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GM-CSF–Licensed CD11b+ Lung Dendritic Cells Orchestrate Th2 Immunity to Blomia tropicalis

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The Blomia tropicalis dust mite is prevalent in tropical and subtropical regions of the world. Although it is a leading cause of asthma, little is known how it induces allergy. Using a novel murine asthma model induced by intranasal exposure to B. tropicalis, we observed that a single intranasal sensitization to B. tropicalis extract induces strong Th2 priming in the lung draining lymph node. Resident CD11b+ dendritic cells (DCs) preferentially transport Ag from the lung to the draining lymph node and are crucial for the initiation of Th2 CD4+ T cell responses. As a consequence, mice selectively deficient in CD11b+ DCs exhibited attenuated Th2 responses and more importantly did not develop any allergic inflammation. Conversely, mice deficient in CD103+ DCs and CCR2-dependent monocyte-derived DCs exhibited similar allergic inflammation compared with their wild-type counterparts. We also show that CD11b+ DCs constitutively express higher levels of GM-CSF receptor compared with CD103+ DCs and are thus selectively licensed by lung epithelial-derived GM-CSF to induce Th2 immunity. Taken together, our study identifies GM-CSF–licensed CD11b+ lung DCs as a key component for induction of Th2 responses and represents a potential target for therapeutic intervention in allergy. The Journal of Immunology, 2014, 193: 496–509.

Asthma has increased in prevalence over the past three decades and currently affects >300 million people worldwide (1). Dust mites represent a significant source of indoor allergens and are responsible for a substantial percentage of asthma cases (2). Experimental animal models using Dermatophagoides pteronyssinus to induce allergic responses through respiratory sensitization and challenge are now widely used and have led to a greater understanding of allergenicity (2–6). However, less attention has been paid to another major mite allergen, Blomia tropicalis, which is prevalent in tropical and subtropical regions of the world (7, 8), where >2 billion people live. High frequencies of sensitivity to B. tropicalis have been described in asthma and rhinitis patients in those regions, and >20 allergens were identified through their IgE-binding activity (9–12). Most of these allergens only have 30–40% sequence identity with their D. pteronyssinus counterparts and possess low IgE cross-reactivity (13). Sensitization to B. tropicalis allergens is thus an independent cause of allergy and asthma. In addition, although experimental mouse models of B. tropicalis extract-induced asthma using s.c. priming have been described (14), a more relevant model of allergic responses elicited by B. tropicalis solely via the respiratory route has yet to be reported, and little is known about regulation of Th2 responses in B. tropicalis extract-induced asthmatic models.

Th2 CD4+ T cells are central to the pathogenesis of asthma through the cytokines they produce and are responsible for driving eosinophilia, goblet cell hyperplasia, airway hyperresponsiveness, and B cell isotype switching to IgE (15–19). Lung-resident dendritic cells (DCs) play a critical role in asthma by orchestrating the priming and polarization of CD4+ T cells (20–23). Although lung DCs have been shown to control the induction of allergic responses to the dust mite (24, 25), there has been considerable debate about which lung DC subset is responsible for priming Th2 CD4+ T cells. Lung DCs, as well as other nonlymphoid tissue DC populations, are distinguished by mutually exclusive surface expression of the integrins CD103 and CD11b at steady state (26). During inflammation, monocytes infiltrating the lung can also differentiate into DCs, termed as inflammatory DCs, and can also contribute to asthma pathogenesis. However, these cells mainly act at the late stage of inflammation by secreting chemokine and presenting Ag within the lung tissue (27). The exact contribution of each lung-resident DC subset to the pathogenesis of dust mite allergy is not conclusive, as studies using ex vivo sorted lung DCs have led to conflicting reports about the contribution of CD103+ and CD11b+ DCs to Th2 priming (27–29). In addition, although mouse models that permit the selective depletion of lung CD103+ DCs exist (30, 31), it has only recently been possible to specifically deplete resident lung CD11b+ DCs. This is most likely due to the heterogeneity of the mouse CD11b+ DC compartment (26), as suggested by their partial dependence on CSF-1R and fms-related
tyrosine kinase 3 ligand (Flt3L) (26). Recent studies showed that CD11b+ DCs are critical to sensitization to *D. pteronyssinus* allergens (27, 32). However, as they did not have a means to deplete CD11b+ lung conventional dendritic cells in vivo, their conclusions were based on the combinatorial use of Flt3L-deficient (DC-deficient) mice and Langerin-diphtheria toxin receptor (DTR) (CD103+ DC-deficient) mice and hence were indirect. Furthermore, Flt3LKO mice not only lack conventional dendritic cells, but also exhibit defects in the B cell and regulatory T cell compartments at steady state (33).

In this study, we describe a murine model of dust mite allergy using *B. tropicalis* extract and demonstrate that intranasal (i.n.) sensitization and challenge result in the development of characteristic features of allergic inflammation, including alveolar eosinophilia, mucus hypersecretion, Th2 cytokine production, and airway hyperresponsiveness. We show that lung CD11b+ DCs are the major DC subset responsible for transporting *B. tropicalis* Ag to the lung-draining lymph nodes (LNs) and demonstrate that these cells are superior at priming Th2 CD4+ T cells compared with CD103+ DCs ex vivo. Using a mouse model (CD11c-cresIFN regulatory factor 4 [IRF4]fox) that specifically lacks lung-resident CD11b+ DCs (34), we show that only lung CD11b+ DCs are critically required for Th2 priming and development of allergy. In contrast, lung CD103+ DCs and CCR2-dependent monocye-derived inflammatory DCs were not essential for development of allergic responses to *B. tropicalis*. Finally, we demonstrate that lung epithelial cell-derived GM-CSF is a key factor that specifically licenses CD11b+ lung DCs to initiate Th2 priming.

### Materials and Methods

#### Mice

Age- and sex-matched C57BL/6 mice (aged 8–10 wk) and BALB/c mice were purchased from the animal breeding center of the National University of Singapore. DO11.10 and 4get mice were purchased from The Jackson Laboratory. 4get × DO11.10 mice were generated by crossing homozygous 4get mice with homozygous DO11.10 mice, and the resulting F1 offspring that expressed both the DO11.10 and 4get transgenes were used for experiments. The above mouse strains were maintained in the satellite animal housing unit of the Centre for Comparative Medicine, National University of Singapore. CCR2 knockout, IRF4-fox, and CD11c-cre mice were purchased from JAX; the Langerin-DTR-enhanced GFP (eGFP) was obtained from B. Malissen (Marseille, France). These strains were bred and maintained at the Biopolis Resource Centre (A*STAR). All mice were maintained under pathogen-free conditions. All experiments were performed in accordance with institutional guidelines and were approved by the National University of Singapore and Biopolis Resource Centre Institutional Animal Care and Use Committees under protocol numbers 029/09 and 090444, respectively.

#### Allergen preparation

Ten grams of frozen *B. tropicalis* (Siriraj Dust Mite Center for Services and Research, Thailand) was extracted overnight with slow stirring at 4˚C in PBS (pH 7.4). The extract was then centrifuged at 13,000 g for 30 min at 4˚C, and the supernatant was filtered through a 0.22-μm filter and stored at −80˚C. Extract was assayed for endotoxin levels using the QCL-1000 kit (Hyglos) and was 20 EU/mg protein.

#### Immunization

Mice were anesthetized with a ketamine-xylazine mixture (100 and 100 mg/kg) and immunized i.n. with 100 μg *B. tropicalis* extract in 25 μl PBS. In some experiments, *B. tropicalis* was mixed with 100 μg endotoxin-free OVA (Protos) or 30 μg Alexa Fluor 647 (AF647) OVA conjugate (Invitrogen). In some experiments, mice were sacrificed 24 h after immunization, and lungs were collected and homogenized with a tissue homogenizer in 250 μl radioimmunoprecipitation assay buffer (Sigma-Aldrich), followed by a centrifugation to pellet debris. Cleared lysate was quantified for protein concentration with Bradford reagent (Bio-Rad), according to the manufacturer’s protocol. Cytokines in lung homogenates were measured by ELISA (R&D Systems). All mice were maintained under pathogen-free conditions. All experiments were performed in accordance with institutional guidelines and were approved by the National University of Singapore and Biopolis Resource Centre Institutional Animal Care and Use Committees under protocol numbers 029/09 and 090444, respectively.

#### Flow cytometry and cell sorting

The above mouse strains were maintained in the satellite animal housing unit of the Centre for Comparative Medicine, National University of Singapore. CCR2 knockout, IRF4-fox, and CD11c-cre mice were purchased from JAX; the Langerin-DTR-enhanced GFP (eGFP) was obtained from B. Malissen (Marseille, France). These strains were bred and maintained at the Biopolis Resource Centre (A*STAR). All mice were maintained under pathogen-free conditions. All experiments were performed in accordance with institutional guidelines and were approved by the National University of Singapore and Biopolis Resource Centre Institutional Animal Care and Use Committees under protocol numbers 029/09 and 090444, respectively.

Mouse and rat cells were labeled with antibodies coupled to a variety of fluorophores. Single cells were analyzed on a FACSCalibur or CyAn ADP (Beckman Coulter). Samples were acquired for at least 10,000 events for each sample.

#### Cell isolation and transfer

To obtain naive CD4+ T cells from 4get × DO11.10 mice, single-cell suspensions from spleens and LNs of mice were layered on Ficoll-Paque (1.064 g/ml; Axis-Shield) and centrifuged at 1700 g for 30 min at 4˚C. To obtain migratory DCs from the lung-draining LN cells, LNs were physically disrupted into a single-cell suspension by passing through a 61-μm cell strainer. To perform intracellular staining of lung-draining LN cells after restimulation, cells were incubated for 10 min at 4˚C before physical disruption into single-cell suspension by passing through a 61-μm cell strainer. To obtain naive CD4+ T cells from 4get × DO11.10 mice, single-cell suspensions from spleens and LNs of mice were layered on Ficoll-Paque (GE Healthcare) and centrifuged at 600 × g for 20 min at room temperature without braking. Cells accumulating at the interface were collected, washed, and isolated with anti-CD4–conjugated MACS beads (Miltenyi Biotec). For adoptive transfer of 4get × DO11.10 T cells, CD4+ T cells were positively selected with CD4 microbeads, followed by purification by magnetic-activated cell separation (Miltenyi Biotec). Purified CD4+ T cells (2.5 × 10⁶) were transferred into each recipient mouse.

The following Abs were used for staining cells: CD11c PerCPCy5.5 or FITC (N418; Bioscience), Ly-6G allophycocyanin (1A8; BioLegend), SiglecF PE (E50–2440; BD Pharmingen), CD45 eFluor450 (17A2; eBioscience), I-A/E eFluor450 (M5/1114.15.2; eBioscience) or V500 (M5/1114.15.2; BD Horizon), CD24 PE-Cy7 or eFluor450 (M1/69; BioLegend), CD46 or allophycocyanin (5×-5/7.1; BioLegend), CD103 biotin or allophycocyanin-PE 2F7; eBioscience), CD80 PerCP Cy5.5 (16-10A1; BioLegend), CD86 PE (GL-1; BD Pharmingen), CD40 PE (1C10; BD Pharmingen), CD11b PerCPCy5.5 or allophycocyanin-eFluor 780 (M1/70; eBioscience), CD4 PB (RM4-5; BD Pharmingen) or allophycocyanin (RM4-5; eBioscience), CD19 PerCPCy5.5 (145–2C11; eBioscience), CD26L FITC (MEL-14; BioLegend), CD44 allophycocyanin (IM7; BD Pharmingen), CD25 PE (PC61; BioLegend), IL-4 allophycocyanin (1B11; BD Pharmingen) or FITC PE (XMG1.2; BD Pharmingen), CD25 PE or allophycocyanin (98/423; R&D Systems), CD45 AP647 (30-F11; BioLegend), DO11.10 clonotypic TCR-PE (KJ1-26; BD Pharmingen), and FcεRIe PE (MAR-1; BioLegend). Fc blocking Ab (2.4G2) was used during all FACs staining. Cells were incubated for 30 min on ice with the appropriate Abs. Live/Dead cells were differentiated by violet or blue live/ dead fixable dye staining, according to the manufacturer’s instruction (Molecular Probes, Invitrogen), or by 7-aminoactinomycin D (Sigma-Aldrich) or DAPI. Streptavidin eFluor 605NC (eBioscience) was used for secondary detection of biotinylated Abs.

To perform intracellular staining of lung-draining LN cells after restimulation, cells were further stimulated with PMA and ionomycin (Sigma-Aldrich) in the presence of GolgiPlug containing brefeldin A (BD Pharmingen) and GolgiStop containing monensin (BD Pharmingen) for 6 h. Cells were then stained for surface markers before addition of fixation/permeabilization buffer (eBioscience) and thereafter stained for intracellular cytokines. Flow cytometric analysis was performed using CyAn ADP (Beckman Coulter), BD Fortessa, or LSR II (BD Pharmingen) and analyzed using FlowJo software (Tree Star, San Carlos, CA). Cell sorting was performed using the MoFlo (Beckman Coulter) or the FACS Aria (BD Pharmingen).
Ag presentation to T cells ex vivo

Sorted CD4+ T cells from 4/T × DO11.10 mice were labeled with violet CFSE (Molecular Probes, Invitrogen) and plated at a density of 100,000 cells/well in the presence of sorted DCs from immunized mice (5 T cells:1 DC). After 4 d, cells were harvested and OVA-specific T cell proliferation was determined by flow cytometry. The 7-aminoactinomycin D was added 5 min before analysis to differentiate live/dead cells.

Lung histology

Lung tissues were obtained after perfusion with PBS and fixation with 0.5 ml 4% paraformaldehyde (Sigma-Aldrich). To perform periodic acid-Schiff’s staining, tissues were further fixed with 4% paraformaldehyde for 3–5 d and then dehydrated and embedded in paraffin. Sections (5 mm thick) were cut and stained with periodic acid-Schiff’s reagent and H&E (Sigma-Aldrich) to identify mucus-secreting goblet cells.

To perform immunofluorescence staining, tissues were fixed with 2% paraformaldehyde with 30% sucrose overnight and washed in PBS buffer for 1 h on a rotator, followed by embedding in optimum cutting temperature compound (Sakura FineTek). Sections 5 μm in thickness were cut on a cryostat (Leica) and were kept at −20°C. Cryosections were air dried, blocked with PBS with 0.2% BSA, and stained with rabbit anti-mouse GM-CSF (1:200 dilution; FL-144; Santa Cruz) and rat anti-mouse epithelial cell adhesion molecule (1:800 dilution; G8.8; eBioscience) in PBS containing 1% normal mouse serum overnight at 4°C. Cy3-conjugated anti-rabbit Ab (1:300 dilution; 711-166-152; Jackson ImmunoResearch Laboratories) and AF647-conjugated anti-rat Ab (1:400; A21247; Invitrogen) were used for detection. Sections were counterstained with DAPI (KPL) for visualization of cell nuclei and mounted with fluorescent mounting medium (Dako) for analysis. Images were taken with a fluorescence microscope (Axio imager.Z1, Axiocam HRM camera; 10×/0.3, original magnification ×100 and ×400/0.75, original magnification ×400 EC Plan-NEOFLUAR objective lenses; Carl Zeiss Micro Imaging, Jena, Germany).

Assessment of lung function

Airway responsiveness was measured as the change in airway resistance to increased concentrations of nebulized methacholine (0.5–8.0 mg/ml; Sigma-Aldrich). Mice were anesthetized, tracheostomized, and mechanically ventilated by using FinePointe Series RC Sites (Buxco Research System, Wilmington, NC), and airway resistance was recorded. Results are expressed as percentages of respective basal values in response to PBS.

**FIGURE 1.** Intranasal sensitization and challenge with *B. tropicalis* induces a characteristic allergic asthma phenotype. (A) Immunization protocol of mice. (B) Differential cell counts in BAL fluid. (C) Representative lung section showing mucus secretion visualized by periodic acid-Schiff’s staining (original magnification ×100). Scale bar, 50 μm. (D) Airway resistance in response to increasing concentrations of nebulized methacholine (0.5–8.0 mg/ml). Results expressed as deviation from respective basal PBS values. (E) Levels of cytokines from lung-draining LN cells after restimulation. MLN cells were plated at a density of 400,000 cells/well in a 96-well plate and restimulated for 5 d with 20 μg/ml *B. tropicalis* extract. Cytokine levels were measured by ELISA. *n* = 12. ND, not detected. All data in figure pooled from three independent experiments. *p < 0.05, ***p < 0.001.
The unpaired Student $t$ test was used for comparison between two groups, and one-way ANOVA was used for multiple group comparison. Data are expressed as means ± SEM, and $p$ values are indicated by asterisks. Flow cytometric profiles and histological pictures are representations of at least three independent experiments with three to four mice per group.

**Results**

**Intranasal sensitization and challenge with *B. tropicalis* induces a characteristic allergic asthma phenotype**

To determine whether direct respiratory exposure to *B. tropicalis* was able to induce an allergic response, mice were i.n. sensitized on day 0 with *B. tropicalis* or PBS, challenged with *B. tropicalis* or PBS from days 9 to 11, and analyzed 72 h after the last challenge (Fig. 1A). We observed that *B. tropicalis*–treated mice displayed classical features of allergic responses, such as eosinophil and lymphocyte migration into the airways (Fig. 1B), and mucus hypersecretion (Fig. 1C), whereas PBS-treated mice did not. We also observed increased airway responsiveness in mice treated with *B. tropicalis* upon methacholine challenge, compared with mice treated with PBS (Fig. 1D). Upon restimulation of cells from the lung MLNs with *B. tropicalis* extracts, CD4$^+$ T cells demonstrated enhanced production of Th2 cytokines IL-4, IL-5, IL-10, and IL-13, but only minute amounts of Th1 cytokine IFN-$\gamma$ (Fig. 1E). Taken together, these results demonstrate that repeated respiratory exposure to *B. tropicalis* extracts induces a robust Th2 response and results in the development of classic pathological features of allergy observed with *D. pteronyssinus* sensitization.

**A single exposure to *B. tropicalis* leads to IL-4–producing CD4$^+$ T cells in MLNs**

To monitor Th2 responses in vivo, we used IL-4-IRES-eGFP (4get) mice, in which a bicistronic construct encoding enhanced GFP (eGFP) was inserted into the IL-4 locus (IL-4-eGFP) (35). To...
investigate the kinetics of the appearance of IL-4–producing CD4+ T cells in the lung-draining LNs, we treated 4get mice with *B. tropicalis* extract and examined them at various time points after sensitization. Importantly, a single i.n. administration of *B. tropicalis* extract resulted in the robust induction of a Th2 response indicated by the accumulation of IL-4-eGFP+ CD4+ T cells in the MLNs by day 3, which increased further by day 7 (Fig. 2A). To confirm that this rapid induction of IL-4-eGFP+ cells by *B. tropicalis* was Ag specific, we crossed 4get mice with DO11.10 mice and adoptively transferred OVA-specific CD4+ T cells into BALB/c mice immunized with OVA or OVA mixed with *B. tropicalis*. Using the KJ1-26 TCR Ab to track the adoptively transferred cells, we observed a robust induction of IL-4-eGFP 3 d after immunization, demonstrating that the IL-4 was induced in an Ag-specific manner (Fig. 2B). This also shows that the *B. tropicalis* extract has a strong adjuvant effect for the priming for Th2 CD4 T cells.

To determine whether the Th2-inducing capacity of *B. tropicalis* was due to protease activity of allergens within the extract, we boiled the *B. tropicalis* extract at 100˚C for 10 min to inactivate enzymatic activity. Interestingly, mice immunized and challenged with heat-treated *B. tropicalis* exhibited a strong Th2 response, similar in magnitude to mice immunized and challenged with untreated *B. tropicalis* extract (Fig. 2C, 2D). As a control, we treated *B. tropicalis* with proteinase K to destroy protein allergens and observed a significant reduction in Th2 responses. Taken together, these data demonstrate that immunization with *B. tropicalis* extract is able to rapidly induce IL-4 production by Ag-specific CD4 T cells and that the protein components within the extract responsible for inducing the Th2 responses are resistant to heat.

**CD11b+ lung DCs are superior at transporting Ag to the MLNs and promoting a Th2 response after *B. tropicalis* challenge**

Next, we examined the effect of *B. tropicalis* administration on the ability of lung DCs to capture and present Ag in the MLNs. We used a novel gating strategy to characterize lung-resident DC subsets as the sole use of CD11b and CD103 in combination with CD11c and MHCII to define the lung DC subset results in substantial contamination of the CD11b+ DC population with interstitial macrophages (34). Using two additional markers, CD24 (heat-stable Ag) and CD64 (high-affinity FcγRI), bona fide Flt3-dependent resident lung CD11b+ DCs can be discriminated as a CD24+CD64neg population and interstitial macrophages as a CD24negCD64+ population (Supplemental Fig. 1A). Within the MLNs, the CD64+CD24neg macrophages were absent and all cells within the lung migratory DCs gate (CD11c+MHCIIhigh) were uniformly positive for CD24+ and negative for CD64 (Supplemental Fig. 1B).

*B. tropicalis* induced a rapid mobilization of lung DCs to the MLNs, and, within 24 h, a significant increase in the number of migratory DCs was observed (Fig. 3A). Next, we analyzed the relative capacity of lung DCs to migrate to the MLNs carrying Ag by i.n. administration of OVA-AF647 with or without *B. tropicalis* (Fig. 3B). In the absence of *B. tropicalis*, we observed preferential uptake of Ag by CD11b+ compared with CD103+ DCs in the MLNs. Coexposure to *B. tropicalis* resulted in enhanced OVA-AF647 uptake by both DC subsets. However, CD11b+ DCs still...
carried more OVA-AF647 compared with CD103+ DCs (Fig. 3B). To examine whether this resulted in an increased capacity to present Ag to CD4+ T cells, we sorted the migratory MLN DCs 3 d after immunization with OVA or OVA plus *B. tropicalis* and cultured them for 4 d with CFSE-labeled naive CD25negCD44low CD62Lhigh OVA-specific CD4+ T cells sorted from 4get x DO11.10 mice. Proliferation of DO11.10 CD4+ T cells was determined by CFSE dilution, and IL-4 production was assessed by eGFP expression. We observed that OVA plus *B. tropicalis*–treated CD11b+ DCs were able to induce robust proliferation and differentiation of naive DO11.10 CD4+ T cells into IL-4–expressing cells. Importantly, CD103+ DCs did not induce proliferation and differentiation of naive DO11.10 CD4+ T cells into IL-4–expressing cells (Fig. 3C), even though they captured OVA (Fig. 3B). Together, these data indicate that lung CD11b+ DCs are the predominant DC subset that transports Ag to the MLNs after *B. tropicalis* challenge and are superior stimulators of naive CD4+ T cell proliferation and differentiation into Th2 cells.

**In vivo depletion of CD11b+ but not CD103+ lung DCs abrogates the development of allergic responses to *B. tropicalis***

Several studies previously established that lung-resident DCs are necessary and sufficient for the development of allergic response to inhaled allergens (20–23). However, due to conflicting reports (27–29), which subset of lung-resident DCs is responsible for the induction of Th2 priming remains a subject of debate.

To address the contribution of CD103+ lung DCs, we used Langerin-DTR mice in which langerin-expressing CD103+ lung DCs are specifically ablated by injection of diptheria toxin (DT) (Supplemental Fig. 2). To examine the contribution of CD103+ lung DCs to the initiation of Th2 immunity after *B. tropicalis* sensitization, mice were injected with DT i.p. before receiving...
a single i.n. administration of \textit{B. tropicalis} and analyzed 3 d later. The MLNs were harvested, restimulated with \textit{B. tropicalis} extract for 5 d, and examined for the induction of Th2 responses. Depletion of CD103+ lung DCs did not alter the magnitude of Th2 response during priming stage of \textit{B. tropicalis} immunization indicated by the unchanged level of IL-4 and IFN-\(\gamma\) production from CD4+ T cells (Fig. 4A). Furthermore, depletion of CD103+ lung DCs had little effect on the magnitude of allergic responses when mice received i.n. sensitization followed by multiple challenges of \textit{B. tropicalis}. Eosinophil and lymphocyte migration into the airways were similar to the PBS-treated group (Fig. 4B), as well as mucus hypersecretion (Fig. 4C). We also observed a similar production of Th2 cytokines IL-4, IL-5, IL-10, and IL-13 after restimulation of MLN cells from CD103+ DC-depleted and non-depleted mice with \textit{B. tropicalis} (Fig. 4D).

Next, we investigated the contribution of lung-resident CD11b+ DCs. To specifically ablate CD11b+ DCs while sparing CD103+ DCs, we used a mouse model (CD11c-cre\textsuperscript{pos} IF4\textsuperscript{flex}) that lacked expression of the \textit{Irf4} gene in CD11c+ cells by crossing CD11c-cre mice (36) with IRF4\textsuperscript{flex} mice (37). Because lung-resident CD11b+ DCs are critically dependent on IRF4, DC-specific ablation of IRF4 led to the selective loss of pulmonary CD11b+ DCs (34). We also observed that resident CD11b+ DCs were still absent in the lungs and in the MLNs 48 h after \textit{B. tropicalis} challenge in CD11c-cre\textsuperscript{pos} IF4\textsuperscript{flex} mice (Fig. 5). Strikingly, during the priming stage of \textit{B. tropicalis} immunization, the percentage of IL-4–positive CD4+ T cells was reduced in CD11b+ DC-deficient mice (CD11c-cre\textsuperscript{pos} IF4\textsuperscript{flex}), whereas production of IFN-\(\gamma\) was enhanced (Fig. 6A). In addition, when CD11c-cre\textsuperscript{pos} IF4\textsuperscript{flex} mice were sensitized and challenged with \textit{B. tropicalis}, we observed significantly reduced BAL eosinophil infiltration (Fig. 6B), mucus secretion (Fig. 6C), and Th2 cytokine production by restimulated MLN cells (Fig. 6D), compared with their littermate controls (CD11c-cre\textsuperscript{neg} IF4\textsuperscript{flex}). To confirm that CD11c-cre\textsuperscript{pos} IF4\textsuperscript{flex} mice did not have spontaneous development of allergy and Th2 cytokine release by T cells, we immunized and challenged both CD11c-cre\textsuperscript{pos} IF4\textsuperscript{flex} and CD11c-cre\textsuperscript{neg} IF4\textsuperscript{flex} mice with PBS. We did not observe any difference in the number of cells in the BAL (Fig. 6E), and the majority of cells in the BAL were macrophages. Overall cell counts were low, similar to the wild-type (WT) mice that were only challenged with PBS in Fig. 1B. Furthermore, we did not detect any Th2 cytokines after restimulating the LN cells with \textit{B. tropicalis} Ag (data not shown), which again is similar to PBS-challenged WT mice in Fig. 1E. This clearly demonstrates that CD11c-cre pos IF4flex mice have a normal lung phenotype in the absence of \textit{B. tropicalis} administration and do not display spontaneous release of Th2 cytokines by T cells.

To demonstrate that the specific loss of CD11b+ DCs in CD11c-cre\textsuperscript{pos} IF4\textsuperscript{flex} mice was the key factor underlying the abrogation of allergic responses after \textit{B. tropicalis} challenge, we performed a DC subset rescue experiment. CD103+ and CD11b+ DCs were sorted from the lungs of WT mice that have been challenged with \textit{B. tropicalis} 16 h earlier, using the strategy outlined in Supplemental Fig. 1A, and transferred intratracheally into the lungs of CD11c-cre\textsuperscript{pos} IF4\textsuperscript{flex} mice. Upon subsequent multiple challenges with \textit{B. tropicalis}, only mice that received CD11b+
DCs exhibited a significant Th2 response, characterized by lung eosinophilia. In contrast, mice that received CD103+ DCs did not exhibit any allergic eosinophilia (Fig. 7A). Restimulation of the MLN cells showed a trend of increased Th2 cytokines in the mice that received CD11b+ DCs (Fig. 7B), and histological analysis showed enhanced mucus secretion only in the CD11b+ DC recip-

**FIGURE 6.** In vivo depletion of CD11b+ lung DCs abrogates the development of allergic responses to *B. tropicalis*. (A) Intracellular staining of IFN-γ and IL-4 in CD4+ T cells from the MLNs of CD11c-cre^neg^IRF4^floxed^ and CD11c-cre^pos^IRF4^floxed^ mice challenged with *B. tropicalis* 3 d earlier. Harvested draining LN cells were restimulated with 20 μg/ml *B. tropicalis* for 5 d. *n* = 10. Data pooled from three independent experiments. (B) Differential cell counts in BAL of CD11c-cre^neg^IRF4^floxed^ and CD11c-cre^pos^IRF4^floxed^ mice challenged multiple times with *B. tropicalis*, as per protocol set out in Fig. 1A. *n* = 12. Data pooled from three independent experiments. (C) Representative lung section showing mucus secretion visualized by periodic acid-Schiff's staining (original magnification ×100). Scale bar, 50 μm. (D) Levels of cytokines from lung-draining LN cells after restimulation. MLN cells were plated at a density of 400,000 cells/well in a 96-well plate and restimulated for 5 d with 20 μg/ml *B. tropicalis* extract. Cytokine levels were measured by ELISA. *n* = 12. Data pooled from three independent experiments. (E) Differential cell counts in BAL of CD11c-cre^neg^IRF4^floxed^ and CD11c-cre^pos^IRF4^floxed^ mice challenged multiple times with PBS, as per protocol set out in Fig. 1A. *n* = 6 for CD11c-cre^neg^IRF4^floxed^ and *n* = 4 for CD11c-cre^pos^IRF4^floxed^.*p* < 0.05, **p** < 0.01.

MLN cells showed a trend of increased Th2 cytokines in the mice that received CD11b+ DCs (Fig. 7B), and histological analysis showed enhanced mucus secretion only in the CD11b+ DC recip-

DCs exhibited a significant Th2 response, characterized by lung eosinophilia. In contrast, mice that received CD103+ DCs did not exhibit any allergic eosinophilia (Fig. 7A). Restimulation of the
ient mice (Fig. 7C). These results suggest that CD11b+ DCs confer the allergic phenotype absent in CD11c-cre<sup>+</sup> IRF4<sup>fl</sup> mice.

Several studies have demonstrated that monocyte-derived DCs can contribute to the pool of CD11c<sup>+</sup>CD11b<sup>+</sup> DCs in the lung at steady state (38, 39). To examine the contribution of monocyte-derived inflammatory DCs to the initiation of allergic inflammation to <i>B. tropicalis</i>, we used CCR2 knockout (CCR2KO) mice. CCR2 is required for the mobilization of monocytes from the bone marrow and therefore for the generation of inflammatory DCs (40). Importantly, we did not observe any difference in IL-4 or IFN-<i>γ</i> production from restimulated MLN cells after initial sensitization of CCR2KO mice with <i>B. tropicalis</i> (Supplemental Fig. 3A). Furthermore, eosinophil infiltration in the BAL (Supplemental Fig. 3B), mucus secretion in the airway (Supplemental Fig. 3C), and the cytokine profile of restimulated MLN cells (Supplemental Fig. 3D) were also comparable between CCR2KO and WT mice after multiple challenges with <i>B. tropicalis</i>. We also did not observe significant recruitment of inflammatory DCs into the lung at day 3 (Supplemental Fig. 3E).

Taken together, these data suggest that resident CD11b+, but not CD103<sup>+</sup> lung DCs or monocyte-derived inflammatory DCs, play an essential role in driving the development of Th2 responses to dust mite Ags in vivo.

**Lung epithelium-derived GM-CSF is a critical regulator of CD11b<sup>+</sup> DC-mediated Th2 cell priming**

GM-CSF is a critical cytokine for the development of allergic asthma as its blockade with neutralizing Abs abrogates the development of Th2 responses (41, 42). However, little is known about how GM-CSF potentiates Th2 priming. We examined the BAL fluid for the presence of pro-Th2 cytokines GM-CSF, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP) (43–45), and observed significant increase of GM-CSF and IL-33, but not IL-25 or TSLP 6 h after <i>B. tropicalis</i> challenge (Fig. 8A). GM-CSF levels remained elevated for 24 h and returned to basal levels by 48 h postchallenge (Fig. 8B). To determine the early source of GM-CSF production following <i>B. tropicalis</i> inoculation, we sorted

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**FIGURE 7.** Upon multiple challenges with <i>B. tropicalis</i>, only mice that received CD11b<sup>+</sup> DCs, not CD103<sup>+</sup> DCs, exhibited a significant Th2 response. (A) Adoptive intratracheal transfer of DC into CD11c-cre<sup>+</sup>IRF4<sup>fl</sup> mice. Both WT and CD11c-cre<sup>+</sup>IRF4<sup>fl</sup> mice were challenged with 100 µg <i>B. tropicalis</i> (day 0), and 16 h later 30,000–40,000 CD11b<sup>+</sup> DCs or CD11b<sup>+</sup> DCs sorted from the lungs of the immunized WT mice were intratracheally transferred into the lungs of CD11c-cre<sup>+</sup>IRF4<sup>fl</sup> mice. Recipient CD11c-cre<sup>+</sup>IRF4<sup>fl</sup> mice were then challenged with 10 µg <i>B. tropicalis</i> on days 9–11 and culled on day 14. n = 7 for CD11b<sup>+</sup> DCs, n = 5 for CD103<sup>+</sup> DCs, and n = 5 for PBS. Data pooled from four independent experiments. *p < 0.05, **p < 0.01. (B) Levels of cytokines from lung-draining LN cells after restimulation. MLN cells were plated at a density of 400,000 cells/well in a 96-well plate and restimulated for 5 d with 20 µg/ml <i>B. tropicalis</i> extract. Cytokine levels were measured by ELISA. n = 7. Data pooled from three independent experiments. (C) Representative lung section showing mucus secretion visualized by periodic acid-Schiff’s staining (original magnification ×100). Scale bar, 50 µm.
FIGURE 8. Lung epithelium-derived GM-CSF mediates allergic responses induced by B. tropicalis. (A) Levels of GM-CSF, IL-33, IL-25, and TSLP in BAL fluid of mice challenged with 100 μg B. tropicalis 6 h earlier. Cytokine levels were measured by ELISA. n = 12. (B) Kinetics of GM-CSF in total lung homogenate of mice treated by B. tropicalis or PBS. Cytokine levels were measured by ELISA. n = 20. (C) GM-CSF levels in the supernatant after overnight culture of CD45neg and CD45pos lung cells sorted from mice immunized with B. tropicalis or PBS 24 h earlier. UD, undetected. (D) Immunofluorescence staining of lung sections from mice immunized with PBS and B. tropicalis 24 h earlier with DAPI (blue), GM-CSF (red), and epithelial cell adhesion molecule (white). Original magnification ×200. Scale bar, 50 μm. (E) Levels of GM-CSF from lung homogenate of mice immunized with B. tropicalis mixed with either anti-GM-CSF or control goat IgG 24 h earlier. Cytokine levels were measured by ELISA. n = 10. (F) Intracellular staining of IFN-γ and IL-4 in T cells from the MLNs of mice challenged with B. tropicalis and anti-GM-CSF or control IgG Ab 3 d earlier. (Figure legend continues)
CD11b+ LUNG DCs PRIME RESPIRATORY Th2 RESPONSES

To investigate the role of epithelial-derived GM-CSF, we administered i.n. 50 μg anti-GM-CSF Ab concurrently with B. tropicalis. This dose of anti–GM-CSF neutralizing Ab was sufficient to block lung-derived GM-CSF at 24 h after B. tropicalis challenge (Fig. 8E). Consistent with a previous report (42), we observed that GM-CSF neutralization significantly ameliorated IL-4 production from restimulated MLN cells after initial sensitization with B. tropicalis (Fig. 8F). Similarly, eosinophil infiltration in the BAL (Fig. 8G), mucus secretion in the airway (Fig. 8H), and Th2 cytokine production of restimulated MLN cells (Fig. 8I) after multiple challenges of B. tropicalis were also diminished with GM-CSF blocking.

As this phenotype was similar to the one we observed in the CD11c-creIRF4flox mice that lacked lung CD11b+ DCs, we hypothesize that GM-CSF was acting directly on CD11b+ DCs. Thus, we examined the expression of GM-CSF receptor α-chain (CSF-2Rα) on lung CD103+ and CD11b+ DCs before and after challenge with B. tropicalis. At both steady state and after B. tropicalis challenge, CD11b+ DCs constitutively expressed higher levels of CSF-2Rα than CD103+ DCs (Fig. 9A), suggesting that GM-CSF may act predominantly on CD11b+ DCs. We next investigated how GM-CSF regulates CD11b+ DC function at the level of their migration capacity, maturation, or Ag presentation.

First, to test whether GM-CSF blockade affected the ability of CD11b+ DCs to migrate to the MLNs, we administered OVA-AF647 plus B. tropicalis with anti–GM-CSF or PBS and quantified the numbers of CD11b+ DCs and CD103+ DCs that were positive for OVA-AF647 in the MLNs. We observed that CD11b+ DCs from anti–GM-CSF–treated mice demonstrated equal potential to acquire OVA-AF647 and migrate to the MLNs (Fig. 9B, 9C). Moreover, anti–GM-CSF treatment did not affect the expression of CD40, CD80, and CD86 costimulatory molecules or MHC II on CD11b+ DCs (Fig. 9D). This suggests that the attenuated Th2 responses that resulted from GM-CSF blockade were not the consequence of reduced migration of DCs to the MLNs or a defect in costimulatory molecule expression.

To determine whether the ability of GM-CSF to potentiate the allergic response was through direct action on the capacity of lung-resident CD11b+ DCs to prime Th2 T cells, we sorted both DC subsets from the MLNs of mice inoculated with OVA plus B. tropicalis treated with either anti–GM-CSF or isotype control Abs and evaluated their ability to prime naïve CD4+ T cells sorted from 4ng × 10D11.10 mice. Importantly, CD11b+ DCs from anti–GM-CSF–treated mice exhibited a reduced capacity for T cell priming and Th2 polarization (Fig. 9E), suggesting that CD11b+ DCs require GM-CSF licensing to effectively prime Th2 cells. Conversely, administration of rGM-CSF together with OVA plus B. tropicalis during inoculation significantly augmented the ability of CD11b+ DCs to prime naïve CD4+ T cells and bias them toward a Th2 phenotype (Fig. 9F), whereas no such effect was observed with CD103+ DCs. Taken together, these data demonstrate that lung epithelium-derived GM-CSF is a key factor in Th2 priming and that this effect is mediated by potentiating the Ag-presenting capacity of lung-resident CD11b+ DCs to prime and polarize Th2 cells.

Discussion

We have established a mouse model of B. tropicalis respiratory allergy in which i.n. sensitization and challenge with B. tropicalis extract induce a characteristic allergic asthma phenotype. Upon challenge with B. tropicalis, CD11b+ DCs were the predominant DC subset that carried Ag to the LN and the only subset to potentially elicit Th2 priming. Finally, we showed that the capacity of CD11b+ DCs to prime and polarize CD4+ Th2 cells was critically dependent on lung epithelium-derived GM-CSF, thus identifying GM-CSF licensing of CD11b+ DCs as a critical mediator of allergic sensitization.

Lung DCs play a fundamental role in initiating the allergic response to inhaled Ags by virtue of their sentinel function and role in bridging the innate and adaptive immune systems. B. tropicalis and D. pteronyssinus are phylogenetically distinct mite species, and their allergens do not cross-react strongly (47); however, it is interesting to note that the underlying mechanism for triggering airway allergy may be similar. Although it was suggested several years ago that basophils were essential for the development of Th2 disease in response to allergens (48–50), it is now unequivocally accepted that DCs play an essential role in Th2 priming (22, 51). There has been some debate about which DC subset in the lung contributes to this process. Using ex vivo isolated DCs from the lungs of D. pteronyssinus–challenged mice, Nakano et al. (29) showed that CD103+ DCs selectively primed Th2 cells, whereas CD11b+ DCs were responsible for Th1 skewing. They substantiated this claim by showing that BTEX2 mice, which possess a point mutation in the IRF8 coding region and lack CD103+ DCs, fail to mount a robust Th2 response after D. pteronyssinus treatment. As the IRF8 mutation is not restricted solely to the DC compartment in the BXH2 model, this may have complicated their findings. In contrast, data from another group showed that CD11b+ DCs isolated from steady state lungs preferentially primed Th2 responses, whereas CD103+ DCs promoted Th1 and Th17 polarization (28). Another study using intratracheal adoptive transfer suggested that only CD11b+ DCs were able to initiate allergic sensitization to D. pteronyssinus (27). However, it has also been demonstrated that the adoptive transfer of bone marrow–derived DCs (21–23) or even splenic DCs (52) into the lung can induce allergic sensitization. Our data showing that mice lacking CD11b+ DCs are refractory to dust mite challenge support the paradigm that CD11b+ DCs are the essential DC subset responsible for allergic sensitization. This is further supported by a recent report that IFR4-dependent PDL2+ CD11b+ DCs mediate the development of allergy after i.n. challenge with papain (53), under-
FIGURE 9. Lung epithelium-derived GM-CSF is a critical regulator of CD11b+ DC priming of Th2 cells. (A) Expression of GM-CSF receptor α-chain on lung CD11b+ and CD103+ DCs 24 h after challenge with PBS or *B. tropicalis*. The mean fluorescent intensity (±SD) from three independent experiments is indicated beside the histograms. (B) Numbers of migratory CD11c+MHCIIhigh CD11b+DCs and CD103+DCs in... (Figure legend continues)
scoring the central role of lung CD11b+ DCs in orchestrating Th2 responses. It should be noted, however, that in the absence of CD11b+ DCs, Th2 responses, although dramatically attenuated, are not completely absent (Fig. 6D). We hypothesize that the reason that some Th2 cytokines are still observed in CD11c−/−IRF4−/− mice is that these mice still retain LN-resident DCs, as can be seen by the presence of the CD11c+MHCII+ population in Fig. 5B. To our knowledge, the role of these LN-resident DCs in priming Th2 cells in dust mite allergy has yet to be described. We speculate that these DCs are able to pick up low levels of B. tropicalis Ag that has drained from the lung to the lymphatics to initiate priming of Th2 CD4+ T cells.

In contrast to CD11b+ DCs that principally act early in sensitization, monocyte-derived DCs appear to be involved at a later stage during allergen challenge as they are recruited in the inflamed tissue (27). Monocyte-derived DCs could compensate for the lack of CD11b+ DCs in Fth3-deficient mice, but only when a high dose of 100 μg D. pteronyssinus was used for both sensitization and challenge. With this protocol, CCR2KO mice developed less allergic airway inflammation when exposed to a high-dose allergen challenge protocol (27). In our B. tropicalis model, however, we did not observe a role for CCR2-dependent monocytederived DCs in the development of Th2 responses, even though a high dose of 100 μg was used during sensitization. However, it should be noted that the definition of “high dose” is defined by protein amount and does not take into account that the actual concentration of the Th2-inducing allergens may vary between D. pteronyssinus and B. tropicalis extracts. Further studies to compare the allergen content of D. pteronyssinus and B. tropicalis extracts are needed.

It has long been known that the lung is a rich source of GM-CSF. Lung-conditioned medium has been widely exploited in the past as a convenient source of GM-CSF, and the cytokine can also be readily detected in cultures of primary human bronchial epithelial cells. However, excessive production of GM-CSF within the airways is able to break tolerance to inhaled Ag and elicit an allergic immune response (54). Consistent with this observation, it was reported that i.n. challenge with D. pteronyssinus resulted in the release of GM-CSF from lung epithelial cells (55) in an IL-1R-dependent mechanism (41), and that local neutralization of lung GM-CSF by Abs markedly reduced lung Th2 inflammation (41, 42). Although lung epithelium-derived GM-CSF has been strongly implicated as a key driver of the Th2 response, it has yet to be formally demonstrated that GM-CSF acts through lung DCs to promote sensitization and not indirectly through other cell types within the lung parenchyma such as lung macrophages (56). In our study, we showed that neutralizing GM-CSF dramatically inhibited the ability of CD11b+ DCs to prime Th2 CD4+ T cells, and conversely, that supplementation of rGM-CSF was able to selectively enhance the proliferation and Th2 skewing of CD4+ T cells by CD11b+ DCs and not CD103+ DCs. Although this evidence points to a key role of GM-CSF in promoting CD11b+ function during B. tropicalis challenge, it remains possible that GM-CSF may act on another bystander cell that in turn promotes CD11b+ DC priming of Th2 CD4+ T cells. Further experiments will be required to elucidate this point.

Although targeting GM-CSF might offer a therapeutic option to inhibit allergic sensitization, GM-CSF performs other tasks, and homeostatic levels of GM-CSF in the lung regulate multiple functions such as alveolar macrophage differentiation and function (57), catabolism of surfactant proteins (58), and even lung DC differentiation (59). By demonstrating a role for GM-CSF in licensing CD11b+ DCs, specific blockade of the CSF-2R signaling pathway on CD11b+ DCs might offer a novel therapeutic target. However, CSF-2R signaling on CD103+ DCs has recently been shown to require GM-CSF for optimal influenza virus clearance (60). To summarize, our study demonstrates a critical role of CD11b+ DCs in the initiation of Th2 responses to inhaled B. tropicalis allergens and that lung-derived GM-CSF is a key mediator that potentiates their ability to prime and polarize Th2 CD4+ T cells.

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Disclosures

The authors have no financial interests of conflict.

References


MLNs 24 h after challenge with B. tropicalis and control goat IgG or anti-GM-CSF Ab. n = 12. Data pooled from three independent experiments. (C) Uptake of OVA-AF647 by DC subsets in MLN cells 24 h after i.n. administration of OVA-AF647 mixed with B. tropicalis and either control goat IgG or anti-GM-CSF Ab. n = 20. Data pooled from four independent experiments. (D) Expression of costimulatory molecules in DC subsets in the MLNs 2 d after i.n. administration of B. tropicalis with either control goat IgG or anti-GM-CSF Ab. Data representative of two independent experiments. (E) Ag presentation and Th2 differentiation capacity of DC subsets in MLNs after GM-CSF blockade. Naive OVA-specific CD4+ T cells were sorted from 4×1010 mice, labeled with CFSE, and cultured with DC subsets sorted from the MLNs of mice immunized with OVA mixed with B. tropicalis plus control goat IgG or anti--GM-CSF Ab 3 d earlier. Cells were cultured for 4 d before analysis. Data representative of three independent experiments. (F) Ag presentation and Th2 differentiation capacity of DC subsets in MLNs after rGM-CSF administration. Naive OVA-specific CD4+ T cells were sorted from 4×1010 mice and cultured with DC subsets sorted from the MLNs of mice immunized with OVA mixed with B. tropicalis, with or without 1 μg GM-CSF. Data shown representative of three independent experiments. *p < 0.05, **p < 0.01.


