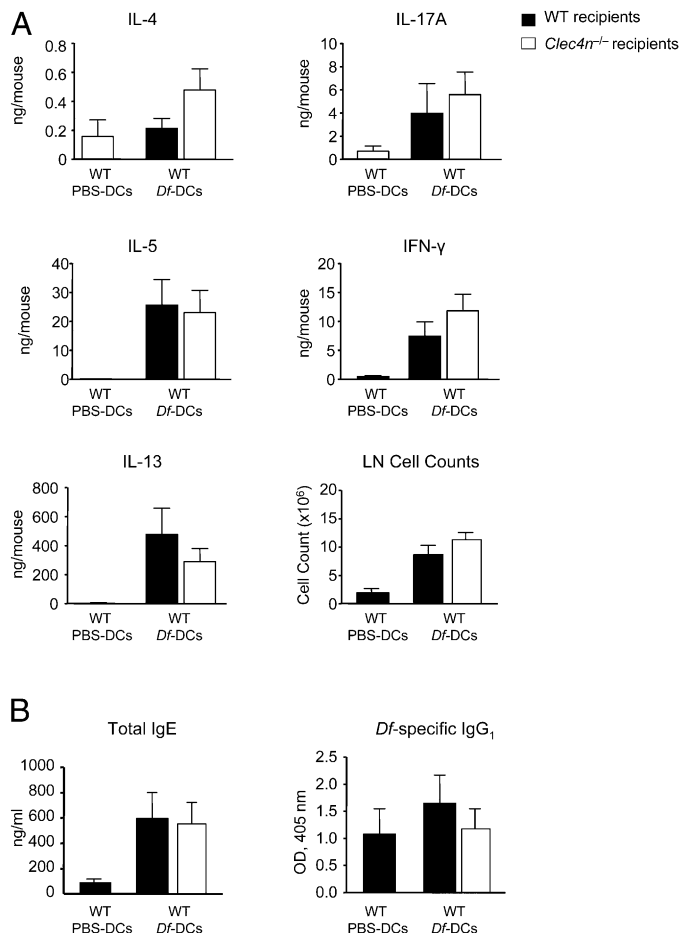


FIGURE 4. Sensitized *Clec4n*^{-/-} mice have intact *D. farinae*-elicited serum Igs and lymph node cytokine production. WT or *Clec4n*^{-/-} mice were sensitized and challenged as in Fig. 3. (A) On day 16, peribronchial lymph node cells were isolated, counted, and restimulated for 72 h with 20 μ g/ml *D. farinae*, and cytokines in the supernatant were measured by ELISA. Data are means $\pm$ SEM ($n = 9$ –11/*Df*-DC; $n = 4$ /PBS-DC) combined from three independent experiments. (B) Serum IgE and serum *D. farinae*-specific IgG₁ were measured by ELISA. Data are means $\pm$ SEM ($n = 9$ –10/*Df*-DC; $n = 4$ /PBS-DC) combined from three independent experiments.



and similar quantities of IL-5, IL-13, IL-17A, and IFN- γ detected, as compared with WT recipients. There was a trend to increased IL-4 in cultures from *Clec4n*^{-/-} recipients that was not statistically significant. Thus, the immunologic responses measured in the lung-draining lymph nodes of *Clec4n*^{-/-} recipients were not impaired, in sharp contrast to the reductions in airway inflammation and T cell recruitment to the lung.

After sensitization with WT *D. farinae*-pulsed BMDCs and challenge with *D. farinae*, WT mice had a significant increase in serum total IgE, as compared with PBS-pulsed BMDC controls (Fig. 4B). There was no increase in *D. farinae*-specific IgG₁. Serum IgE and *D. farinae*-specific IgG₁ titers were not reduced in *Clec4n*^{-/-} mice sensitized with WT *D. farinae*-pulsed BMDCs and challenged with *D. farinae*. The intact serum Ab production and lung-draining lymph node responses seen in *Clec4n*^{-/-} recipients contrast with their impaired airway and pulmonary inflammation, indicating a specific role for Dectin-2 in the pulmonary microenvironment.

Dectin-2 is specifically expressed on CD11c⁺ cells in the lung of naive and D. farinae-sensitized and -challenged mice

To identify the cell types expressing Dectin-2 that might regulate pulmonary inflammation at *D. farinae* challenge, we characterized Dectin-2 expression in naive and in sensitized and challenged lung from C57BL/6 WT and *Clec4n*^{-/-} mice. Single-cell suspensions from lungs were stained with Ab for Dectin-2 and with Abs to discriminate lymphocytes, granulocytes, monocytes, and macrophage/DC populations. In naive lung, Dectin-2 was expressed on autofluorescent CD11c⁺CD11b⁻ macrophages and on non-autofluorescent CD11c⁺CD11b⁻ and CD11c⁺CD11b⁺ DCs (Fig.

5A). Dectin-2 was not expressed on non-autofluorescent CD11c⁺CD103⁺ DCs (data not shown). Nearly 100% of Dectin-2⁺ cells were CD11c⁺, because the small numbers of CD11c⁻ cells stained with the Dectin-2 Ab were detected both in WT and *Clec4n*^{-/-} mice. In *D. farinae*-sensitized and -challenged WT lung, Dectin-2 was expressed on CD11c⁺CD11b⁻ and CD11c⁺CD11b⁺ cells and additionally on CD11c⁺CD11b⁺Ly6C⁺ MDDCs (Fig. 5B), which accounted for 35, 40, and 20% of Dectin-2⁺CD11c⁺ cells, respectively. Although CD11c⁺CD11b⁺Ly6C⁺ cells were newly recruited to *D. farinae*-challenged lung, Dectin-2⁺CD11c⁺CD11b⁻ cells and Dectin-2⁺CD11c⁺CD11b⁺ cells also increased by 2- and 10-fold over that of naive lung. Again, nearly 100% of Dectin-2⁺ cells were CD11c⁺. CD11c⁻CD115⁺Ly6C⁺ monocytes did not express Dectin-2. CD3⁺ T cells, CD19⁺ B cells, NK1.1⁺ NK cells, and SSC^{hi}Ly6G^{hi}CCR3⁻ neutrophils and SSC^{hi}Ly6G^{hi}CCR3⁺ eosinophils also were negative for Dectin-2 (data not shown). All CD11c⁺ subsets identified in sensitized and challenged WT mice were present in comparable numbers in treated *Clec4n*^{-/-} mice (Fig. 5C), suggesting that Dectin-2 has no role in the development or recruitment of any specific subpopulation of CD11c⁺ cells to the lung.

Dectin-2 regulates pulmonary chemokine generation from MDDCs

We next sought to determine whether the absence of Dectin-2 on CD11c⁺ cell populations influenced *D. farinae*-induced generation of inflammatory mediators in the lung at challenge. We first assessed the lung transcript levels of inflammatory cytokines known to be expressed by BMDCs and induced by Dectin-2 signaling in vitro (6, 13, 17). WT and *Clec4n*^{-/-} mice sensitized with

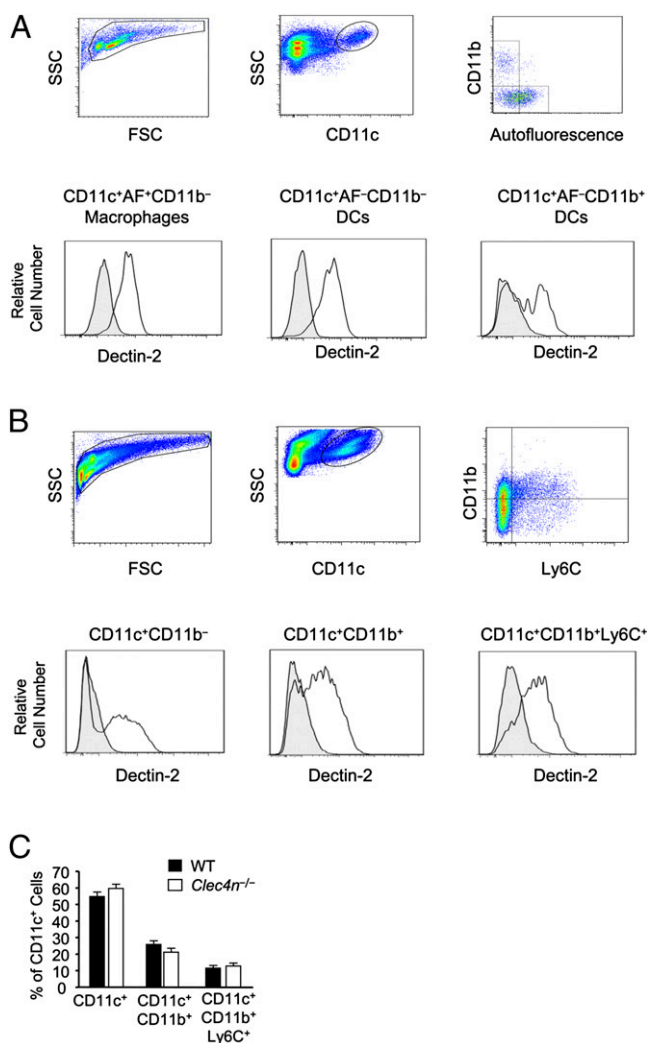


FIGURE 5. Dectin-2 is specifically expressed on CD11c⁺ cells in the lung of naive and *D. farinae*-sensitized and -challenged mice. Single-cell suspensions from the lung of WT C57BL/6 mice were generated and stained with the indicated Abs or isotype controls. (**A**) Top row shows gating of viable cells from naive mice using forward light scatter/side scatter (of light) (FSC/SSC), staining for CD11c, and autofluorescence (AF)/staining for CD11b. Bottom row shows Dectin-2 expression (black lines) and isotype control (shaded gray) on CD11c⁺AF⁺CD11b⁻ macrophages (left panel), CD11c⁺AF⁺CD11b⁻ DCs (middle panel), and CD11c⁺AF⁺CD11b⁺ DCs (right panel). (**B**) Top row shows gating of viable cells from WT C57BL/6 mice sensitized to 1 μ g *D. farinae* on days 0 and 1, challenged to 1 μ g *D. farinae* on days 14 and 15, and killed on day 17. FSC/SSC and stainings for CD11c, and CD11b/Ly6C are shown. Bottom row shows Dectin-2 expression (black lines) and isotype control (shaded gray) on CD11c⁺CD11b⁻ (left panel), CD11c⁺CD11b⁺ (middle panel), and CD11c⁺CD11b⁺Ly6C⁺ (right panel) populations. (**C**) CD11c⁺ subsets from WT and *Clec4n*^{-/-} lung after sensitization and challenge as in (B). Data are means $\pm$ SEM ($n = 4$ –7 mice/group) combined from two independent experiments.

D. farinae-pulsed BMDCs and challenged with *D. farinae* had comparable levels of TNF- α , IL-6, IL-10, and IL-23 transcripts (Fig. 6A). To examine whether cys-LT production in the lung may be altered in *Clec4n*^{-/-} mice, BAL fluid was obtained 24 h after the last challenge. After methanol extraction and HPLC separation, LTC₄, LTD₄, and LTE₄ from BAL fluid were quantified by ELISA. *Clec4n*^{-/-} mice sensitized with WT *D. farinae*-pulsed BMDCs and challenged with *D. farinae* had BAL fluid cys-LT levels (combined contents of LTC₄, LTD₄, and LTE₄) comparable to WT recipients (Fig. 6B). Because CD11c⁺ cells are major

sources of pulmonary chemokines in allergic inflammation (29), we reasoned that Dectin-2 may regulate the generation of these mediators and assessed transcript levels of several chemokines from the lungs of each treatment group (Fig. 6C). WT recipients sensitized with *D. farinae*-pulsed BMDCs and challenged with *D. farinae* had increased transcript levels of CCL2, CCL4, CCL8, CCL12, CCL17, and CCL22 in the lung, as compared with PBS controls. *Clec4n*^{-/-} recipients sensitized with *D. farinae*-pulsed BMDCs and challenged with *D. farinae* had reduced levels of CCL2, CCL4, and CCL8 but similar levels of CCL12, CCL17, CCL22, CXCL1, and CXCL2 in the lung, as compared with WT recipients (Fig. 6C; data not shown). ELISA on lung homogenates (Fig. 6D) confirmed a significant reduction of CCL4 (32%) and of CCL8 (40%) protein in *Clec4n*^{-/-} recipients, as compared with WT controls.

To understand which cell produced CCL4 and CCL8, each Dectin-2-expressing pulmonary CD11c⁺ population was isolated by flow cytometric sorting from WT and *Clec4n*^{-/-} recipients that had been sensitized with *D. farinae*-pulsed BMDCs and challenged with *D. farinae*. Fig. 7A demonstrates a representative dot plot of each sorted population: CD11c⁺CD11b⁻ (upper left), CD11c⁺CD11b⁺ (upper right), CD11c⁺CD11b⁺Ly6C⁺ MDDCs (lower left), and an additional subpopulation of MDDCs, which are CD11c⁺CD11b⁺Ly6C⁺CD64⁺ (lower right) (30). After a 24-h culture, neither CCL4 nor CCL8 was detected in the supernatants of CD11c⁺CD11b⁻ cells from WT recipients (Fig. 7B), despite their capacity to make TNF- α with zymosan stimulation (data not shown). WT CD11c⁺CD11b⁺ cells produced CCL4 but no detectable CCL8. Although WT CD11c⁺CD11b⁺Ly6C⁺ MDDCs produced nanogram quantities of CCL4 and CCL8, CD11c⁺CD11b⁺Ly6C⁺CD64⁺ MDDCs produced the largest quantity of each chemokine. Production of CCL4 and CCL8 was significantly reduced in MDDC cultures from *Clec4n*^{-/-} recipients, suggesting that Dectin-2 regulates CC chemokine production from recruited MDDCs. *D. farinae*-induced CCL4 generation also was demonstrated in WT BMDCs but absent from *Clec4n*^{-/-} cultured BMDCs (Fig. 7C), suggesting that Dectin-2 can directly regulate *D. farinae*-induced chemokine generation. There was no generation of CCL8 from BMDCs.

CCL8 is a ligand for CCR8 found on Th2 cells and plays a critical role in promoting allergic inflammation in the skin (31). To determine whether restoring CCL8 in the airway of deficient *Clec4n*^{-/-} recipients would be sufficient to reconstitute allergic airway inflammation, we sensitized WT and *Clec4n*^{-/-} mice by adoptive transfer of WT *D. farinae*-pulsed BMDCs, challenged them with *D. farinae*, and provided 5 ng CCL8 by intranasal injection at the time of each *D. farinae* challenge (Fig. 7D). WT recipients had recruitment of inflammatory cells to the BAL fluid including lymphocytes, eosinophils, and neutrophils, whereas these were nearly absent in *Clec4n*^{-/-} mice (Fig. 7E). However, addition of CCL8 to *D. farinae* airway challenge restored BAL fluid inflammation with recruitment of macrophages, lymphocytes, eosinophils, and neutrophils, as compared with *Clec4n*^{-/-} mice challenged with *D. farinae* alone.

Pulmonary mononuclear cells from each treatment group were isolated, stimulated with PMA and ionomycin, and assessed for the numbers of CD3⁺ cells that produced IL-5, IL-17A, and IFN- γ (Fig. 7F). WT mice sensitized with *D. farinae*-pulsed BMDCs and challenged with *D. farinae* recruited more CD3⁺IL-5⁺, CD3⁺IL-17A⁺, and CD3⁺IFN- γ ⁺ subpopulations, as compared with *Clec4n*^{-/-} recipients. Addition of CCL8 to the *D. farinae* airway challenge of *Clec4n*^{-/-} mice significantly increased the CD3⁺IL-5⁺ cell population to 3-fold that of *Clec4n*^{-/-} mice injected with *D. farinae* alone. There was a trend to increasing the CD3⁺IL-17A⁺ pop-

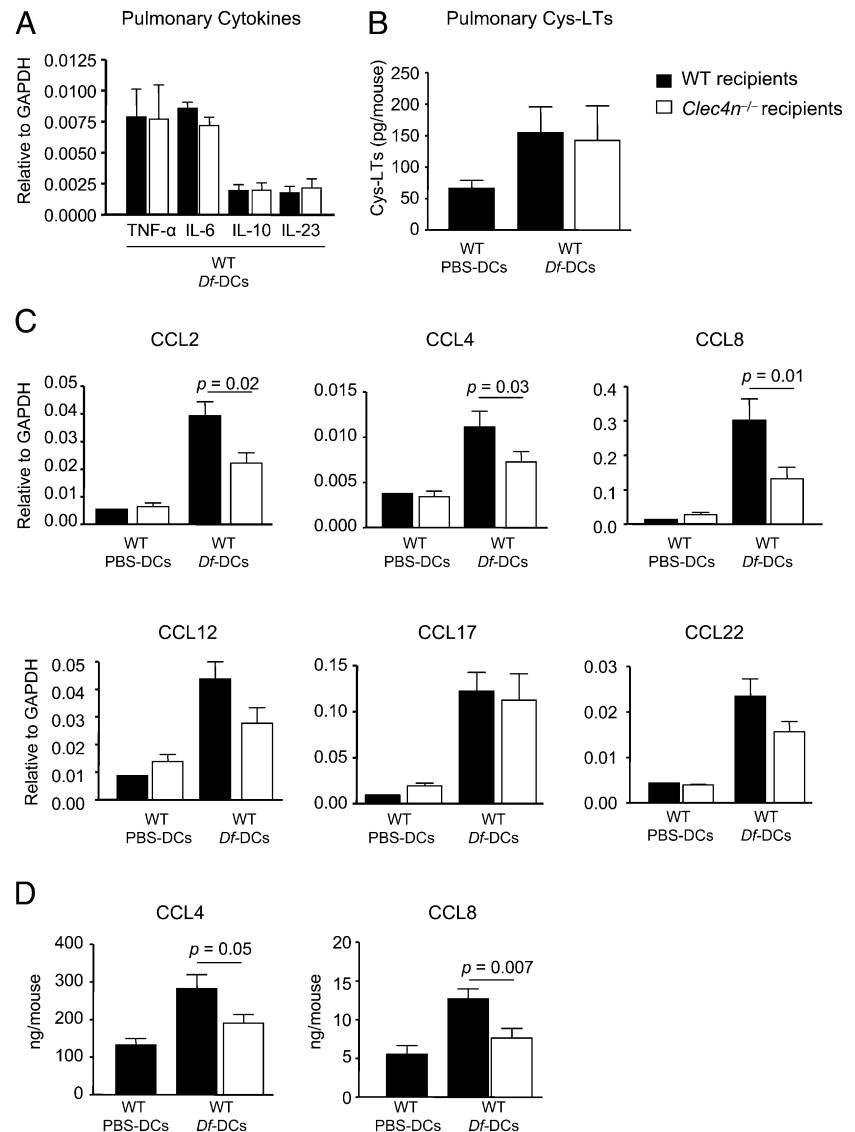


FIGURE 6. Sensitized *Clec4n*^{-/-} mice have impaired *D. farinae*-elicited pulmonary chemokines. WT or *Clec4n*^{-/-} mice were sensitized with WT BMDCs and challenged with *D. farinae*, as in Figs. 3 and 4. **(A)** Cytokine transcripts in lung 48 h after the last *D. farinae* challenge. **(B)** Cys-LTs from BAL 24 h after the last *D. farinae* challenge. **(C)** Chemokine transcripts from WT and *Clec4n*^{-/-} recipients 48 h after the last *D. farinae* challenge. Data are means $\pm$ SEM ($n = 9-11$ /Df-DCs; $n = 3$ /PBS-DCs) combined from three independent experiments. **(D)** CCL4 and CCL8 from lung homogenates were measured by ELISA. Data are means $\pm$ SEM ($n = 13-15$ /Df-DCs; $n = 4$ /PBS-DCs) combined from four independent experiments.

ulation that was not statistically significant and no increase in CD3⁺IFN- γ ⁺ cells. These results demonstrate that restoration of CCL8 alone is sufficient to reconstitute BAL fluid inflammation and Th2 cell recruitment to the lung in deficient *Clec4n*^{-/-} mice.

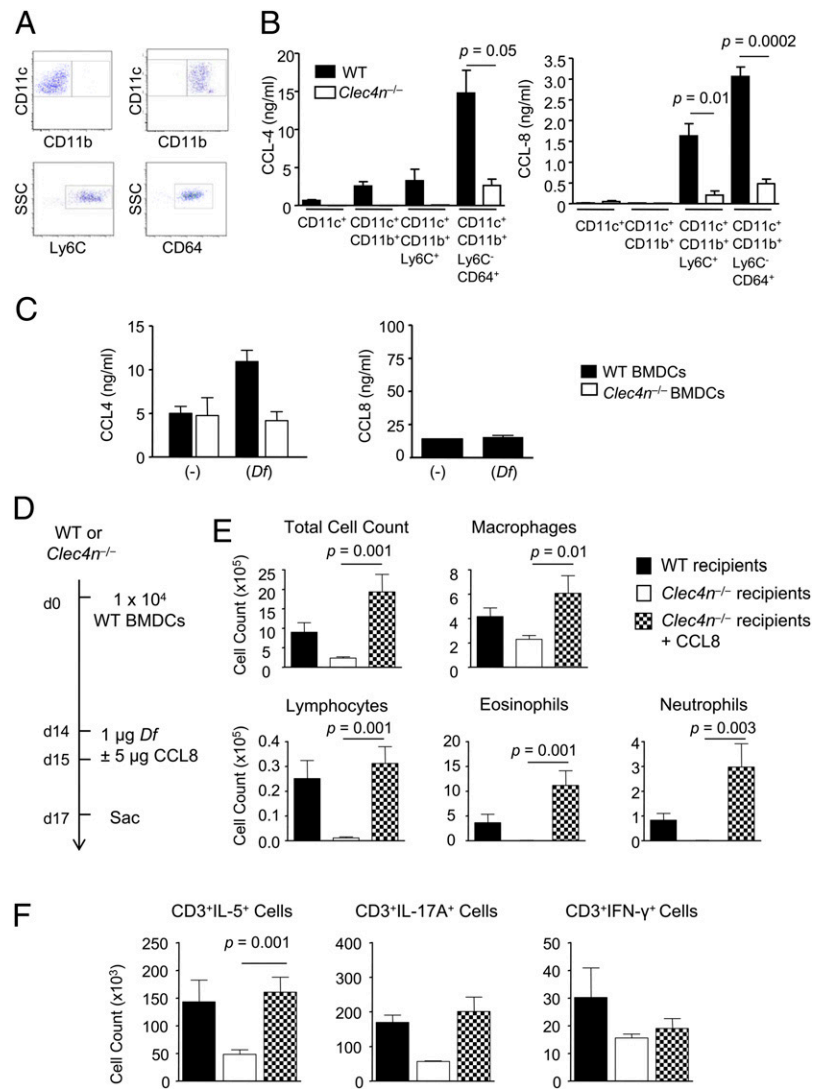
Discussion

Despite recent attention, the inflammatory pathways activated by native allergens are still incompletely understood and the magnitude of the contribution of innate signaling to allergic pulmonary inflammation remains unclear. We previously identified a role for the Dectin-2/cys-LT pathway in DC priming for Th2 immunity to the HDM *D. farinae* (6). Because Dectin-2 can initiate inflammatory signaling via both the cys-LT pathway and the CARD9/cytokine pathway, we considered that Dectin-2 may have a role not only in canonical Th2 sensitization but also in the inflammatory events in the effector phase of *D. farinae*-elicited pulmonary inflammation. By using Dectin-2 blocking Ab during *D. farinae* challenges of sensitized WT mice and by using the adoptive transfer of WT *D. farinae*-pulsed BMDCs to sensitize *Clec4n*^{-/-} mice, we established a role for Dectin-2 in the effector phase of *D. farinae*-elicited pulmonary inflammation. Furthermore, in each model, deficiency of Dectin-2 only in the effector phase caused striking reductions in pulmonary Th1, Th2, and Th17 cells, but

little or no reduction in systemic immunity, as characterized by serum levels of IgE and by the number of lung-draining lymph node cells and their cytokine generation. These findings indicate that Dectin-2 regulation of allergen-induced effector phase responses is specific to the pulmonary microenvironment.

In the lung of naive mice, we found Dectin-2 expression on multiple CD11c⁺ cell populations. Dectin-2 was expressed on non-autofluorescent CD11c⁺CD11b⁻ and CD11c⁺CD11b⁺ DCs and on autofluorescent CD11c⁺CD11b⁻ macrophages but not on CD11c⁺CD103⁺ DCs. A prior study demonstrated Dectin-2 uniquely expressed on tissue macrophages in naive lung using immunohistochemistry with the same Ab on fixed tissue (32). Our detection of additional Dectin-2-expressing cell populations is likely due to the increased sensitivity of flow cytometry on unfixed cells and the availability of *Clec4n*^{-/-} mice as negative controls. The absence of Dectin-2 on CD11c⁺CD103⁺ DCs in the lung is consistent with the distinct developmental lineage of this uniquely Batf1-dependent DC subset (33). The CD11c⁺CD11b⁻ lung macrophage population is reported to control the generation of Ag-induced T regulatory cells, and this function is abrogated by dust mite activation via the MyD88/Trif pathway (34). Although we found that this population expresses high levels of Dectin-2, the role of Dectin-2 signaling in macrophage-mediated tolerance is unknown.

FIGURE 7. Sensitized *Clec4n^{-/-}* mice have impaired MDDC chemokine production and restoration of airway CCL8 reconstitutes allergic airway inflammation. (**A** and **B**) WT or *Clec4n^{-/-}* mice were sensitized with WT BMDCs and challenged with *D. farinae*, as in Figs. 3 and 4. Single-cell suspensions from the lung were generated, stained with the indicated Abs or isotype controls, and sorted. (**A**) Top row shows staining from sorted CD11c⁺CD11b⁻ cells (left panel) and CD11c⁺CD11b⁺ cells (right panel). Bottom row shows staining from sorted CD11c⁺CD11b⁺ Ly6C⁺ cells (left panel) and from CD11c⁺CD11b⁺ Ly6C⁺CD64⁺ cells (right panel). (**B**) CCL-4 (left panel) and CCL-8 (right panel) from cultured supernatant of each population at 24 h. Data are means $\pm$ SEM ($n = 4$ –6/group) combined from two independent experiments. (**C**) CCL-4 (left panel) and CCL-8 (right panel) from cultured supernatant of BMDCs with and without *D. farinae* stimulation for 12 h. Data are means $\pm$ SEM ($n = 4$) combined from two independent experiments. (**D**–**F**) WT or *Clec4n^{-/-}* mice were sensitized with WT BMDCs and challenged with *D. farinae* with or without addition of 5 μ g CCL8 30 min prior to each *D. farinae* challenge. (**D**) Experimental diagram. (**E**) Cytospins from BAL fluid were stained and counted. Data are means $\pm$ SEM ($n = 7$ –9 mice/group) combined from two independent experiments. (**F**) Pulmonary mononuclear cells were isolated, stimulated, fixed, stained for CD3 and intracellular cytokines, and analyzed by flow cytometry, as in Fig. 1C and 1D. Data are means $\pm$ SEM ($n = 4$ –7 mice/group) combined from two independent experiments.



In sensitized and challenged mice, we found Dectin-2 on CD11c⁺CD11b⁻, CD11c⁺CD11b⁺, and CD11c⁺CD11b⁺Ly6C⁺ MDDCs but not on the CD11c⁺CD11b⁺Ly6C⁺ monocytes from which they derive (30, 35, 36), suggesting that Dectin-2 is up-regulated during the maturation of these monocytes in the inflammatory setting of the lung. This is consistent with a study of Dectin-2 expression in a zymosan peritonitis model, where Dectin-2 was noted to be absent on newly recruited inflammatory monocytes, upregulated on maturing inflammatory monocytes, and later downregulated on the monocyte-derived macrophage population (32). This tight regulation was reproduced in the peritoneum by injection of thioglycollate and of BIOgel and in the spleen by i.v. injections of LPS and pam3Cys. In murine BMDCs, we have found that Dectin-2 cell surface expression is upregulated by IL-33 in a dose-dependent and ST2-dependent fashion (data not shown), suggesting again that Dectin-2 is coordinately expressed during the maturation of inflammatory DCs (37, 38). Further studies on IL-33 regulation of Dectin-2 function will be important, because IL-33 is recently recognized as a critical regulator of DC function in allergic inflammation induced by HDM (39, 40).

In cultured DCs and macrophages, Dectin-2 signaling triggers the generation of IL-6, IL-23, and TNF- α via a Syk/CARD9-dependent pathway (13, 17, 18) and cys-LTs via a Syk-dependent/CARD9-independent pathway (16) (data not shown). Nonetheless,

we found that *Clec4n^{-/-}* mice sensitized with WT *D. farinae*-pulsed BMDCs and challenged with *D. farinae* had levels of lung transcript for TNF- α , IL-6, and IL-23 that were comparable to that of WT recipients, despite their impaired inflammatory response. Although TNF- α and IL-6 in *Clec4n^{-/-}* mice may be generated by activation of the TLR4 pathway in DCs (41) by HDM, normal *D. farinae*-induced IL-23 levels in *Clec4n^{-/-}* mice indicates signaling from an additional receptor system, which may be Dectin-1 or the C3a receptor (42, 43). WT and *Clec4n^{-/-}* recipients sensitized with *D. farinae*-pulsed BMDCs had similar levels of cys-LTs in the BAL 24 h after the last *D. farinae* challenge, although the levels detected were not greatly elevated above PBS control, and likely reflect the rapid metabolism of cys-LTs in tissue (44) or their inactivation by the respiratory burst of myeloid cells (45).

We found impairments in pulmonary chemokine production in the lung of *Clec4n^{-/-}* mice after *D. farinae*-pulsed BMDC sensitization and *D. farinae* challenge, including CCL2, CCL4, and CCL8, ligands for monocytes, CCR5-expressing Th1 cells, and CCR8-expressing Th2 cells, respectively (46). Furthermore, deficient CCL4 and CCL8 chemokine production in the lung of *Clec4n^{-/-}* mice was mapped to pulmonary CD11c⁺CD11b⁺ Ly6C⁺ and CD11c⁺CD11b⁺ Ly6C⁺CD64⁺ MDDCs. This confirms and extends a prior study demonstrating chemokine generation in the lung of HDM-challenged mice is specific to recruited CD11c⁺CD11b⁺Ly6C⁺

MDDCs (30). We found *D. farinae*-induced CCL4 production from BMDCs also was dependent on Dectin-2, demonstrating that Dectin-2 can directly mediate chemokine production. In the lung of *Clec4n*^{-/-} mice, we did not find significant differences in the generation of CCL17 or CCL22, generated from CD103⁺ DCs (30), and we did not see a difference in CXCL1 or CXCL2, chemokines generated from another Fc γ -chain-associated CLR, Mincle (47). These results suggest that one way in which Dectin-2 promotes inflammation is via the specific activation of CC chemokines.

Prior studies on the role of CCL8 in allergic pulmonary inflammation have come to discrepant conclusions. The receptor for CCL8, CCR8, is expressed on both murine and human Th2 cells (48, 49) and participates in Th2 cell recruitment to CCL8-expressing skin in a model of atopic dermatitis (31). CCL8 is not expressed in naive lung (31). Moreover, pulmonary models of allergic inflammation elicited with OVA, which failed to demonstrate a role for CCR8, did not establish induction of CCL8 in the tissue (50, 51). In our studies using HDM, we observed robust generation of CCL8 in both whole-lung and isolated MDDCs. Moreover, pharmacologic addition of CCL8 to the airways of *Clec4n*^{-/-} mice increased both eosinophilic pulmonary inflammation and Th2 cell recruitment. This is consistent with models of allergic pulmonary inflammation elicited by cockroach or *Aspergillus* where a role for CCR8 was shown (52, 53) along with the induction of the ligand, CCL8 (52). Although our results do establish a role for Dectin-2 in regulating allergic pulmonary inflammation at the effector stage and in regulating chemokine production from MDDCs, we cannot exclude the possibility that Dectin-2 mediates additional functions of CD11c⁺ populations in the effector phase.

Although our studies showed a dominant effect of Dectin-2 in specifically regulating lung inflammation, we did note some reduction in lymph node cell numbers and their generation of IL-5, IL-13, and IL-17A with Dectin-2 mAb during the challenge phase. This presumably reflects an additional influence of Dectin-2 on memory Th2 and Th17 responses to *D. farinae* challenge, in accordance with our earlier work on Dectin-2, in which Ab blockade during both sensitization and challenge dramatically reduced lymph node cell expansion and cytokine production elicited by *D. farinae* (6).

In sum, our findings suggest that Dectin-2 on CD11c⁺ cells regulates the effector phase of allergic pulmonary inflammation by promoting polarized T cell recruitment and CC chemokine generation in the lung. Furthermore, these findings indicate that native allergens such as HDM may induce significant pulmonary inflammation by activating innate signaling pathways independently from their role in sensitization and that therapies directed to these innate activities may be a novel approach for allergic asthma.

Disclosures

The authors have no financial conflicts of interest.

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