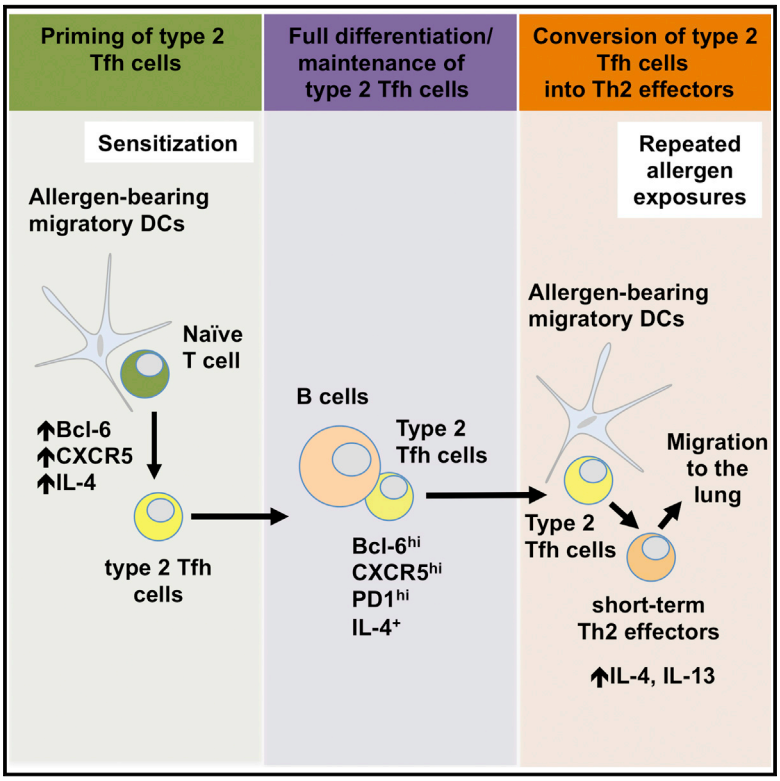
Article

Immunity

T Follicular Helper Cell Plasticity Shapes Pathogenic T Helper 2 Cell-Mediated Immunity to Inhaled House Dust Mite

Graphical Abstract



Highlights

- HDM sensitization does not prime Th2 cells, but induces IL-4-committed Tfh cells
- Ag-presenting B cells synergize with DCs to prime Tfh cells during HDM sensitization
- IL-4-committed Tfh cells are precursors of effector Th2 cells after HDM rechallenge
- Depletion of IL-4-committed Tfh cells prevents Th2 cell-mediated immunity to HDM

Authors

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In Brief

Follicular helper T (Tfh) cells are specialized providers of T cell help to B cells. León and colleagues demonstrate that interleukin-4-committed Tfh cells are precursors of effector T helper type 2 cells after intranasal challenge with house dust mites.

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CellPress

T Follicular Helper Cell Plasticity Shapes Pathogenic T Helper 2 Cell-Mediated Immunity to Inhaled House Dust Mite

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SUMMARY

Exposure to environmental antigens, such as house dust mite (HDM), often leads to T helper 2 (Th2) cell-driven allergic responses. However, the mechanisms underlying the development of these responses are incompletely understood. We found that the initial exposure to HDM did not lead to Th2 cell development but instead promoted the formation of interleukin-4 (IL-4)-committed T follicular helper (Tfh) cells. Following challenge exposure to HDM, Tfh cells differentiated into IL-4 and IL-13 double-producing Th2 cells that accumulated in the lung and recruited eosinophils. B cells were required to expand IL-4-committed Tfh cells during the sensitization phase, but did not directly contribute to disease. Impairment of Tfh cell responses during the sensitization phase or Tfh cell depletion prevented Th2 cell-mediated responses following challenge. Thus, our data demonstrate that Tfh cells are precursors of HDM-specific Th2 cells and reveal an unexpected role of B cells and Tfh cells in the pathogenesis of allergic asthma.

INTRODUCTION

Cytokines produced by T helper 2 (Th2) cells in the lungs of asthma patients promote inflammation, eosinophil accumulation, and mucus hyperproduction, which ultimately lead to recurrent bronchoconstriction, a characteristic of allergic asthma (Hamid and Tulic, 2009). Given the critical role of Th2 cells in the development of allergic inflammatory responses, it is essential that we understand the mechanisms that control Th2 cell development to commonly encountered respiratory allergens, so that we can design therapeutic strategies. Although the exact mechanism by which allergen-specific Th2 cell responses are initiated is incompletely defined, it is thought to require antigen (Ag) presentation by pulmonary dendritic cells (DCs), which capture allergen-derived Ags in the lung and migrate into the lung-draining mediastinal lymph node (mLN), where they prime allergen-specific $CD4^+$ T cells (van Helden and Lambrecht, 2013). In fact, conditional depletion of lung DCs precludes Th2 cell-mediated immunity to house dust mite (HDM) (Hammad et al., 2010). Nevertheless, commitment of primed $CD4^+$ T cells to the Th2 cell pathway might also require complex interactions with other cell types, including epithelial cells (Lambrecht and Hammad, 2013) and type 2 innate lymphoid cells (ILC2s) (Halim et al., 2014).

B cells also contribute to Th2 cell development by multiple mechanisms (León et al., 2014). Indeed, Ag presentation by B cells promotes the accumulation of Th2 cells in the lungs of mice exposed to cockroach Ags (Lindell et al., 2008). B cells are also important for the development and maintenance of T follicular helper (Tfh) cells (Crotty, 2014). Certainly, the development of Th2 and Tfh cells share some developmental requirements. For example, both Th2 and Tfh cell responses require B cell help, ICOS, IL-21R, OX40-OX40L, and CD28 signaling (Coquet et al., 2015; Crotty, 2014; Lane, 2000; León et al., 2014) and are primed within the interfollicular areas of the LN (Kerfoot et al., 2011; León et al., 2012).

Tfh cells retain substantial developmental plasticity (Lu et al., 2011) and, upon secondary challenge, can differentiate into effector T cells that migrate into non-lymphoid tissues (Lüthje et al., 2012). Moreover, Tfh cells can be an important source of IL-4 (King and Mohrs, 2009). Particularly, Th2 responses to airborne antigens uniquely require an initial phase of antigen sensitization that does not cause Th2-mediated airway inflammation, but is required for the development of effector Th2 cells following challenge (Galli et al., 2008; Gelfand et al., 2004). However, the identity of the Th2 cell-precursors elicited during the sensitization phase is not yet known.

Here we show that sensitization to inhaled HDM in mice did not directly result in Th2 cell development, but instead elicited IL-4-committed Tfh cells that were confined to the mLN. Following HDM challenge, Tfh cells generated during the sensitization phase differentiated into Th2 cells and homed to the lungs, where they recruited eosinophils. The differentiation of IL-4-committed Tfh cells required Ag presentation by both DCs and B cells. As a consequence, Th2 cell-mediated immunity after HDM rechallenge was impaired in B cell deficient mice and in mice in which B cells or DCs were unable to present Ags. Moreover, the depletion of Tfh cells after HDM sensitization prevented Th2 cell-mediated immunity upon challenge exposure. Thus,

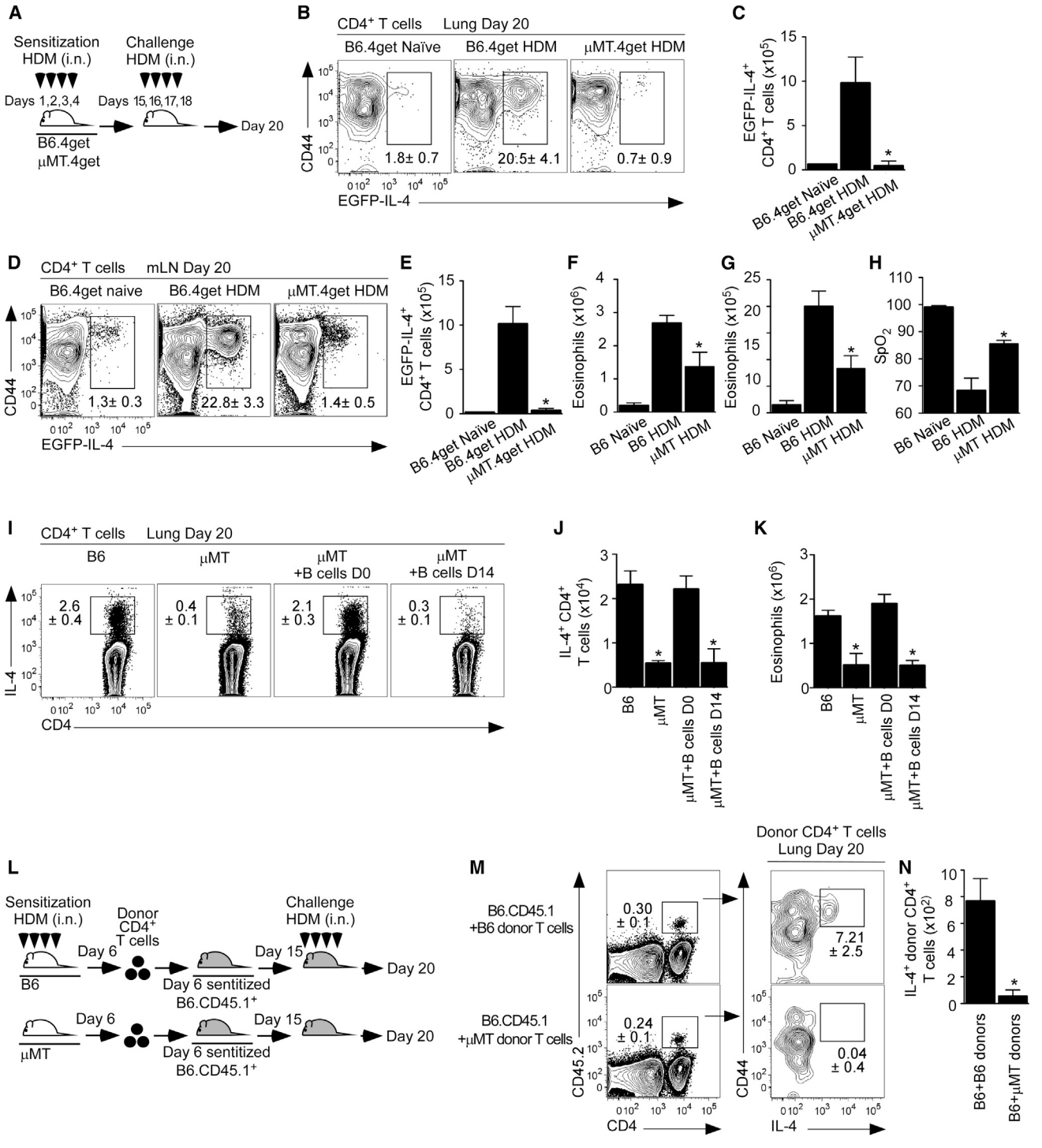


Figure 1. B Cells Control HDM-Induced Pulmonary Th2 Cell Responses

(A–E) B6.4get and µMT.4get mice were i.n. treated with 25 µg of HDM for 4 consecutive days starting on day 1. On day 15, mice were i.n. challenged with 25 µg of HDM daily for 4 days and analyzed on day 20 by flow cytometry (A). As a control, naive B6.4get mice were also analyzed. The frequency (B) and (D) and number (C) and (E) of IL-4-expressing (EGFP$^+$) CD4$^+$ T cells from the lungs (B) and (C) and mLN (D) and (E) are shown. (F-G) The number of eosinophils in the lungs (F) and BAL (G) of naive and HDM-challenged B6 and µMT mice are shown. (H) Arterial oxygen saturation (SpO2) of hemoglobin, measured with pulse oximeter, 2 hr after final challenge.

(I–K) B6 and µMT mice were i.n. treated with 25 µg of HDM for 4 consecutive days starting on day 1. Some µMT mice received 50 × 10^6 naive B cells i.v. on day 0, or on day 14. On day 15, all mice were i.n. challenged with 25 µg of HDM daily for 4 consecutive days. Mice were analyzed on day 20. The frequency (I) and number

(legend continued on next page)

IL-4-committed Tfh cells are the precursors to pathogenic Th2 cells in allergic airway disease.

RESULTS

B Cells Are Necessary for Th2 Cell-Mediated Immunity to Inhaled HDM

To test whether B cells influenced the Th2 cell response to inhaled HDM, we intranasally (i.n.) sensitized IL-4 reporter B6.4get mice and B cell-deficient 4get (μMT.4get) mice (Mohrs et al., 2001) with 25 μg of HDM extract on 4 consecutive days (sensitization phase, Figure 1A), challenged them i.n. with HDM extract on days 15, 16, 17, and 18 (challenge phase, Figure 1A) and determined the frequency (Figure 1B) and number (Figure 1C) of GFP$^+$CD4$^+$ T cells in the lungs on day 20. As expected, GFP$^+$CD4$^+$ T cells accumulated in the lungs of HDM-challenged B6.4get mice relative to naive B6.4get animals (Figures 1B and 1C), whereas GFP$^+$CD4$^+$ T cells failed to accumulate in the lungs of HDM-challenged μMT.4get mice. We observed similar results in the lung-draining mLN (Figures 1D and 1E).

We next sensitized and challenged C57BL/6J (B6) and C57BL/6J.μMT (μMT) mice and quantified IL-4-producing and IL-13-producing cells by intracellular cytokine staining (ICS). We found that both IL-4-producing (Figures S1A and S1B) and IL-13-producing (Figure S1C and S1D) CD4$^+$ T cells were reduced in μMT mice compared to B6 mice. We also enumerated eosinophils in the lungs and bronchoalveolar lavage (BAL) of sensitized and challenged mice and observed that the number of eosinophils was significantly reduced in μMT mice compared to those in B6 mice (Figures 1F and 1G). Arterial oxygen saturation of hemoglobin (SpO2) was measured. HDM-sensitized and challenged B6 mice displayed reduced SpO2 compared to μMT mice (Figure 1H). These results indicated that B cells were necessary for the development of pulmonary Th2 cell responses to HDM.

We next tested whether the pulmonary Th2 cell response required B cells during the sensitization phase or following challenge, by transferring purified B cells from naive B6 into μMT mice one day before HDM sensitization, or one day before HDM challenge. As controls, we included μMT and B6 mice that were HDM sensitized but did not receive B cells at any time. We enumerated IL-4-producing CD4$^+$ T cells on day 6 after challenge and found similar numbers of IL-4$^+$CD4$^+$ cells in the lungs of B6 and μMT mice that received B cells before the sensitization phase (Figures 1I and 1J), but fewer IL-4$^+$CD4$^+$ cells in μMT mice and in μMT mice that received B cells prior to HDM challenge. We also found that the numbers of eosinophils were similarly high in the lungs of B6 and μMT mice that received B cells before the sensitization phase (Figure 1K), but were similarly low in μMT mice and in μMT mice that received B cells prior to HDM challenge. These data suggest that the Th2 cell response to HDM sensitization and challenge is dependent on B cells during the sensitization, but not the challenge phase of the response.

To confirm these results, we transferred equivalent numbers of purified CD4$^+$ T cells from the mLN of day-6 HDM-sensitized B6 or μMT mice (CD45.2$^+$) into day-6 HDM-sensitized CD45.1$^+$ B6 mice (Figure 1L). Recipient mice were challenged with HDM on days 15–18 and we enumerated IL-4$^+$CD44^{hi}CD4$^+$ cells from the donor (CD45.2$^+$) and the host (CD45.1$^+$) on day 20. We found that the frequency (Figure S1E) and number (Figure S1F) of host IL-4$^+$CD44^{hi}CD4$^+$ cells were similar in the two groups. However, the frequency (Figure 1M) and number (Figure 1N) of donor IL-4$^+$CD44^{hi}CD4$^+$ cells were reduced in recipients of cells from μMT donors compared to cells from B6 donors. Collectively, these data indicate that the absence of B cells during the sensitization phase compromises the development of Th2 cell responses after challenge, regardless of whether B cells were present following challenge.

To better understand the role of B cells during the sensitization phase, we enumerated GFP$^+$CD4$^+$ T cells in the mLN of B6.4get and μMT.4get mice 6 days after HDM sensitization. We found that HDM-sensitized B6.4get had higher frequency (Figure 2A) and number (Figure 2B) of GFP$^+$CD4$^+$ T cells compared to non-sensitized B6.4get mice, but that HDM-sensitized μMT.4get mice failed to accumulate GFP$^+$CD4$^+$ T cells in the mLN. These data indicate that B cells are required for the differentiation of IL-4-commited CD4$^+$ T cells after HDM sensitization.

We next generated bone marrow (BM) chimeras in which B cells selectively lacked MHC class II (MHC II) (Figure 2C), by irradiating μMT.4get mice and reconstituted them with an 80:20 mix of BM obtained from μMT.4get and *H2-Ab1*$^{-/-}$ donors (B-MHC II-deficient chimeras), or with an 80:20 mix of μMT.4get and B6 mice (B-WT chimeras). Following reconstitution, chimeric mice were i.n. sensitized with HDM and the mLN analyzed 6 days later. We found that the frequency (Figure 2D) and number (Figure 2E) of B cells were similar in the two groups of chimeras. As expected, however, we found that B cells in B-WT chimeras expressed MHC II, but that B cells in B-MHC II-deficient chimeras did not (Figure 2F).

Next, we enumerated GFP$^+$CD44^{hi}CD4$^+$ T cells in the mLN 6 days later. We found that the frequency (Figure 2G) and number (Figure 2H) of GFP$^+$CD44^{hi}CD4$^+$ T cells was higher in the mLN of B-WT chimeras compared to B-MHC II-deficient chimeras. These results indicate that Ag-presenting B cells are required for the differentiation of IL-4-committed CD4$^+$ T cells during the HDM sensitization phase.

B Cell and DCs Synergize to Prime Specific CD4$^+$ T Cells to Inhaled HDM

We next tested whether Ag presentation by DCs was important for the priming of CD4$^+$ T cells following HDM sensitization. For

(J) of IL-4$^+$ CD4$^+$ T cells in the lungs were determined by intracellular staining after restimulation for 4 hr with anti-CD3 and BFA. (K) The number of eosinophils in the lungs are shown.

(L and M) B6 and μMT mice were i.n. treated with 25 μg of HDM for 4 consecutive days starting on day 1. Six days later, CD4$^+$ T cells were purified from the mLN and 2 × 10^6 of purified CD4$^+$ T cells from B6 or μMT donor mice were transferred into day 6 HDM-sensitized B6.CD45.1$^+$ recipient mice. Recipient mice were i.n. challenged on day 15 with 25 μg of HDM daily for 4 consecutive days and analyzed on day 20 (L). The frequency (M) and number (**N**) of donor IL-4$^+$ CD4$^+$ T cells in the lungs of recipient mice were determined by intracellular staining after restimulation with anti-CD3$^+$ BFA for 4 hr. *$p < 0.01$ versus HDM-treated B6 (unpaired Student's t test). Data are representative of three independent experiments (mean and SD of 4–5 mice per group). See also Figure S1.

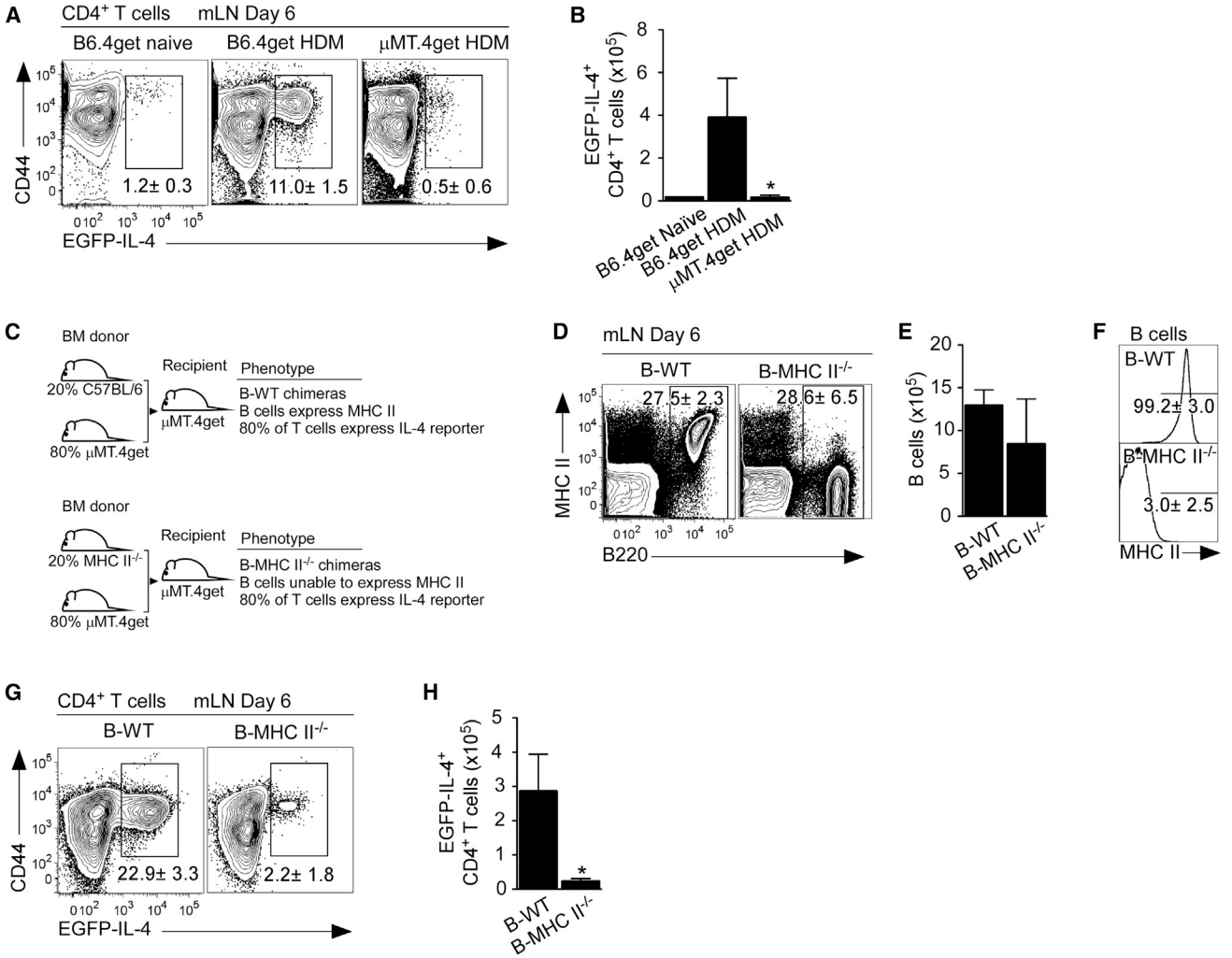


Figure 2. Ag Presentation by B Cells Is Necessary to Prime of IL-4-Commited CD4$^+$ T Cell Responses to HDM

(A and B) IL-4 reporter B6.4get and μMT.4get mice were i.n. treated with 25 μg of HDM or PBS for 4 consecutive days starting on day 1. The frequency (A) and number (B) of IL-4-expressing (EGFP$^+$) CD4$^+$ T cells in the mLN of treated mice were calculated by flow cytometry on day 6.

(C–H) μMT.4get mice were irradiated and reconstituted with an 80:20 mixture of BM from μMT.4get and B6 donors (B-WT), or with an 80:20 mixture of BM from μMT.4get and MHC II deficient donors (B-MHC II-deficient). Eight weeks later, reconstituted-chimeric mice were i.n. treated with 25 μg of HDM of PBS for 4 consecutive days starting on day 1 and mice were analyzed at day 6. The frequency (D) and number (E) of B cells in the mLN were calculated by flow cytometry. (F) Expression of MHC II on B cells. Frequency (G) and number (H) of IL-4-expressing (EGFP$^+$) CD4$^+$ T cells in the mLN. *$p < 0.001$ versus HDM-treated B6 (unpaired Student's t test). Data are representative of three independent experiments (mean and SD of 4–5 mice per group).

this purpose, we generated BM chimeric mice in which DCs selectively lacked MHC II by reconstituting irradiated B6 mice with an 80:20 mix of *Itgax*-DTR BM and *H2-Ab1*$^{-/-}$ BM (DC-MHC II-deficient chimeras), or with an 80:20 mix of *Itgax*-DTR BM and B6 BM (DC-WT chimeras) (Figure 3A). Two months later, we adoptively transferred CD45.1$^+$OTII T cells into the chimeric mice and treated the recipients with diphtheria toxin (DT) to ablate CD11c$^+$ cells derived from the *Itgax*-DTR BM (León et al., 2012). One day later, recipient mice were i.n. sensitized with a mixture of HDM and ovalbumin (HDM+OVA) for 4 consecutive days. Mice received an additional injection of DT on day 3 and were analyzed on day 6. As expected, DCs in the DC-WT chimeras expressed MHC II, whereas DCs in the DT-treated DC-MHC II-deficient chimeras lacked MHC II expression (Figure 3B). However, the frequency (Figure 3C) and number (Figure 3D) of DCs were similar in the two groups. Importantly, we found a higher frequency (Figure 3E) and number (Figure 3F) of donor OTII cells in the mLN of DC-WT mice than in DC-MHC II-deficient mice. Furthermore, OTII cells in DC-WT mice expressed high amounts of the activation markers CD44 (Figure 3G) and CD69 (Figure 3H). In contrast, the phenotype of OTII cells in DC-MHC II-deficient mice was consistent with naive T cells (Figures 3G and 3H). These results suggest that Ag presentation by DCs is also required to prime CD4$^+$ T cell responses to inhaled HDM and that DCs and B cells synergize to expand specific CD4$^+$ T cells in the mLN following pulmonary HDM sensitization.

To understand how B cells gained access to lung-derived HDM antigens following HDM sensitization, we administered

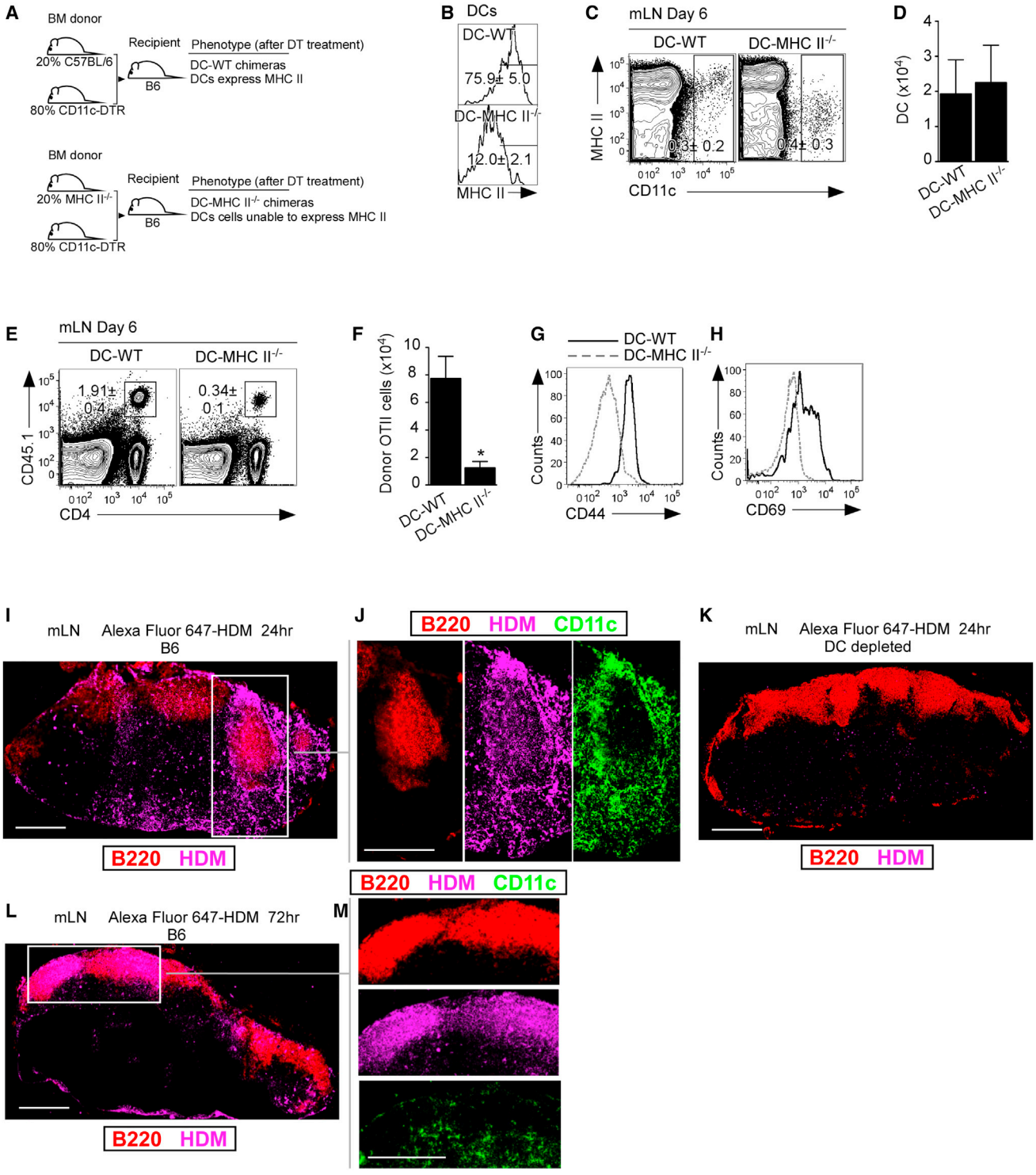


Figure 3. Ag Presentation by DC Cells Is Necessary for the Priming of Specific CD4⁺ T Cells after Allergic Sensitization

(A–H) B6 mice were irradiated and reconstituted with an 80:20 mixture of BM from CD11c-DTR and B6 donors (DC-WT) or from CD11c-DTR and MHC II-deficient donors (DC-MHC II-deficient) (A). Eight weeks later, 2.5 × 10^4 naive OTII.CD45.1⁺ CD4⁺ T cells were transferred into reconstituted chimeric mice on day 0. Recipient mice were treated with HDM+OVA (from day 1 to 4), injected with PBS or DT at day 0 and 3, and analyzed on day 6. The expression of MHC II on DC (B) and the frequency (C) and number (D) of DCs in the mLN are shown. Frequency (E) and number (F) of donor OTII T cells from the mLN. Expression of CD44 (G) and CD69 (H) in donor OTII T cells from the mLN.

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Alexa Fluor 647-labeled HDM to B6 mice and examined sections of mLN by fluorescence microscopy 24 hr after HDM inoculation (Figures 3I–3K). We found that labeled HDM was predominately located in the interfollicular area and co-localized with cells expressing CD11c (Figures 3I and 3J). Importantly, labeled HDM was not detectable in the mLNs of DT-treated *Itgax*-DTR mice (Figure 3K), consistent with the idea that HDM is transported to the mLN by lung-migratory DCs (Plantinga et al., 2013). Notably, some labeled HDM was also detected in B cell follicles (Figures 3I and 3J), particularly by 72 hr after HDM inoculation (Figures 3L and 3M). These data indicate that lung-migratory, HDM-bearing DCs preferentially localize within the periphery of the B cell follicles early after HDM sensitization, and that Ag is subsequently transferred to cells in the follicle.

Sensitization with HDM Elicits IL-4-Committed Tfh Cells, but Not Effector Th2 Cells

Given that Tfh cells can produce IL-4 (King and Mohrs, 2009) and require cognate interactions with Ag-presenting B cells and DCs (Ballesteros-Tato and Randall, 2014), we hypothesized that B cell deficiency compromised Th2 cell-mediated responses to HDM by preventing the initial development of IL-4-commited Tfh cells. To test this possibility, we analyzed the phenotype of IL-4-committed $CD4^+$ T cells in day 6 HDM-sensitized B6.4get mice. We found that GFP^+CD4^+ T cells did not accumulate in the lungs of HDM-sensitized mice relative to naive mice (Figure 4A). In contrast, we found a much higher frequency of GFP^+CD4^+ T cells in the mLN of HDM-sensitized mice than in naive mice (Figure 4B). Moreover, the GFP^+CD4^+ T cells in the mLN of HDM-sensitized mice consisted of a homogenous population of $CXCR5^{hi}PD\text{-}1^{hi}$ T cells (Figure 4C) that also expressed high amounts of the transcription factor, BCL-6, the Tfh marker, GL7 and the inducible costimulator ICOS, but were CD25 and FOXP3 negative and GATA-3-low (Figure 4D and data not shown). Flow cytometric analysis of intracellular cytokine production showed expression of IL-21 and IL-4 but little or no production of IL-2 or the Th2 cell-associated cytokines IL-13 and IL-5 (Figure 4E and data not shown). Similar results were obtained from analyzing the phenotype of IL-4-committed $CD4^+$ T cells in day 14 HDM-sensitized B6.4get mice (Figures S2A–S2C). Thus, we conclude that these cells are IL-4-committed Tfh cells.

Corresponding with this conclusion, we next examined B cell responses. We found that HDM sensitization induced immunoglobulin E (IgE)-secreting antibody secreting cells (ASC) and the formation of germinal centers (GCs) in the mLN (Figures 4F–4H and Figure S2D). Both responses were boosted after challenge.

We next analyzed the phenotype of IL-4-committed $CD4^+$ T cells in the lung and mLN of B6.4get mice following challenge with HDM. We found that the GFP^+CD4^+ T cells in the lungs of challenged mice were $CXCR5^{lo}PD\text{-}1^{lo}$ (Figure 4I), expressed low amounts of BCL-6 and GL7 and intermediate amounts of CD25, high amounts of GATA-3 (Figure 4J), and produced IL-2, IL-4, IL-13, and IL-5 (Figure 4K and data not shown), consistent with the phenotype of effector Th2 cells (Liang et al., 2012). In contrast, the GFP^+CD4^+ T cells in the mLN consisted of a mix of $CXCR5^{lo}PD\text{-}1^{lo}BCL\text{-}6^{lo}GL7^{lo}CD25^{int}$ $GATA\text{-}3^{hi}$ effector Th2 cells, which produced IL-2, IL-4, and IL-13 and $CXCR5^{hi}PD\text{-}1^{hi}BCL\text{-}6^{hi}GL7^{hi}CD25^{-}GATA\text{-}3^{lo}$ Tfh cells, which produced IL-21 (Figures 4L–4N). We also analyzed the phenotype of IL-4-committed $CD4^+$ T cells in the peripheral blood of B6.4get mice during the HDM sensitization and challenge phases. We found that IL-4-committed $CD4^+$ T cells were only detected after challenge (Figure S2 A) and displayed an effector phenotype (Figure S2 B).

Taken together, these data indicated that HDM sensitization elicit IL-4-committed Tfh cells, which are confined to the mLN, whereas challenge with HDM expands effector Th2 cells that home to the lung.

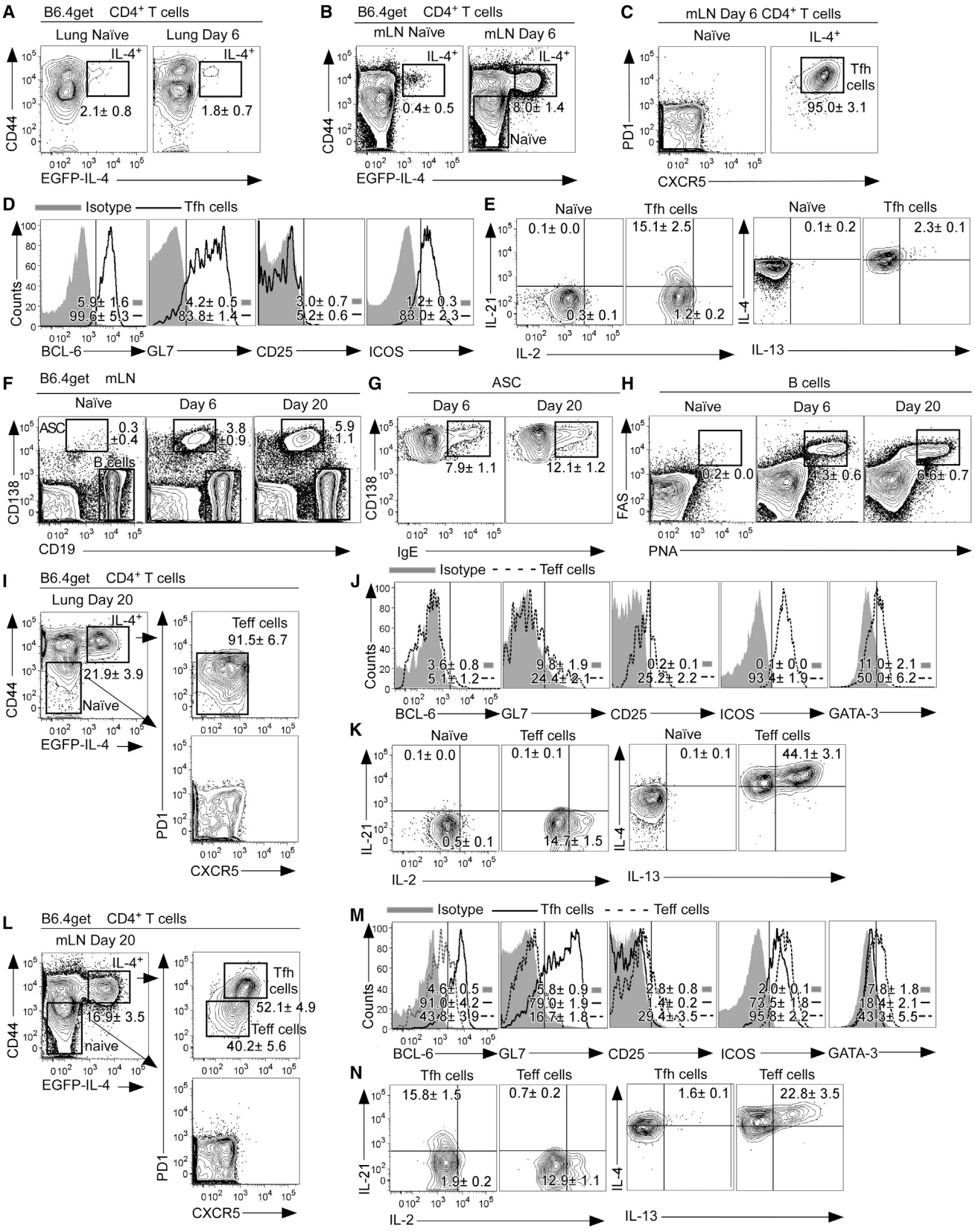
To further confirm this conclusion, we adoptively transferred $CD45.1^+$ OTII T cells into naive B6 and μMT mice. One day later, mice were sensitized with HDM-OVA for 4 consecutive days and analyzed the donor OTII cells in the mLNs of recipient mice on day 6 (Figures 5A–5F) and day 14 (Figure S3A-C). We found that the frequencies (Figure 5A) and numbers (Figure 5B) of donor OTII cells were increased in the mLNs of HDM-sensitized B6 mice compared to HDM-sensitized μMT mice or non-sensitized B6 mice. Importantly, OTII cells in the HDM-sensitized B6 mice expressed high amounts of the activation markers CD44 (Figure 5C) and CD69 (Figure 5D), uniformly co-expressed CXCR5 and PD-1 (Figure 5E), resulted in $BCL\text{-}6^{hi}$ (Figure 5F), and expressed GL7 and IL-4 transcripts (Figure S3C), thus resembling IL-4-committed Tfh cells. Similar results were obtained when donor OTII cells in the mLN were analyzed on day 14 (Figures S3A–S3C). As expected, donor OTII cells in the mLN of HDM-sensitized μMT mice resulted in $CXCR5^{lo}PD\text{-}1^{lo}BCL\text{-}6^{lo}$ (Figures 5C–5F), and resembled naive OTII cells in non-sensitized B6 mice. We also failed to detect donor OTII cells in the lungs of either B6 or μMT recipient mice following HDM sensitization (data not shown). Finally, immunofluorescence staining of mLN sections from HDM-OVA-sensitized mice on day 6 (Figure S3D) and day 14 (Figure S3E) showed donor $CD45.1^+$ $CD4^+$ OTII cells inside GCs. These data provide further evidence that OTII cells differentiated into Tfh cells following HDM sensitization.

Taken together, these data demonstrate that HDM sensitization does not induce effector Th2 cells, but rather expands IL-4-committed Tfh cells that are retained in the mLN.

IL-4-Committed Tfh Cells Differentiate into Effector Th2 Cells and Home to the Lungs after HDM Challenge

Recent studies indicate that Tfh cells maintain the flexibility to differentiate into Teff cells upon secondary challenge (Lüthje et al., 2012). To test whether Tfh cells elicited by HDM sensitization differentiated into Th2 effector cells following HDM

(I–M) B6 mice were i.n. treated with 25 μg of Alexa Fluor 647-labeled HDM. Cryosections from the mLN were stained with anti-B220 (red) and CD11c (green) and analyzed by fluorescent microscopy on days 1 (I–K) and 3 (L and M). The regions within the box in (I) and (L) were enlarged in (J) and (M), respectively. In the same experiment, CD11c-DTR mice were injected i.p. with 60 ng DT and i.n. treated with 25 μg of Alexa Fluor 647-labeled HDM 24 hr later. Cryosections from the mLN one day 1 after HDM inoculation are shown (K). Scale bars represent 400 μm. *$p < 0.005$ (unpaired Student's t test). Data are representative of three independent experiments (mean and SD of 4–5 mice per group).



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challenge, we adoptively transferred equivalent numbers of naive CD45.1$^+$OTII cells or day 6 HDM-OVA-sensitized CD45.1$^+$OTII cells from the mLNs of donor mice into day 6 HDM-sensitized recipients and challenged the recipients on day 15 (Figure. 5G). Importantly, the OTII cells transferred from naive mice did not express CXCR5 or PD-1 (Figure 5H) or BCL-6 (Figure 5I), whereas those transferred from day 6 HDM sensitized mice were uniformly CXCR5^{hi}PD-1^{hi}BCL-6^{hi} Tfh cells (Figures 5H and 5I). Following challenge, we found that progeny of the naive OTII donor cells in the mLN had differentiated into CXCR5^{hi}PD-1^{hi}BCL-6^{hi} Tfh cells (Figure 5J), whereas the progeny of the donor Tfh OTII cells had differentiated into a mixed population of CXCR5^{hi}PD-1^{hi}BCL-6^{hi} Tfh cells and CXCR5^{lo}PD-1^{lo}BCL-6^{lo} effector CD4$^+$ T cells (Figure 5J). We also found that the progeny of naive OTII donor cells poorly accumulated in the lung (Figures 5K and 5L) and failed to differentiate into IL-4 and IL-13 double-producing effector Th2 cells (Figures 5M and 5N). In contrast, the progeny of Tfh OTII donor cells efficiently accumulated in the lung (Figures 5K and 5L), and many of them differentiated into IL-4 and IL-13 double-producing effector Th2 cells (Figures 5M and 5N). As a control, we examined the frequency (Figures S3F) and number (Figure S3G) of host IL-4-producing CD4$^+$ T cells and found that they were similar in the recipients of naive and Tfh OTII donor cells. Importantly, donor Tfh OTII cells poorly accumulated in the lung (Figures S3H–S3K) and differentiated into IL-4 and IL-13 double-positive effector Th2 cells (Figures S3L–S3M) when the recipients were not previously sensitized with allergen.

Taken together, these data indicate that IL-4-committed Tfh cells generated by HDM sensitization rapidly differentiate into secondary effector Th2 cells and home to the lung upon HDM challenge.

To further confirm this conclusion, we used *Il21*-mCherry and *Il2*-emGFP BAC dual reporter Tg mice (Wang et al., 2011). Analysis of mLNs of dual reporter BM chimeric mice on day 6 after HDM sensitization identified a population of CD44^{hi}CD4$^+$ T cells that were *Il21*-mCherry$^+$*Il2*-emGFP$^-$ (mCherry-IL-21$^+$) (Figure 6A). These cells were not detected in naive mice (Figure 6A) or in the lungs of mice following HDM sensitization (Figure 6C). Further analysis showed that mCherry-IL-21$^+$ cells in the mLN expressed intracellular IL-21 and the markers PD1 and CXCR5 (Figure 6B) thus resembling Tfh cells (Lüthje et al., 2012). To analyze whether Tfh cells can differentiate into Th2 effector cells following HDM challenge, *Il21*-mCherry$^+$*Il2*-emGFP$^-$ (mCherry-IL-21$^+$) Tfh cells, *Il21*-mCherry$^-$*Il2*-emGFP$^+$ (GFP-IL-2$^+$) non-Tfh cells (Figure 6D) and naive CD44^{lo}CD4$^+$ T cells were sorted from mLNs of day 6 HDM-sensitized dual reporter BM chimeric mice and equal numbers of cells were adoptively transferred into day 6 HDM-sensitized recipients. Recipient mice were then challenged on day 15 and analyzed on day 20. Following challenge, we found significant expansion of donor-derived cells in the mLNs of mice that had received mCherry-IL-21$^+$ Tfh cells compared with those receiving naive or GFP-IL-2$^+$ non-Tfh cells (Figures 6E and 6F). Furthermore, the progeny of the donor mCherry-IL-21$^+$ Tfh cells differentiated into a mixed population of CXCR5^{hi}PD-1^{hi} Tfh cells and CXCR5^{lo}PD-1^{lo} effector CD4$^+$ T cells. (Figure 6G). As expected, CXCR5^{hi}PD-1^{hi} Tfh derived from donor mCherry-IL-21$^+$ cells expressed mCherry-IL-21 but were GFP-IL-2 negative. In contrast, the CXCR5^{lo}PD-1^{lo} effector CD4$^+$ T cells derived from donor mCherry-IL-21$^+$ cells dowregulated the expression of mCherry-IL-21 and upregulated the expression of GFP-IL-2 (Figure 6H).

We also found that the progeny of naive or GFP-IL-2$^+$ non-Tfh cells donor cells poorly accumulated in the lung (Figures 6I and 6J) and failed to differentiate into IL-4 and IL-13 double-producing effector Th2 cells (Figures 6M and 6N). In contrast, the progeny of the donor mCherry-IL-21$^+$ Tfh cells efficiently accumulated in the lung (Figures 6I and 6J), where they expressed markers consistent with an effector Th2 phenotype (such as CXCR5^{lo}PD-1^{lo} Figure 6K). Furthermore, these cells had downregulated the expression of mCherry-IL-21 and completely failed to produce IL-21 after stimulation (Figure 6L). By contrast, mCherry-IL-21$^+$ donor-derived cells in the lung upregulated the expression of GFP-IL-2 (Figure 6L) and differentiated into IL-4 and IL-13 double-producing effector Th2 cells (Figures 6M and 6N). Together, these experiments showed that IL-4-committed Tfh cells generated upon HDM sensitization were able to differentiate effector Th2 cells when recalled.

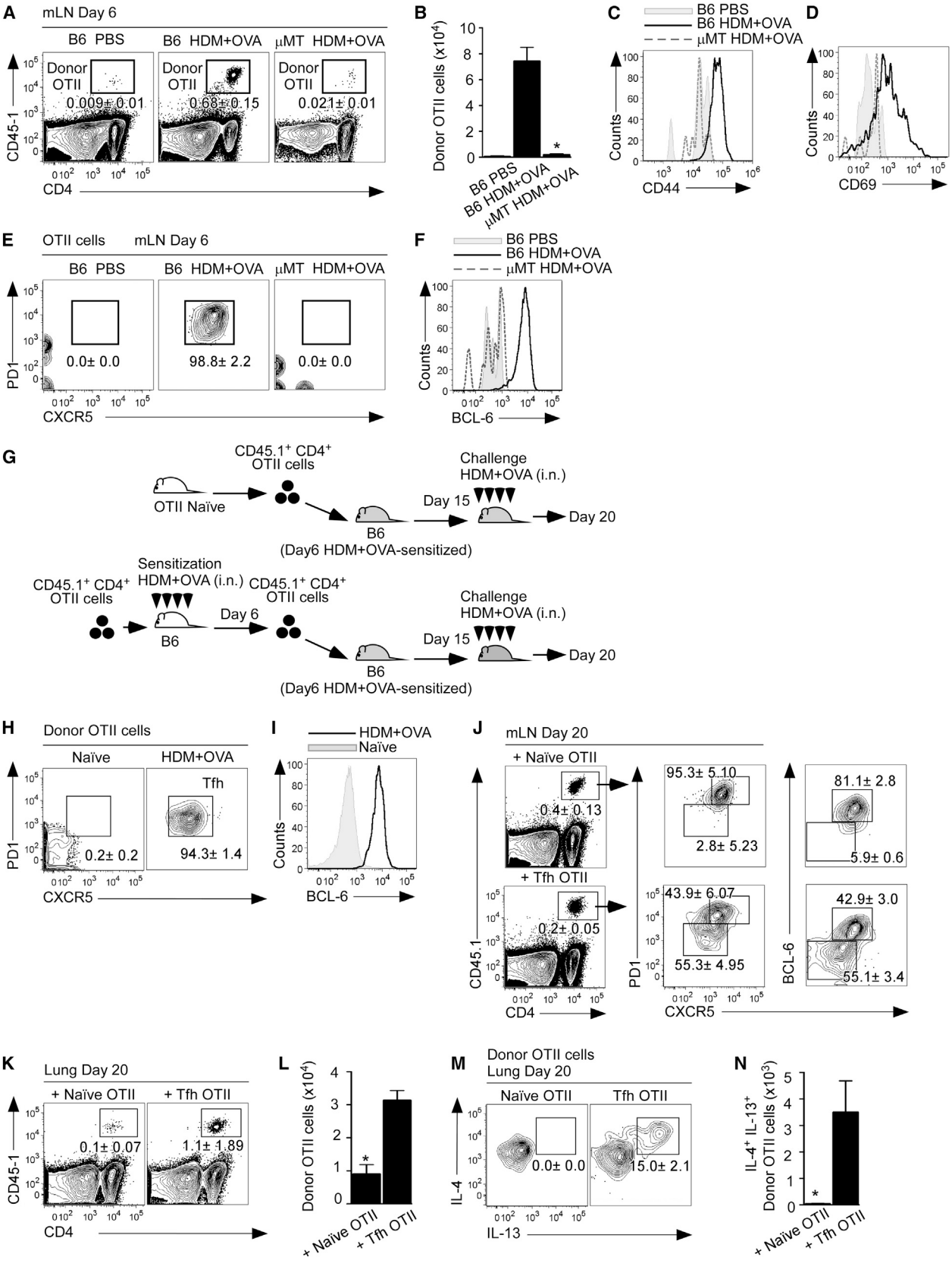
Depletion of Pre-existing IL-4-Committed Tfh Prevents Th2-Teff Cell Responses after HDM Challenge

We next tested whether blockade of Tfh development during HDM sensitization precluded effector Th2 responses after HDM challenge. To address this possibility, we treated HDM-sensitized B6.4get mice with either 50 mg/Kg BCL-6 inhibitor 79-6 (Cerchietti et al., 2010) per day or vehicle (10% DMSO) on days 4 to 11 and enumerated GFP-IL-4$^+$ Tfh cells in the mLN on day 12. We found that the number (Figure 7B) of GFP-IL-4$^+$ CD4 T cells with a PD-1^{hi}CXCR5^{hi} Tfh cell phenotype was reduced in 79-6 treated mice compared to PBS-treated controls. We next purified CD4$^+$ T cells from the mLN of day 12 HDM-sensitized, 79-6-treated and control mice and transferred equivalent numbers into day 12 HDM-sensitized CD45.1$^+$ B6 mice (Figure 7A), challenged the recipients with HDM on days 13–16 and enumerated effector Th2 cells from the donor (CD45.2$^+$) on day 18 in the lungs. We found that the frequency

Figure 4. HDM Sensitization Induces IL-4-Expressing Tfh Cells but Not Effector Th2 Cells

(A–E) B6.4get mice were i.n treated with 25 μg of HDM of PBS for 4 consecutive days starting on day 1 and analyzed by flow cytometry on day 6. The frequency of IL-4-expressing (EGFP$^+$) on the CD4$^+$ T cells from the lungs (A) and mLNs (B) are shown. Expression of PD-1 and CXCR5 (C), BCL-6, GL7, CD25, and ICOS (D) and intracellular IL-2 and IL-21 (E) on naive (CD44^{lo}EGFP$^-$) and antigen-experienced (CD44^{hi}EGFP$^+$) CD4$^+$ T cells in the mLN of HDM-treated mice are shown at day 6. (F–H) B6 mice were i.n. HDM sensitized on day 1 and analyzed on day 6 or HDM sensitized on day 1, challenged on day 15 and analyzed on day 20. The frequency of CD138$^+$ antibody secreting cells (ASC) (F), IgE$^+$CD138$^+$ ACS (G), and PNA$^+$FAS$^+$ GC cells (H) in the mLN are shown.

(I–N) B6.4get mice were i.n. treated with 25 μg of HDM for 4 consecutive days starting on day 1. On day 15, mice were i.n. challenged with 25 μg HDM daily for 4 consecutive days. Mice were sacrificed and analyzed by flow cytometry on day 20. The expression of PD-1 and CXCR5 (I), BCL-6, GL7, CD25, ICOS, and GATA-3 (J) and intracellular IL-2 and IL-21 (K) on naive (CD44^{lo}EGFP$^-$) and antigen-experienced (CD44^{hi}EGFP$^+$) CD4$^+$ T cells in the lung are shown. Expression of PD-1 and CXCR5 (L), BCL-6, GL7, CD25, ICOS, and GATA-3 (M) and intracellular IL-2 and IL-21 (**N**) on naive (CD44^{lo}EGFP$^-$) and antigen-experienced (CD44^{hi}EGFP$^+$) CD4$^+$ T cells in the mLN. Data are representative of three independent experiments (mean and SD of 4-5 mice per group). See also Figure S2.



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(Figures 7C and 7E) and number (Figures 7D and 7F) of donor GFP$^+$ (Figures 7C and 7D) and IL-13$^+$ (Figures 7E and 7F) effector Th2 cells were reduced in recipients that had received CD4$^+$ T cell from 79-6-treated mice compared to control mice. As a control, we showed that donor GFP$^+$ and IL-13$^+$ effector Th2 cells accumulated normally in mice that received 79-6 starting 1 day after challenge (Figures 7C–7F). These results suggest that BCL-6 inhibitor therapy prevents Tfh cell responses after HDM sensitization, which in turn precludes effector Th2 cell-mediated responses following challenge.

IL-2 directly inhibits the differentiation and maintenance of Tfh cells (Ballesteros-Tato et al., 2012). To test whether recombinant IL-2 (rIL-2) treatment after HDM sensitization could impair Th2 effector cell responses after HDM challenge, we adoptively transferred CD45.1$^+$ OTII cells into naive CD45.2$^+$ B6 recipient mice, sensitized the recipients one day later with HDM-OVA, treated them with 30,000 units of human rIL-2 or PBS twice a day on days 10 and 11 (Figure 7G), and enumerated CD45.1$^+$ Tfh cells in the mLN on day 12. We found that the number (Figure 7H) of donor OTII cells with a PD-1^{hi}CXCR5^{hi} Tfh cell phenotype was reduced in rIL-2-treated mice compared to PBS-treated controls. In agreement with these results, the number of CD19$^+$BCL-6$^+$ GC B cells was reduced in rIL-2-treated mice as well (Figure 7I).

To evaluate whether the lack of Tfh cells in rIL-2-treated mice could affect subsequent Th2 effector cell responses, we challenged rIL-2-treated and control mice with HDM-OVA and characterized the Th2 effector cell response in the lungs on day 20. As a control, some mice were treated with rIL-2 starting 1 day after HDM-OVA challenge (Figure 7G). We found that the frequency (Figure 7J) and number (Figure 7K) of donor OTII cells as well as the frequency (Figure 7L) and number (Figure 7M) of IL-4-producing donor Th2 effector cells was dramatically reduced in mice treated with rIL-2 after HDM sensitization. In contrast, IL-4-producing donor Th2 effector cells accumulated to even higher amounts in mice that received rIL-2 during HDM challenge (Figures 7J–7M), consistent with the idea that IL-2 signaling promotes Th2 effector cell responses (Liao et al., 2008). We also found that the number of eosinophils in the lungs and BAL was reduced in mice treated with rIL-2 after HDM sensitization, but was increased in mice treated with rIL-2 during HDM challenge (Figures 7N and 7O). Importantly, rIL-2 treatment did not alter the frequency or number of Foxp3$^+$ regulatory T cells at any time point or organ analyzed (Figure S4A-F). Taken together, these results indicate that depletion of IL-4-committed Tfh cells prevents the development of Th2 effector cell-mediated allergic responses upon secondary HDM challenge.

DISCUSSION

Our data show that, following pulmonary sensitization with HDM, naive CD4$^+$ T cells differentiate into IL-4-committed Tfh cells in the mLN, but fail to differentiate into pathogenic effector Th2 cells that home to the lung. As a result, initial sensitization with HDM does not cause Th2 cell-mediated symptoms or pulmonary pathology. However, following HDM challenge, the IL-4-committed Tfh cells rapidly differentiate into effector Th2 cells that home to the lung, produce IL-4, and recruit eosinophils. Our results are consistent with studies showing that Tfh cells have the capacity for long-term persistence and the ability to differentiate into potentially-pathogenic effector T cells upon secondary challenge (Choi et al., 2013; Lüthje et al., 2012) and offer a perspective for how Th2-cell-mediated pathology to inhaled HDM is initiated.

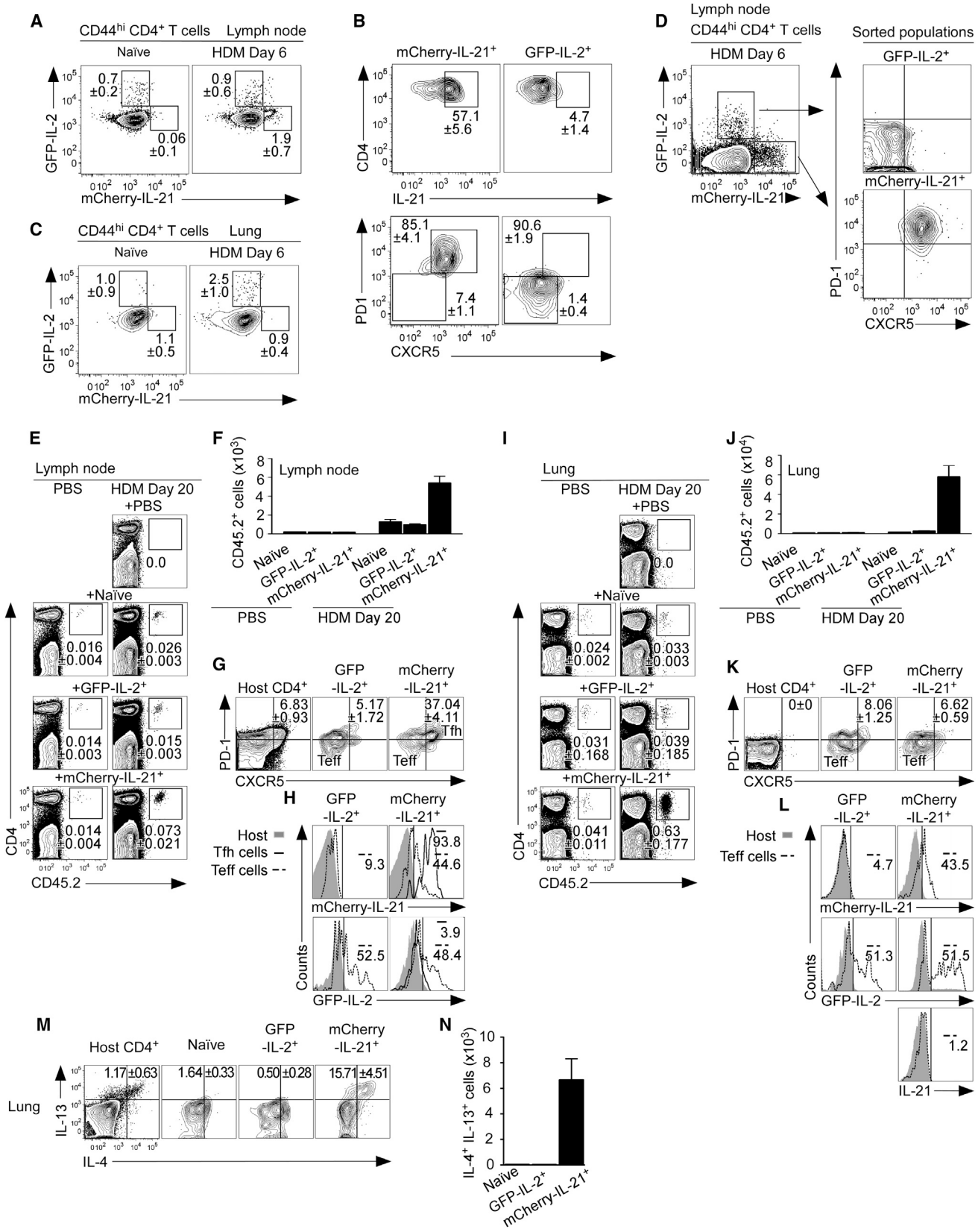
Our results also demonstrate that Th2 cell responses following sensitization and challenge with HDM are dependent on both DCs and B cells. Although other studies show that DCs are essential for Th2 cell responses to pulmonary allergens (Hammad et al., 2010; Plantinga et al., 2013), our data confirm that DCs are important in two ways; first for carrying antigen from the lung to the mLN and second for priming allergen-specific CD4$^+$ T cell via MHC II-dependent cognate-interactions. However, unlike some studies suggesting that Ag-presentation by DCs alone is sufficient for Th2 cell responses to inhaled HDM (Hammad et al., 2010), our data demonstrate that Th2 cell responses to HDM also require additional cognate interactions with Ag-presenting B cells. Rather than directly promoting the differentiation of effector Th2 cells however, B cells synergize with DCs to promote the differentiation of IL-4-commited Tfh cells during the HDM-sensitization phase. Although DCs are sufficient to prime Tfh cell responses, Ag-presentation by B cells is necessary to complete the Tfh cell differentiation program (Baumjohann et al., 2013; Choi et al., 2011; Deenick et al., 2010; Goenka et al., 2011). Thus, our results are consistent with the current models for how Tfh cell responses are initiated (Ballesteros-Tato and Randall, 2014).

Importantly, although the exact mechanisms by which DCs prime Tfh cells is incompletely defined the initiation of the Tfh cell responses seems to occur within the interfollicular area of the LN, where responding CD4$^+$ T cell first upregulate BCL-6

Figure 5. CD4 T Cells Activated after Sensitization Develop into Effector Th2 Cells after HDM Challenge

(A–F) 2.5 × 10^4 naive OTII.CD45.1$^+$ CD4$^+$ T cells were transferred into naive B6 and μMT mice. One day later, recipient mice were i.n. treated with 25 μg of HDM + 25μg of OVA (HDM+OVA), or PBS for 4 consecutive days. Mice were sacrificed and analyzed by flow cytometry 2 days after the last HDM inoculation. Frequency (A) and number (B) of donor OTII T cells from the mLN. Expression of CD44 (C), CD69 (D), CXCR5 and PD1 (E), and BCL-6 (F) in donor OTII T cells from the mLN.

(G–N) B6 (CD45.2$^+$) mice were i.n. treated with 25 μg of HDM + 25 μg of OVA (HDM+OVA) for 4 consecutive days. On day 6, mice were adoptively transferred with 2.5 × 10^4 CD45.1$^+$ OTII cells obtained from naive OTII donor mice, or 2.5 × 10^4 CD45.1$^+$ OTII cells obtained from the mLN of day 6 HDM+OVA-sensitized B6 mice that received CD45.1$^+$ OTII cells one day before HDM+OVA administration (G). (H and I) The expression of CXCR5 and PD1 (H) and BCL-6 (I) in purified donor OTII T cells are shown. Recipient mice were then challenged on day 15 with 25 μg HDM+OVA and analyzed on day 20. (J) Donor CD45.1$^+$ OTII T cells were identified by flow cytometry and the percentages of donor CD4$^+$ T cells with a CXCR5^{hi}PD1^{hi}BCL-6^{hi} and CXCR5^{lo}PD1^{lo}BCL-6^{lo} phenotype were determined in the mLN. (K and L) The frequency (K) and number (L) of donor CD45.1$^+$ OTII T cells in the lungs are shown. (M and N) The frequency (M) and number (**N**) of IL-4-producing cells among the donor CD45.1$^+$ OTII T cells were determined in the lungs by intracellular staining after restimulation for 4 hr with anti-CD3 and BFA. *$p < 0.001$ versus HDM+OVA-treated B6 (unpaired Student's t test). Data are representative of two or more independent experiments (mean and SD of 4–5 mice per group). See also Figure S3.



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and CXCR5 (Kerfoot et al., 2011). Thus, positioning of DCs within the interfollicular zone of the secondary lymphoid organs is likely to be instrumental for their ability to prime Tfh cell responses to HDM. In agreement with this idea, we found that lung-migratory, HDM-bearing DCs preferentially localize within the interfollicular area of the mLN early after HDM sensitization, where they hand off Ag to cells in the B cell follicle.

Recently, we showed that DCs responding to nematode infection upregulate CXCR5, downregulate CCR7, and home to the interfollicular areas of the mesenteric LN in response to CXCL13, where they prime effector Th2 cell responses (León et al., 2012). However, we failed to detected effector Th2 cells in the mLN and lung of HDM sensitized mice, perhaps because concomitant signals provided by HDM-presenting B cells promote the more efficient differentiation of Tfh cells. Alternatively, engagement of DCs and B cells by particular HDM-derived products might condition them to preferentially promote the differentiation of IL-4-committed Tfh cells during the sensitization phase, perhaps by favoring the expression of cytokines or costimulatory ligands that promote Tfh cell formation. In any case, we suggest a two-step differentiation model in which HDM-specific IL-4-committed $CD4^+$ T cells are first primed by lung-migratory DCs in the border of the B cell follicle where they begin to proliferate and acquire a Tfh like-signature. IL-4-committed pre-Tfh cells subsequently encounter Ag-bearing B cells that acquired HDM-derived Ags from the lung-migratory DCs. In the B cell follicle, activated B cells replace DCs as the primary APCs and provide additional survival and differentiation signals that allow HDM-specific IL-4-committed Tfh cells to survive as HDM-specific memory cells, which further differentiate into effector Th2 cells after re-challenge. This model is supported by recent studies showing that sustained antigenic stimulation by B cells is required for the maintenance of the Tfh cell responses (Baumjohann et al., 2013; Choi et al., 2011; Deenick et al., 2010; Goenka et al., 2011) and data indicating that Tfh cells are able to become memory cells (Choi et al., 2013; Lüthje et al., 2012) but retain the capacity to differentiate into effector T cells during a recall response (Lüthje et al., 2012).

The fact that HDM challenge induces IL-4-committed Tfh cells to differentiate into effector Th2 cells is at odds with a recent paper reaching the opposite conclusion. This work has concluded that although HDM-specific Tfh cells are required for normal Th2 responses to HDM, they do not convert into effector Th2 cells (Coquet et al., 2015). The authors largely base their conclusion on the fact that IL-21^+ Tfh cells from HDM sensitized mice failed to differentiate into Th2 cells upon adoptive transfer into naive mice. However, we have demonstrated here that Tfh to Th2 cell conversion only occurs in recipients that had been previously exposed to the allergen. As a consequence, Tfh cells transferred into naive recipients poorly differentiated into Th2 cells. Thus, differences in the experimental design might have led to opposing conclusions compared with our study. In any case, our findings demonstrate that an inability to generate Tfh cell responses during the HDM sensitization phase precludes Th2 cell-mediated immunity after secondary HDM challenge. These data suggest that targeting Tfh cells might be a good therapeutic strategy to prevent Th2-cell mediated immunity to HDM. In support of this, we found that depletion of pre-existing Tfh cells following rIL-2 treatment prevents Th2-cell mediated immunity after HDM re-exposure. Therefore, knowing the factors that control Tfh cell maintenance in response to HDM and the mechanisms by which Tfh cells convert into effector Th2 cells following repeated allergen encounter, will provide valuable information to design therapeutic strategies to prevent allergic responses to HDM.

In conclusion, our findings offer insights into how Th2-cell-mediated immunity to HDM is initiated, reveal an important role for B cells and Tfh cells in this process, and expose the potential therapeutic benefits of targeting Tfh cells to prevent allergic asthma.

EXPERIMENTAL PROCEDURES

Mice and Immunizations

The mouse strains used in these experiments include: C57BL/6J (B6), B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1^+ B6 congenics), B6.129S2-*Ighm*^{tm1Cgn}/J (μMT), B6.FVB-Tg (*Itgax*-DTR-EGFP)57Lan/J (CD11c-DTR), B6.129S-H2$^{dlAb1\text{-}Ea}$ (MHC II-deficient), C57BL/6-Tg(*TcraTcrb*)425Cbn/J (OTII), B6.129-Il4^{tm1Lky}/J (B6.4get IL-4 reporter mice), B6.129-Il4^{tm1Lky}/J.IgHm^{tm1Cgn}/J (μMT.4get), B6.129-Il4^{tm1Lky}/J. C57BL/6-Tg(*TcraTcrb*)425Cbn/J (OTII.4get), and *Il21*-mCherry and *Il2*-emGFP dual-reporter transgenic mice. B6.4get mice and μMT.4get mice were originally obtained from M. Mohrs (Trudeau Institute). *Il21*-mCherry and *Il2*-emGFP dual-reporter mice were originally obtained from Dr. Warren J. Leonard (NHLBI). All other mice were originally obtained from The Jackson Laboratory and were bred in the University of Alabama at Birmingham animal facility. HDM (Dermatophagoides and teronyssinus) extracts were obtained from Greer laboratories. Adult mice were administered intranasally (i.n.) with 25 μg of HDM extract or 25 μg of HDM extract and 25 μg of OVA (Sigma-Aldrich) daily for 4 days and challenged (i.n.) with 25 μg of HDM or 25 μg of HDM extract and 25 μg of OVA 14 days later. In some experiments, HDM extract was labeled with AF647 labeling kit (Invitrogen) prior to administration to mice. The University of Alabama at Birmingham Institutional and NHLBI Animal Care and Use Committees approved all procedures involving animals.

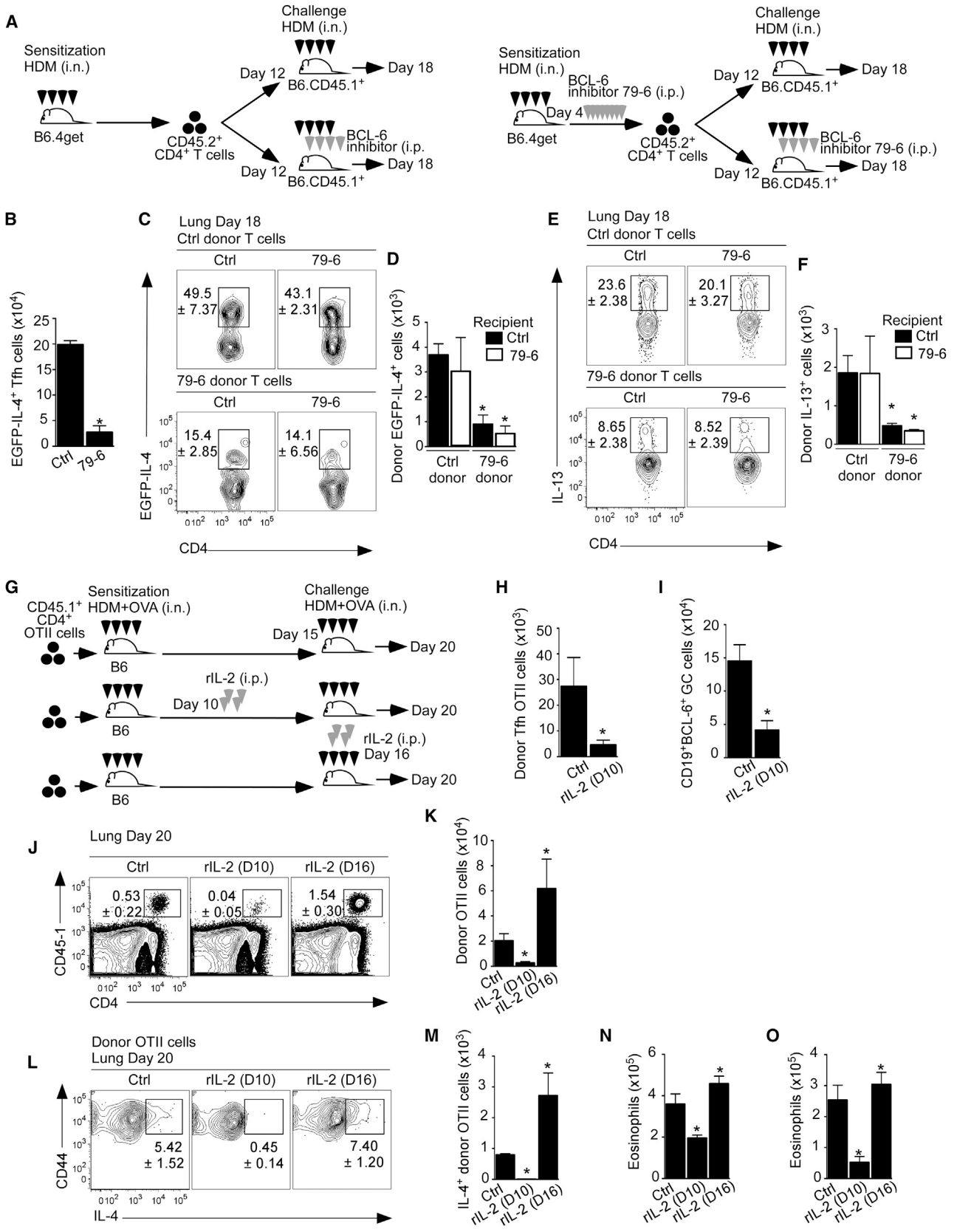
BM Chimeras

Recipient mice were irradiated with 950 Rads from a high-energy X-rays source delivered in a split dose and reconstituted with 10^7 total BM cells. To

Figure 6. Tfh Cells Develop into Effector Th2 Cells after HDM Challenge

(A–C) CD45.1^+ B6 mice were irradiated and reconstituted with BM from *Il21*-mCherry and *Il2*-emGFP dual-reporter mice. Eight weeks later, reconstituted-chimeric mice were i.n. HDM sensitized on day 1 and analyzed on day 6. The frequency of IL-21-expressing (mCherry$^+$) and IL-2-expressing (GFP$^+$) cells in CD44^{hi}CD4$^+$ T cells from the mLN (A) and lung (C) and are shown. Expression of intracellular IL-21, CXCR5, and PD1 in mCherry$^+$ GFP$^-$ single-positive and mCherry$^-$ GFP$^+$ single-positive CD4$^+$ T cells from the mLN (B) is shown.

(D–N) 1 × 10^5 mCherry$^+$ GFP$^-$ single-positive (CXCR5$^+$PD1$^+$) or mCherry$^-$ GFP$^+$ single-positive (CXCR5$^-$PD1$^-$) CD44^{hi}CD4$^+$ T cells (D) or naive CD44^{lo}CD4$^+$ T cells, sorted by flow cytometry on day 6 after sensitization, were adoptively transferred into day 6 HDM sensitized CD45.1^+ B6 mice. Recipient mice were then challenged with HDM or PBS on day 15 and analyzed on day 20. The frequency (E) and (I) and number (F) and (J) of donor CD45.2^+ T cells in the mLN (E and F) and lung (I and J) are shown. The frequency of CXCR5 and PD1 (G) and (K) and mCherry-IL-21, GFP-IL-2, and intracellular IL-21 (H) and (L) in donor CXCR5^{hi}PD1^{hi} (Tfh) and CXCR5^{lo}PD1^{lo} (Teff) CD4$^+$ T cells from mLN (G and H) and lung (K and L) are shown. (M and N) The frequency (M) and number (**N**) of IL-4 and IL-13 double-producing cells among the donor CD45.2^+ T cells were determined in the lungs by intracellular staining after restimulation for 4 hr with anti-CD3 and BFA (mean and SD of 4–5 mice per group).



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generate mice that express *Il21*-mCherry and *Il2*-emGFP dual-reporter gene in BM cells, CD45.1$^+$ B6 recipients were reconstituted with 100% of *Il21*-mCherry and *Il2*-emGFP BM. To generate mice that lacked MHC II expression specifically in the DC compartment, we reconstituted B6 recipients with 80% *Itgax*-DTR BM + 20% MHC II-deficient BM. For controls, B6 recipients were reconstituted with 80% *Itgax*-DTR BM + 20% B6 BM. To generate mice that lacked MHC II expression specifically in the B cell compartment, we reconstituted μMT recipients with 80% μMT.4get BM + 20% MHC II-deficient BM. For controls, μMT recipients were reconstituted with 80% μMT.4get BM + 20% B6 BM. Mice were allowed to reconstitute for at least 8–12 weeks before HDM treatment.

In Vivo Treatments

To deplete CD11c$^+$ cells in vivo, we treated CD11c-DTR mice and *Itgax*-DTR BM chimeras intraperitoneally (i.p.) with 60 ng DT (Sigma-Aldrich). Chimeras received additional injections of DT every 3 days. For rIL-2 treatment, mice were intraperitoneally administered 30,000 U of human rIL-2 (National Cancer Institute) twice a day for two days. To block BCL-6 activity, we treated mice daily by i.p. injection with 50 mg/Kg BCL-6 inhibitor 79-6 (Calbiochem) or vehicle (10% DMSO).

Cell Preparation and Flow Cytometry

Lungs were isolated, cut into small fragments and digested for 45 min at 37°C with 0.6 mg/ml collagenase A (Sigma) and 30 μg/ml DNase I (Sigma) in RPMI-1640 medium (GIBCO). Digested lungs, mLNs, or spleens were mechanically disrupted by passage through a wire mesh. Red blood cells were lysed with 150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA. Fc receptors were blocked with anti-mouse CD16/32 (5 μg/ml; BD-Biosciences), followed by staining with fluorochrome-conjugated Ab. Fluorochrome-labeled anti-B220 (RA3-6B2), anti-BCL-6 (K112.91), anti-CD3 (17A2) anti-CD4 (GK1.5), anti-CD19 (1D3), anti-CD11c (HL3), anti-CD25 (7D4), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD69 (H1-2F3), anti-CD138 (281-2), anti-CXCR5 (2G8), anti-FAS (CD95; Jo2), anti-GATA-3 (L50-823), GL7, anti-ICOS (7E.17G9), anti-IgE (R35-72), anti-Siglec F (E50-2440), and anti-MHC II (AF6-120.1) were from BD Biosciences. Fluorochrome-labeled anti-PD1 (J43) and anti-Foxp3 (FJK-16 s) were from eBioscience. Fluorochrome-labeled PNA was from LifeTech. For intracellular cytokine staining, cell suspensions were stimulated on anti-CD3-coated plates (2 μg/ml) in the presence of Brefeldin-A (BFA, 10 μg/ml) for 4 hr. Restimulated cells were surface stained, fixed in 4% paraformaldehyde, permeabilized with 0.1% saponin, and stained with Abs against IL-2 (JE86-5H4), IL-4 (BVD4-1D11), and IL-13 (13A) (BD-Biosciences) or IL-21 (FFA21; eBioscience). BCL-6, GATA-3, and Foxp3 intracellular staining was performed using the MOUSE regulatory T cell staining kit (eBioscience). Flow cytometry was performed on FACSCanto II (BD-Biosciences) instrument.

Cell Purifications and T Cell Transfers

CD4$^+$ T cells were isolated by MACs from the spleens of naive CD45.1$^+$ OTII mice or from the mLNs of HDM-treated B6 and μMT mice. B220$^+$ B cells were isolated by MACs from the spleens of naive B6 mice. All T and B cell preparations were more than 95% pure. An aliquot of purified CD4$^+$ T cells from mLN of HDM-treated mice previously transferred with naive CD45.1$^+$ CD4$^+$ OTII cells was stained with anti-CD45.1 mAb to calculate the number of OTII cells present in the purified population. Equivalent numbers (2.5×10^4) of naive and primed OTII cells were transferred (i.v.) into naive or HDM-treated B6 recipient mice. 1×10^5 sorted Il21-mCherry$^+$ Il2-emGFP$^-$ CD44^{hi}CD4$^+$ Tfh cells, *Il21*-mCherry$^-$ *Il2*-emGFP$^+$ CD44^{hi}CD4$^+$ non-Tfh cells and naive CD44^{lo} CD4$^+$ T cells were i.v. transferred into congenic HDM-treated CD45.1$^+$ B6 recipient mice.

Immunofluorescence

Frozen sections (10 μm), prepared from OCT (Sakura Finetek)-embedded LNs, were incubated with 10 μg/ml of Fc block and 5% normal donkey serum in PBS and then stained with FITC-labeled anti-IgDb (217-170), FITC-labeled anti-CD11c (HL3), FITC-labeled anti-CD45.1 (A20), biotin-labeled or Alexa Fluor 647-labeled anti-B220 (RA3-6B2), biotin-labeled anti-CD4 (GK1.5), or biotin-labeled anti-CD35/CD21 (8D9). Primary antibodies were detected with Alexa Fluor 488-labeled goat anti-FITC and streptavidin-Alexa Fluor 555 (Invitrogen Life Sciences). Slides were mounted with Slow Fade Gold Antifade (Invitrogen). Images were collected with an Eclipse Ti-E Nikon inverted microscope and recorded with a Clara interline CCD camera (Andor). The images were taken with a 20× objective for 200× final magnification. Images were collected using NIS Elements Image software and saved as JPEG files.

Statistical Analyses

GraphPad Prism software (Version 5.0a) was used for data analysis. Data were analyzed using the unpaired Student's t test. Values of $p < 0.05$ were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.11.017.

AUTHOR CONTRIBUTIONS

B.L. and A.B.-T. wrote the manuscript. B.L. designed and performed all experiments with help from A.B.-T. T.D.R., F.E.L., R.S., and W.J.L. provided advice, discussion, and reagents that were critical to this work. All authors reviewed the manuscript before submission.

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Figure 7. Blockade of Tfh Cell Development Impairs Effector Th2 Cell Responses to HDM

(A–F) B6.4get mice were i.n. HDM sensitized on day 1 and daily i.p treated with 50 mg/Kg of BCL-6 inhibitor 79-6 or vehicle (10% DMSO) from day 4 to day 11. Number of IL-4-expressing (EGFP$^+$) Tfh cells from the mLN on day 12 (B). CD4$^+$ T cells were purified from the mLN on day 12 and 2×10^6 of purified CD4$^+$ T cells were transferred into day 12 HDM-sensitized B6.CD45.1$^+$ recipient mice. Recipient mice were i.n. challenged on day 13 and analyzed on day 18 (A). Frequency (C) and number (D) of donor IL-4-expressing (EGFP$^+$) CD4$^+$ T cells in the lung. Frequency (E) and number (F) of donor IL-13$^+$ CD4$^+$ T cells in the lung.
(G–O) 2.5×10^4 naive OTII.CD45.1$^+$ CD4$^+$T cells were transferred into naive congenic B6 mice on day 0. Mice were i.n. HDM+OVA sensitized on day 1 and challenged on day 15. Mice were treated with 30,000 U of rIL-2 or PBS given twice a day for 2 days starting on day 10 or on day 16 (G). Number of donor Tfh OTII cells (H) and GC cells (I) in the mLN on day 12. Frequency (J) and number (K) of donor OTII cells from the lungs on day 20. Frequency (L) and number (M) of IL-4-producing cells among the donor OTII T cells determined in the lungs by intracellular staining after restimulation for 4 hr with anti-CD3 and BFA. Number of eosinophils from day 20 lungs (N) and BAL (O) is shown. *$p < 0.005$ versus Ctrl (unpaired Student's t test). Data are representative of two independent experiments (mean and SD of 4–5 mice per group) See also Figure S4.

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Immunity

Supplemental Information

T Follicular Helper Cell Plasticity Shapes Pathogenic T Helper 2 Cell-Mediated Immunity to Inhaled House Dust Mite

André Ballesteros-Tato, Troy D. Randall, Frances E. Lund, Rosanne Spolski, Warren J. Leonard, and Beatriz León

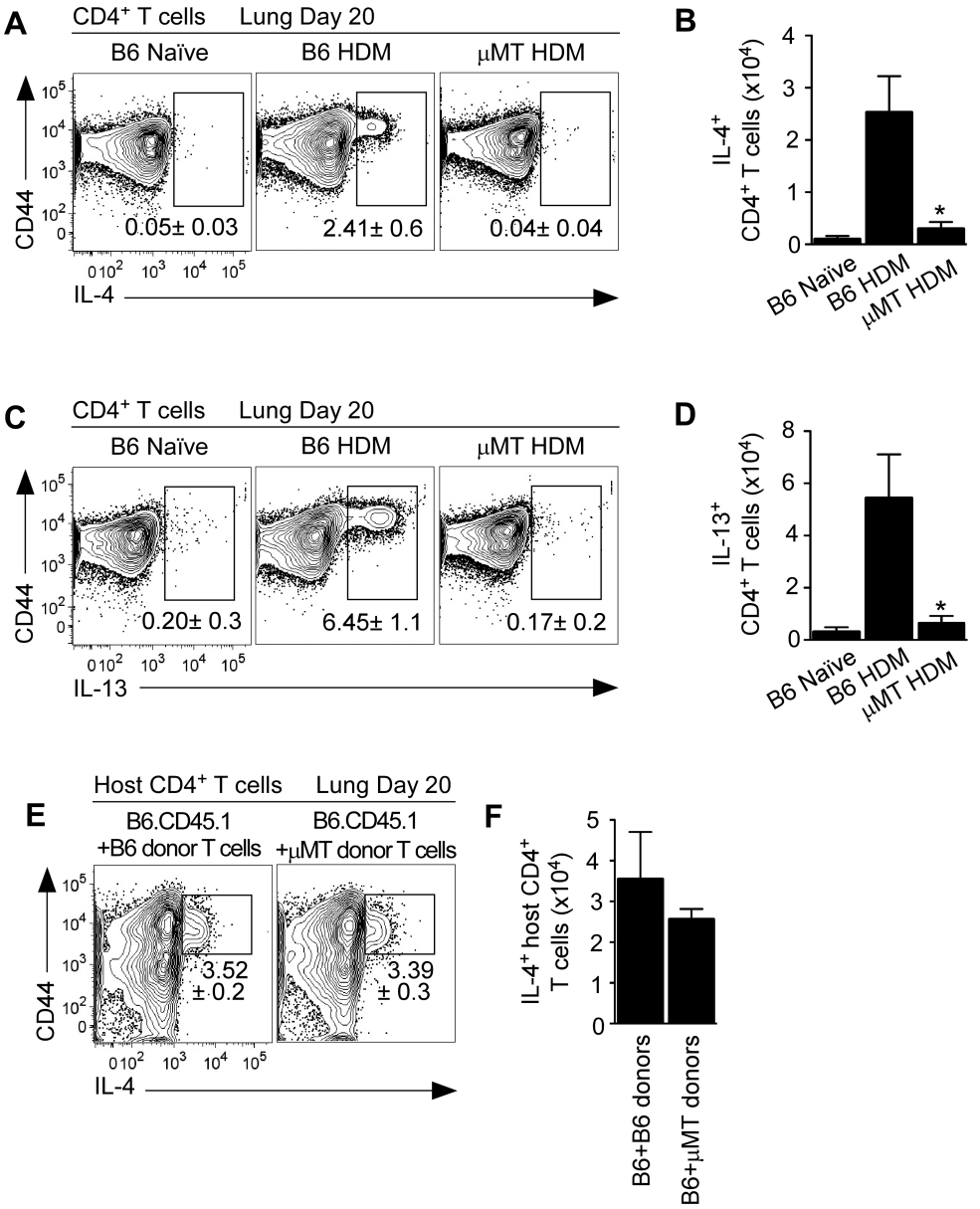


Figure S1. B cells are required for HDM-specific Th2 cytokine production by lung CD4⁺ T cells. (Related to Figure 1).

(**A-D**) B6 and μMT mice were i.n treated with 25μg of HDM for 4 consecutive days starting on day 1. On day 15, mice were i.n challenged with 25μg of HDM daily for 4 days. The frequency and number of IL-4 (**A** and **B**) and IL-13 (**C** and **D**) producing CD4⁺ T cells in the lungs were determined on day 20 by intracellular staining after restimulation for 4 h with anti-CD3 and BFA. As a control, naïve non-treated B6 mice were also analyzed. (**E-F**) HDM-sensitized B6.CD45.1⁺ recipient mice were i.n challenged on day 15 with 25μg of HDM daily for 4 consecutive days and analyzed on day 20. The frequency (**E**) and number (**F**) of host IL-4+ CD4⁺ T cells were determined by intracellular staining after restimulation with anti-CD3+ BFA for 4 h. *P < 0.001 vs. HDM-treated B6. (unpaired Student's t test). Data are representative of three independent experiments (mean and S.D. of 4-5 mice per group).

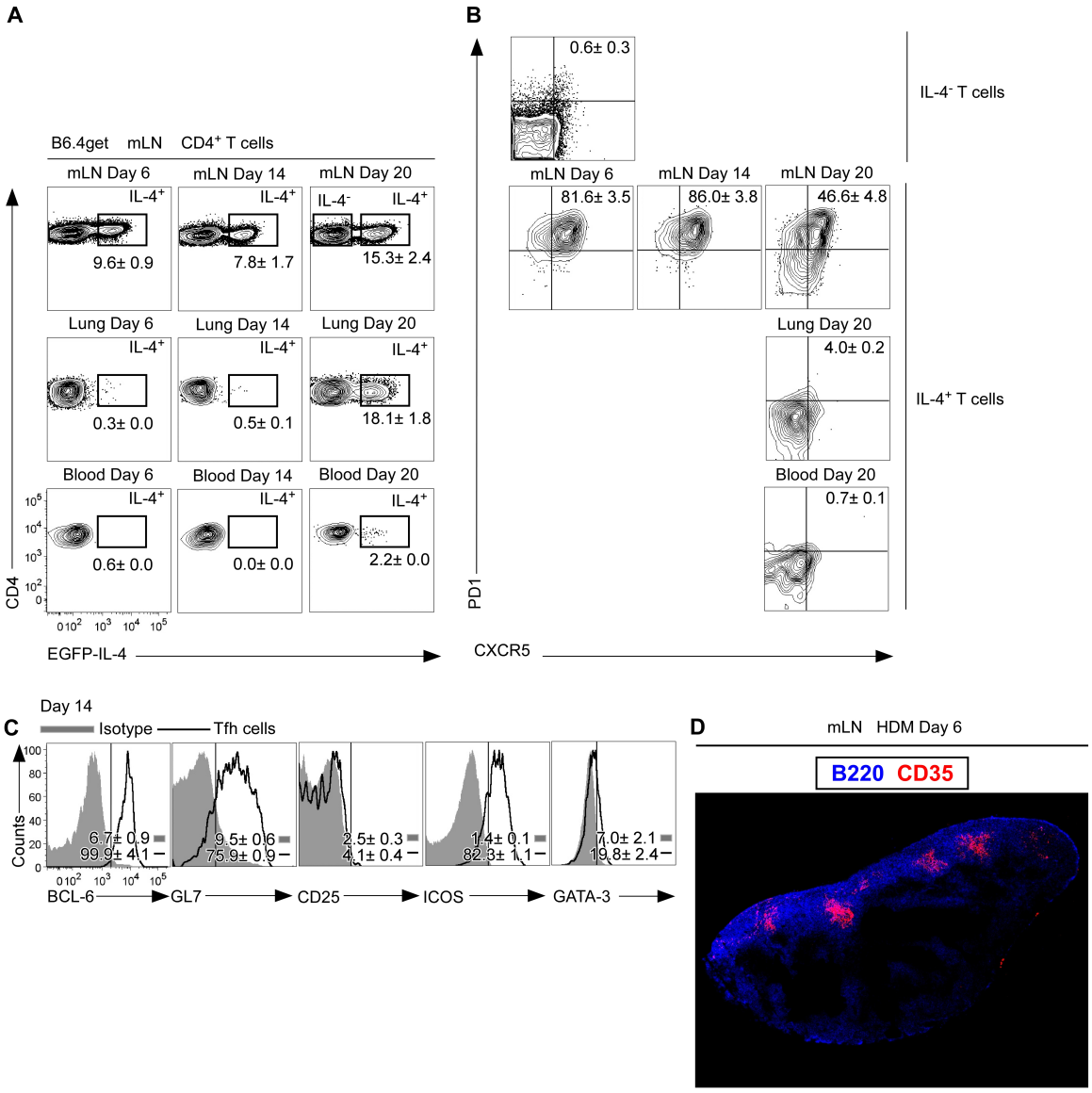


Figure S2. Kinetic analysis of IL-4-expressing (EGFP$^+$) CD4$^+$ T cells after administration of HDM (Related to Figure 4).

(**A-C**) B6.4get mice were i.n HDM sensitized on day 1 and analyzed on day 6 and day 14 or HDM sensitized on day 1, challenged on day 15 and analyzed on day 20. The frequency of IL-4-expressing (EGFP$^+$) on the CD4$^+$ T cells from the mLNs, lungs and peripheral blood are shown (**A**). Expression of PD-1 and CXCR5 on EGFP$^-$ and CD44^{hi}EGFP$^+$ CD4$^+$ T cells are shown (**B**). Expression of BCL-6, GL7, CD25, ICOS and GATA-3 on CD44^{hi}EGFP$^+$ CD4$^+$ T cells in the mLN of HDM-treated mice are shown at day 14 (**C**). (**D**) B6 mice were i.n HDM sensitized on day 1 and analyzed on day 6. Cryosections from the mLN were stained with anti-B220 (blue) and CD35 (red) and analyzed by fluorescent microscopy. Data are representative of two independent experiments (mean and S.D. of 4-5 mice per group).

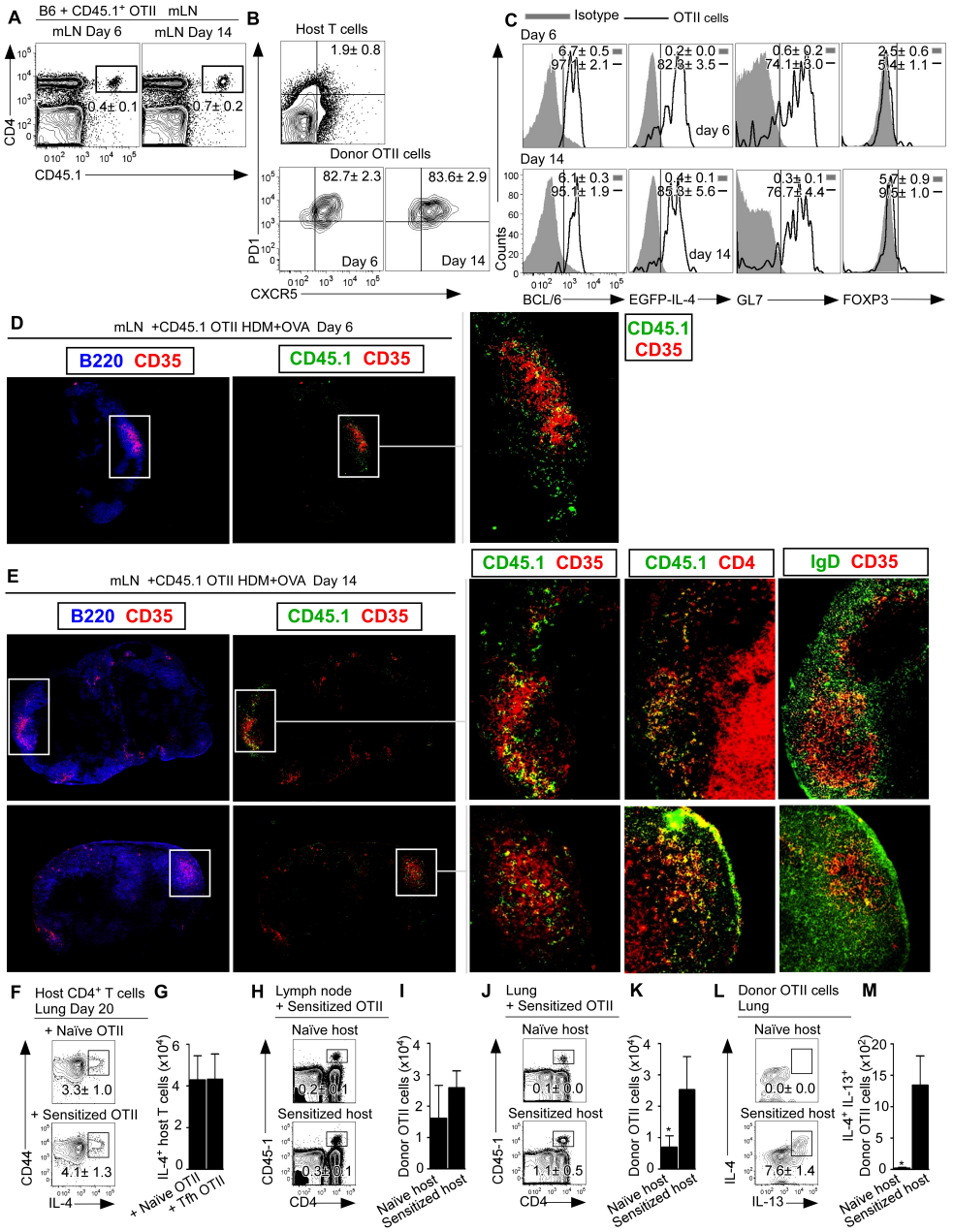


Figure S3. Kinetic analysis of the phenotype and intranodal localization of donor OTII cells after sensitization (Related to Figure 5).

(**A-C**) 25x10^3 naïve OTII. 4get. CD45.1$^+$. CD4$^+$ T cells were transferred into naive B6 mice. One day later, recipient mice were i.n treated with HDM+OVA for 4 consecutive days and analyzed 2 days after the last HDM inoculation. Frequency of donor OTII T cells from the mLN on day 6 and 14 (**A**). Expression of CXCR5 and PD1 (**B**) and BCL-6, EGFP-IL-4, GL7 and FOXP3 (**C**) in donor OTII T cells from the mLN on day 6 and 14.
(**D-E**) 25x10^3 naïve OTII. CD45.1$^+$. CD4$^+$ T cells were transferred into naive B6 mice. One day later, recipient mice were i.n HDM+OVA sensitized and analyzed on day 6 (**D**) and day 14 (**E**). Cryosections from the mLN were stained with anti-B220 (blue), CD35 (red) and CD45.1 (green), with anti-B220 (blue), CD4 (red) and CD45.1 (green) or with anti-B220 (blue), CD35 (red) and IgD (green) and analyzed by fluorescent microscopy. (**F-G**) HDM-sensitized B6 recipient mice were i.n challenged on day 15 and analyzed on day 20. The frequency (**F**) and number (**G**) of host (CD45.2+) IL-4 producing CD4+ T cells in the lungs were determined by intracellular staining after restimulation for 4 h with anti-CD3 and BFA. (**H-M**) 25x10^3 OTII CD45.1$^+$ CD4$^+$ T cells obtained from hosts 6 days after HDM+OVA sensitization were transferred into naive B6 mice or day 6 HDM+OVA-sensitized B6 mice. 8 days later, recipient mice were i.n treated with HDM+OVA for 4 consecutive days and analyzed 2 days after the last HDM inoculation. The frequency (**H,J**) and number (**I,K**) of donor CD45.1+ OTII T cells in the mLN (**H-I**) and lungs (**J-K**) are shown. The frequency (**L**) and number (**M**) of IL-4/IL-13-producing cells among the donor CD45.1+ OTII T cells were determined in the lungs by intracellular staining after restimulation for 4 h with anti-CD3 and BFA. *P < 0.01 (unpaired Student's t test). Data are representative of two or more independent experiments (mean and S.D. of 4-5 mice per group).

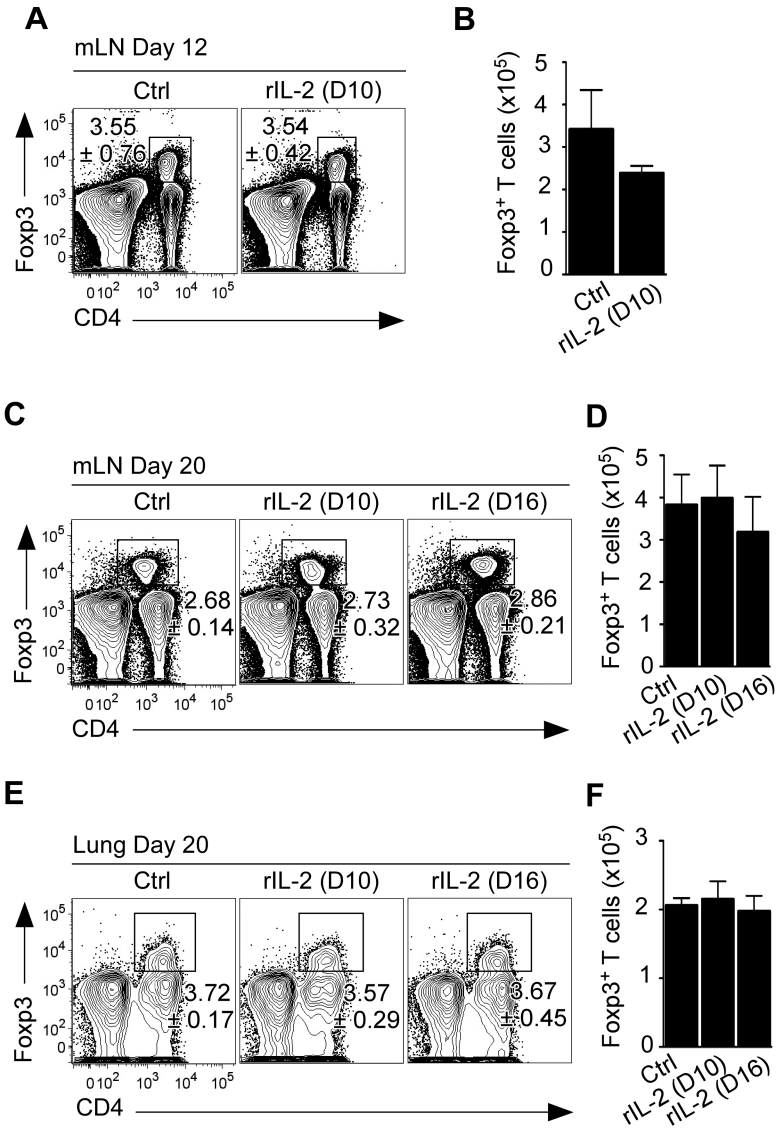


Figure S4. rIL-2 treatment in HDM-treated mice does not increase the number of Foxp3$^+$ Treg cells (related to Figure 7).

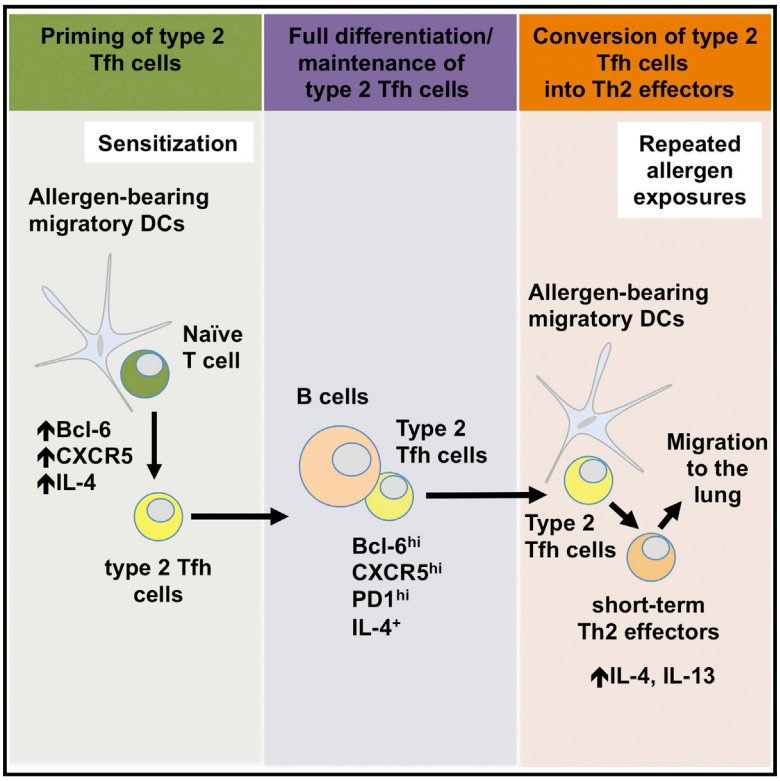
(**A-B**) Frequency (**A**) and number (**B**) of host Foxp3$^+$ Treg cells from day 12 mLN of HDM+OVA-treated B6 mice injected with rIL-2 or PBS according to the schematic in **Figure 7E**. (**C-F**) Frequency and number of host Foxp3$^+$ Treg cells from day 20 mLN (**C-D**) and lungs (**E-F**) of HDM-treated B6 mice injected with IL-2 or PBS according to the schematic in **Figure 7E**.

Article

Immunity

T Follicular Helper Cell Plasticity Shapes Pathogenic T Helper 2 Cell-Mediated Immunity to Inhaled House Dust Mite

Graphical Abstract



Highlights

- HDM sensitization does not prime Th2 cells, but induces IL-4-committed Tfh cells
- Ag-presenting B cells synergize with DCs to prime Tfh cells during HDM sensitization
- IL-4-committed Tfh cells are precursors of effector Th2 cells after HDM rechallenge
- Depletion of IL-4-committed Tfh cells prevents Th2 cell-mediated immunity to HDM

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In Brief

Follicular helper T (Tfh) cells are specialized providers of T cell help to B cells. León and colleagues demonstrate that interleukin-4-committed Tfh cells are precursors of effector T helper type 2 cells after intranasal challenge with house dust mites.

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T Follicular Helper Cell Plasticity Shapes Pathogenic T Helper 2 Cell-Mediated Immunity to Inhaled House Dust Mite

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SUMMARY

Exposure to environmental antigens, such as house dust mite (HDM), often leads to T helper 2 (Th2) cell-driven allergic responses. However, the mechanisms underlying the development of these responses are incompletely understood. We found that the initial exposure to HDM did not lead to Th2 cell development but instead promoted the formation of interleukin-4 (IL-4)-committed T follicular helper (Tfh) cells. Following challenge exposure to HDM, Tfh cells differentiated into IL-4 and IL-13 double-producing Th2 cells that accumulated in the lung and recruited eosinophils. B cells were required to expand IL-4-committed Tfh cells during the sensitization phase, but did not directly contribute to disease. Impairment of Tfh cell responses during the sensitization phase or Tfh cell depletion prevented Th2 cell-mediated responses following challenge. Thus, our data demonstrate that Tfh cells are precursors of HDM-specific Th2 cells and reveal an unexpected role of B cells and Tfh cells in the pathogenesis of allergic asthma.

INTRODUCTION

Cytokines produced by T helper 2 (Th2) cells in the lungs of asthma patients promote inflammation, eosinophil accumulation, and mucus hyperproduction, which ultimately lead to recurrent bronchoconstriction, a characteristic of allergic asthma (Hamid and Tulic, 2009). Given the critical role of Th2 cells in the development of allergic inflammatory responses, it is essential that we understand the mechanisms that control Th2 cell development to commonly encountered respiratory allergens, so that we can design therapeutic strategies. Although the exact mechanism by which allergen-specific Th2 cell responses are initiated is incompletely defined, it is thought to require antigen (Ag) presentation by pulmonary dendritic cells (DCs), which capture allergen-derived Ags in the lung and migrate into the lung-draining mediastinal lymph node (mLN), where they prime allergen-specific $CD4^+$ T cells (van Helden and Lambrecht, 2013). In fact, conditional depletion of lung DCs precludes Th2 cell-mediated immunity to house dust mite (HDM) (Hammad et al., 2010). Nevertheless, commitment of primed $CD4^+$ T cells to the Th2 cell pathway might also require complex interactions with other cell types, including epithelial cells (Lambrecht and Hammad, 2013) and type 2 innate lymphoid cells (ILC2s) (Halim et al., 2014).

B cells also contribute to Th2 cell development by multiple mechanisms (León et al., 2014). Indeed, Ag presentation by B cells promotes the accumulation of Th2 cells in the lungs of mice exposed to cockroach Ags (Lindell et al., 2008). B cells are also important for the development and maintenance of T follicular helper (Tfh) cells (Crotty, 2014). Certainly, the development of Th2 and Tfh cells share some developmental requirements. For example, both Th2 and Tfh cell responses require B cell help, ICOS, IL-21R, OX40-OX40L, and CD28 signaling (Coquet et al., 2015; Crotty, 2014; Lane, 2000; León et al., 2014) and are primed within the interfollicular areas of the LN (Kerfoot et al., 2011; León et al., 2012).

Tfh cells retain substantial developmental plasticity (Lu et al., 2011) and, upon secondary challenge, can differentiate into effector T cells that migrate into non-lymphoid tissues (Lüthje et al., 2012). Moreover, Tfh cells can be an important source of IL-4 (King and Mohrs, 2009). Particularly, Th2 responses to airborne antigens uniquely require an initial phase of antigen sensitization that does not cause Th2-mediated airway inflammation, but is required for the development of effector Th2 cells following challenge (Galli et al., 2008; Gelfand et al., 2004). However, the identity of the Th2 cell-precursors elicited during the sensitization phase is not yet known.

Here we show that sensitization to inhaled HDM in mice did not directly result in Th2 cell development, but instead elicited IL-4-committed Tfh cells that were confined to the mLN. Following HDM challenge, Tfh cells generated during the sensitization phase differentiated into Th2 cells and homed to the lungs, where they recruited eosinophils. The differentiation of IL-4-committed Tfh cells required Ag presentation by both DCs and B cells. As a consequence, Th2 cell-mediated immunity after HDM rechallenge was impaired in B cell deficient mice and in mice in which B cells or DCs were unable to present Ags. Moreover, the depletion of Tfh cells after HDM sensitization prevented Th2 cell-mediated immunity upon challenge exposure. Thus,

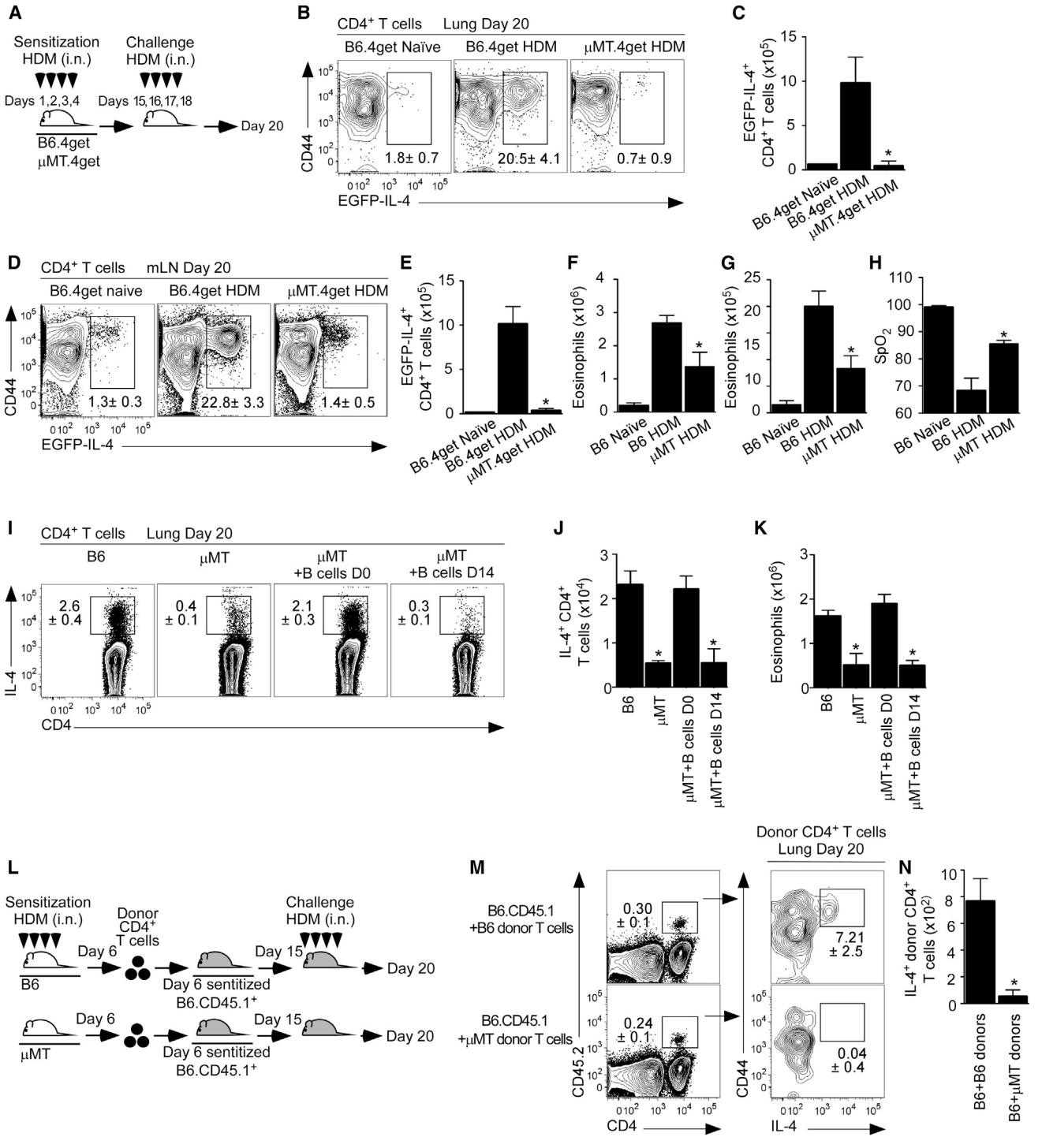


Figure 1. B Cells Control HDM-Induced Pulmonary Th2 Cell Responses

(A–E) B6.4get and µMT.4get mice were i.n. treated with 25 µg of HDM for 4 consecutive days starting on day 1. On day 15, mice were i.n. challenged with 25 µg of HDM daily for 4 days and analyzed on day 20 by flow cytometry (A). As a control, naive B6.4get mice were also analyzed. The frequency (B) and (D) and number (C) and (E) of IL-4-expressing (EGFP$^+$) CD4$^+$ T cells from the lungs (B) and (C) and mLN (D) and (E) are shown. (F-G) The number of eosinophils in the lungs (F) and BAL (G) of naive and HDM-challenged B6 and µMT mice are shown. (H) Arterial oxygen saturation (SpO2) of hemoglobin, measured with pulse oximeter, 2 hr after final challenge.

(I–K) B6 and µMT mice were i.n. treated with 25 µg of HDM for 4 consecutive days starting on day 1. Some µMT mice received 50 × 10^6 naive B cells i.v. on day 0, or on day 14. On day 15, all mice were i.n. challenged with 25 µg of HDM daily for 4 consecutive days. Mice were analyzed on day 20. The frequency (I) and number

(legend continued on next page)

IL-4-committed Tfh cells are the precursors to pathogenic Th2 cells in allergic airway disease.

RESULTS

B Cells Are Necessary for Th2 Cell-Mediated Immunity to Inhaled HDM

To test whether B cells influenced the Th2 cell response to inhaled HDM, we intranasally (i.n.) sensitized IL-4 reporter B6.4get mice and B cell-deficient 4get (μMT.4get) mice (Mohrs et al., 2001) with 25 μg of HDM extract on 4 consecutive days (sensitization phase, Figure 1A), challenged them i.n. with HDM extract on days 15, 16, 17, and 18 (challenge phase, Figure 1A) and determined the frequency (Figure 1B) and number (Figure 1C) of GFP$^+$CD4$^+$ T cells in the lungs on day 20. As expected, GFP$^+$CD4$^+$ T cells accumulated in the lungs of HDM-challenged B6.4get mice relative to naive B6.4get animals (Figures 1B and 1C), whereas GFP$^+$CD4$^+$ T cells failed to accumulate in the lungs of HDM-challenged μMT.4get mice. We observed similar results in the lung-draining mLN (Figures 1D and 1E).

We next sensitized and challenged C57BL/6J (B6) and C57BL/6J.μMT (μMT) mice and quantified IL-4-producing and IL-13-producing cells by intracellular cytokine staining (ICS). We found that both IL-4-producing (Figures S1A and S1B) and IL-13-producing (Figure S1C and S1D) CD4$^+$ T cells were reduced in μMT mice compared to B6 mice. We also enumerated eosinophils in the lungs and bronchoalveolar lavage (BAL) of sensitized and challenged mice and observed that the number of eosinophils was significantly reduced in μMT mice compared to those in B6 mice (Figures 1F and 1G). Arterial oxygen saturation of hemoglobin (SpO2) was measured. HDM-sensitized and challenged B6 mice displayed reduced SpO2 compared to μMT mice (Figure 1H). These results indicated that B cells were necessary for the development of pulmonary Th2 cell responses to HDM.

We next tested whether the pulmonary Th2 cell response required B cells during the sensitization phase or following challenge, by transferring purified B cells from naive B6 into μMT mice one day before HDM sensitization, or one day before HDM challenge. As controls, we included μMT and B6 mice that were HDM sensitized but did not receive B cells at any time. We enumerated IL-4-producing CD4$^+$ T cells on day 6 after challenge and found similar numbers of IL-4$^+$CD4$^+$ cells in the lungs of B6 and μMT mice that received B cells before the sensitization phase (Figures 1I and 1J), but fewer IL-4$^+$CD4$^+$ cells in μMT mice and in μMT mice that received B cells prior to HDM challenge. We also found that the numbers of eosinophils were similarly high in the lungs of B6 and μMT mice that received B cells before the sensitization phase (Figure 1K), but were similarly low in μMT mice and in μMT mice that received B cells prior to HDM challenge. These data suggest that the Th2 cell response to HDM sensitization and challenge is dependent on B cells during the sensitization, but not the challenge phase of the response.

To confirm these results, we transferred equivalent numbers of purified CD4$^+$ T cells from the mLN of day-6 HDM-sensitized B6 or μMT mice (CD45.2$^+$) into day-6 HDM-sensitized CD45.1$^+$ B6 mice (Figure 1L). Recipient mice were challenged with HDM on days 15–18 and we enumerated IL-4$^+$CD44^{hi}CD4$^+$ cells from the donor (CD45.2$^+$) and the host (CD45.1$^+$) on day 20. We found that the frequency (Figure S1E) and number (Figure S1F) of host IL-4$^+$CD44^{hi}CD4$^+$ cells were similar in the two groups. However, the frequency (Figure 1M) and number (Figure 1N) of donor IL-4$^+$CD44^{hi}CD4$^+$ cells were reduced in recipients of cells from μMT donors compared to cells from B6 donors. Collectively, these data indicate that the absence of B cells during the sensitization phase compromises the development of Th2 cell responses after challenge, regardless of whether B cells were present following challenge.

To better understand the role of B cells during the sensitization phase, we enumerated GFP$^+$CD4$^+$ T cells in the mLN of B6.4get and μMT.4get mice 6 days after HDM sensitization. We found that HDM-sensitized B6.4get had higher frequency (Figure 2A) and number (Figure 2B) of GFP$^+$CD4$^+$ T cells compared to non-sensitized B6.4get mice, but that HDM-sensitized μMT.4get mice failed to accumulate GFP$^+$CD4$^+$ T cells in the mLN. These data indicate that B cells are required for the differentiation of IL-4-commited CD4$^+$ T cells after HDM sensitization.

We next generated bone marrow (BM) chimeras in which B cells selectively lacked MHC class II (MHC II) (Figure 2C), by irradiating μMT.4get mice and reconstituted them with an 80:20 mix of BM obtained from μMT.4get and *H2-Ab1*$^{-/-}$ donors (B-MHC II-deficient chimeras), or with an 80:20 mix of μMT.4get and B6 mice (B-WT chimeras). Following reconstitution, chimeric mice were i.n. sensitized with HDM and the mLN analyzed 6 days later. We found that the frequency (Figure 2D) and number (Figure 2E) of B cells were similar in the two groups of chimeras. As expected, however, we found that B cells in B-WT chimeras expressed MHC II, but that B cells in B-MHC II-deficient chimeras did not (Figure 2F).

Next, we enumerated GFP$^+$CD44^{hi}CD4$^+$ T cells in the mLN 6 days later. We found that the frequency (Figure 2G) and number (Figure 2H) of GFP$^+$CD44^{hi}CD4$^+$ T cells was higher in the mLN of B-WT chimeras compared to B-MHC II-deficient chimeras. These results indicate that Ag-presenting B cells are required for the differentiation of IL-4-committed CD4$^+$ T cells during the HDM sensitization phase.

B Cell and DCs Synergize to Prime Specific CD4$^+$ T Cells to Inhaled HDM

We next tested whether Ag presentation by DCs was important for the priming of CD4$^+$ T cells following HDM sensitization. For

(J) of IL-4$^+$ CD4$^+$ T cells in the lungs were determined by intracellular staining after restimulation for 4 hr with anti-CD3 and BFA. (K) The number of eosinophils in the lungs are shown.

(L and M) B6 and μMT mice were i.n. treated with 25 μg of HDM for 4 consecutive days starting on day 1. Six days later, CD4$^+$ T cells were purified from the mLN and 2 × 10^6 of purified CD4$^+$ T cells from B6 or μMT donor mice were transferred into day 6 HDM-sensitized B6.CD45.1$^+$ recipient mice. Recipient mice were i.n. challenged on day 15 with 25 μg of HDM daily for 4 consecutive days and analyzed on day 20 (L). The frequency (M) and number (**N**) of donor IL-4$^+$ CD4$^+$ T cells in the lungs of recipient mice were determined by intracellular staining after restimulation with anti-CD3$^+$ BFA for 4 hr. *$p < 0.01$ versus HDM-treated B6 (unpaired Student's t test). Data are representative of three independent experiments (mean and SD of 4–5 mice per group). See also Figure S1.

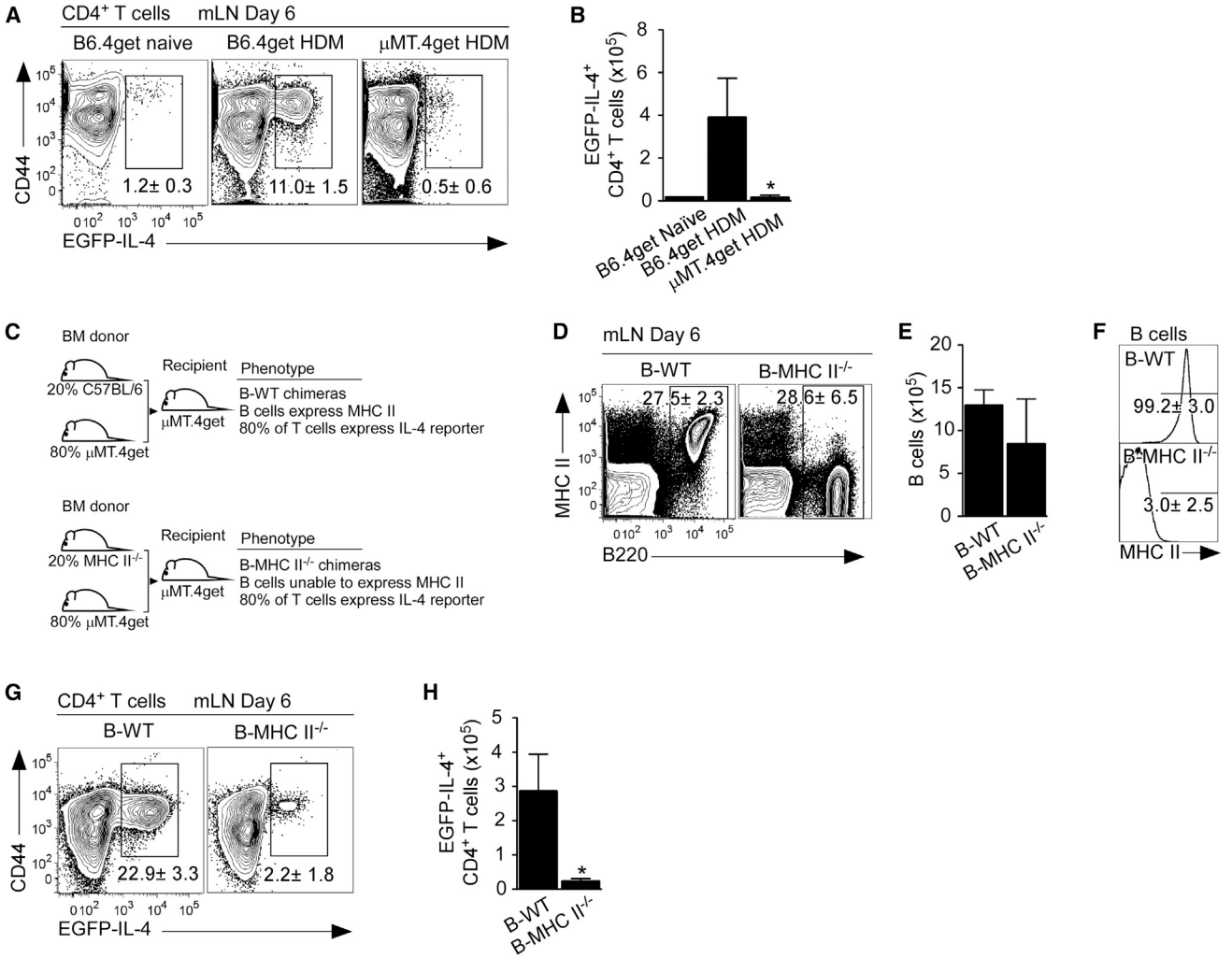


Figure 2. Ag Presentation by B Cells Is Necessary to Prime of IL-4-Commited CD4$^+$ T Cell Responses to HDM

(A and B) IL-4 reporter B6.4get and μMT.4get mice were i.n. treated with 25 μg of HDM or PBS for 4 consecutive days starting on day 1. The frequency (A) and number (B) of IL-4-expressing (EGFP$^+$) CD4$^+$ T cells in the mLN of treated mice were calculated by flow cytometry on day 6.

(C–H) μMT.4get mice were irradiated and reconstituted with an 80:20 mixture of BM from μMT.4get and B6 donors (B-WT), or with an 80:20 mixture of BM from μMT.4get and MHC II deficient donors (B-MHC II-deficient). Eight weeks later, reconstituted-chimeric mice were i.n. treated with 25 μg of HDM of PBS for 4 consecutive days starting on day 1 and mice were analyzed at day 6. The frequency (D) and number (E) of B cells in the mLN were calculated by flow cytometry. (F) Expression of MHC II on B cells. Frequency (G) and number (H) of IL-4-expressing (EGFP$^+$) CD4$^+$ T cells in the mLN. *$p < 0.001$ versus HDM-treated B6 (unpaired Student's t test). Data are representative of three independent experiments (mean and SD of 4–5 mice per group).

this purpose, we generated BM chimeric mice in which DCs selectively lacked MHC II by reconstituting irradiated B6 mice with an 80:20 mix of *Itgax*-DTR BM and *H2-Ab1*$^{-/-}$ BM (DC-MHC II-deficient chimeras), or with an 80:20 mix of *Itgax*-DTR BM and B6 BM (DC-WT chimeras) (Figure 3A). Two months later, we adoptively transferred CD45.1$^+$OTII T cells into the chimeric mice and treated the recipients with diphtheria toxin (DT) to ablate CD11c$^+$ cells derived from the *Itgax*-DTR BM (León et al., 2012). One day later, recipient mice were i.n. sensitized with a mixture of HDM and ovalbumin (HDM+OVA) for 4 consecutive days. Mice received an additional injection of DT on day 3 and were analyzed on day 6. As expected, DCs in the DC-WT chimeras expressed MHC II, whereas DCs in the DT-treated DC-MHC II-deficient chimeras lacked MHC II expression (Figure 3B). However, the frequency (Figure 3C) and number (Figure 3D) of DCs were similar in the two groups. Importantly, we found a higher frequency (Figure 3E) and number (Figure 3F) of donor OTII cells in the mLN of DC-WT mice than in DC-MHC II-deficient mice. Furthermore, OTII cells in DC-WT mice expressed high amounts of the activation markers CD44 (Figure 3G) and CD69 (Figure 3H). In contrast, the phenotype of OTII cells in DC-MHC II-deficient mice was consistent with naive T cells (Figures 3G and 3H). These results suggest that Ag presentation by DCs is also required to prime CD4$^+$ T cell responses to inhaled HDM and that DCs and B cells synergize to expand specific CD4$^+$ T cells in the mLN following pulmonary HDM sensitization.

To understand how B cells gained access to lung-derived HDM antigens following HDM sensitization, we administered

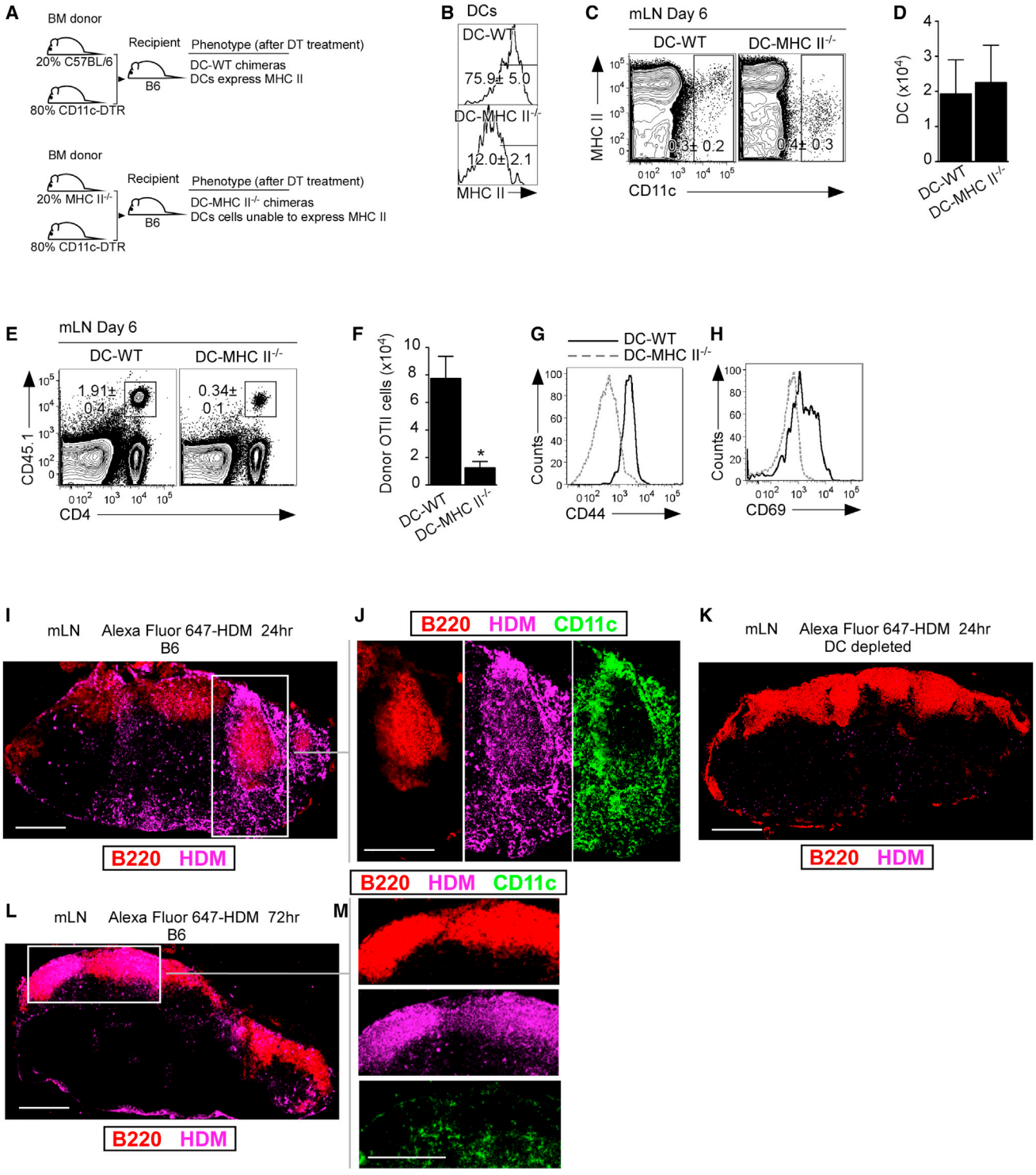


Figure 3. Ag Presentation by DC Cells Is Necessary for the Priming of Specific CD4⁺ T Cells after Allergic Sensitization

(A–H) B6 mice were irradiated and reconstituted with an 80:20 mixture of BM from CD11c-DTR and B6 donors (DC-WT) or from CD11c-DTR and MHC II-deficient donors (DC-MHC II-deficient) (A). Eight weeks later, 2.5 × 10^4 naive OTII.CD45.1⁺ CD4⁺ T cells were transferred into reconstituted chimeric mice on day 0. Recipient mice were treated with HDM+OVA (from day 1 to 4), injected with PBS or DT at day 0 and 3, and analyzed on day 6. The expression of MHC II on DC (B) and the frequency (C) and number (D) of DCs in the mLN are shown. Frequency (E) and number (F) of donor OTII T cells from the mLN. Expression of CD44 (G) and CD69 (H) in donor OTII T cells from the mLN.

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Alexa Fluor 647-labeled HDM to B6 mice and examined sections of mLN by fluorescence microscopy 24 hr after HDM inoculation (Figures 3I–3K). We found that labeled HDM was predominately located in the interfollicular area and co-localized with cells expressing CD11c (Figures 3I and 3J). Importantly, labeled HDM was not detectable in the mLNs of DT-treated *Itgax*-DTR mice (Figure 3K), consistent with the idea that HDM is transported to the mLN by lung-migratory DCs (Plantinga et al., 2013). Notably, some labeled HDM was also detected in B cell follicles (Figures 3I and 3J), particularly by 72 hr after HDM inoculation (Figures 3L and 3M). These data indicate that lung-migratory, HDM-bearing DCs preferentially localize within the periphery of the B cell follicles early after HDM sensitization, and that Ag is subsequently transferred to cells in the follicle.

Sensitization with HDM Elicits IL-4-Committed Tfh Cells, but Not Effector Th2 Cells

Given that Tfh cells can produce IL-4 (King and Mohrs, 2009) and require cognate interactions with Ag-presenting B cells and DCs (Ballesteros-Tato and Randall, 2014), we hypothesized that B cell deficiency compromised Th2 cell-mediated responses to HDM by preventing the initial development of IL-4-commited Tfh cells. To test this possibility, we analyzed the phenotype of IL-4-committed $CD4^+$ T cells in day 6 HDM-sensitized B6.4get mice. We found that GFP^+CD4^+ T cells did not accumulate in the lungs of HDM-sensitized mice relative to naive mice (Figure 4A). In contrast, we found a much higher frequency of GFP^+CD4^+ T cells in the mLN of HDM-sensitized mice than in naive mice (Figure 4B). Moreover, the GFP^+CD4^+ T cells in the mLN of HDM-sensitized mice consisted of a homogenous population of $CXCR5^{hi}PD\text{-}1^{hi}$ T cells (Figure 4C) that also expressed high amounts of the transcription factor, BCL-6, the Tfh marker, GL7 and the inducible costimulator ICOS, but were CD25 and FOXP3 negative and GATA-3-low (Figure 4D and data not shown). Flow cytometric analysis of intracellular cytokine production showed expression of IL-21 and IL-4 but little or no production of IL-2 or the Th2 cell-associated cytokines IL-13 and IL-5 (Figure 4E and data not shown). Similar results were obtained from analyzing the phenotype of IL-4-committed $CD4^+$ T cells in day 14 HDM-sensitized B6.4get mice (Figures S2A–S2C). Thus, we conclude that these cells are IL-4-committed Tfh cells.

Corresponding with this conclusion, we next examined B cell responses. We found that HDM sensitization induced immunoglobulin E (IgE)-secreting antibody secreting cells (ASC) and the formation of germinal centers (GCs) in the mLN (Figures 4F–4H and Figure S2D). Both responses were boosted after challenge.

We next analyzed the phenotype of IL-4-committed $CD4^+$ T cells in the lung and mLN of B6.4get mice following challenge with HDM. We found that the GFP^+CD4^+ T cells in the lungs of challenged mice were $CXCR5^{lo}PD\text{-}1^{lo}$ (Figure 4I), expressed low amounts of BCL-6 and GL7 and intermediate amounts of CD25, high amounts of GATA-3 (Figure 4J), and produced IL-2, IL-4, IL-13, and IL-5 (Figure 4K and data not shown), consistent with the phenotype of effector Th2 cells (Liang et al., 2012). In contrast, the GFP^+CD4^+ T cells in the mLN consisted of a mix of $CXCR5^{lo}PD\text{-}1^{lo}BCL\text{-}6^{lo}GL7^{lo}CD25^{int}$ $GATA\text{-}3^{hi}$ effector Th2 cells, which produced IL-2, IL-4, and IL-13 and $CXCR5^{hi}PD\text{-}1^{hi}BCL\text{-}6^{hi}GL7^{hi}CD25^{-}GATA\text{-}3^{lo}$ Tfh cells, which produced IL-21 (Figures 4L–4N). We also analyzed the phenotype of IL-4-committed $CD4^+$ T cells in the peripheral blood of B6.4get mice during the HDM sensitization and challenge phases. We found that IL-4-committed $CD4^+$ T cells were only detected after challenge (Figure S2 A) and displayed an effector phenotype (Figure S2 B).

Taken together, these data indicated that HDM sensitization elicit IL-4-committed Tfh cells, which are confined to the mLN, whereas challenge with HDM expands effector Th2 cells that home to the lung.

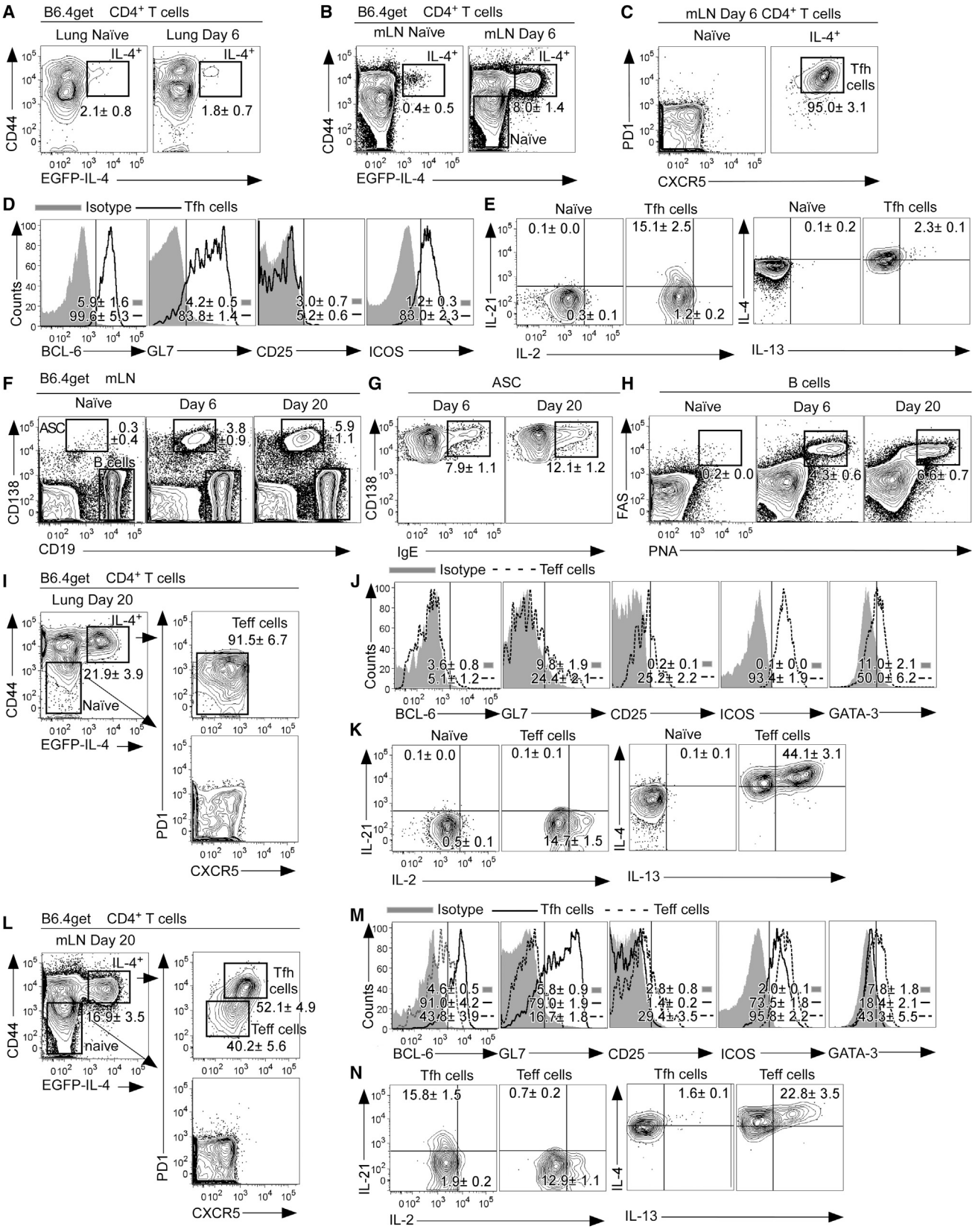
To further confirm this conclusion, we adoptively transferred $CD45.1^+$ OTII T cells into naive B6 and μMT mice. One day later, mice were sensitized with HDM-OVA for 4 consecutive days and analyzed the donor OTII cells in the mLNs of recipient mice on day 6 (Figures 5A–5F) and day 14 (Figure S3A-C). We found that the frequencies (Figure 5A) and numbers (Figure 5B) of donor OTII cells were increased in the mLNs of HDM-sensitized B6 mice compared to HDM-sensitized μMT mice or non-sensitized B6 mice. Importantly, OTII cells in the HDM-sensitized B6 mice expressed high amounts of the activation markers CD44 (Figure 5C) and CD69 (Figure 5D), uniformly co-expressed CXCR5 and PD-1 (Figure 5E), resulted in $BCL\text{-}6^{hi}$ (Figure 5F), and expressed GL7 and IL-4 transcripts (Figure S3C), thus resembling IL-4-committed Tfh cells. Similar results were obtained when donor OTII cells in the mLN were analyzed on day 14 (Figures S3A–S3C). As expected, donor OTII cells in the mLN of HDM-sensitized μMT mice resulted in $CXCR5^{lo}PD\text{-}1^{lo}BCL\text{-}6^{lo}$ (Figures 5C–5F), and resembled naive OTII cells in non-sensitized B6 mice. We also failed to detect donor OTII cells in the lungs of either B6 or μMT recipient mice following HDM sensitization (data not shown). Finally, immunofluorescence staining of mLN sections from HDM-OVA-sensitized mice on day 6 (Figure S3D) and day 14 (Figure S3E) showed donor $CD45.1^+$ $CD4^+$ OTII cells inside GCs. These data provide further evidence that OTII cells differentiated into Tfh cells following HDM sensitization.

Taken together, these data demonstrate that HDM sensitization does not induce effector Th2 cells, but rather expands IL-4-committed Tfh cells that are retained in the mLN.

IL-4-Committed Tfh Cells Differentiate into Effector Th2 Cells and Home to the Lungs after HDM Challenge

Recent studies indicate that Tfh cells maintain the flexibility to differentiate into Teff cells upon secondary challenge (Lüthje et al., 2012). To test whether Tfh cells elicited by HDM sensitization differentiated into Th2 effector cells following HDM

(I–M) B6 mice were i.n. treated with 25 μg of Alexa Fluor 647-labeled HDM. Cryosections from the mLN were stained with anti-B220 (red) and CD11c (green) and analyzed by fluorescent microscopy on days 1 (I–K) and 3 (L and M). The regions within the box in (I) and (L) were enlarged in (J) and (M), respectively. In the same experiment, CD11c-DTR mice were injected i.p. with 60 ng DT and i.n. treated with 25 μg of Alexa Fluor 647-labeled HDM 24 hr later. Cryosections from the mLN one day 1 after HDM inoculation are shown (K). Scale bars represent 400 μm. *$p < 0.005$ (unpaired Student's t test). Data are representative of three independent experiments (mean and SD of 4–5 mice per group).



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challenge, we adoptively transferred equivalent numbers of naive CD45.1$^+$OTII cells or day 6 HDM-OVA-sensitized CD45.1$^+$OTII cells from the mLNs of donor mice into day 6 HDM-sensitized recipients and challenged the recipients on day 15 (Figure. 5G). Importantly, the OTII cells transferred from naive mice did not express CXCR5 or PD-1 (Figure 5H) or BCL-6 (Figure 5I), whereas those transferred from day 6 HDM sensitized mice were uniformly CXCR5^{hi}PD-1^{hi}BCL-6^{hi} Tfh cells (Figures 5H and 5I). Following challenge, we found that progeny of the naive OTII donor cells in the mLN had differentiated into CXCR5^{hi}PD-1^{hi}BCL-6^{hi} Tfh cells (Figure 5J), whereas the progeny of the donor Tfh OTII cells had differentiated into a mixed population of CXCR5^{hi}PD-1^{hi}BCL-6^{hi} Tfh cells and CXCR5^{lo}PD-1^{lo}BCL-6^{lo} effector CD4$^+$ T cells (Figure 5J). We also found that the progeny of naive OTII donor cells poorly accumulated in the lung (Figures 5K and 5L) and failed to differentiate into IL-4 and IL-13 double-producing effector Th2 cells (Figures 5M and 5N). In contrast, the progeny of Tfh OTII donor cells efficiently accumulated in the lung (Figures 5K and 5L), and many of them differentiated into IL-4 and IL-13 double-producing effector Th2 cells (Figures 5M and 5N). As a control, we examined the frequency (Figures S3F) and number (Figure S3G) of host IL-4-producing CD4$^+$ T cells and found that they were similar in the recipients of naive and Tfh OTII donor cells. Importantly, donor Tfh OTII cells poorly accumulated in the lung (Figures S3H–S3K) and differentiated into IL-4 and IL-13 double-positive effector Th2 cells (Figures S3L–S3M) when the recipients were not previously sensitized with allergen.

Taken together, these data indicate that IL-4-committed Tfh cells generated by HDM sensitization rapidly differentiate into secondary effector Th2 cells and home to the lung upon HDM challenge.

To further confirm this conclusion, we used *Il21*-mCherry and *Il2*-emGFP BAC dual reporter Tg mice (Wang et al., 2011). Analysis of mLNs of dual reporter BM chimeric mice on day 6 after HDM sensitization identified a population of CD44^{hi}CD4$^+$ T cells that were *Il21*-mCherry$^+$*Il2*-emGFP$^-$ (mCherry-IL-21$^+$) (Figure 6A). These cells were not detected in naive mice (Figure 6A) or in the lungs of mice following HDM sensitization (Figure 6C). Further analysis showed that mCherry-IL-21$^+$ cells in the mLN expressed intracellular IL-21 and the markers PD1 and CXCR5 (Figure 6B) thus resembling Tfh cells (Lüthje et al., 2012). To analyze whether Tfh cells can differentiate into Th2 effector cells following HDM challenge, *Il21*-mCherry$^+$*Il2*-emGFP$^-$ (mCherry-IL-21$^+$) Tfh cells, *Il21*-mCherry$^-$*Il2*-emGFP$^+$ (GFP-IL-2$^+$) non-Tfh cells (Figure 6D) and naive CD44^{lo}CD4$^+$ T cells were sorted from mLNs of day 6 HDM-sensitized dual reporter BM chimeric mice and equal numbers of cells were adoptively transferred into day 6 HDM-sensitized recipients. Recipient mice were then challenged on day 15 and analyzed on day 20. Following challenge, we found significant expansion of donor-derived cells in the mLNs of mice that had received mCherry-IL-21$^+$ Tfh cells compared with those receiving naive or GFP-IL-2$^+$ non-Tfh cells (Figures 6E and 6F). Furthermore, the progeny of the donor mCherry-IL-21$^+$ Tfh cells differentiated into a mixed population of CXCR5^{hi}PD-1^{hi} Tfh cells and CXCR5^{lo}PD-1^{lo} effector CD4$^+$ T cells. (Figure 6G). As expected, CXCR5^{hi}PD-1^{hi} Tfh derived from donor mCherry-IL-21$^+$ cells expressed mCherry-IL-21 but were GFP-IL-2 negative. In contrast, the CXCR5^{lo}PD-1^{lo} effector CD4$^+$ T cells derived from donor mCherry-IL-21$^+$ cells dowregulated the expression of mCherry-IL-21 and upregulated the expression of GFP-IL-2 (Figure 6H).

We also found that the progeny of naive or GFP-IL-2$^+$ non-Tfh cells donor cells poorly accumulated in the lung (Figures 6I and 6J) and failed to differentiate into IL-4 and IL-13 double-producing effector Th2 cells (Figures 6M and 6N). In contrast, the progeny of the donor mCherry-IL-21$^+$ Tfh cells efficiently accumulated in the lung (Figures 6I and 6J), where they expressed markers consistent with an effector Th2 phenotype (such as CXCR5^{lo}PD-1^{lo} Figure 6K). Furthermore, these cells had downregulated the expression of mCherry-IL-21 and completely failed to produce IL-21 after stimulation (Figure 6L). By contrast, mCherry-IL-21$^+$ donor-derived cells in the lung upregulated the expression of GFP-IL-2 (Figure 6L) and differentiated into IL-4 and IL-13 double-producing effector Th2 cells (Figures 6M and 6N). Together, these experiments showed that IL-4-committed Tfh cells generated upon HDM sensitization were able to differentiate effector Th2 cells when recalled.

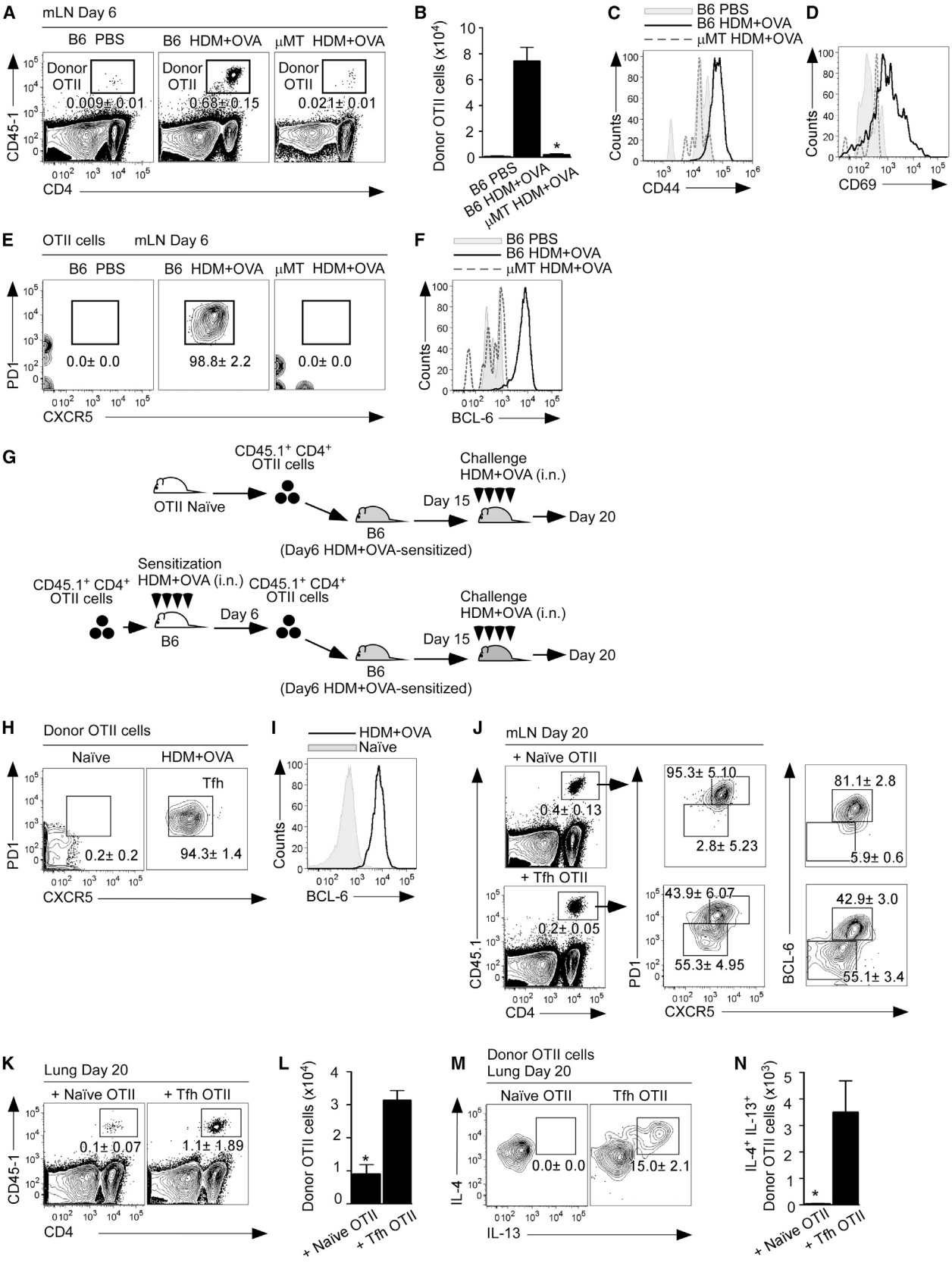
Depletion of Pre-existing IL-4-Committed Tfh Prevents Th2-Teff Cell Responses after HDM Challenge

We next tested whether blockade of Tfh development during HDM sensitization precluded effector Th2 responses after HDM challenge. To address this possibility, we treated HDM-sensitized B6.4get mice with either 50 mg/Kg BCL-6 inhibitor 79-6 (Cerchietti et al., 2010) per day or vehicle (10% DMSO) on days 4 to 11 and enumerated GFP-IL-4$^+$ Tfh cells in the mLN on day 12. We found that the number (Figure 7B) of GFP-IL-4$^+$ CD4 T cells with a PD-1^{hi}CXCR5^{hi} Tfh cell phenotype was reduced in 79-6 treated mice compared to PBS-treated controls. We next purified CD4$^+$ T cells from the mLN of day 12 HDM-sensitized, 79-6-treated and control mice and transferred equivalent numbers into day 12 HDM-sensitized CD45.1$^+$ B6 mice (Figure 7A), challenged the recipients with HDM on days 13–16 and enumerated effector Th2 cells from the donor (CD45.2$^+$) on day 18 in the lungs. We found that the frequency

Figure 4. HDM Sensitization Induces IL-4-Expressing Tfh Cells but Not Effector Th2 Cells

(A–E) B6.4get mice were i.n treated with 25 μg of HDM of PBS for 4 consecutive days starting on day 1 and analyzed by flow cytometry on day 6. The frequency of IL-4-expressing (EGFP$^+$) on the CD4$^+$ T cells from the lungs (A) and mLNs (B) are shown. Expression of PD-1 and CXCR5 (C), BCL-6, GL7, CD25, and ICOS (D) and intracellular IL-2 and IL-21 (E) on naive (CD44^{lo}EGFP$^-$) and antigen-experienced (CD44^{hi}EGFP$^+$) CD4$^+$ T cells in the mLN of HDM-treated mice are shown at day 6. (F–H) B6 mice were i.n. HDM sensitized on day 1 and analyzed on day 6 or HDM sensitized on day 1, challenged on day 15 and analyzed on day 20. The frequency of CD138$^+$ antibody secreting cells (ASC) (F), IgE$^+$CD138$^+$ ACS (G), and PNA$^+$FAS$^+$ GC cells (H) in the mLN are shown.

(I–N) B6.4get mice were i.n. treated with 25 μg of HDM for 4 consecutive days starting on day 1. On day 15, mice were i.n. challenged with 25 μg HDM daily for 4 consecutive days. Mice were sacrificed and analyzed by flow cytometry on day 20. The expression of PD-1 and CXCR5 (I), BCL-6, GL7, CD25, ICOS, and GATA-3 (J) and intracellular IL-2 and IL-21 (K) on naive (CD44^{lo}EGFP$^-$) and antigen-experienced (CD44^{hi}EGFP$^+$) CD4$^+$ T cells in the lung are shown. Expression of PD-1 and CXCR5 (L), BCL-6, GL7, CD25, ICOS, and GATA-3 (M) and intracellular IL-2 and IL-21 (**N**) on naive (CD44^{lo}EGFP$^-$) and antigen-experienced (CD44^{hi}EGFP$^+$) CD4$^+$ T cells in the mLN. Data are representative of three independent experiments (mean and SD of 4-5 mice per group). See also Figure S2.



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(Figures 7C and 7E) and number (Figures 7D and 7F) of donor GFP$^+$ (Figures 7C and 7D) and IL-13$^+$ (Figures 7E and 7F) effector Th2 cells were reduced in recipients that had received CD4$^+$ T cell from 79-6-treated mice compared to control mice. As a control, we showed that donor GFP$^+$ and IL-13$^+$ effector Th2 cells accumulated normally in mice that received 79-6 starting 1 day after challenge (Figures 7C–7F). These results suggest that BCL-6 inhibitor therapy prevents Tfh cell responses after HDM sensitization, which in turn precludes effector Th2 cell-mediated responses following challenge.

IL-2 directly inhibits the differentiation and maintenance of Tfh cells (Ballesteros-Tato et al., 2012). To test whether recombinant IL-2 (rIL-2) treatment after HDM sensitization could impair Th2 effector cell responses after HDM challenge, we adoptively transferred CD45.1$^+$ OTII cells into naive CD45.2$^+$ B6 recipient mice, sensitized the recipients one day later with HDM-OVA, treated them with 30,000 units of human rIL-2 or PBS twice a day on days 10 and 11 (Figure 7G), and enumerated CD45.1$^+$ Tfh cells in the mLN on day 12. We found that the number (Figure 7H) of donor OTII cells with a PD-1^{hi}CXCR5^{hi} Tfh cell phenotype was reduced in rIL-2-treated mice compared to PBS-treated controls. In agreement with these results, the number of CD19$^+$BCL-6$^+$ GC B cells was reduced in rIL-2-treated mice as well (Figure 7I).

To evaluate whether the lack of Tfh cells in rIL-2-treated mice could affect subsequent Th2 effector cell responses, we challenged rIL-2-treated and control mice with HDM-OVA and characterized the Th2 effector cell response in the lungs on day 20. As a control, some mice were treated with rIL-2 starting 1 day after HDM-OVA challenge (Figure 7G). We found that the frequency (Figure 7J) and number (Figure 7K) of donor OTII cells as well as the frequency (Figure 7L) and number (Figure 7M) of IL-4-producing donor Th2 effector cells was dramatically reduced in mice treated with rIL-2 after HDM sensitization. In contrast, IL-4-producing donor Th2 effector cells accumulated to even higher amounts in mice that received rIL-2 during HDM challenge (Figures 7J–7M), consistent with the idea that IL-2 signaling promotes Th2 effector cell responses (Liao et al., 2008). We also found that the number of eosinophils in the lungs and BAL was reduced in mice treated with rIL-2 after HDM sensitization, but was increased in mice treated with rIL-2 during HDM challenge (Figures 7N and 7O). Importantly, rIL-2 treatment did not alter the frequency or number of Foxp3$^+$ regulatory T cells at any time point or organ analyzed (Figure S4A-F). Taken together, these results indicate that depletion of IL-4-committed Tfh cells prevents the development of Th2 effector cell-mediated allergic responses upon secondary HDM challenge.

DISCUSSION

Our data show that, following pulmonary sensitization with HDM, naive CD4$^+$ T cells differentiate into IL-4-committed Tfh cells in the mLN, but fail to differentiate into pathogenic effector Th2 cells that home to the lung. As a result, initial sensitization with HDM does not cause Th2 cell-mediated symptoms or pulmonary pathology. However, following HDM challenge, the IL-4-committed Tfh cells rapidly differentiate into effector Th2 cells that home to the lung, produce IL-4, and recruit eosinophils. Our results are consistent with studies showing that Tfh cells have the capacity for long-term persistence and the ability to differentiate into potentially-pathogenic effector T cells upon secondary challenge (Choi et al., 2013; Lüthje et al., 2012) and offer a perspective for how Th2-cell-mediated pathology to inhaled HDM is initiated.

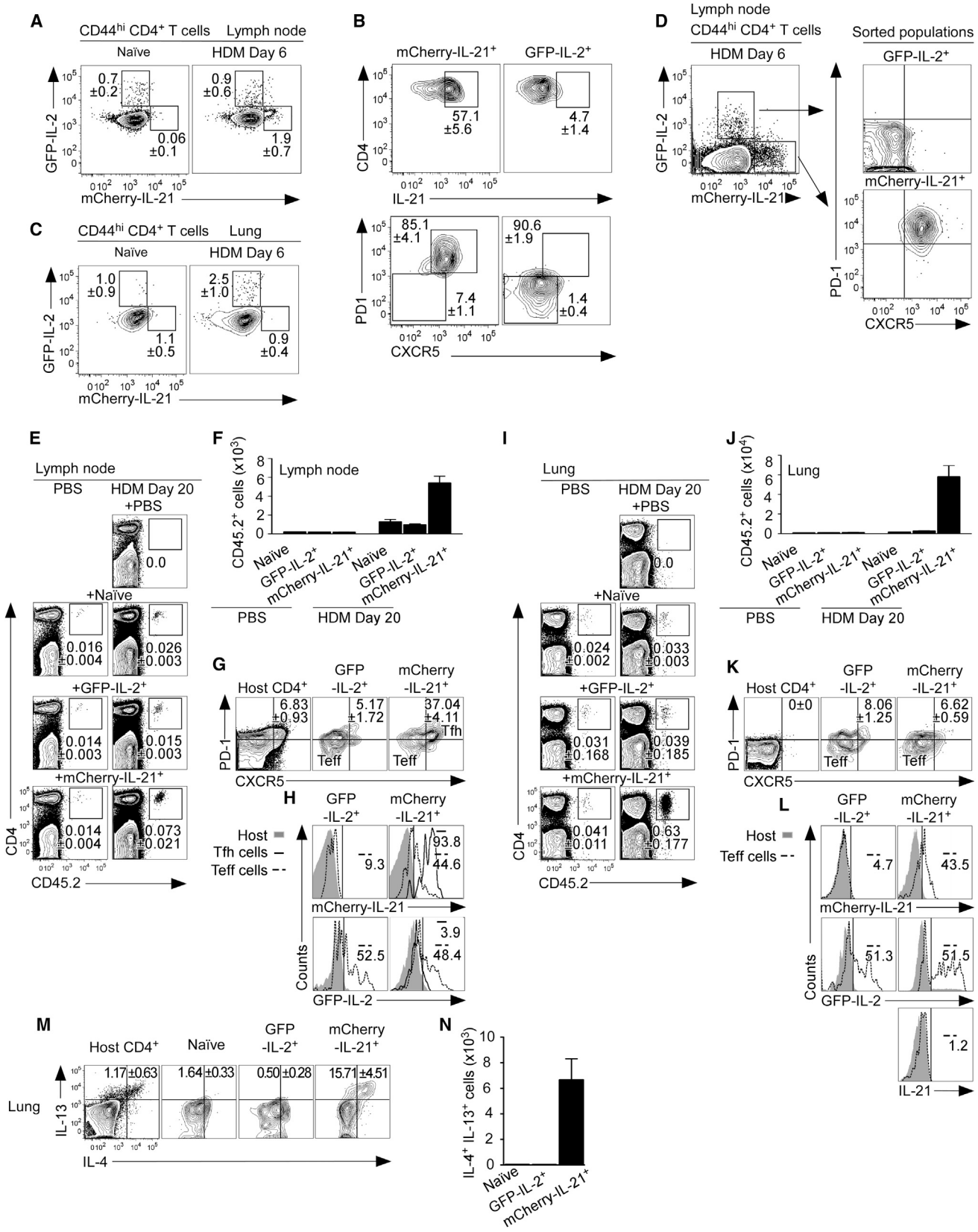
Our results also demonstrate that Th2 cell responses following sensitization and challenge with HDM are dependent on both DCs and B cells. Although other studies show that DCs are essential for Th2 cell responses to pulmonary allergens (Hammad et al., 2010; Plantinga et al., 2013), our data confirm that DCs are important in two ways; first for carrying antigen from the lung to the mLN and second for priming allergen-specific CD4$^+$ T cell via MHC II-dependent cognate-interactions. However, unlike some studies suggesting that Ag-presentation by DCs alone is sufficient for Th2 cell responses to inhaled HDM (Hammad et al., 2010), our data demonstrate that Th2 cell responses to HDM also require additional cognate interactions with Ag-presenting B cells. Rather than directly promoting the differentiation of effector Th2 cells however, B cells synergize with DCs to promote the differentiation of IL-4-commited Tfh cells during the HDM-sensitization phase. Although DCs are sufficient to prime Tfh cell responses, Ag-presentation by B cells is necessary to complete the Tfh cell differentiation program (Baumjohann et al., 2013; Choi et al., 2011; Deenick et al., 2010; Goenka et al., 2011). Thus, our results are consistent with the current models for how Tfh cell responses are initiated (Ballesteros-Tato and Randall, 2014).

Importantly, although the exact mechanisms by which DCs prime Tfh cells is incompletely defined the initiation of the Tfh cell responses seems to occur within the interfollicular area of the LN, where responding CD4$^+$ T cell first upregulate BCL-6

Figure 5. CD4 T Cells Activated after Sensitization Develop into Effector Th2 Cells after HDM Challenge

(A–F) 2.5 × 10^4 naive OTII.CD45.1$^+$ CD4$^+$ T cells were transferred into naive B6 and μMT mice. One day later, recipient mice were i.n. treated with 25 μg of HDM + 25μg of OVA (HDM+OVA), or PBS for 4 consecutive days. Mice were sacrificed and analyzed by flow cytometry 2 days after the last HDM inoculation. Frequency (A) and number (B) of donor OTII T cells from the mLN. Expression of CD44 (C), CD69 (D), CXCR5 and PD1 (E), and BCL-6 (F) in donor OTII T cells from the mLN.

(G–N) B6 (CD45.2$^+$) mice were i.n. treated with 25 μg of HDM + 25 μg of OVA (HDM+OVA) for 4 consecutive days. On day 6, mice were adoptively transferred with 2.5 × 10^4 CD45.1$^+$ OTII cells obtained from naive OTII donor mice, or 2.5 × 10^4 CD45.1$^+$ OTII cells obtained from the mLN of day 6 HDM+OVA-sensitized B6 mice that received CD45.1$^+$ OTII cells one day before HDM+OVA administration (G). (H and I) The expression of CXCR5 and PD1 (H) and BCL-6 (I) in purified donor OTII T cells are shown. Recipient mice were then challenged on day 15 with 25 μg HDM+OVA and analyzed on day 20. (J) Donor CD45.1$^+$ OTII T cells were identified by flow cytometry and the percentages of donor CD4$^+$ T cells with a CXCR5^{hi}PD1^{hi}BCL-6^{hi} and CXCR5^{lo}PD1^{lo}BCL-6^{lo} phenotype were determined in the mLN. (K and L) The frequency (K) and number (L) of donor CD45.1$^+$ OTII T cells in the lungs are shown. (M and N) The frequency (M) and number (**N**) of IL-4-producing cells among the donor CD45.1$^+$ OTII T cells were determined in the lungs by intracellular staining after restimulation for 4 hr with anti-CD3 and BFA. *$p < 0.001$ versus HDM+OVA-treated B6 (unpaired Student's t test). Data are representative of two or more independent experiments (mean and SD of 4–5 mice per group). See also Figure S3.



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and CXCR5 (Kerfoot et al., 2011). Thus, positioning of DCs within the interfollicular zone of the secondary lymphoid organs is likely to be instrumental for their ability to prime Tfh cell responses to HDM. In agreement with this idea, we found that lung-migratory, HDM-bearing DCs preferentially localize within the interfollicular area of the mLN early after HDM sensitization, where they hand off Ag to cells in the B cell follicle.

Recently, we showed that DCs responding to nematode infection upregulate CXCR5, downregulate CCR7, and home to the interfollicular areas of the mesenteric LN in response to CXCL13, where they prime effector Th2 cell responses (León et al., 2012). However, we failed to detected effector Th2 cells in the mLN and lung of HDM sensitized mice, perhaps because concomitant signals provided by HDM-presenting B cells promote the more efficient differentiation of Tfh cells. Alternatively, engagement of DCs and B cells by particular HDM-derived products might condition them to preferentially promote the differentiation of IL-4-committed Tfh cells during the sensitization phase, perhaps by favoring the expression of cytokines or costimulatory ligands that promote Tfh cell formation. In any case, we suggest a two-step differentiation model in which HDM-specific IL-4-committed $CD4^+$ T cells are first primed by lung-migratory DCs in the border of the B cell follicle where they begin to proliferate and acquire a Tfh like-signature. IL-4-committed pre-Tfh cells subsequently encounter Ag-bearing B cells that acquired HDM-derived Ags from the lung-migratory DCs. In the B cell follicle, activated B cells replace DCs as the primary APCs and provide additional survival and differentiation signals that allow HDM-specific IL-4-committed Tfh cells to survive as HDM-specific memory cells, which further differentiate into effector Th2 cells after re-challenge. This model is supported by recent studies showing that sustained antigenic stimulation by B cells is required for the maintenance of the Tfh cell responses (Baumjohann et al., 2013; Choi et al., 2011; Deenick et al., 2010; Goenka et al., 2011) and data indicating that Tfh cells are able to become memory cells (Choi et al., 2013; Lüthje et al., 2012) but retain the capacity to differentiate into effector T cells during a recall response (Lüthje et al., 2012).

The fact that HDM challenge induces IL-4-committed Tfh cells to differentiate into effector Th2 cells is at odds with a recent paper reaching the opposite conclusion. This work has concluded that although HDM-specific Tfh cells are required for normal Th2 responses to HDM, they do not convert into effector Th2 cells (Coquet et al., 2015). The authors largely base their conclusion on the fact that IL-21^+ Tfh cells from HDM sensitized mice failed to differentiate into Th2 cells upon adoptive transfer into naive mice. However, we have demonstrated here that Tfh to Th2 cell conversion only occurs in recipients that had been previously exposed to the allergen. As a consequence, Tfh cells transferred into naive recipients poorly differentiated into Th2 cells. Thus, differences in the experimental design might have led to opposing conclusions compared with our study. In any case, our findings demonstrate that an inability to generate Tfh cell responses during the HDM sensitization phase precludes Th2 cell-mediated immunity after secondary HDM challenge. These data suggest that targeting Tfh cells might be a good therapeutic strategy to prevent Th2-cell mediated immunity to HDM. In support of this, we found that depletion of pre-existing Tfh cells following rIL-2 treatment prevents Th2-cell mediated immunity after HDM re-exposure. Therefore, knowing the factors that control Tfh cell maintenance in response to HDM and the mechanisms by which Tfh cells convert into effector Th2 cells following repeated allergen encounter, will provide valuable information to design therapeutic strategies to prevent allergic responses to HDM.

In conclusion, our findings offer insights into how Th2-cell-mediated immunity to HDM is initiated, reveal an important role for B cells and Tfh cells in this process, and expose the potential therapeutic benefits of targeting Tfh cells to prevent allergic asthma.

EXPERIMENTAL PROCEDURES

Mice and Immunizations

The mouse strains used in these experiments include: C57BL/6J (B6), B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ (CD45.1^+ B6 congenics), B6.129S2-*Ighm*^{tm1Cgn}/J (μMT), B6.FVB-Tg (*Itgax*-DTR-EGFP)57Lan/J (CD11c-DTR), B6.129S-H2$^{dlAb1\text{-}Ea}$ (MHC II-deficient), C57BL/6-Tg(*TcraTcrb*)425Cbn/J (OTII), B6.129-Il4^{tm1Lky}/J (B6.4get IL-4 reporter mice), B6.129-Il4^{tm1Lky}/J.IgHm^{tm1Cgn}/J (μMT.4get), B6.129-Il4^{tm1Lky}/J. C57BL/6-Tg(*TcraTcrb*)425Cbn/J (OTII.4get), and *Il21*-mCherry and *Il2*-emGFP dual-reporter transgenic mice. B6.4get mice and μMT.4get mice were originally obtained from M. Mohrs (Trudeau Institute). *Il21*-mCherry and *Il2*-emGFP dual-reporter mice were originally obtained from Dr. Warren J. Leonard (NHLBI). All other mice were originally obtained from The Jackson Laboratory and were bred in the University of Alabama at Birmingham animal facility. HDM (Dermatophagoides and teronyssinus) extracts were obtained from Greer laboratories. Adult mice were administered intranasally (i.n.) with 25 μg of HDM extract or 25 μg of HDM extract and 25 μg of OVA (Sigma-Aldrich) daily for 4 days and challenged (i.n.) with 25 μg of HDM or 25 μg of HDM extract and 25 μg of OVA 14 days later. In some experiments, HDM extract was labeled with AF647 labeling kit (Invitrogen) prior to administration to mice. The University of Alabama at Birmingham Institutional and NHLBI Animal Care and Use Committees approved all procedures involving animals.

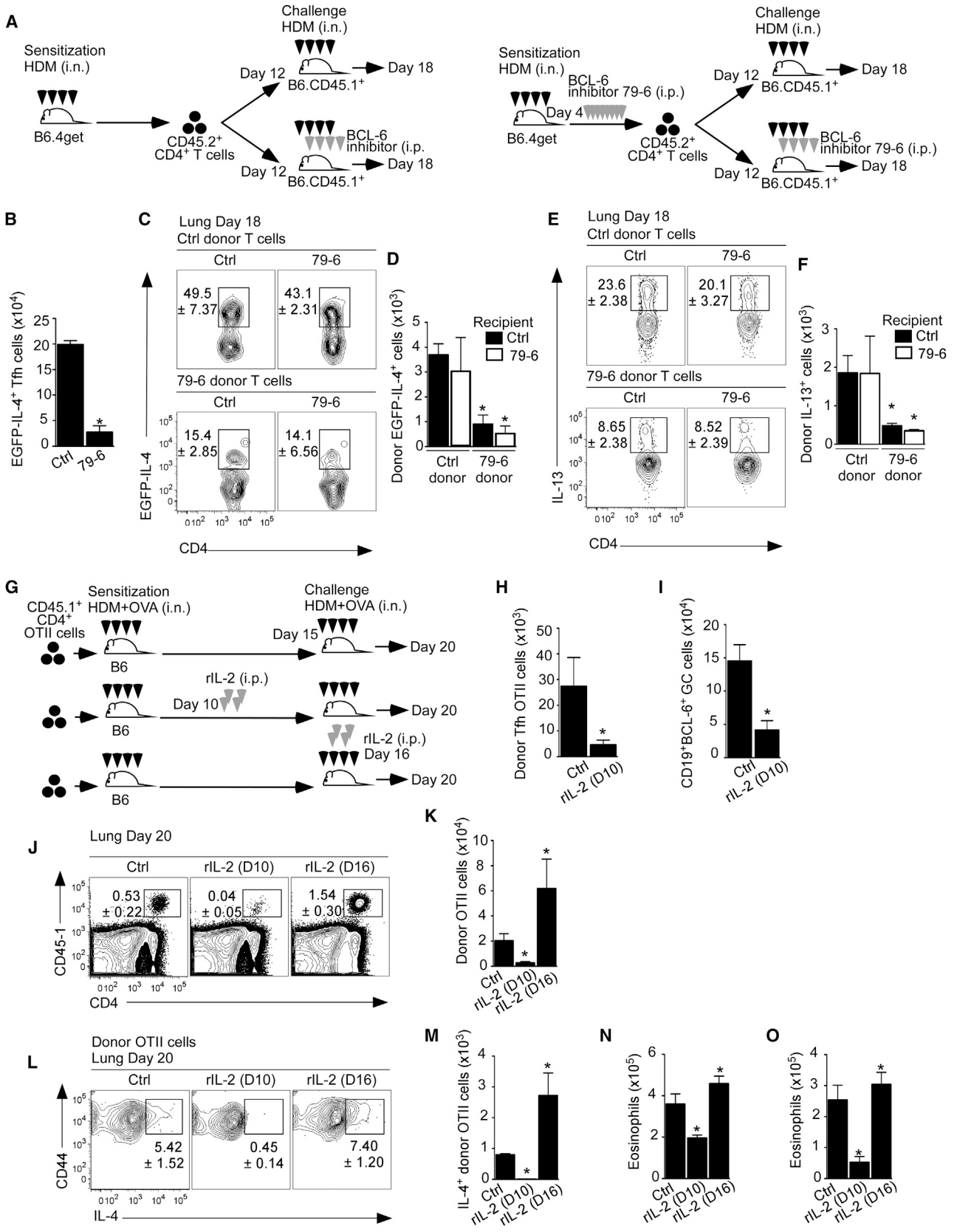
BM Chimeras

Recipient mice were irradiated with 950 Rads from a high-energy X-rays source delivered in a split dose and reconstituted with 10^7 total BM cells. To

Figure 6. Tfh Cells Develop into Effector Th2 Cells after HDM Challenge

(A–C) CD45.1^+ B6 mice were irradiated and reconstituted with BM from *Il21*-mCherry and *Il2*-emGFP dual-reporter mice. Eight weeks later, reconstituted-chimeric mice were i.n. HDM sensitized on day 1 and analyzed on day 6. The frequency of IL-21-expressing (mCherry$^+$) and IL-2-expressing (GFP$^+$) cells in CD44^{hi}CD4$^+$ T cells from the mLN (A) and lung (C) and are shown. Expression of intracellular IL-21, CXCR5, and PD1 in mCherry$^+$ GFP$^-$ single-positive and mCherry$^-$ GFP$^+$ single-positive CD4$^+$ T cells from the mLN (B) is shown.

(D–N) 1 × 10^5 mCherry$^+$ GFP$^-$ single-positive (CXCR5$^+$PD1$^+$) or mCherry$^-$ GFP$^+$ single-positive (CXCR5$^-$PD1$^-$) CD44^{hi}CD4$^+$ T cells (D) or naive CD44^{lo}CD4$^+$ T cells, sorted by flow cytometry on day 6 after sensitization, were adoptively transferred into day 6 HDM sensitized CD45.1^+ B6 mice. Recipient mice were then challenged with HDM or PBS on day 15 and analyzed on day 20. The frequency (E) and (I) and number (F) and (J) of donor CD45.2^+ T cells in the mLN (E and F) and lung (I and J) are shown. The frequency of CXCR5 and PD1 (G) and (K) and mCherry-IL-21, GFP-IL-2, and intracellular IL-21 (H) and (L) in donor CXCR5^{hi}PD1^{hi} (Tfh) and CXCR5^{lo}PD1^{lo} (Teff) CD4$^+$ T cells from mLN (G and H) and lung (K and L) are shown. (M and N) The frequency (M) and number (**N**) of IL-4 and IL-13 double-producing cells among the donor CD45.2^+ T cells were determined in the lungs by intracellular staining after restimulation for 4 hr with anti-CD3 and BFA (mean and SD of 4–5 mice per group).



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generate mice that express *Il21*-mCherry and *Il2*-emGFP dual-reporter gene in BM cells, CD45.1$^+$ B6 recipients were reconstituted with 100% of *Il21*-mCherry and *Il2*-emGFP BM. To generate mice that lacked MHC II expression specifically in the DC compartment, we reconstituted B6 recipients with 80% *Itgax*-DTR BM + 20% MHC II-deficient BM. For controls, B6 recipients were reconstituted with 80% *Itgax*-DTR BM + 20% B6 BM. To generate mice that lacked MHC II expression specifically in the B cell compartment, we reconstituted μMT recipients with 80% μMT.4get BM + 20% MHC II-deficient BM. For controls, μMT recipients were reconstituted with 80% μMT.4get BM + 20% B6 BM. Mice were allowed to reconstitute for at least 8–12 weeks before HDM treatment.

In Vivo Treatments

To deplete CD11c$^+$ cells in vivo, we treated CD11c-DTR mice and *Itgax*-DTR BM chimeras intraperitoneally (i.p.) with 60 ng DT (Sigma-Aldrich). Chimeras received additional injections of DT every 3 days. For rIL-2 treatment, mice were intraperitoneally administered 30,000 U of human rIL-2 (National Cancer Institute) twice a day for two days. To block BCL-6 activity, we treated mice daily by i.p. injection with 50 mg/Kg BCL-6 inhibitor 79-6 (Calbiochem) or vehicle (10% DMSO).

Cell Preparation and Flow Cytometry

Lungs were isolated, cut into small fragments and digested for 45 min at 37°C with 0.6 mg/ml collagenase A (Sigma) and 30 μg/ml DNase I (Sigma) in RPMI-1640 medium (GIBCO). Digested lungs, mLNs, or spleens were mechanically disrupted by passage through a wire mesh. Red blood cells were lysed with 150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA. Fc receptors were blocked with anti-mouse CD16/32 (5 μg/ml; BD-Biosciences), followed by staining with fluorochrome-conjugated Ab. Fluorochrome-labeled anti-B220 (RA3-6B2), anti-BCL-6 (K112.91), anti-CD3 (17A2) anti-CD4 (GK1.5), anti-CD19 (1D3), anti-CD11c (HL3), anti-CD25 (7D4), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD69 (H1-2F3), anti-CD138 (281-2), anti-CXCR5 (2G8), anti-FAS (CD95; Jo2), anti-GATA-3 (L50-823), GL7, anti-ICOS (7E.17G9), anti-IgE (R35-72), anti-Siglec F (E50-2440), and anti-MHC II (AF6-120.1) were from BD Biosciences. Fluorochrome-labeled anti-PD1 (J43) and anti-Foxp3 (FJK-16 s) were from eBioscience. Fluorochrome-labeled PNA was from LifeTech. For intracellular cytokine staining, cell suspensions were stimulated on anti-CD3-coated plates (2 μg/ml) in the presence of Brefeldin-A (BFA, 10 μg/ml) for 4 hr. Restimulated cells were surface stained, fixed in 4% paraformaldehyde, permeabilized with 0.1% saponin, and stained with Abs against IL-2 (JE86-5H4), IL-4 (BVD4-1D11), and IL-13 (13A) (BD-Biosciences) or IL-21 (FFA21; eBioscience). BCL-6, GATA-3, and Foxp3 intracellular staining was performed using the MOUSE regulatory T cell staining kit (eBioscience). Flow cytometry was performed on FACSCanto II (BD-Biosciences) instrument.

Cell Purifications and T Cell Transfers

CD4$^+$ T cells were isolated by MACs from the spleens of naive CD45.1$^+$ OTII mice or from the mLNs of HDM-treated B6 and μMT mice. B220$^+$ B cells were isolated by MACs from the spleens of naive B6 mice. All T and B cell preparations were more than 95% pure. An aliquot of purified CD4$^+$ T cells from mLN of HDM-treated mice previously transferred with naive CD45.1$^+$ CD4$^+$ OTII cells was stained with anti-CD45.1 mAb to calculate the number of OTII cells present in the purified population. Equivalent numbers (2.5×10^4) of naive and primed OTII cells were transferred (i.v.) into naive or HDM-treated B6 recipient mice. 1×10^5 sorted Il21-mCherry$^+$ Il2-emGFP$^-$ CD44^{hi}CD4$^+$ Tfh cells, *Il21*-mCherry$^-$ *Il2*-emGFP$^+$ CD44^{hi}CD4$^+$ non-Tfh cells and naive CD44^{lo} CD4$^+$ T cells were i.v. transferred into congenic HDM-treated CD45.1$^+$ B6 recipient mice.

Immunofluorescence

Frozen sections (10 μm), prepared from OCT (Sakura Finetek)-embedded LNs, were incubated with 10 μg/ml of Fc block and 5% normal donkey serum in PBS and then stained with FITC-labeled anti-IgDb (217-170), FITC-labeled anti-CD11c (HL3), FITC-labeled anti-CD45.1 (A20), biotin-labeled or Alexa Fluor 647-labeled anti-B220 (RA3-6B2), biotin-labeled anti-CD4 (GK1.5), or biotin-labeled anti-CD35/CD21 (8D9). Primary antibodies were detected with Alexa Fluor 488-labeled goat anti-FITC and streptavidin-Alexa Fluor 555 (Invitrogen Life Sciences). Slides were mounted with Slow Fade Gold Antifade (Invitrogen). Images were collected with an Eclipse Ti-E Nikon inverted microscope and recorded with a Clara interline CCD camera (Andor). The images were taken with a 20× objective for 200× final magnification. Images were collected using NIS Elements Image software and saved as JPEG files.

Statistical Analyses

GraphPad Prism software (Version 5.0a) was used for data analysis. Data were analyzed using the unpaired Student's t test. Values of $p < 0.05$ were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.11.017.

AUTHOR CONTRIBUTIONS

B.L. and A.B.-T. wrote the manuscript. B.L. designed and performed all experiments with help from A.B.-T. T.D.R., F.E.L., R.S., and W.J.L. provided advice, discussion, and reagents that were critical to this work. All authors reviewed the manuscript before submission.

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Figure 7. Blockade of Tfh Cell Development Impairs Effector Th2 Cell Responses to HDM

(A–F) B6.4get mice were i.n. HDM sensitized on day 1 and daily i.p treated with 50 mg/Kg of BCL-6 inhibitor 79-6 or vehicle (10% DMSO) from day 4 to day 11. Number of IL-4-expressing (EGFP$^+$) Tfh cells from the mLN on day 12 (B). CD4$^+$ T cells were purified from the mLN on day 12 and 2×10^6 of purified CD4$^+$ T cells were transferred into day 12 HDM-sensitized B6.CD45.1$^+$ recipient mice. Recipient mice were i.n. challenged on day 13 and analyzed on day 18 (A). Frequency (C) and number (D) of donor IL-4-expressing (EGFP$^+$) CD4$^+$ T cells in the lung. Frequency (E) and number (F) of donor IL-13$^+$ CD4$^+$ T cells in the lung.
(G–O) 2.5×10^4 naive OTII.CD45.1$^+$ CD4$^+$T cells were transferred into naive congenic B6 mice on day 0. Mice were i.n. HDM+OVA sensitized on day 1 and challenged on day 15. Mice were treated with 30,000 U of rIL-2 or PBS given twice a day for 2 days starting on day 10 or on day 16 (G). Number of donor Tfh OTII cells (H) and GC cells (I) in the mLN on day 12. Frequency (J) and number (K) of donor OTII cells from the lungs on day 20. Frequency (L) and number (M) of IL-4-producing cells among the donor OTII T cells determined in the lungs by intracellular staining after restimulation for 4 hr with anti-CD3 and BFA. Number of eosinophils from day 20 lungs (N) and BAL (O) is shown. *$p < 0.005$ versus Ctrl (unpaired Student's t test). Data are representative of two independent experiments (mean and SD of 4–5 mice per group) See also Figure S4.

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Immunity

Supplemental Information

T Follicular Helper Cell Plasticity Shapes Pathogenic T Helper 2 Cell-Mediated Immunity to Inhaled House Dust Mite

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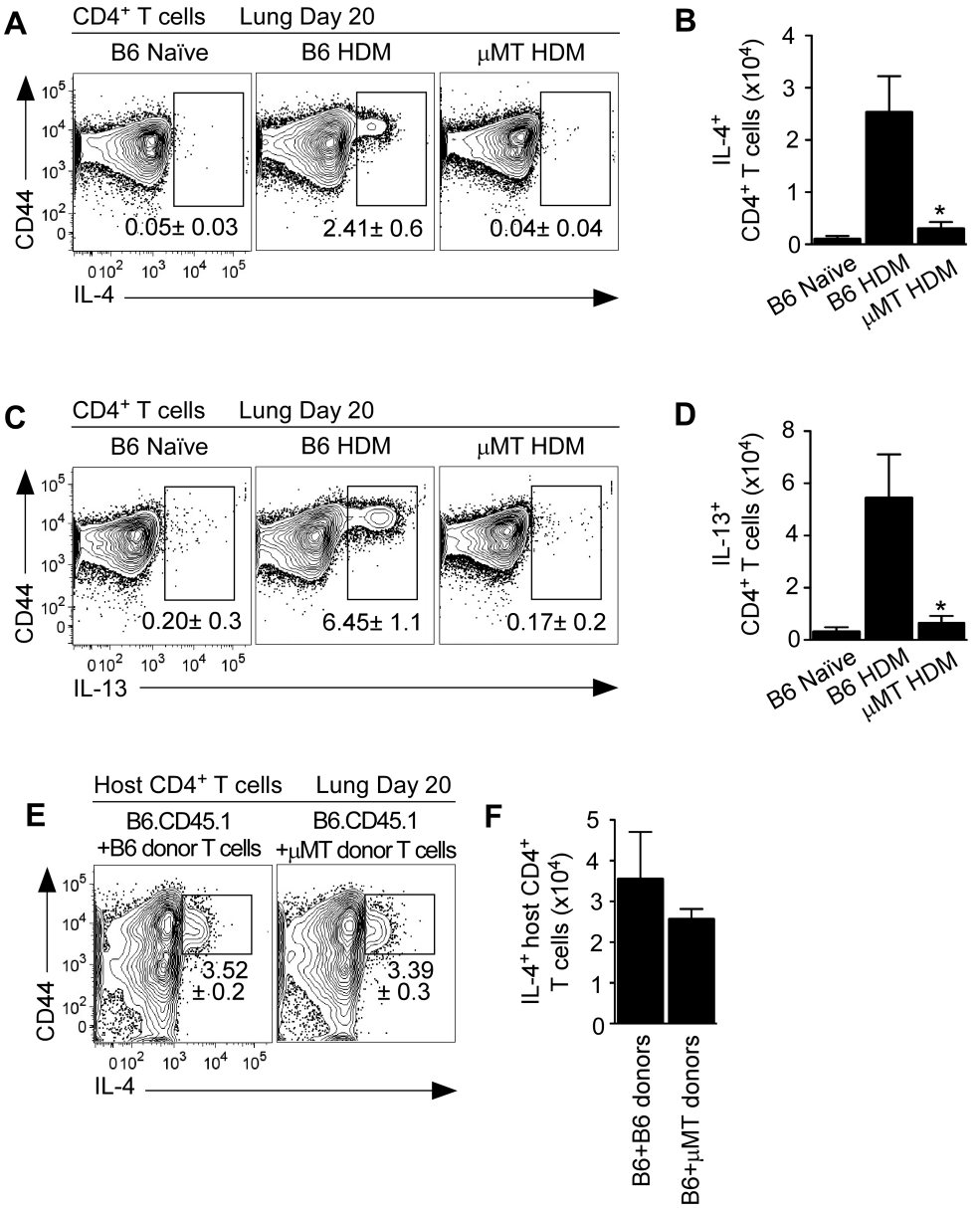


Figure S1. B cells are required for HDM-specific Th2 cytokine production by lung CD4⁺ T cells. (Related to Figure 1).

(**A-D**) B6 and μMT mice were i.n treated with 25μg of HDM for 4 consecutive days starting on day 1. On day 15, mice were i.n challenged with 25μg of HDM daily for 4 days. The frequency and number of IL-4 (**A** and **B**) and IL-13 (**C** and **D**) producing CD4⁺ T cells in the lungs were determined on day 20 by intracellular staining after restimulation for 4 h with anti-CD3 and BFA. As a control, naïve non-treated B6 mice were also analyzed. (**E-F**) HDM-sensitized B6.CD45.1⁺ recipient mice were i.n challenged on day 15 with 25μg of HDM daily for 4 consecutive days and analyzed on day 20. The frequency (**E**) and number (**F**) of host IL-4+ CD4⁺ T cells were determined by intracellular staining after restimulation with anti-CD3+ BFA for 4 h. *P < 0.001 vs. HDM-treated B6. (unpaired Student's t test). Data are representative of three independent experiments (mean and S.D. of 4-5 mice per group).

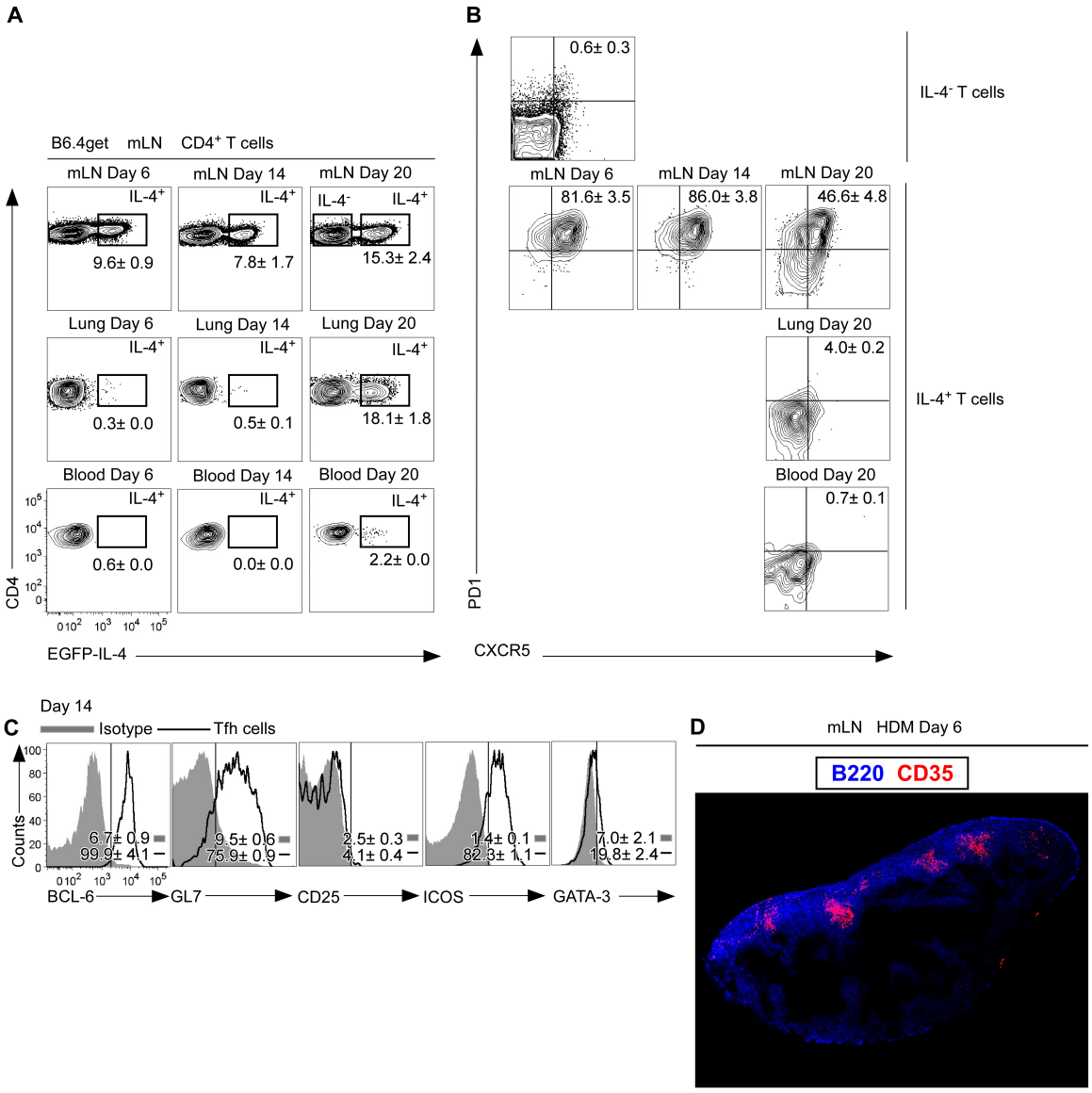


Figure S2. Kinetic analysis of IL-4-expressing (EGFP$^+$) CD4$^+$ T cells after administration of HDM (Related to Figure 4).

(**A-C**) B6.4get mice were i.n HDM sensitized on day 1 and analyzed on day 6 and day 14 or HDM sensitized on day 1, challenged on day 15 and analyzed on day 20. The frequency of IL-4-expressing (EGFP$^+$) on the CD4$^+$ T cells from the mLNs, lungs and peripheral blood are shown (**A**). Expression of PD-1 and CXCR5 on EGFP$^-$ and CD44^{hi}EGFP$^+$ CD4$^+$ T cells are shown (**B**). Expression of BCL-6, GL7, CD25, ICOS and GATA-3 on CD44^{hi}EGFP$^+$ CD4$^+$ T cells in the mLN of HDM-treated mice are shown at day 14 (**C**). (**D**) B6 mice were i.n HDM sensitized on day 1 and analyzed on day 6. Cryosections from the mLN were stained with anti-B220 (blue) and CD35 (red) and analyzed by fluorescent microscopy. Data are representative of two independent experiments (mean and S.D. of 4-5 mice per group).

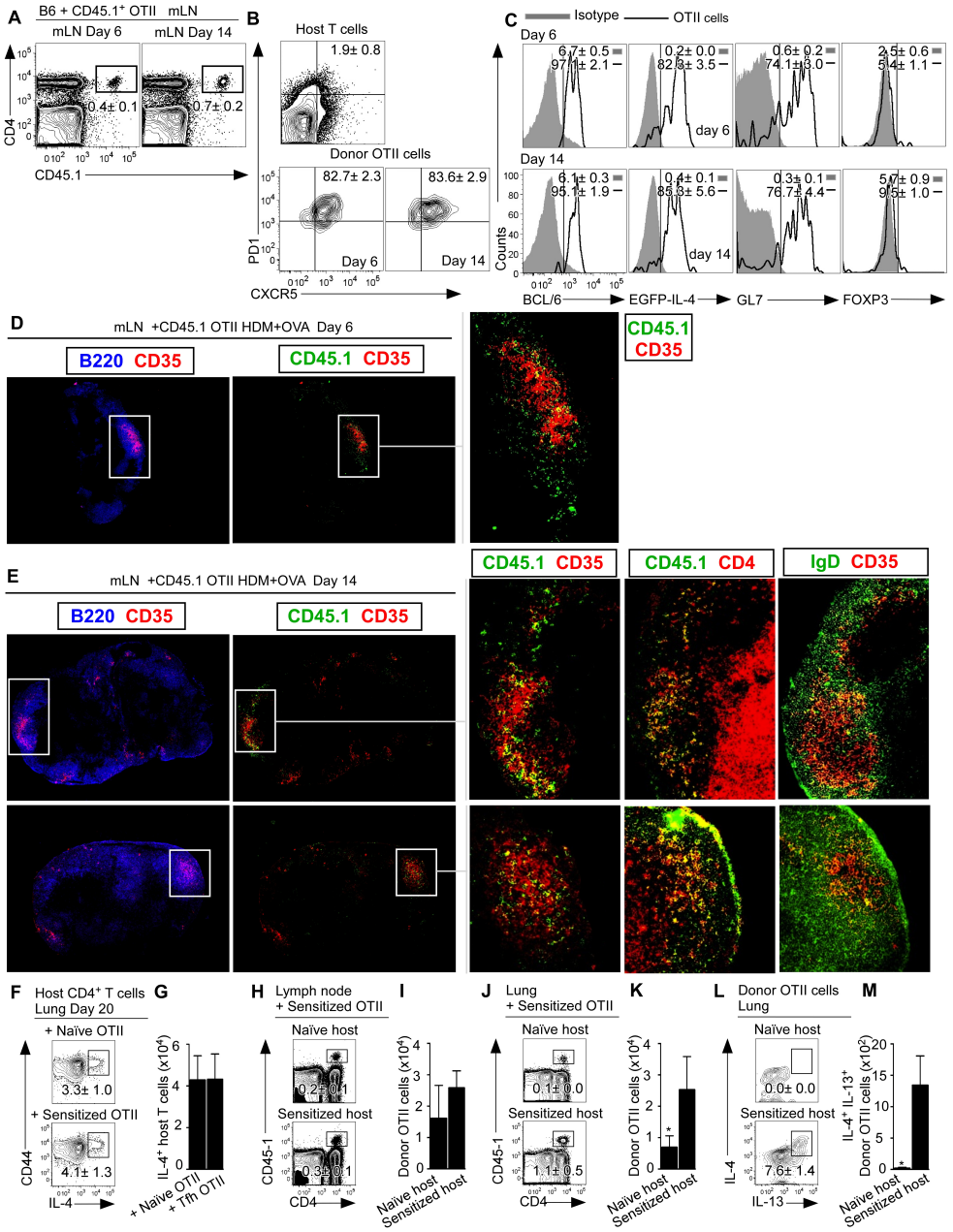


Figure S3. Kinetic analysis of the phenotype and intranodal localization of donor OTII cells after sensitization (Related to Figure 5).

(**A-C**) 25x10^3 naïve OTII. 4get. CD45.1$^+$. CD4$^+$ T cells were transferred into naive B6 mice. One day later, recipient mice were i.n treated with HDM+OVA for 4 consecutive days and analyzed 2 days after the last HDM inoculation. Frequency of donor OTII T cells from the mLN on day 6 and 14 (**A**). Expression of CXCR5 and PD1 (**B**) and BCL-6, EGFP-IL-4, GL7 and FOXP3 (**C**) in donor OTII T cells from the mLN on day 6 and 14.
(**D-E**) 25x10^3 naïve OTII. CD45.1$^+$. CD4$^+$ T cells were transferred into naive B6 mice. One day later, recipient mice were i.n HDM+OVA sensitized and analyzed on day 6 (**D**) and day 14 (**E**). Cryosections from the mLN were stained with anti-B220 (blue), CD35 (red) and CD45.1 (green), with anti-B220 (blue), CD4 (red) and CD45.1 (green) or with anti-B220 (blue), CD35 (red) and IgD (green) and analyzed by fluorescent microscopy. (**F-G**) HDM-sensitized B6 recipient mice were i.n challenged on day 15 and analyzed on day 20. The frequency (**F**) and number (**G**) of host (CD45.2+) IL-4 producing CD4+ T cells in the lungs were determined by intracellular staining after restimulation for 4 h with anti-CD3 and BFA. (**H-M**) 25x10^3 OTII CD45.1$^+$ CD4$^+$ T cells obtained from hosts 6 days after HDM+OVA sensitization were transferred into naive B6 mice or day 6 HDM+OVA-sensitized B6 mice. 8 days later, recipient mice were i.n treated with HDM+OVA for 4 consecutive days and analyzed 2 days after the last HDM inoculation. The frequency (**H,J**) and number (**I,K**) of donor CD45.1+ OTII T cells in the mLN (**H-I**) and lungs (**J-K**) are shown. The frequency (**L**) and number (**M**) of IL-4/IL-13-producing cells among the donor CD45.1+ OTII T cells were determined in the lungs by intracellular staining after restimulation for 4 h with anti-CD3 and BFA. *P < 0.01 (unpaired Student's t test). Data are representative of two or more independent experiments (mean and S.D. of 4-5 mice per group).

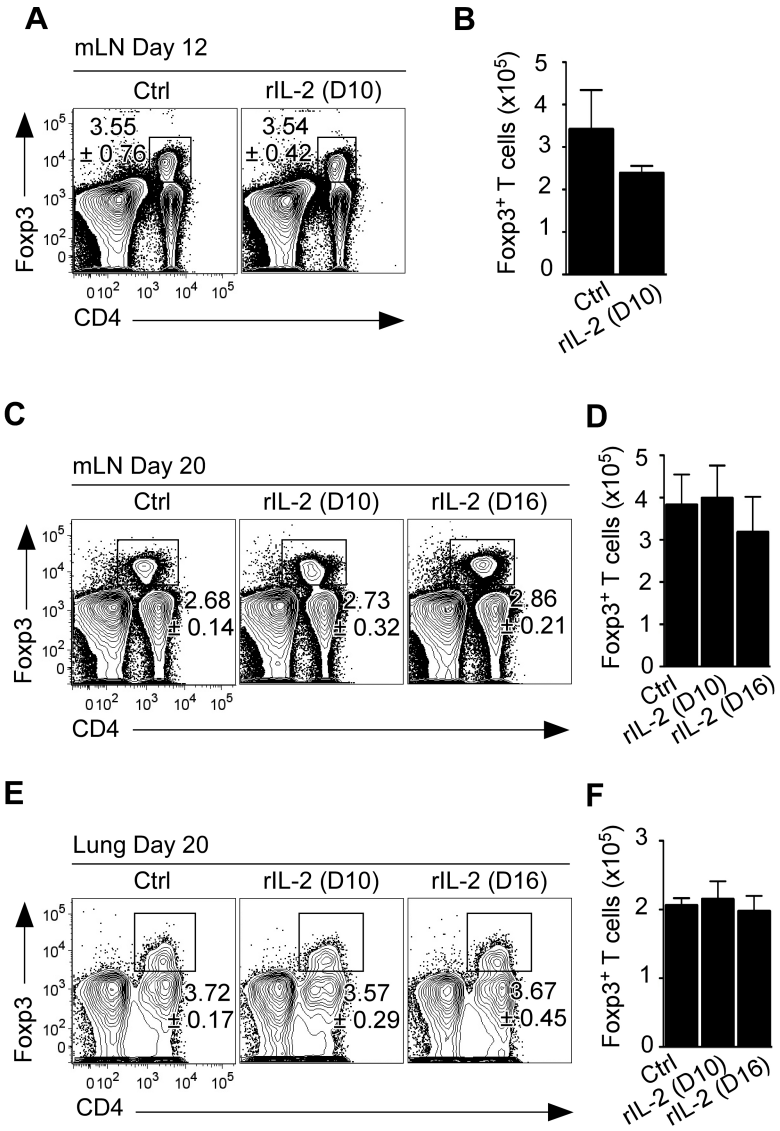


Figure S4. rIL-2 treatment in HDM-treated mice does not increase the number of Foxp3$^+$ Treg cells (related to Figure 7).

(**A-B**) Frequency (**A**) and number (**B**) of host Foxp3$^+$ Treg cells from day 12 mLN of HDM+OVA-treated B6 mice injected with rIL-2 or PBS according to the schematic in **Figure 7E**. (**C-F**) Frequency and number of host Foxp3$^+$ Treg cells from day 20 mLN (**C-D**) and lungs (**E-F**) of HDM-treated B6 mice injected with IL-2 or PBS according to the schematic in **Figure 7E**.