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Dectin-2 mediates Th2 immunity through the generation of cysteinyl leukotrienes

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The innate signaling pathways for Th2 immunity activated by inhaled antigens are not well defined. We previously identified Dectin-2 as a receptor for glycans in allergen extracts from the house dust mite *Dermatophagoides farinae* (*Df*) that mediates cysteinyl leukotriene (cys-LT) generation from pulmonary CD11c$^+$ cells and from GM-CSF–cultured bone marrow cells (BMCs^{GM-CSF}). Using lentiviral knockdown of Dectin-2 in BMCs^{GM-CSF} and adoptive transfer of *Df*-pulsed BMCs^{GM-CSF} to sensitize naive mice, we now report that Dectin-2 is critical for the development of *Df*-elicited eosinophilic and neutrophilic pulmonary inflammation and Th2 cytokine generation in the lungs and restimulated lymph nodes. Sensitization with *Df*-pulsed BMCs^{GM-CSF} from LTC_4 synthase (LTC_4S)–deficient mice or type 1 cys-LT receptor ($CysLT_1R$)–deficient mice demonstrated that both proteins were required for *Df*-elicited eosinophilic pulmonary inflammation and Th2 cytokine generation in the lungs and restimulated lymph nodes. Direct sensitization and challenge of *Ltc4s*$^{-/-}$ and *CysItr1*$^{-/-}$ mice confirmed that cys-LTs mediate these parameters of *Df*-elicited Th2 pulmonary inflammation. Thus, the Dectin-2–cys-LT pathway is critical for the induction of Th2 immunity to a major allergen, in part through $CysLT_1R$. These findings identify a previously unrecognized link between a myeloid C-type lectin receptor and Th2 immunity.

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Abbreviations used: BAL, bronchoalveolar lavage; BMC^{GM-CSF}, GM-CSF–cultured BM cell; CLR, C-type lectin receptor; cys-LT, cysteinyl leukotriene; $CysLT_1R$, type 1 cys-LT receptor; *Df*, *Dermatophagoides farinae*; *Dp*, *Dermatophagoides pteronyssinus*; HDM, house dust mite; LTC_4S, LTC_4 synthase; NLR, Nod-like receptor; ntcBMC^{GM-CSF}, nontargeting control BMC^{GM-CSF}; PRR, pattern recognition receptor; SEA, *Schistosoma mansoni* egg antigen; Syk, spleen tyrosine kinase; TLR, toll-like receptor.

Innate host responses to microbes rely on germline-encoded pattern recognition receptors (PRRs) that recognize conserved groups of molecules, PAMPs (pathogen-associated molecular patterns), that are found in microbes. PRR signaling during antigen presentation plays a critical role in influencing DC activation and cytokine production for tailoring the subsequent adaptive immune response (Iwasaki and Medzhitov, 2004). DCs express four families of PRRs: the toll-like receptors (TLRs), the Nod-like receptors (NLRs), the RIG-I and Mda5 helicases, and the membrane-associated C-type lectin receptors (CLRs). Whereas DC signaling through TLRs in response to some bacterial species is linked to the induction of IL-12 and Th1 immunity (Iwasaki and Medzhitov, 2004), and signaling through CLRs in response to fungi is linked to the generation of IL-6, IL-23, and Th17 immunity (LeibundGut-Landmann et al., 2007; Robinson et al., 2009), only TLR4 on lung stromal cells is clearly linked to the preferential generation of Th2 immunity (Hammad et al., 2009).

Nonmammalian glycans in helminths, fungi, and plants can act as potent Th2 adjuvants. *Schistosoma mansoni* egg antigen (SEA) elicits Th2 cytokines and antigen-specific IgE in BALB/c mice. This activity is lost with SEA deglycosylation, whereas conjugation of the germane α-fucosylated oligosaccharide, lacto-*N*-fucopentaose III, to human albumin promotes albumin-specific Th2 cytokine production and albumin-specific IgE (Okano et al., 1999, 2001). Ara h 1, the immunodominant peanut allergen containing xylosylated complex mannose glycans, such as $Man_{3(-4)}XylGlcNAc_2$, confers human monocyte-derived DCs with the capacity to generate IL-4– and IL-13–producing CD4$^+$ T cells from naive CD45RA$^+$

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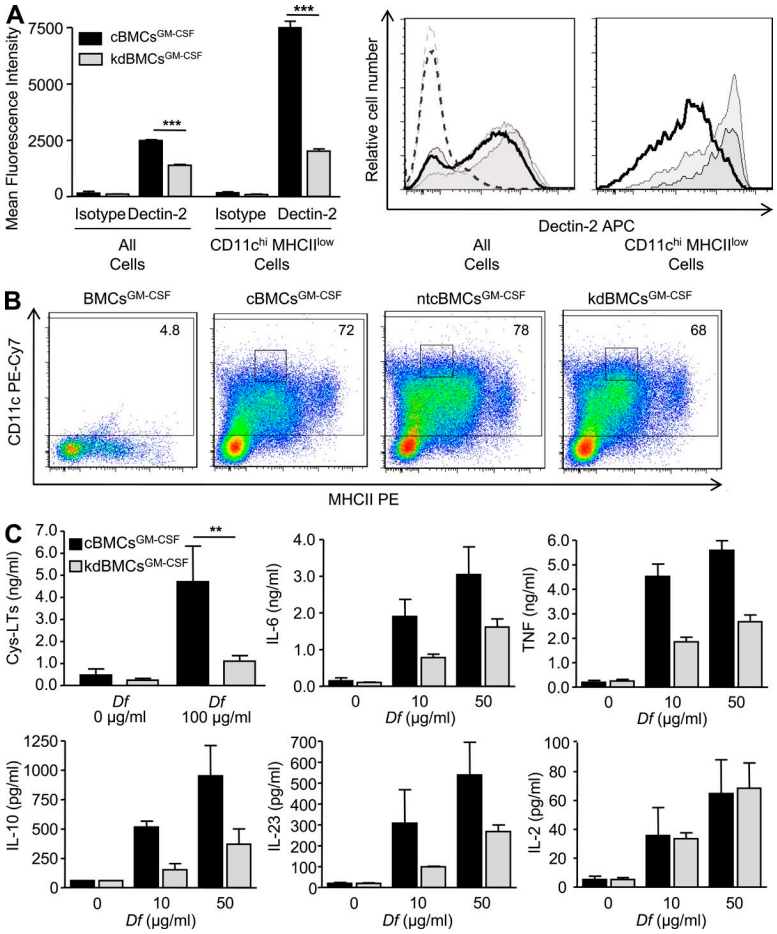

Figure 1. Dectin-2 mediates *Df*-elicited cytokine production by BMCs^{GM-CSF}. BMCs^{GM-CSF} were infected with viral particles containing Dectin-2 shRNA (kdBMCs^{GM-CSF}), a vector control (cBMCs^{GM-CSF}), or a nontargeting vector control (ntcBMC^{GM-CSF}), selected with puromycin, and harvested at day 7. (A) Dectin-2 expression on all BMCs^{GM-CSF} and on CD11c^{hi} MHCII^{low} cells was assessed by flow cytometry. Left, results are means ± SEM (n = 4–6 mice per group) from three independent experiments. ***, P = 0.0001. Significance was determined with an unpaired Student's t test. Right, a representative histogram of Dectin-2 expression on all BMCs^{GM-CSF} and on CD11c^{hi}MHCII^{low} cells from cBMCs^{GM-CSF} (gray), ntcBMC^{GM-CSF} (light gray), and kdBMCs^{GM-CSF} (heavy black line) cultures is compared. Isotype control staining on all BMCs^{GM-CSF} is also shown for cBMCs^{GM-CSF} (gray dotted), ntcBMCs^{GM-CSF} (light gray dotted), and kdBMCs^{GM-CSF} (heavy black line dotted). (B) Representative histograms are shown of CD11c expression and MHCII expression on cBMCs^{GM-CSF}, ntcBMCs^{GM-CSF}, and kdBMCs^{GM-CSF}, with the percentage of CD11c positive cells noted, as gated by the isotype control staining on a mixture of cBMCs^{GM-CSF}, ntcBMCs^{GM-CSF}, and kdBMCs^{GM-CSF}, designated BMCs^{GM-CSF}. MHCII staining was performed with nonsaturating antibody concentrations. Gating on a CD11c^{hi}MHCII^{low} Dectin-2^{hi} subset is also indicated (small boxes). (C) BMCs^{GM-CSF} were stimulated with *Df* at the indicated concentrations and the supernatant contents were assessed by ELISA for cys-LTs at 60 min and for cytokines at 4 h. Results for cys-LT generation are means ± SEM (n = 6–8 mice per group) from four independent experiments. **, P = 0.02. Significance was determined with an unpaired Student's t test. Results for cytokines are means ± SEM (n = 5–6 mice per group) from three independent experiments. P = 0.01 for IL-6, P = 0.04 for IL-10, P = 0.03 for IL-23, and P = 0.0001 for TNF. Significance was determined with two-way ANOVA (p-values refer to the integrated differences in cBMCs^{GM-CSF} and kdBMCs^{GM-CSF} over the varied doses).

T cells (Shreffler et al., 2006). In mice, the intratracheal administration of soluble β-glucan (β-1,3/1,6-linked) from *Candida albicans* with OVA augments the levels of elicited pulmonary eosinophilia, pulmonary Th2 cytokine induction, and total and OVA-specific serum IgE produced by the administration of OVA alone (Inoue et al., 2009), and β-1,3/1,6-glucan from baker's yeast can boost serum levels of OVA-specific IgE and IgG1, but not OVA-specific IgG2a, when added to an OVA footpad injection (Instanes et al., 2004). Thus, N-linked core α-fucosylated and/or β-xylosylated complex mannose glycans found widely in plants, insects, and helminths and β-1,3/1,6-glucans found in fungi appear to be nonmammalian molecular patterns important in triggering Th2 immunity (Altmann, 2007; Wills-Karp et al., 2010). However, although members of the CLR family recognize these motifs—e.g., L-SIGN (liver/lymph node–specific intercellular adhesion molecule 3–grabbing nonintegrin) recognizes α-fucosylated oligosaccharides in SEA (Meyer et al., 2007), DC-SIGN (DC-specific intercellular adhesion molecule 3–grabbing nonintegrin) recognizes xylosylated complex mannose in Ara h 1 (Shreffler et al., 2006), and Dectin-1 recognizes fungal β-glucans (Brown and Gordon, 2001)—a direct role for a myeloid CLR in the generation of Th2 immunity has not been demonstrated.

Dectin-2 is a member of the myeloid CLR family that couples to the FcRγ chain to initiate ITAM (immunoreceptor tyrosine-based activation motif)-dependent signaling and the recruitment and activation of spleen tyrosine kinase (Syk; Ariizumi et al., 2000; Sato et al., 2006). The Dectin-2–Syk pathway activates the CARD9 adaptor protein in DCs to generate NF-κB–dependent IL-2, IL-10, TNF, and IL-23p19 and to promote Th17 immune responses to *C. albicans* (Robinson et al., 2009). We have previously demonstrated that the Dectin-2–Syk pathway also triggers the generation of cysteinyl leukotrienes (cys-LTs) from pulmonary CD11c^{+} cells and from GM-CSF–cultured BM cells (BMCs^{GM-CSF}) in response to glycans in allergen extracts from the house dust mite (HDM) species *Dermatophagoides farinae* (*Df*) and *Dermatophagoides pteronyssinus* (*Dp*; Barrett et al., 2009), the most common aeroallergens to elicit sensitization in humans and potent triggers of Th2- and Th17-dependent bronchial inflammation in mice (Cates et al., 2004; Krishnamoorthy et al., 2008; Hammad et al., 2009; Phipps et al., 2009). Thus, we hypothesized that Dectin-2 is a PRR coupled to the generation of Th2 immune responses via cys-LT signaling.

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CD11c$^+$ DCs in the bronchial mucosa sample inhaled antigens and then migrate to the lung-draining lymph nodes while simultaneously activating a maturation program that results in antigen-driven T cell division. This sensitization can be mimicked by the intratracheal or intranasal adoptive transfer of antigen-pulsed myeloid DCs to naive recipient mice and subsequent antigen challenge (Lambrecht et al., 2000a,b,c). We took advantage of this adoptive transfer system and the ability to knock down Dectin-2 in BMCs$^{GM\text{-}CSF}$ to examine the role of the Dectin-2–cys-LT pathway in *Df*-induced Th2 and Th17 pulmonary inflammation. In this paper, we demonstrate that Dectin-2 mediates *Df*-elicited generation of cys-LTs and cytokines, including TNF, IL-6, IL-10, and IL-23, using lentiviral knockdown of Dectin-2 in BMCs$^{GM\text{-}CSF}$ (kdBMCs$^{GM\text{-}CSF}$). Moreover, WT mice sensitized with *Df*-pulsed kdBMCs$^{GM\text{-}CSF}$ and challenged intranasally with *Df* had significantly reduced pulmonary inflammation and Th2 cytokine generation from lung and restimulated parabronchial lymph nodes as compared with mice sensitized with *Df*-pulsed BMCs$^{GM\text{-}CSF}$ infected with a vector control (cBMCs$^{GM\text{-}CSF}$), thereby identifying Dectin-2 as a critical PRR for Th2 immunity elicited by HDM. Furthermore, we show that the genetic absence of either LTC_4 synthase (LTC_4S), the critical enzyme in cys-LT generation, or the type 1 cys-LT receptor ($CysLT_1R$) on *Df*-pulsed BMCs$^{GM\text{-}CSF}$ similarly impairs *Df*-induced Th2 pulmonary inflammation in lung and lymph nodes, thereby demonstrating that cys-LT production and $CysLT_1R$-mediated function promote Th2 sensitization.

RESULTS

Dectin-2 mediates *Df*-elicited production of IL-6, IL-10, IL-23, and TNF by BMCs$^{GM\text{-}CSF}$

Before assessing the role of Dectin-2 in *Df*-initiated cytokine responses in vitro and in vivo using GM-CSF–expanded BM cultures, we further characterized the BMCs$^{GM\text{-}CSF}$ from WT BALB/c mice. At day 7, BMCs$^{GM\text{-}CSF}$ were 74.9 ± 0.7% CD11c$^+$ by flow cytometry. The CD11c$^+$ cell population expressed CD11b (97.7 ± 0.4%) and MHCII (88.5 ± 0.2%; Fig. S1 A and not depicted). Although 89 ± 3.6% of CD11c$^+$ cells expressed Dectin-2, the range of expression was broad with CD11c^{hi}MHCII^{low} cells expressing the highest levels (Fig. S1 A). 92.4 ± 0.2% of Dectin-2$^+$ cells were CD11c$^+$ (Fig. S1 B). 7.6 ± 0.2% of Dectin-2$^+$ cells were CD11c$^-$ and expressed CD11b and the monocyte marker Ly6C (Fig. S1 B). Analysis of stained cytospins revealed that 12.4 ± 0.5% of BMCs$^{GM\text{-}CSF}$ were granulocytes (not depicted), all of which were Dectin-2$^-$ by immunocytochemistry (Fig. S1 C).

To assess the role of Dectin-2 in *Df*-induced cytokine production, we infected day-1 BMCs$^{GM\text{-}CSF}$ with lentivirus expressing a short hairpin (sh) RNA for Dectin-2 (kd-BMCs$^{GM\text{-}CSF}$), a nontargeting sequence (nontargeting control BMC$^{GM\text{-}CSF}$ [ntcBMC$^{GM\text{-}CSF}$]), or an empty vector alone (cBMCs$^{GM\text{-}CSF}$). After puromycin selection of infected cells, kdBMCs$^{GM\text{-}CSF}$ demonstrated a significant 43.9% reduction in Dectin-2 expression by flow cytometry, as compared with cBMCs$^{GM\text{-}CSF}$ (Fig. 1 A, left). A CD11c^{hi} MHCII^{low} subgroup,

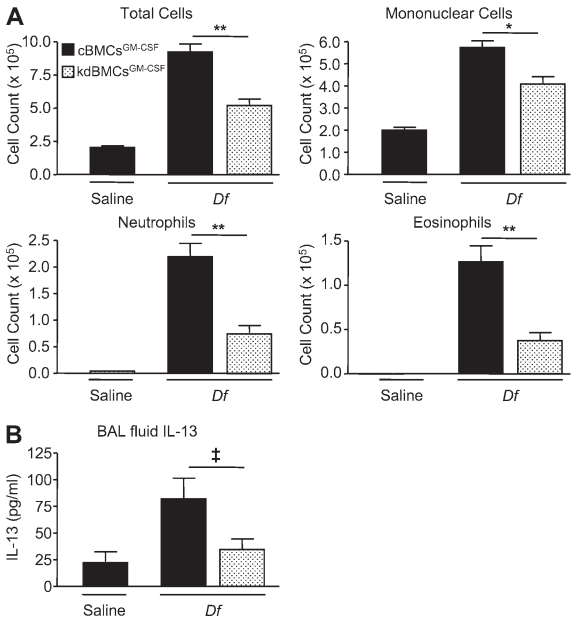

Figure 2. BMC$^{GM\text{-}CSF}$ Dectin-2 is required for *Df*-elicited Th2 pulmonary inflammation. WT mice were sensitized by intranasal administration of 10^4 saline-pulsed or *Df*-pulsed kdBMCs$^{GM\text{-}CSF}$ or cBMCs$^{GM\text{-}CSF}$ at day 8 of culture, challenged with 2 µg *Df* on day 22 and day 23, and analyzed on day 25. (A) Cells from the BAL fluid were counted, cytospin preparations were stained, and 400 cells/slide were counted for specific cell types. Results are means ± SEM (n = 13–14 mice per group) from three independent experiments. **, P = 0.0001; *, P = 0.001. Significance was determined with an unpaired Student's t test. (B) ELISA was performed on BAL fluid supernatants. Results are means ± SEM (n = 14 mice per group) from three independent experiments. ‡, P = 0.05.

which was 5 ± 0.8% of total cells, expressed high levels of Dectin-2 in control vector BMC$^{GM\text{-}CSF}$ cultures and demonstrated a significant 73.6% reduction in Dectin-2 expression in knockdown BMC$^{GM\text{-}CSF}$ cultures (Fig. 1 A, left). Dectin-2 expression on all BMCs$^{GM\text{-}CSF}$ and on CD11c^{hi} MHCII^{low} cells from ntcBMC$^{GM\text{-}CSF}$ was comparable to cBMCs$^{GM\text{-}CSF}$ and distinct from the reduction observed in kdBMCs$^{GM\text{-}CSF}$ (Fig. 1 A, right). Day-7 cBMCs$^{GM\text{-}CSF}$, ntcBMCs$^{GM\text{-}CSF}$, and kdBMCs$^{GM\text{-}CSF}$ showed similar expression of CD11c and MHCII (Fig. 1 B). The knockdown was confirmed by bioassay, in which kdBMCs$^{GM\text{-}CSF}$ demonstrated a 79.6% reduction in cys-LT generation after stimulation for 1 h with 100 µg/ml *Df* (Fig. 1 C). In parallel, we stimulated cells at 0, 10, or 50 µg/ml *Df* for 4 or 30 h. At 4 h, kdBMCs$^{GM\text{-}CSF}$ stimulated with *Df* had significant reductions in the generation of IL-6, IL-10, IL-23, and TNF, as compared with cBMCs$^{GM\text{-}CSF}$ (Fig. 1 C). There was no reduction in IL-2. At 30 h, kd-BMCs$^{GM\text{-}CSF}$ had significant reductions in IL-6, IL-10, and TNF, as compared with cBMCs$^{GM\text{-}CSF}$, and a lower level of IL-23 that did not reach statistical significance (Fig. S2 A). kdBMCs$^{GM\text{-}CSF}$ had no reduction in TNF generation in response to LPS or to curdlan (Fig. S2 B) and no reduction in cys-LT generation in response to curdlan (Fig. S2 B), indicating the functional integrity of TLR4 and Dectin-1.

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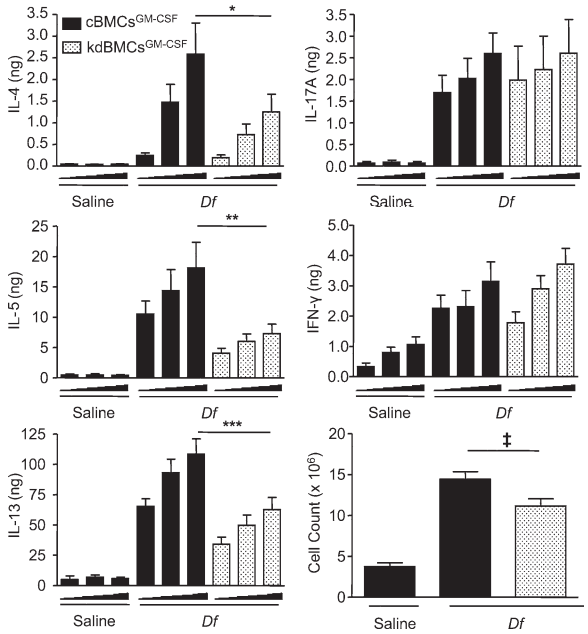

Figure 3. BMC^{GM-CSF} Dectin-2 is required for lymph node Th2 cytokine production elicited by *Df*. WT mice were sensitized with saline-pulsed or *Df*-pulsed kdBMCs^{GM-CSF} or cBMCs^{GM-CSF} and challenged with *Df* as described in the Fig. 2 legend. Parabronchial lymph node cells were isolated, counted, and restimulated for 72 h with 0, 1, or 5 µg/ml *Df*, and cytokines in the supernatant were measured by ELISA. Total cytokine production per mouse is shown. Results are means ± SEM (n = 10–13 mice per group) from three independent experiments. Triangles under x axes indicate 0, 1, and 5 µg/ml *Df*, from left to right. *, P = 0.04; **, P = 0.01; ***, P = 0.007; ‡, P = 0.03. Significance was determined with an unpaired Student's *t* test.

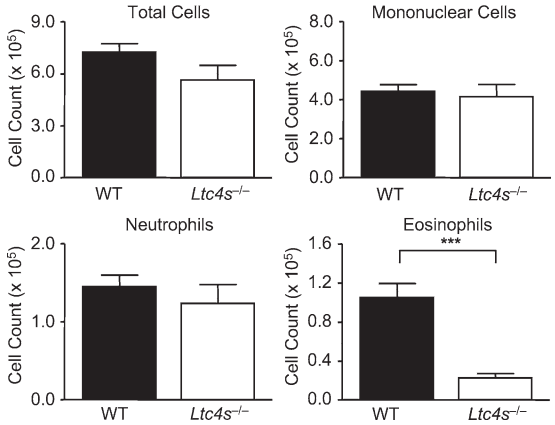

Figure 4. BMC^{GM-CSF} LTC$_4$S is required for *Df*-elicited eosinophilic pulmonary inflammation. WT mice were sensitized by intranasal administration of 10^4 *Df*-pulsed WT or *Ltc4s*$^{-/-}$ BMCs^{GM-CSF}, challenged with 2 µg *Df* on day 22 and day 23, and analyzed on day 25. Cells from the BAL fluid were counted, cytospin preparations were stained, and 400 cells/slide were counted for specific cell types. Results are means ± SEM (n = 13–14 mice per group) from four independent experiments. ***, P = 0.0001. Significance was determined with an unpaired Student's *t* test.

BMC^{GM-CSF} Dectin-2 mediates *Df*-elicited Th2 pulmonary inflammation

We next assessed whether Dectin-2 influenced the ability of BMCs^{GM-CSF} to initiate a *Df*-induced immune response in an adoptive transfer model. We stimulated day-7 kdBMCs^{GM-CSF} and cBMCs^{GM-CSF} with 100 µg/ml *Df* or saline for 24 h, adoptively transferred 10^4 cells into WT recipients by intranasal injection on day 8, challenged recipients with 2 µg *Df* intranasally on day 22 and day 23, and euthanized the recipients for assay on day 25. *Df*-pulsed cBMCs^{GM-CSF} were able to sensitize recipients for *Df* challenge, inducing a robust inflammatory response with recruitment of mononuclear cells, neutrophils, and eosinophils in bronchoalveolar lavage (BAL) fluid. After *Df* challenge, recipients sensitized with kd-BMCs^{GM-CSF} had significant reductions in total cell counts of 43.6%, in mononuclear cells of 28.9%, in neutrophils of 65.9%, and in eosinophils of 70.3%, as compared with recipients of cBMCs^{GM-CSF} (Fig. 2 A). To determine whether the reduction in BAL fluid inflammation was associated with a reduction in T cell cytokines, ELISA was performed on the BAL fluid supernatants for IL-13, IL-17A, and IFN-γ (Fig. 2 B). Recipients sensitized with kdBMCs^{GM-CSF} had a significant 59.7% reduction in BAL fluid IL-13, as compared with mice sensitized with cBMCs^{GM-CSF}. The levels of IL-17A and IFN-γ were below the limits of detection.

To determine whether the reduction in BAL fluid inflammatory cells and cytokines reflected an impairment in the generation of Th2 and/or Th17 immune responses in the lymph nodes, we assessed cytokine generation from the lung-draining lymph nodes of the three groups of mice. Upon ex vivo restimulation with 0, 1, or 5 µg/ml *Df* for 72 h, recipients sensitized with *Df*-pulsed cBMCs^{GM-CSF} generated robust production of T cell cytokines, as compared with recipients of saline-pulsed cBMCs^{GM-CSF}. Lymph node cells from recipients sensitized with *Df*-pulsed kdBMCs^{GM-CSF} had significant reductions at the 5-µg/ml dose in the total generation of IL-4, IL-5, and IL-13 per mouse of 51.7, 59.6, and 42.2%, respectively, compared with recipients of *Df*-pulsed cBMCs^{GM-CSF} (Fig. 3). IL-17A and IFN-γ were not different between the *Df*-pulsed groups. The reduction in IL-4, IL-5, and IL-13 generated by recipients of *Df*-pulsed kdBMCs^{GM-CSF} was the result of both a reduction in concentration of each cytokine per million cells plated and an 18.9% reduction in the total lymph node cell count (Fig. 3). The reductions in concentration were significant for IL-5 and IL-13, whereas the trend for IL-4 did not reach significance. Thus, the cytokine findings of a reduced Th2 response in the restimulated nodal cells reflected the reduced eosinophil count and the reduced IL-13 level in the BAL fluid. There was no reduction in IL-17A generation to account for the notable neutrophil reduction in the BAL fluid of mice sensitized with kdBMCs^{GM-CSF}.

BMC^{GM-CSF} cys-LT generation mediates *Df*-elicited Th2 pulmonary inflammation

As Dectin-2 can elicit generation of both cys-LTs and proinflammatory cytokines, we next determined the contribution of these lipid mediators to *Df*-induced pulmonary inflammation in the adoptive transfer model. We generated BMCs^{GM-CSF}

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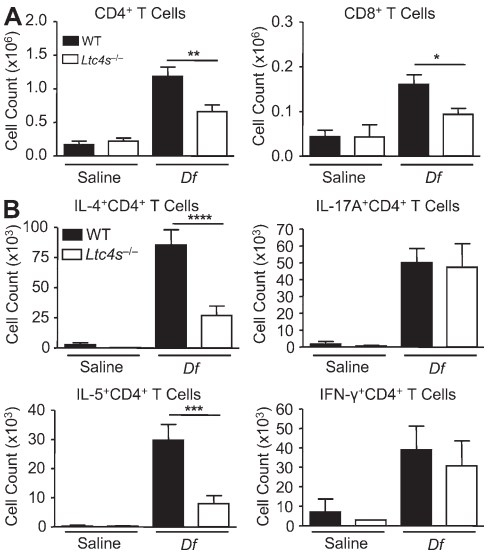

Figure 5. BMC$^{GM\text{-}CSF}$ LTC$_4$S is required for *Df*-elicited CD4$^+$ Th2 cell recruitment to the lung. WT mice were sensitized with saline-pulsed or *Df*-pulsed WT or *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ and challenged with *Df* as described in the legend to Fig. 4. Pulmonary mononuclear cells were isolated, stimulated with 50 ng/ml PMA and 1 µM ionomycin for 10 h in the presence of 2.5 µM monensin, stained for cell surface expression of CD4 and CD8 (A) and for intracellular expression of IL-4, IL-5, IL-17A, and IFN-γ (B), and analyzed by flow cytometry. Gating on cell size, on CD4 and CD8 expression, the total number of cells recruited to the lung is shown. Results are means ± SEM (n = 11 mice per group) from two experiments. *, P = 0.02; **, P = 0.005; ***, P = 0.002; ****, P = 0.0008. Significance was determined with an unpaired Student's t test.

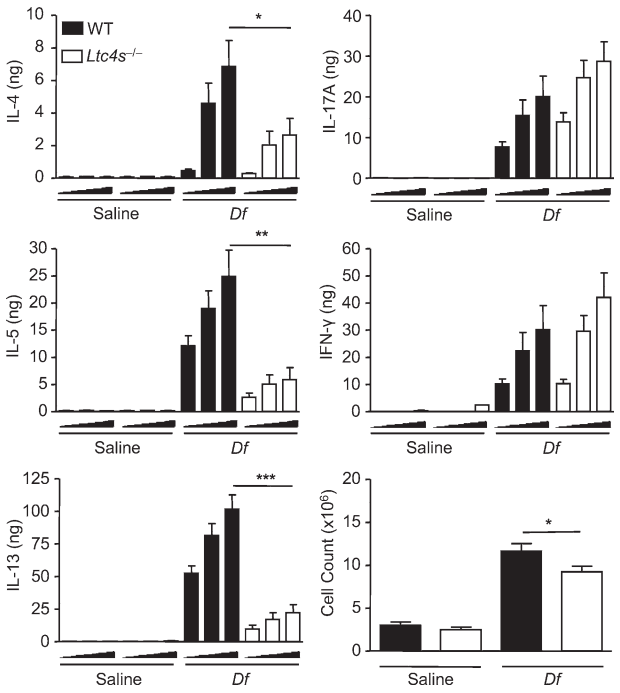

Figure 6. BMC$^{GM\text{-}CSF}$ LTC$_4$S is required for lymph node Th2 cytokine production elicited by *Df*. WT mice were sensitized with saline-pulsed or *Df*-pulsed WT or *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ and challenged with *Df* as described in the legend to Fig. 4. Parabronchial lymph node cells were isolated, counted, and restimulated for 72 h with 0, 1, or 5 µg/ml *Df*, and cytokines in the supernatant were measured by ELISA. Total cytokine production per mouse is shown. Results are means ± SEM (n = 12–15 mice per group) from three experiments. Triangles under x axes indicate 0, 1, and 5 µg/ml *Df*, from left to right. *, P = 0.04; **, P = 0.001; ***, P = 0.0001. Significance was determined with an unpaired Student's t test.

from BALB/c WT and LTC$_4$S-deficient (*Ltc4s*$^{-/-}$) mice, pulsed them with *Df*, sensitized WT recipients via intranasal transfer of *Df*-pulsed BMCs$^{GM\text{-}CSF}$, and challenged recipients intranasally with *Df* to elicit memory responses. Whereas WT BMCs$^{GM\text{-}CSF}$ sensitized recipients for robust pulmonary inflammation with recruitment of mononuclear cells, neutrophils, and eosinophils to the airway, mice sensitized with *Df*-pulsed *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ had a selective 78.4% reduction in the recruitment of eosinophils to the BAL fluid with *Df* challenge (Fig. 4).

To determine whether the attenuation of eosinophil recruitment in mice sensitized with *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ reflected a decrement in Th2 cells in the lung, we characterized the CD4$^+$ and CD8$^+$ T cell populations recruited to the lung in another cohort sensitized and challenged in the same manner. Pulmonary mononuclear cells were isolated, stimulated with PMA and ionomycin in the presence of monensin, permeabilized, and stained for CD4, CD8, IL-4, IL-5, IL-17A, and IFN-γ. Fig. 5 A shows that mice sensitized with *Df*-pulsed WT BMCs$^{GM\text{-}CSF}$ and challenged with *Df* had recruitment of both CD4$^+$ and CD8$^+$ T cells to the lung, as compared with mice sensitized with saline-pulsed WT BMCs$^{GM\text{-}CSF}$. Recruitment of CD4$^+$ and CD8$^+$ T cells was significantly reduced by 44.4 and 41.9%, respectively, in mice sensitized with *Df*-pulsed *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$. Recruited CD4$^+$ T cells produced IL-4, IL-5, IL-17A, and IFN-γ (Fig. 5 B and Fig. S3). There was no significant induction of these cytokines from CD8$^+$ T cells. Recipients of *Df*-pulsed *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ had a significant 68.6% reduction in the number of IL-4$^+$ CD4$^+$ T cells and a significant 73.0% reduction in the number of IL-5$^+$CD4$^+$ T cells, as compared with mice sensitized with *Df*-pulsed WT BMCs$^{GM\text{-}CSF}$, whereas the numbers of IL-17A$^+$ and IFN-γ$^+$CD4$^+$ T cells were unchanged. Thus, the selective decline in BAL fluid eosinophils was paralleled by a selective reduction in Th2 cells recruited to lung.

We next assessed the lung-draining lymph nodes to determine whether the profile of cytokine production was similarly impaired. Cells from the lung-draining lymph nodes of sensitized and challenged recipients were restimulated ex vivo with *Df* at 0, 1, or 5 µg/ml, and cytokines in the supernatant were measured at 72 h. Recipients sensitized with *Df*-pulsed WT BMCs$^{GM\text{-}CSF}$ generated robust production of T cell cytokines, as compared with recipients of saline-pulsed WT BMCs$^{GM\text{-}CSF}$. Mice sensitized with *Df*-pulsed *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ had significant reductions in the total generation of IL-4, IL-5, and IL-13 per mouse of 61.5, 76.4, and 78.2%, respectively, as compared with mice sensitized with

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Df-pulsed WT BMCs$^{GM\text{-}CSF}$ (Fig. 6). The reduction in IL-4, IL-5, and IL-13 generation by recipients of *Df*-pulsed *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ was the result of both a reduction in concentration of each cytokine per million cells plated and a 20.5% reduction in the total lymph node cell count (Fig. 6). The reductions in concentration were significant for IL-5 and IL-13, whereas the trend for IL-4 did not reach significance. There was no difference in the total generation of IL-17A or IFN-γ per mouse. Thus, the attenuation in pulmonary eosinophil and Th2 cell recruitment to the lung in mice sensitized with *Df*-pulsed *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ appeared to reflect a selective defect in Th2 immunity to *Df*.

To confirm that MHCII-dependent antigen presentation by BMCs$^{GM\text{-}CSF}$ was required to sensitize recipient mice for the generation of eosinophilic pulmonary inflammation and Th2 cytokine induction after *Df* challenge, we sensitized WT C57BL/6 mice with *Df*-pulsed WT or MHCII-deficient (MHCII$^{\Delta/\Delta}$) BMCs$^{GM\text{-}CSF}$. After *Df* challenge, recipients of *Df*-pulsed MHCII$^{\Delta/\Delta}$ BMCs$^{GM\text{-}CSF}$ had significant reductions in BAL fluid total cells of 88.3%, in monocytes of 66.9%, in neutrophils of 97%, and in eosinophils of 99%, as compared with recipients of *Df*-pulsed WT BMCs$^{GM\text{-}CSF}$ (Fig. S4 A). Lymph node cells were isolated, stimulated with PMA and ionomycin in the presence of monensin, permeabilized, and stained for CD4, CD8, IL-4, IL-5, IL-17A, and IFN-γ. As shown for a representative plot (Fig. S4 B), IL-4, IL-5, and IL-17A were induced in CD4$^+$ lymphocytes from recipients of WT, but not MHCII$^{\Delta/\Delta}$, BMCs$^{GM\text{-}CSF}$. We did not detect IL-4, IL-5, or IL-17A in any other cell population. Thus, MHCII-mediated antigen presentation from transferred BMCs$^{GM\text{-}CSF}$ was necessary to sensitize WT recipients for the appearance of a cellular infiltrate in the BAL fluid and CD4$^+$ Th2 cells in the draining lymph nodes.

Th2 differentiation may be altered by the maturation of DCs, their expression of costimulatory molecules, their production of proinflammatory cytokines, or their ability to migrate to the lung-draining lymph nodes. Flow cytometry revealed comparable cell surface expression of CD80, CD86, CD40, OX40L, CCR7, PD-L1, and ICOSL on CD11c$^+$MHCII$^+$ WT and *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ after *Df* loading (Fig. S5). We assessed cytokine production by WT and *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ stimulated with *Df* and found no significant difference in the ability to generate TNF, IL-10, IL-6, IL-2, or IL-23 (Fig. S6). IL-12p70 was not detected from WT or *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ (unpublished data). BMCs$^{GM\text{-}CSF}$ can chemotax to LTD$_4$ in a CysLT$_1$R-dependent fashion, and cys-LTs have been reported to enhance DC migration to the DC chemotactic ligand for CCR7, CCL19 (Robbiani et al., 2000). We conjugated *Df* to Alexa Fluor 647 and assessed the migration of intranasally transferred Alexa Fluor 647–*Df*-pulsed BMCs$^{GM\text{-}CSF}$ to the regional lymph nodes 24 h later. We found that Alexa Fluor 647–*Df*-pulsed WT and *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ trafficked to the regional lymph nodes to a similar extent and comprised 7.9 ± 0.75 and 13.5 ± 4.7% of CD11c^{hi}MHCII^{hi} cells, respectively, at 24 h over two experiments (Fig. S7).

CysLT$_1$R function conditions BMCs$^{GM\text{-}CSF}$ for Th2 immune responses

We next sought to determine whether the Th2 priming function of cys-LTs was mediated through CysLT$_1$R. WT recipients were sensitized by intranasal transfer of *Df*-pulsed WT or CysLT$_1$R-deficient (*Cysltr1*$^{-/-}$) BMCs$^{GM\text{-}CSF}$ and directly challenged with *Df*. Although *Df*-pulsed WT BMCs$^{GM\text{-}CSF}$ initiated a characteristic robust pulmonary inflammation with the recruitment of mononuclear cells, neutrophils, and eosinophils to the airway, mice sensitized with *Df*-pulsed *Cysltr1*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ had a selective 45.1% reduction in eosinophil recruitment to the BAL fluid (Fig. 7). Restimulation of lung-draining lymph node cells demonstrated that recipients sensitized with *Df*-pulsed WT BMCs$^{GM\text{-}CSF}$ generated robust production of T cell cytokines, as compared with recipients of saline-pulsed WT BMCs$^{GM\text{-}CSF}$. At 5 μg/ml *Df*, mice sensitized with *Df*-pulsed *Cysltr1*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ had significant reductions in the total generation of IL-5 and IL-13 per mouse of 53.7 and 47.4%, respectively, as compared with recipients of *Df*-pulsed WT BMCs$^{GM\text{-}CSF}$, and a 69.3% fall in IL-4 that did not reach statistical significance (Fig. 8). The reductions in IL-5 and IL-13 were the result of a significant 36.7% reduction in the total lymph node cell count (Fig. 8). There was no significant difference in the generation of IL-17A or IFN-γ between mice sensitized with WT BMCs$^{GM\text{-}CSF}$ or *Cysltr1*$^{-/-}$ BMCs$^{GM\text{-}CSF}$. Thus, *Cysltr1*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ had a selective impairment in the generation of Th2 immunity that was similar to the findings with *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$.

Cys-LTs mediate *Df*-elicited active sensitization and Th2 pulmonary inflammation

To demonstrate that cys-LT generation and CysLT$_1$R function are each required for Th2 pulmonary inflammation to *Df* in a direct immunization model, we sensitized and challenged

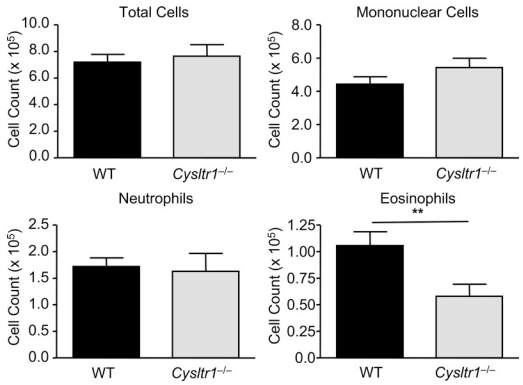

Figure 7. *Df*-elicited eosinophilic pulmonary inflammation is dependent on BMC$^{GM\text{-}CSF}$ CysLT$_1$R. WT mice were sensitized with intranasal administration of 10^4 *Df*-pulsed WT or *Cysltr1*$^{-/-}$ BMCs$^{GM\text{-}CSF}$, challenged with 2 μg *Df* on day 22 and day 23, and analyzed on day 25. Cells from the BAL fluid were counted, cytospin preparations were stained, and 400 cells/slide were counted for specific cell types. Results are means ± SEM (n = 6–7 mice per group) from two experiments. **, P = 0.02. Significance was determined with an unpaired Student's t test.

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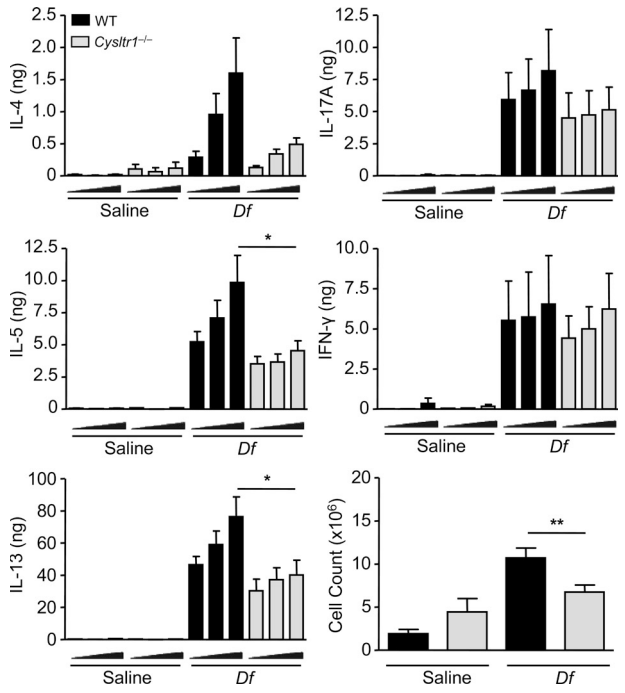

Figure 8. *Df*-elicited Th2 cytokine generation is dependent on BMC^{GM-CSF} CysLT$_1$R. WT mice were sensitized with saline-pulsed or *Df*-pulsed WT or *Cysltr1*$^{-/-}$ BMCs^{GM-CSF} and challenged with *Df* as described in the legend to Fig. 7. Parabronchial lymph node cells were isolated, counted, and restimulated for 72 h with 0, 1, or 5 µg/ml *Df*, and cytokines in the supernatant were measured by ELISA. Total cytokine production per mouse is shown. Results are means ± SEM (n = 7 mice per group) from two experiments. Triangles under x axes indicate 0, 1, and 5 µg/ml *Df*, from left to right. *, P = 0.03; **, P = 0.02. Significance was determined with an unpaired Student's t test.

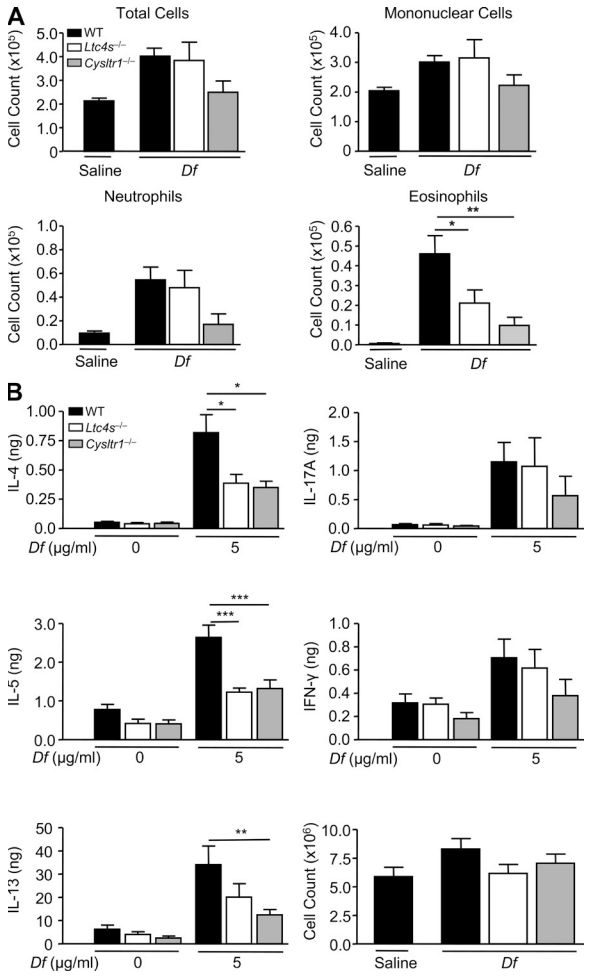

Figure 9. *Df*-elicited eosinophilic pulmonary inflammation and Th2 cytokine generation is dependent on LTC$_4$S and CysLT$_1$R. WT (black), *Ltc4s*$^{-/-}$ (white), or *Cysltr1*$^{-/-}$ (gray) mice were sensitized with 10 µg *Df* or saline on days 0 and 1, challenged with 10 µg *Df* on days 15 and 16, and analyzed on day 18. (A) Cells from the BAL fluid were counted, cytospin preparations were stained, and 400 cells/slide were counted for specific cell types. Results are means ± SEM (n = 7–12 mice per group) from three experiments. *, P = 0.05; **, P = 0.01. (B) Parabronchial lymph node cells were isolated, counted, and restimulated for 72 h with 0 or 5 µg/ml *Df*, and cytokines in the supernatant were measured by ELISA. Total cytokine production per mouse is shown. Results are means ± SEM (n = 7–12 mice per group) from three experiments. *, P = 0.05; **, P = 0.01; ***, P = 0.001. Significance was determined with a one-way ANOVA.

WT, *Ltc4s*$^{-/-}$, and *Cysltr1*$^{-/-}$ mice on a BALB/c background with intranasal *Df* on days 0, 1, 15, and 16 and assessed pulmonary inflammation in the BAL fluid and Th2 cytokine production from restimulated lung-draining lymph node cells. Although WT mice developed a neutrophilic and eosinophilic inflammatory response in the BAL fluid (Fig. 9 A), *Ltc4s*$^{-/-}$ and *Cysltr1*$^{-/-}$ mice had significant reductions in eosinophil recruitment by 48 and 76%, respectively. *Cysltr1*$^{-/-}$ mice had an additional reduction in neutrophil recruitment to the BAL fluid that did not reach statistical significance. Cytokines from restimulated lung-draining lymph node cells showed significant 53 and 57% reductions in IL-4 and significant 54 and 50% reductions in IL-5 in *Ltc4s*$^{-/-}$ and *Cysltr1*$^{-/-}$ mice, respectively, as compared with WT (Fig. 9 B). *Cysltr1*$^{-/-}$ mice had a significant 63% reduction in IL-13, whereas in *Ltc4s*$^{-/-}$ mice the reduction did not reach statistical significance. There were additional reductions in IL-17A and in IFN-γ from *Cysltr1*$^{-/-}$ mice that did not reach statistical significance.

Dectin-2 mediates *Df*-elicited active sensitization and Th2 pulmonary inflammation

Finally, to assess the role of Dectin-2 in *Df*-elicited Th2 pulmonary inflammation in the direct immunization model, we sensitized and challenged BALB/c mice with intranasal *Df* and treated them with a monoclonal antibody to Dectin-2 or the rat IgG2a isotype control on days 0, 2, 15, and 17. Mice treated with the Dectin-2 antibody had significant reductions in BAL fluid total cells (64%), mononuclear cells (61%), neutrophils (78%), and eosinophils (80%), as compared with mice treated with the isotype control (Fig. 10 A). Cytokines from restimulated lung-draining lymph node cells demonstrated that mice treated with the Dectin-2 antibody had significant reductions in the total generation of IL-4, IL-5, IL-13, and IL-17A per mouse of 47, 55, 44, and 90%, respectively, as

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compared with mice treated with the isotype control (Fig. 10 B). The reduction in IL-4, IL-5, and IL-13 generation by mice treated with the Dectin-2 antibody was the result of a 72% reduction in the total lymph node cell count, whereas the reduction in IL-17A was the result of both a reduction in the concentration of each cytokine per million cells plated and the reduction in the total lymph node cell count (Fig. 10 B).

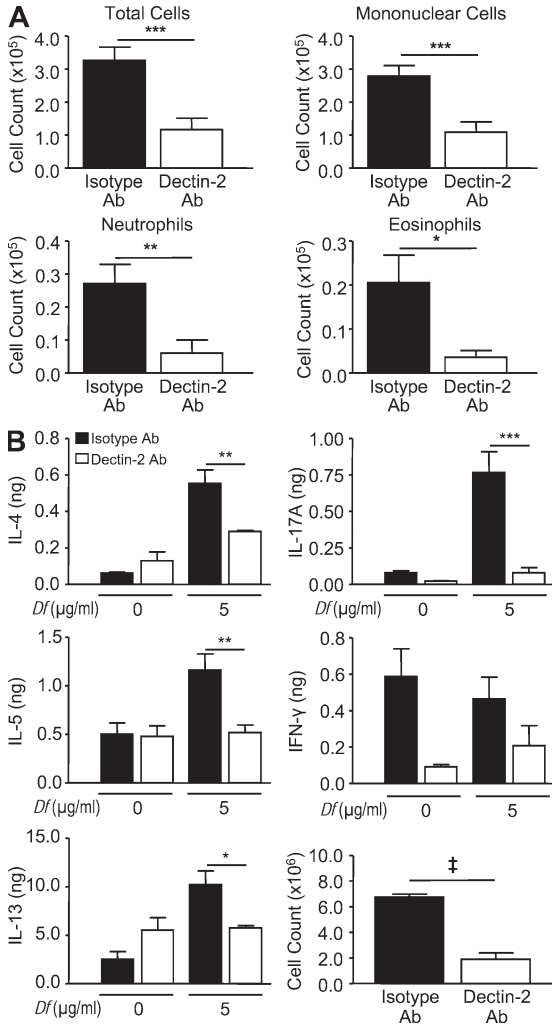

Figure 10. *Df*-elicited pulmonary inflammation and Th2 cytokine generation is dependent on Dectin-2. WT mice were sensitized and challenged as described in the Fig. 9 legend. Mice were injected intraperitoneally with 200 µg Dectin-2 antibody or a rat IgG2a isotype control on days 0, 2, 15, and 17, and analyzed on day 18. (A) Cells from the BAL fluid were counted, cytospin preparations were stained, and 400 cells/slide were counted for specific cell types. (B) Parabronchial lymph node cell counts. Results are means ± SEM (n = 4 mice per group) and are representative of two independent experiments. *, P = 0.04; **, P = 0.02; ***, P = 0.01. (B) Parabronchial lymph node cells were isolated, counted, and restimulated for 72 h with 0 or 5 µg/ml *Df*, and cytokines in the supernatant were measured by ELISA. Total cytokine production per mouse is shown. Results are means ± SEM (n = 4 mice per group) and are representative of two independent experiments. *, P = 0.04; **, P = 0.03; ***, P = 0.01; ‡, P = 0.0001. Significance was determined with an unpaired Student's t test.

DISCUSSION

PRR signaling is central to the initiation of Th1 and Th17 adaptive immune responses. We have previously identified Dectin-2 on pulmonary CD11c$^+$ cells and on BMCs$^{GM\text{-}CSF}$ as a PRR for clinically relevant allergens including *Df*, *Dp*, and *Aspergillus fumigatus* extract (Barrett et al., 2009). In this study, we demonstrate that Dectin-2 activation elicited by *Df* in both adoptive transfer and direct sensitization and challenge models triggers Th2 pulmonary inflammation and that this coupling is mediated through the specific actions of cys-LTs. Notably, TLR4 on lung stromal cells is the only PRR previously identified that links HDM recognition to Th2 pulmonary inflammation (Hammad et al., 2009). Arachidonic acid release and cys-LT generation can be elicited by signaling from other myeloid CLRs, such as Dectin-1 and DC-SIGN (Olynych et al., 2006; Suram et al., 2006; Valera et al., 2007), and although these receptors recognize glycans that can serve as adjuvants for a Th2 response (Shreffler et al., 2006; Inoue et al., 2009), no mechanism for this linkage has been suggested. Herein, we observe a link to Th2 immunity not only via Dectin-2–mediated generation of cys-LTs from BMCs$^{GM\text{-}CSF}$ but also via CysLT$_1$R function on these cells.

Both human DCs and mouse BMCs$^{GM\text{-}CSF}$ express the CysLT$_1$R, and two prior pharmacologic studies with CysLT$_1$R antagonists show that cys-LTs can augment Th2 sensitization in BALB/c mice (Machida et al., 2004; Okunishi et al., 2004). In WT mice sensitized by the adoptive transfer of *Df*-pulsed BMCs$^{GM\text{-}CSF}$ and challenged with *Df*, the recruitment of eosinophils to lung and the levels of IL-5 in BAL fluid were increased by costimulation with LTD$_4$ and suppressed by the presence of a CysLT$_1$R antagonist during the in vitro *Df* pulsing (Machida et al., 2004). Systemic administration of a CysLT$_1$R antagonist during OVA sensitization reduced the levels of BAL fluid IL-4, IL-5, and IFN-γ elicited by OVA challenge (Okunishi et al., 2004). However, in the current study, the reductions in Th2 cytokines in restimulated lymph node cells were not accompanied by a reduction in IFN-γ production in WT recipients sensitized with BMCs$^{GM\text{-}CSF}$ lacking LTC$_4$S or CysLT$_1$R.

Our findings for a CysLT$_1$R function on BMCs$^{GM\text{-}CSF}$ in Th2 sensitization prompt a consideration of possible pathways. We did not find evidence for impaired generation of cytokines such as TNF from *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ in response to *Df* (Fig. S6). Nor did we detect any difference in maturation or expression of costimulatory markers between *Df*-pulsed WT and *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ (Fig. S5). Cys-LTs can specifically modulate human and mouse DC migration (Robbiani et al., 2000; Thivierge et al., 2006), but we did not see any impairment in the migration of Alexa Fluor 647–*Df*-pulsed *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ to the lung-draining lymph nodes, as compared with WT controls (Fig. S7). The CysLT$_1$R ligand, LTD$_4$, can stimulate human neutrophil adhesion to intercellular adhesion molecule 1 in a manner that is dependent on the β2 integrin CD18 and is blocked by the selective CysLT$_1$R antagonist montelukast (Meliton et al., 2010). Moreover, LTD$_4$ can promote α4β1 and α5β1 integrin–dependent adhesion of

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human CD34$^+$ hematopoietic progenitor cells to endothelial cells, vascular cell adhesion molecule 1, and fibronectin, and such adhesion is blocked by CysLT$_1$R antagonism (Boehmler et al., 2009). As CD18/CD11a and the α4β1 integrin are critical membrane proteins recruited to the DC–T cell immune synapse, CysLT$_1$R facilitation of cell–cell adhesion and the organization of the immune synapse may be a mechanism by which CysLT$_1$R can promote nascent Th2 immune responses.

GM-CSF–expanded BM cultures at day 7 provide a mixed population of cells, including Dectin-2$^-$ granulocytes (12.4 ± 0.5%; Fig. S1 C) and Dectin-2$^+$CD11c$^-$ cells (7.0 ± 0.5%) that express CD11b and Ly6C, presumably identifying Dectin-2$^+$ monocytes in this culture. The dominant CD11c$^+$ cell population in these cultures expresses CD11b (97.7 ± 0.4%) and MHCII (88.5 ± 2%). Such cells, termed inflammatory DCs, were recently identified as the critical antigen-presenting cells to drive Th2 responses to HDM (Hammad et al., 2010), and our findings suggest that Dectin-2 expression on these cells may be central to this function. As Dectin-2 is highly expressed on immature CD11c^{hi}MHCII^{low} cells (Fig. 1 A and Fig. S1 A), further studies are needed to determine whether Dectin-2 expression identifies a particular subset of inflammatory DCs in vivo that prime for Th2 immunity.

We found a significant role for Dectin-2–mediated cys-LT production and CysLT$_1$R function in the generation of Th2 immunity to *Df* in both the adoptive transfer studies with *Df*-pulsed BMCs$^{GM\text{-}CSF}$ and the direct sensitization and challenge of LTC$_4$S- and CysLT$_1$R-deficient strains. However, our studies do not exclude a contribution from the *Df*-elicited Dectin-2–dependent cytokines. The pharmacologic addition of TNF to OVA/LPS sensitization can restore pulmonary DC activation and migration and the induction of eosinophilic pulmonary inflammation that is impaired in BALB/c mice with defective TLR4 signaling (Eisenbarth et al., 2002). Mice with a targeted deletion of the IL-23α subunit p19 have attenuated Th2 pulmonary inflammation and specific IgE levels after i.p. sensitization with OVA/alum and intranasal challenge with OVA, which are associated with an impairment in T cell GATA-3 induction (Peng et al., 2010). Antigen-presenting cell–derived IL-6 can drive IL-4 production from naive murine CD4$^+$ T cells (Rincón et al., 1997), and neutralization of IL-6 in ex vivo lymph node cell cultures from mice sensitized with HDM-pulsed BMCs$^{GM\text{-}CSF}$ diminished the production of IL-13 (Krishnamoorthy et al., 2008). As the generation of TNF, IL-23, and IL-6 was blunted in kdBMCs$^{GM\text{-}CSF}$, these mediators may also contribute to *Df*-induced Dectin-2–dependent Th2 immunopathology.

We found a robust Th17 immune response in mice receiving *Df*-pulsed WT BMCs$^{GM\text{-}CSF}$ and challenged with *Df*, as has been noted by others in protocols of intranasal sensitization and challenge with HDM (Krishnamoorthy et al., 2008; Phipps et al., 2009), but we did not find a significant attenuation in lymph node cell production of IL-17A in mice sensitized with *Df*-pulsed *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$, *Df*-pulsed *Cysltr1*$^{-/-}$ BMCs$^{GM\text{-}CSF}$, or *Df*-pulsed kdBMCs$^{GM\text{-}CSF}$. The level of recruitment of Th17 cells to the lung in mice sensitized with *Df*-pulsed *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ was also comparable to that in mice sensitized with *Df*-pulsed WT BMCs$^{GM\text{-}CSF}$. Th17 immunity to dust mite may be generated by signaling from other PRRs during sensitization. After intranasal sensitization and challenge with *Dp*, airway neutrophilia and *Dp*-specific IL-17A production by restimulated lymph node cells was attenuated in BALB/c mice lacking MyD88 (Phipps et al., 2009). Thus, signaling from another TLR or an IL-1 family member may contribute to the *Df*-elicited Th17 priming in our adoptive transfer model. In addition, HDM extracts may activate other Syk/CARD9-dependent CLRs to elicit a Th17 response, as the addition of the Dectin-1 agonist curdlan to OVA immunization augments IL-17 production from restimulated lymphocytes in WT but not Dectin-1–null mice (LeibundGut-Landmann et al., 2007). Interestingly, Dectin-2 may have a more significant role in *Df*-elicited Th17 immunity at the challenge phase, as we did find a reduction in IL-17A production in restimulated lymphocytes from mice treated with the Dectin-2 antibody during both the sensitization and challenge.

In the adoptive transfer model, although the reduction in eosinophil recruitment to the BAL fluid in mice sensitized with *Df*-pulsed kdBMCs$^{GM\text{-}CSF}$ paralleled the reduction in lung and lymph node Th2 responses and was dependent on BMCs$^{GM\text{-}CSF}$ cys-LT production and CysLT$_1$R-mediated function, the significant fall in Dectin-2–dependent neutrophil recruitment to the BAL fluid was not accompanied by a reduction in IL-17A and was not dependent on cys-LTs. Dectin-2 activates the FcRγ chain–, Syk-, and CARD9-dependent signaling pathway in DCs. This pathway is shared with macrophage-inducible C-type lectin for the production of neutrophil chemoattractants, such as CXCL2, in mouse macrophages (Yamasaki et al., 2008), suggesting a mechanism by which Dectin-2 signaling may recruit neutrophils independently of IL-17A.

Earlier in vivo studies identified a role for Dectin-2 signaling in the generation of Th17 immunity to *C. albicans* (Robinson et al., 2009; Saijo et al., 2010) and in regulatory T cell function in a model of UV radiation–induced tolerance (Aragane et al., 2003). Our study not only extends the biology of Dectin-2 to Th2 cells but also provides a link via the cys-LT pathway to pathological Th2 inflammation elicited by HDM, the most common aeroallergen worldwide.

MATERIALS AND METHODS

Reagents and mice. Lyophilized extracts of *Df* (Greer Laboratories) were reconstituted in endotoxin-free PBS (Sigma-Aldrich). DNase-1 (Roche), ionomycin (Calbiochem), and collagenase IV (Worthington Biochemical) were obtained as noted. LPS from *E. coli* O55:B5, curdlan, PMA, monensin, and puromycin were obtained from Sigma-Aldrich. *Ltc4s*$^{-/-}$ mice on a BALB/c background (N10 and N11), *Cysltr1*$^{-/-}$ mice on a BALB/c background (N10), and WT littermate controls were generated and maintained in our laboratory (Kanaoka et al., 2001; Maekawa et al., 2002). MHCII$^{\Delta/\Delta}$ mice and the C57BL/6 controls were obtained from The Jackson Laboratory (Madsen et al., 1999). All studies were approved by the Institutional Animal Care and Use Committee of the Dana-Farber Cancer Institute.

BMCs$^{GM\text{-}CSF}$ generation and *Df* stimulation. BMCs$^{GM\text{-}CSF}$ were generated with GM-CSF, as previously described (Barrett et al., 2009), according

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to the protocol for BM-derived DC generation by Lutz et al. (1999). In brief, BM cells were harvested and resuspended at 4×10^5 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 40 ng/ml of recombinant mouse GM-CSF (Peprotech). 10 ml of this suspension was plated in each Petri dish and cultured at 37°C in a 5% CO_2 incubator. On day 3 of culture, 10 ml of complete media with GM-CSF was added to each plate. On day 6 of culture, 10 ml of media with GM-CSF was exchanged from each plate. The floating cell population was harvested at day 7, washed, and counted. For cell stimulations, *Df* was used at the concentrations noted with a final cell concentration of 10^6 cells/ml.

Cytospin and immunocytochemistry. To assess the cell composition of day-7 cultures, 1.5×10^5 cells in 200 μl were cytospun, fixed, and stained with Hema 3 Stain Set (Thermo Fisher Scientific). Cells were identified by morphological criteria. 400 cells per slide were counted. To assess Dectin-2 expression by immunocytochemistry, cells were fixed in 4% paraformaldehyde, washed, cytospun, and stained using the rat ABC staining system (Santa Cruz Biotechnology, Inc.), according to the manufacturer's instructions. In brief, the slides were blocked with 1.5% goat serum, incubated with rat anti–Dectin-2 (AbD Serotec) or a rat IgG2a isotype control Ab (BD) at 1:20 at 25°C for 1 h, washed, and incubated with biotin-conjugated goat anti–rat antibody for 30 min. Detection was accomplished using avidin-biotinylated horseradish peroxidase and diaminobenzidine substrate. Hematoxylin was used for counterstaining. The pictures were taken by a digital camera (DXM 1200; Nikon) with ACT-1 (version 2.70; Nikon) image acquisition software. 500 cells per sample were assessed.

Flow cytometry. To assess Dectin-2 expression on day 7, $BMCs^{GM\text{-}CSF}$ were harvested, washed, blocked in PBS containing 1 mM EDTA and 10% donkey serum (Jackson ImmunoResearch Laboratories, Inc.), and serially stained with rat anti–Dectin-2 (Abcam) or rat IgG2a isotype control Ab (BD) at 1 μg/0.2 ml/10^6 cells, donkey anti–rat Ig-allophycocyanin (Jackson ImmunoResearch Laboratories, Inc.) at 1:100 dilution, and either rat anti–mouse CD11c-PECy7, MHCII-PE, CD80-PE, CD86-PE, CD40-PE, OX40L-PE, CCR7-PE, PD-L1-PE, and ICOSL-PE (BD) or rat anti–mouse CD11c-Pacific Blue, MHCII-FITC, CD11b-PECy7, and Ly6c-APCCy7 (eBioscience). MHCII-PE staining was performed at nonsaturating antibody concentrations. Analyses were performed on a FACSCanto II flow cytometer (BD), and data were analyzed with FlowJo (7.5; Tree Star, Inc.).

Lentiviral knockdown of Dectin-2 in $BMCs^{GM\text{-}CSF}$. Infectious viral particles were prepared and titered as previously described (Barrett et al., 2009) using the shRNA construct TRCN0000066785 for mouse Dectin-2 in the pLKO1 lentiviral vector (Open Biosystems), an empty vector control (Open Biosystems), and a nontargeting sequence (Sigma-Aldrich). $BMC^{GM\text{-}CSF}$ cultures were begun on day 0, infected on day 1 at a multiplicity of infection of 10, and selected with 2 μg/ml puromycin from days 4–7. $BMC^{GM\text{-}CSF}$ cultures infected with lentivirus contained a mean of 76.8 ± 5% $CD11c^+$ cells at day 7, as detected by flow cytometry.

Cys-LT and cytokine measurement. Cys-LTs in the supernatants of stimulated cells were measured by enzyme immunoassay according to the manufacturer's protocol (Cayman Chemical). The lower limit of detection was 3.9 pg/ml. IL-2, IL-6, IL-10, IL-23, and TNF were measured by ELISA (eBioscience) with lower limits of detection at 8, 78, 7.8, 7.8, and 156 pg/ml, respectively.

Sensitization and challenge protocols. For adoptive transfer of *Df*-pulsed $BMCs^{GM\text{-}CSF}$ into WT recipients and subsequent *Df* challenge, $BMCs^{GM\text{-}CSF}$ were grown to day 7 and stimulated with saline or *Df* at 100 μg/ml for 24 h. On day 8, nonadherent cells were washed twice in PBS, counted, and resuspended in PBS. WT recipient mice were sensitized intranasally with 10^4 cells/50 μl, challenged with 2 μg *Df* intranasally on day 22 and day 23, and euthanized 48 h after the last challenge with an overdose of pentobarbital for analysis of BAL fluid, pulmonary mononuclear cell harvest, and lung-draining lymph node restimulation. After adoptive transfer of $BMCs^{GM\text{-}CSF}$ from $MHCII^{\Delta/\Delta}$ mice or C57BL/6 WT mice, recipient C57BL/6 mice were challenged with 10 μg *Df* intranasally on days 20, 22, and 24 and euthanized on day 25. For the active sensitization and challenge protocol, $Ltc4s^{-/-}$, $Cysltr1^{-/-}$, or BALB/c WT mice were sensitized (days 0 and 1) and challenged (days 15 and 16) with 10 μg *Df* intranasally and euthanized on day 18. For antibody blocking, 200 μg Dectin-2 antibody (clone D2.11E4; Thermo Fisher Scientific) or a rat IgG2a control (BioLegend) was injected intraperitoneally into BALB/c WT mice on days 0, 2, 15, and 17. On days 0 and 15 the antibody was injected 6 h before *Df*.

BAL. Mice were killed, the trachea was exposed and cannulated with a 22-gauge angiocatheter, and the bronchoalveolar space was lavaged three times with 0.75 ml PBS with 1 mM EDTA. The BAL fluid was centrifuged, and the pelleted cells were resuspended in PBS with 1% FCS and counted. Then, 1.5×10^5 cells in 200 μl were cytospun, fixed, and stained with Hema 3 Stain Set (Thermo Fisher Scientific). Cells were identified by morphological criteria. 400 cells per slide were counted. IL-13, IL-17A, and IFN-γ levels in the BAL fluid supernatants were measured by ELISA (eBioscience) with lower limits of detection at 7.8, 7.8, and 31.2 pg/ml, respectively.

Intracellular cytokine staining of pulmonary mononuclear cells and lymph node cells. For pulmonary mononuclear cell assessment, mice were killed, and the lungs were perfused with 10 ml of sterile PBS, homogenized with sterile scalpels, and digested in a shaker for 30 min with 500 U/ml of type IV collagenase and 0.02 mg/ml DNase I at 37°C. The cells were washed, filtered through a 70-μm cell strainer, and separated on a NycoPrep gradient (Axis-Shield) at 600 *g* for 20 min at 4°C. Harvested mononuclear cells were washed; resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, 0.043 mM 2-ME, 2 mM L-glutamine, 0.025 M Hepes buffer, and 1 mM sodium pyruvate; plated at 2×10^6 cells/ml in 24-well plate; and stimulated with 50 ng/ml PMA and 1 μM ionomycin for 2 h at 37°C. 2.5 μM monensin was added and the cells were incubated for an additional 8 h. Cells were harvested, treated with DNase I at a concentration of 60 μg/ml for 15 min at 37°C, washed, fixed with fixation buffer (eBioscience) for 15 min, washed again, and blocked in 300 μl permeabilization buffer (eBioscience) with 1% mouse IgG (Sigma-Aldrich) and 1% anti–mouse CD16/CD32 (BD) for 20 min. Cells were then stained with anti–mouse CD4-PE-Cy7 (clone RM4-5; BD), anti–mouse CD8α-allophycocyanin (clone 53–6.7; BD), anti–mouse IL-4–PE (clone 11B11; BD), anti–mouse IL-5-PE (clone TRFK5; BD), anti–mouse IL-17A–PE (clone TC11-18H10; BD), anti–mouse IFN-γ–PE (clone XMG1.2; eBioscience), or rat IgG1-PE isotype control Ab (BD) at a concentration of 0.2 μg/0.1 ml/2×10^6 cells. Analyses were performed on a FACSCanto flow cytometer, and data were analyzed with FlowJo. For lymph node cell assessment, the parabronchial lymph nodes were excised from each mouse. Single cell suspensions were generated, filtered through a 70-μm cell strainer, washed, resuspended in the RPMI medium described in this section, and counted. Cell stimulation, washing, fixation, permeabilization, and staining were as in this section with the exception that PMA, ionomycin, and then monensin treatment was done for a total of 6 h, and cells were stained with anti–mouse CD4-allophycocyanin (clone RM4-5; BD) and anti–mouse CD8α–PE-Cy7 (clone 53–6.7; BD).

Cytokine assays from lung-draining lymph nodes. Single cell suspensions from the parabronchial lymph nodes were generated. Lymph node cells were cultured at 4×10^6 cells/ml with 0, 1, or 5 μg/ml *Df* for 72 h at 37°C in RPMI medium with 10% FCS. The concentrations of IL-4, IL-5, IL-13, IL-17A, and IFN-γ in the supernatants were measured with ELISA kits (eBioscience) with lower limits of detection at 39 pg/ml, 117 pg/ml, 390 pg/ml, 39 pg/ml, and 156 pg/ml, respectively. The total concentration of cytokine generated per mouse is reported.

$BMC^{GM\text{-}CSF}$ migration. *Df* was conjugated to Alexa Fluor 647 with a Protein Labeling kit (Invitrogen) according to the manufacturer's protocol and

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was dialyzed with a 10K membrane (Thermo Fisher Scientific) against PBS for 6 h. Day-7 BMCs$^{GM\text{-}CSF}$ were stimulated with 100 μg/ml Alexa Fluor 647–*Df* for 18 h, and 10^4, 10^5, or 10^6 cells were transferred intranasally into mice. 24 and 48 h later, the lymph nodes were harvested, digested with collagenase IV and DNase I for 30 min at 37°C, and filtered. Single cell suspensions were blocked in PBS supplemented with 0.5% heat-inactivated FCS, 0.05% NaN_3, 1% mouse IgG, and 1% anti–mouse CD16/CD32. The cells were stained with anti–mouse CD11c-PE-Cy7 (clone HL3; BD) and anti–mouse MHCII-PE (clone M5/114.15.2; BD) at 0.2 μg/100 μl/10^6 cells.

Statistical analysis. All experiments were repeated at least three times, except where noted. Results are expressed as means ± SEM. The Student's *t* test was used for statistical analysis, except where noted. A value of $P < 0.05$ was considered significant.

Online supplemental material. Fig. S1 depicts CD11c, MHCII, Dectin-2, CD11b, and Ly6C expression on BMCs$^{GM\text{-}CSF}$. Fig. S2 shows *Df*-elicited cytokine production by BMCs$^{GM\text{-}CSF}$ at 30 h and the generation of cys-LTs and TNF in response to curdlan and LPS. Fig. S3 shows a representative plot of intracellular cytokine staining in $CD4^+$ lymphocytes from the lung of WT recipients sensitized with *Df*-pulsed WT and *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ and challenged with *Df*. Fig. S4 shows the difference in BAL fluid cell infiltrate and in intracellular cytokine staining in $CD4^+$ lymph node cells from WT recipients sensitized with *Df*-pulsed WT or MHCII$^{\Delta/\Delta}$ BMCs$^{GM\text{-}CSF}$. Fig. S5 shows comparable cell surface expression of costimulatory markers in WT and *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$. Fig. S6 shows comparable production of *Df*-elicited cytokines at 4 and 24 h by WT and *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$. Fig. S7 shows migration of Alexa Fluor 647–*Df*-pulsed WT and *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ to the lung-draining lymph nodes in WT mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100793/DC1.

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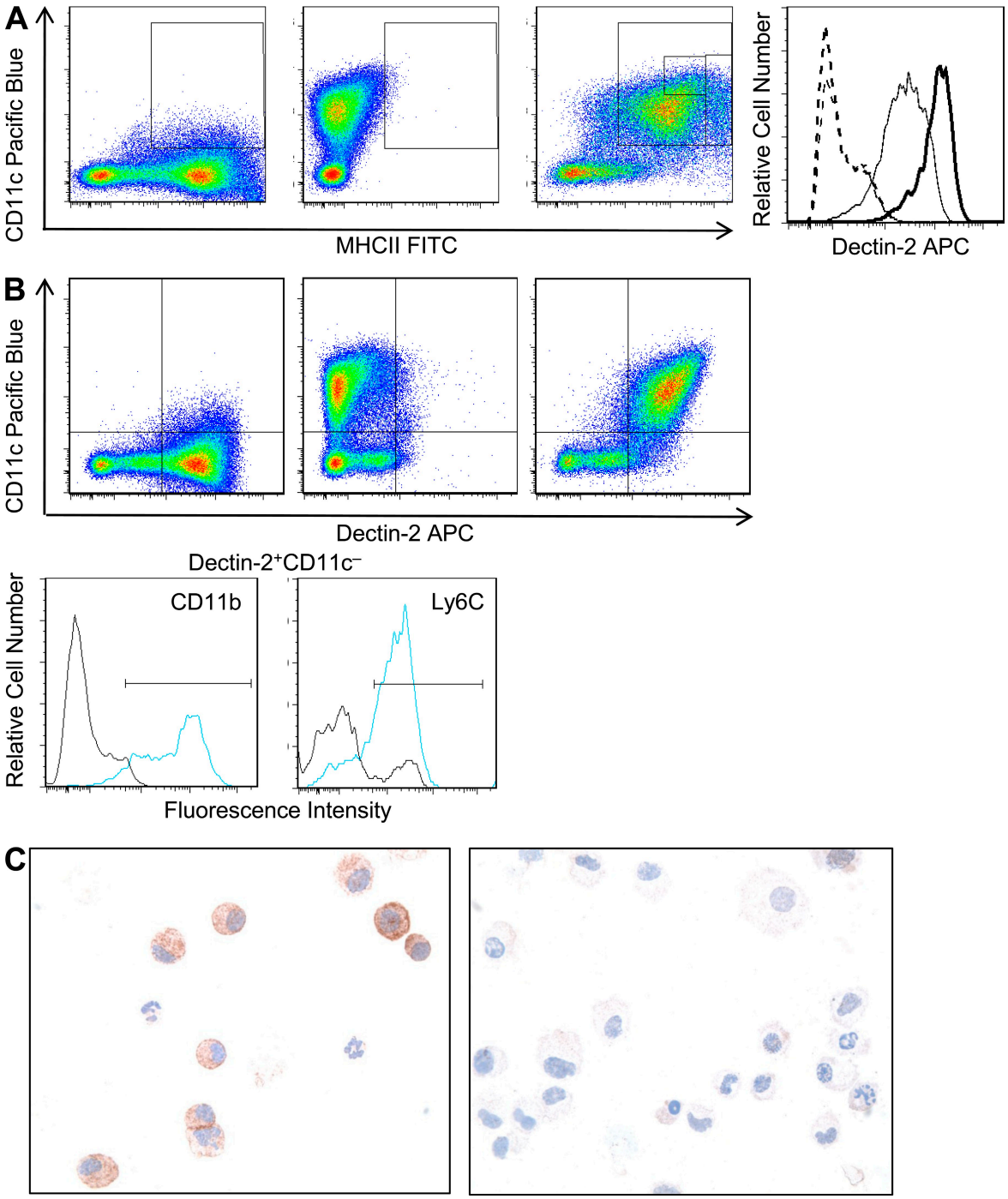

Figure S1. Dectin-2 expression in GM-CSF–expanded BM cultures. Day-7 BMCs$^{GM\text{-}CSF}$ were harvested and stained for the cell surface expression of CD11c, MHCII, and Dectin-2 and analyzed by flow cytometry. (A) Left, a representative histogram is shown of CD11c and MHCII expression, as gated by the isotype controls. Gating on a CD11c^{hi}MHCII^{low} and a CD11c^{+}MHCII^{hi} subset is also indicated (small box and small rectangle, respectively). MHCII staining was performed with saturating antibody concentrations. Right, Dectin-2 expression on the CD11c^{hi}MHCII^{low} subset (thick black line) and the CD11c^{+}MHCII^{hi} subset (thin black line) is shown, as well as the isotype control staining for each population (thick dashed and thin dashed lines, respectively). (B) Top, a representative histogram is shown of CD11c and Dectin-2 expression, as gated by the isotype controls. Bottom, CD11b and Ly6C expression on the Dectin-2^{+}CD11c^{-} population. (C) Dectin-2 expression on cytospin preparations from day-7 BMCs$^{GM\text{-}CSF}$ detected by immunocytochemistry. Positive staining is indicated in brown. Left, anti–Dectin-2. Right, rat IgG2a control. Results are representative of four cultures from two independent experiments.

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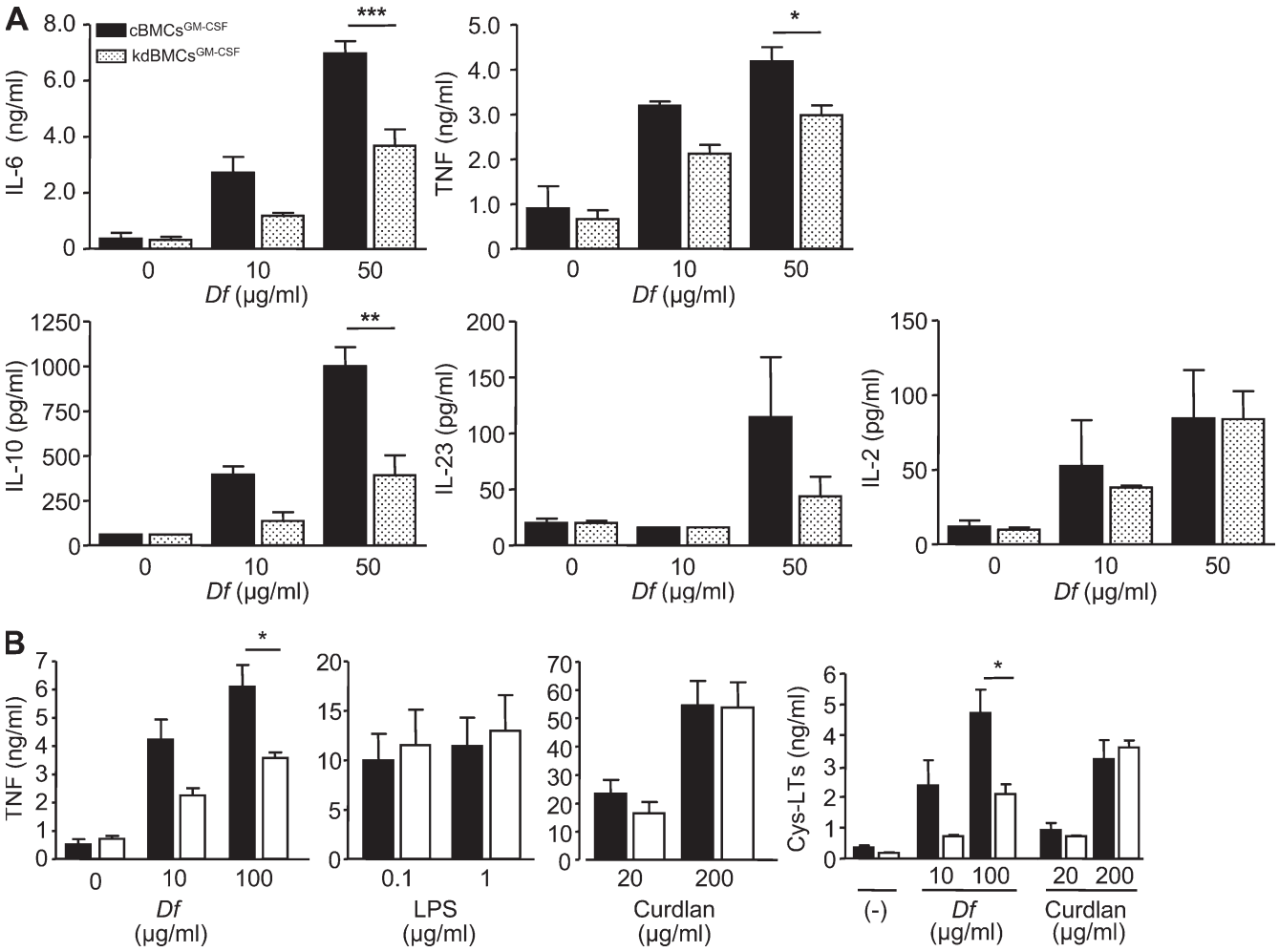

Figure S2. Cys-LT and cytokine production by BMCs^{GM-CSF} after lentiviral knockdown. BMCs^{GM-CSF} were infected with viral particles containing Dectin-2 shRNA (kdBMCs^{GM-CSF}), an empty vector control (cBMCs^{GM-CSF}), or a nontargeting vector control (ntcBMC^{GM-CSF}), selected with puromycin, and harvested at day 7. (A) BMCs^{GM-CSF} were stimulated for 30 h with *Df* at the indicated concentrations, and cytokines in the supernatant were measured by ELISA. Results are means ± SEM (n = 5–6 mice per group) from three independent experiments. *, P = 0.01; **, P = 0.004; ***, P = 0.002. Significance was determined with an unpaired Student's *t* test. (B) Left, TNF production from cBMCs^{GM-CSF} (black) or kdBMCs^{GM-CSF} (white) in response to *Df*, curdlan, or LPS at 4h. Results are means ± SEM (n = 5–6) from three independent experiments. *, P = 0.04. Significance was determined with an unpaired Student's *t* test. Right, Cys-LT production from cBMCs^{GM-CSF} (black) or kdBMCs^{GM-CSF} (white) in response to 10 and 100 μg/ml *Df* or 20 and 200 μg/ml curdlan at 60 min. Results are means ± SEM (n = 4–5) from two independent experiments. *, P = 0.02. Significance was determined with an unpaired Student's *t* test.

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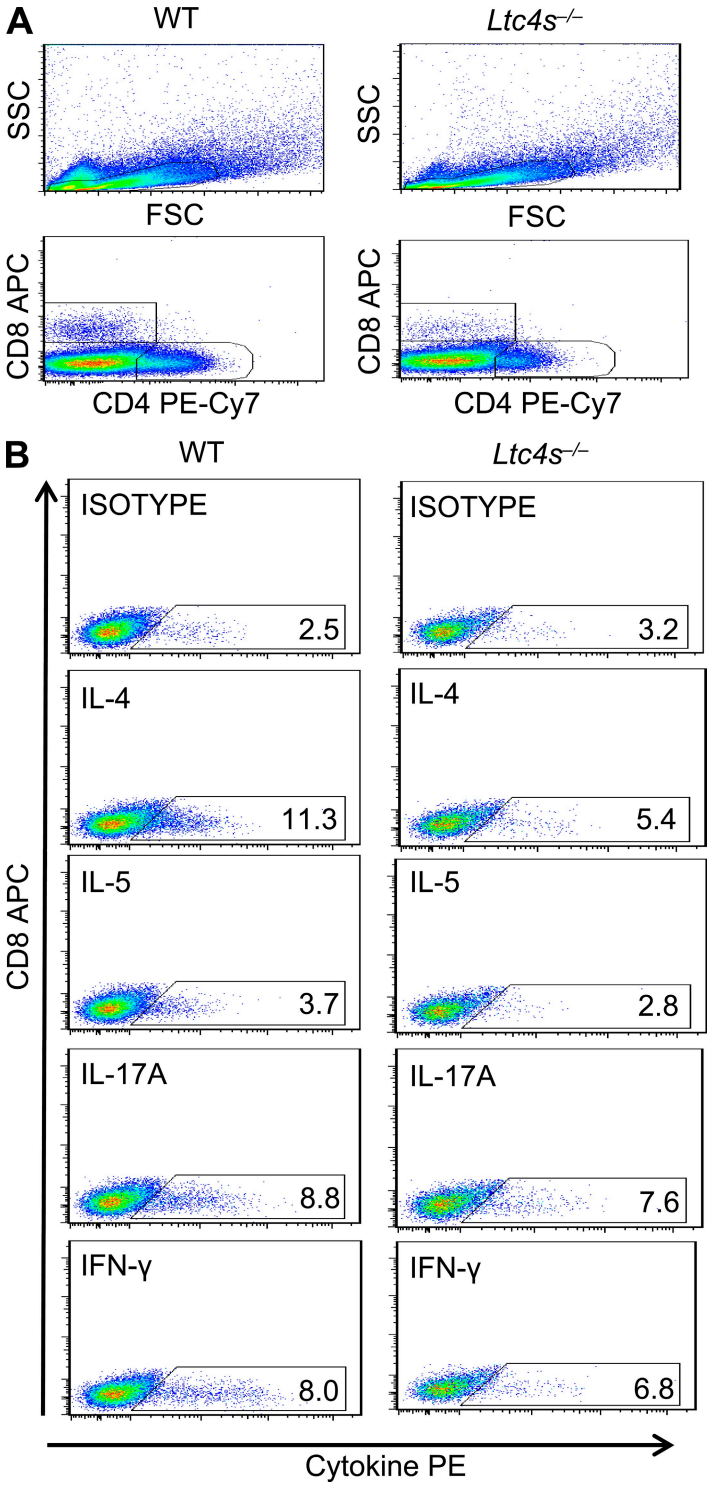

Figure S3. *Df*-elicited CD4⁺ Th2 cell recruitment to the lung. WT mice were sensitized with saline-pulsed or *Df*-pulsed WT or *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ and challenged with *Df* as described in the Fig. 4 legend. Pulmonary mononuclear cells were isolated and stimulated with 50 ng/ml PMA and 1 μM ionomycin for 2 h. 2.5 μM monensin was added and cells were stimulated for an additional 8 h, fixed, permeabilized, 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. (A) Cell size (top) and CD4 and CD8 expression (bottom) from mice sensitized with *Df*-pulsed WT (left) or *Ltc4s*$^{-/-}$ (right) BMCs$^{GM\text{-}CSF}$. (B) Intracellular cytokine staining in CD4⁺ cells in the lymphocyte gate from mice sensitized with *Df*-pulsed WT (left) or *Ltc4s*$^{-/-}$ (right) BMCs$^{GM\text{-}CSF}$ with the percentage of cytokine-positive cells noted.

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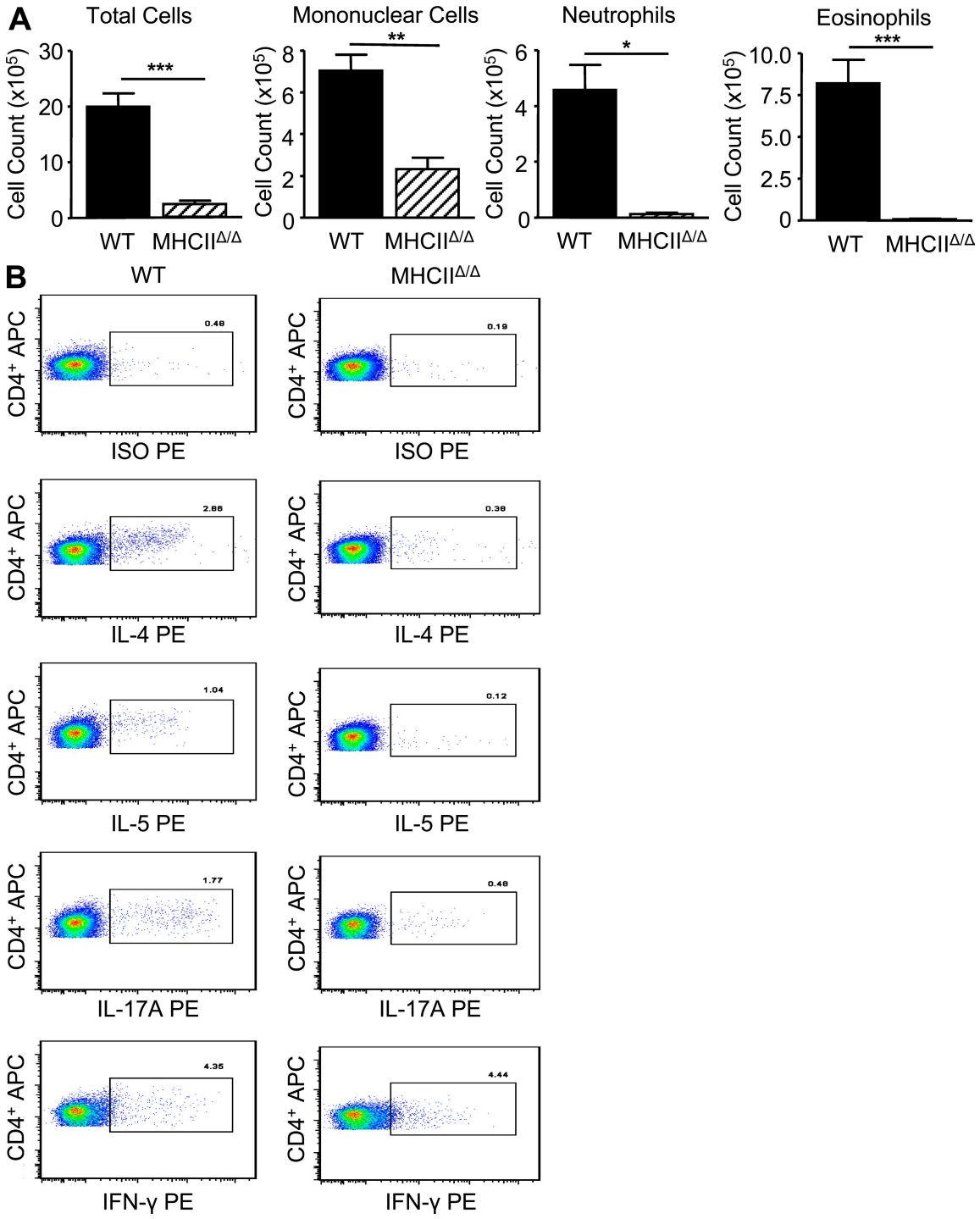

Figure S4. BMCs$^{GM\text{-}CSF}$ MHCII expression is required for *Df*-elicited pulmonary inflammation and CD4+ T cell cytokine generation. WT C57BL/6 mice were sensitized with intranasal administration of 10^4 *Df*-pulsed WT or MHCII$^{\Delta/\Delta}$ BMCs$^{GM\text{-}CSF}$, challenged with 10 µg *Df* on day 22, day 24, and day 26, and analyzed on day 27. (A) Cells from the BAL fluid were counted and 400 cells/slide in cytospins were counted for specific cell types. Results are means ± SEM (n = 8–10 mice per group) from two experiments. *, P = 0.0005; **, P = 0.0002; ***, P = 0.0001. Significance was determined with an unpaired Student's t test. (B) Parabronchial lymph node cells were isolated, stimulated as in Fig. S3 for a total of 6 h, 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. Representative histograms show intracellular cytokine staining on CD4+ cells in the lymphocyte gate from WT recipients of *Df*-pulsed WT (left) or MHCII$^{\Delta/\Delta}$ (right) BMCs$^{GM\text{-}CSF}$.

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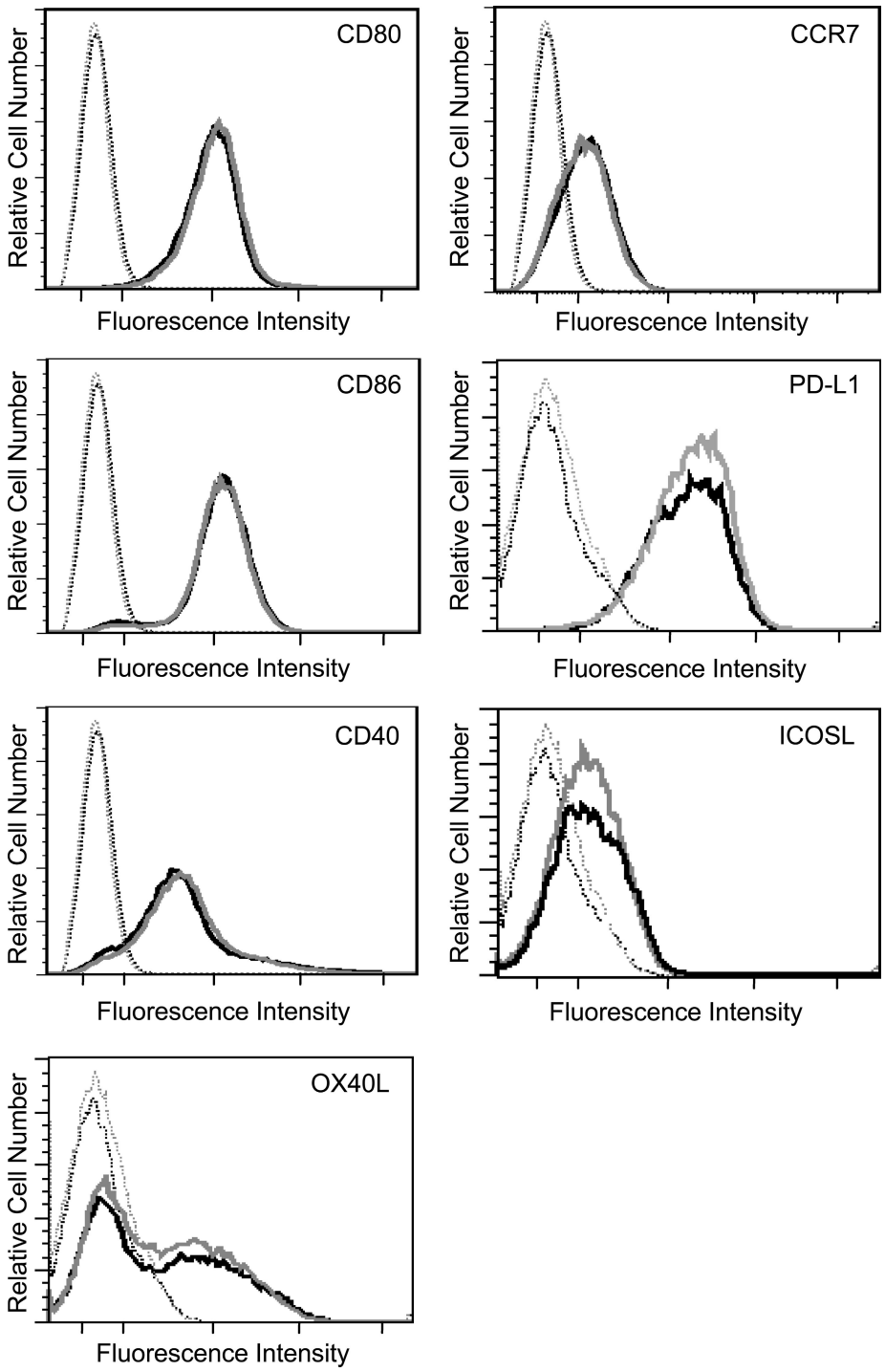

Figure S5. WT and *Ltc4s*$^{-/-}$ BMCs^{GM-CSF} have comparable cell surface expression of costimulatory markers. WT and *Ltc4s*$^{-/-}$ BMCs^{GM-CSF} were grown to day 7, pulsed with 100 µg/ml *Df* for 24 h, harvested, and stained for the cell surface expression of CD11c, MHCII, CD80, CD86, CD40, OX40L, CCR7, PD-L1, and ICOSL, and analyzed by flow cytometry. Representative histograms are shown of costimulatory marker expression on CD11c^{+}MHCII^{+} BMCs^{GM-CSF}. Specific stainings are shown for WT (thick black lines) and *Ltc4s*$^{-/-}$ (thick gray lines) BMCs^{GM-CSF}, and isotype control stainings are shown for WT (dotted black lines) and *Ltc4s*$^{-/-}$ (dotted gray lines) BMCs^{GM-CSF}.

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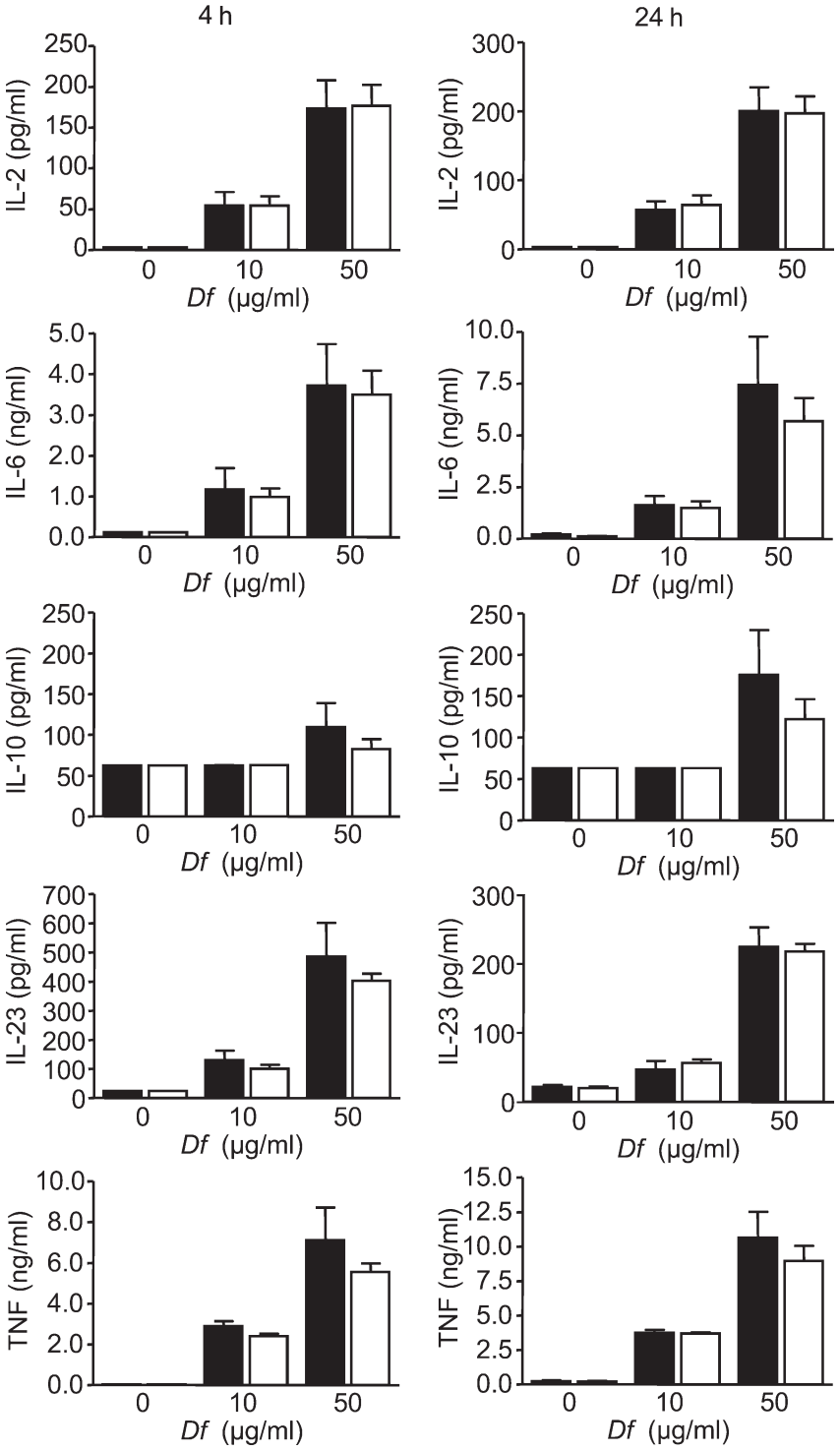

Figure S6. WT and *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ generate comparable concentrations of *Df*-elicited cytokines at 4 and 24 h. WT (black bars) and *Ltc4s*$^{-/-}$ (white bars) BMCs$^{GM\text{-}CSF}$ were grown to day 7 and stimulated with *Df* at the indicated concentrations for either 4 or 24 h, and cytokines in the supernatants were measured by ELISA. Results are means ± SEM (n = 4 mice per group) from two independent experiments.

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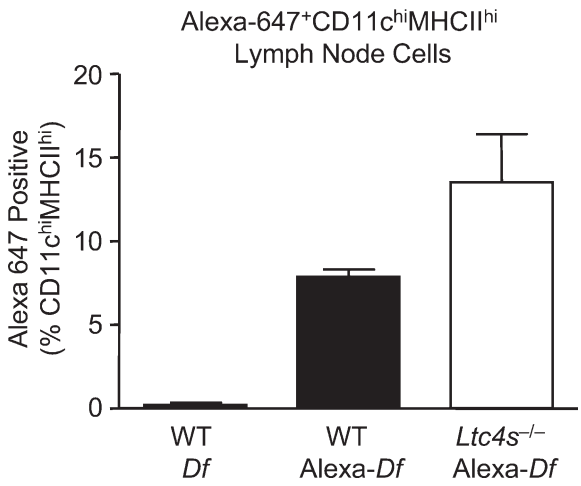

Figure S7. WT and *Ltc4s*$^{-/-}$ BMCs$^{GM\text{-}CSF}$ show comparable migration to the lung-draining lymph nodes. Day-7 BMCs$^{GM\text{-}CSF}$ were stimulated with 100 µg/ml of unlabeled *Df* or Alexa Fluor 647–*Df* for 18 h, and 10^6 cells were transferred intranasally into WT recipients. 24 h later, the draining lymph nodes were harvested and digested, and single cell suspensions were stained for CD11c-PE-Cy7 and MHCII-PE and analyzed by flow cytometry. The percentage of CD11c^{hi}MHCII^{hi} cells that were Alexa Fluor 647 positive is shown. Results are means ± SEM (n = 4–5 mice per group) from two independent experiments.

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