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RESEARCH ARTICLE

Lung CD103$^+$ dendritic cells restrain allergic airway inflammation through IL-12 production

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DCs are necessary and sufficient for induction of allergic airway inflammation. CD11b$^+$ DCs direct the underlying Th2 immunity, but debate surrounds the function of CD103$^+$ DCs in lung immunity and asthma after an allergic challenge. We challenged *Batf3*$^{-/-}$ mice, which lacked lung CD103$^+$ DCs, with the relevant allergen house dust mite (HDM) as a model to ascertain their role in asthma. We show that acute and chronic HDM exposure leads to defective Th1 immunity in Batf3-deficient mice. In addition, chronic HDM challenge in *Batf3*$^{-/-}$ mice results in increased Th2 and Th17 immune responses and exacerbated airway inflammation. Mechanistically, Batf3 absence does not affect induction of Treg or IL-10 production by lung CD4$^+$ T cells following acute HDM challenge. Batf3-dependent CD103$^+$ migratory DCs are the main source of IL-12p40 in the mediastinal lymph node DC compartment in the steady state. Moreover, CD103$^+$ DCs selectively increase their IL-12p40 production upon HDM administration. In vivo IL-12 treatment reverts exacerbated allergic airway inflammation upon chronic HDM challenge in *Batf3*$^{-/-}$ mice, restraining Th2 and Th17 responses without triggering Th1 immunity. These results suggest a protective role for lung CD103$^+$ DCs to HDM allergic airway inflammation through the production of IL-12.

Introduction

Asthma is a chronic inflammatory disease that identifies a wide spectrum of respiratory-related symptoms, typically associated to eosinophilia, with variable and often reversible airway obstruction accompanied by airway hyperreactivity (AHR). Asthma is a major cause of disability and the most common chronic disease among children and young adults. DCs play a pivotal role in the immune response to inhaled allergens by taking them up, transporting them to the draining mediastinal lymph nodes (mLN), and presenting antigens to initiate the CD4$^+$ Th response (1). The main DC subsets in the steady state in the lung are plasmacytoid DCs and conventional DCs, the latter subdivided into 2 functionally distinct subsets that have been recently renamed as classical type 1 DCs (cDC1, Batf3-dependent) and classical type 2 DCs (cDC2, IRF4-dependent) and that are defined by surface marker expression and development in the airways (2–5). Attending to their migratory properties, cDCs are lymphoid organ–resident DCs (res-DCs) (CD8α$^+$, CD24$^+$, and XCR1$^+$ cDC1s and CD11b$^+$ cDC2s) and tissue-derived or migratory DCs (mig-DCs) consisting of CD103$^+$XCR1$^+$ cDC1s and CD11b$^+$SIRPα$^+$ cDC2s, present in the lung and trafficking to the mLNs (6, 7). Upon lung inflammation, the lungs are also infiltrated by monocyte-derived DCs (1). Much effort in the field is currently directed at elucidating the division of labor among different DC subsets in the context of asthma, and contributions by several DC subsets have been defined. It was suggested that mouse lung cDC1s predominantly prime Th1 and Th17 responses in vitro, whereas mouse lung cDC2s foster a Th2 response (8). Plasmacytoid DCs play a key role in the induction of Tregs that control exacerbated airway inflammation (9). CD11b$^+$ cDC2s mediate Th2 priming in response to house dust mite (HDM) and to *Blomia tropicalis* mites (10–12), leading to eosinophilic airway inflammation, mucus production, and AHR. CD11b$^+$ cDC2s also promote a Th17 response upon lung fungal infection with *Aspergillus fumigatus* (13). Th17 immunity contributes to neutrophilic inflammation associated with some severe forms of asthma (14). Monocyte-derived DCs play a major proinflammatory function, contributing significantly to the immunopathology of asthma through the production of chemokines (10). However, debate still surrounds the function of CD103$^+$ cDC1s in Th2 induction and the development of allergic asthma (10, 15–17).

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The development and function of some DC subsets is mediated by the basic leucine zipper transcription factor ATF-like 3 (Batf3) (18–20). In the C57BL/6 background, the main subset affected in tissues and draining LNs is CD103$^+$ cDC1s, while functionally and developmentally related CD8α$^+$ res-cDC1s are partially impaired in the LNs (18–20). Batf3-dependent lung CD103$^+$ cDC1s are required for transport of influenza virus to the mLNs, for the generation of CD8$^+$ T cell immunity against influenza or Sendai virus (19, 21, 22), and for priming of resident memory CD8$^+$ T cells against vaccinia virus (23). Moreover, *Aspergillus*-germinated morphotypes induce IL-2 in lung CD103$^+$ DCs, leading to an optimal Th17 response (24). Here, we explore the debated role of CD103$^+$ DCs in HDM-induced allergic asthma. We report that Batf3-deficient mice develop exacerbated Th2- and Th17-driven allergic asthma when chronically exposed to HDM. CD103$^+$ DCs are key regulators that control Th2 and Th17 responses in allergen-exposed mice not by inducing Tregs, but rather by increasing IL-12p40 production.

Results

Batf3$^{-/-}$ mice show severely impaired CD103$^+$ cDC1 compartment. To evaluate the role of Batf3-dependent cDC1s during asthma, we analyzed Batf3-deficient mice after sensitization and challenge with HDM. We first assessed Batf3-dependent cDC1s in both the mLN and lung, since those are the tissues involved in the initiation and the effector phase, respectively, in asthma. To analyze DCs in the lung, WT and *Batf3$^{-/-}$* mice were sensitized (i.n.) to HDM on day 0 (1 μg) and then challenged i.n. on days 7–11 with 10 μg HDM (Figure 1A and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.90420DS1) (10, 25). When defining our gating strategy for DCs, we first gated out alveolar macrophages based on their CD11c and Siglec-F expression and their strong autofluorescence (10) (Figure 1B and Supplemental Figure 1B). The lungs of WT mice had a reduced frequency of CD103$^+$XCR1$^+$SIRPα$^-$CD24^{hi}CD11b^{lo} cDC1s in the CD11c$^+$MHCII$^+$ compartment on day 14 following HDM sensitization and challenge (Figure 1C and Supplemental Figure 1C), probably due to the recruitment of CD11b$^+$ cDC2s and monocyte-derived DCs (moDCs) to the lung (10). *Batf3$^{-/-}$* mice exposed to PBS lacked CD103$^+$XCR1$^+$ cDC1s, a subset that was not restored following HDM challenge.

For the assessment of early DC response in the mLN to allergen challenge, mice were exposed to 100 μg HDM (Figure 1D and Supplemental Figure 1D), which is the dose administered in previous studies where HDM uptake and migration by pulmonary myeloid DCs was assessed (10, 26). WT mice exposed to a single HDM dose had a decreased percentage of CD8α$^+$ res-cDC1s and CD103$^+$ mig-cDC1s in the DC compartment in the mLNs (Figure 1, E–G), but the total number was not decreased on day 3 after challenge compared with PBS controls (data not shown). *Batf3$^{-/-}$* mice lacked CD103$^+$XCR1$^+$SIRPα$^-$CD24^{hi}CD11b^{lo} cDC1s in the mLNs and showed reduced res-CD8α$^+$ cDC1 in the mLNs (Figure 1, E–G, and Supplemental Figure 1, E–G), the remaining res-CD8α$^+$ cDC1 being XCR1$^-$ (27). The similar DC profile in the mLN of *Batf3$^{-/-}$* mice, regardless of HDM challenge, suggests that the treatment does not restore CD103$^+$ DCs in the lungs of *Batf3$^{-/-}$* mice, in contrast with the effect of aerosol exposure to *Mycobacterium tuberculosis* (28).

Impaired Th1 immunity in Batf3$^{-/-}$ mice upon acute HDM challenge. Next, WT and *Batf3$^{-/-}$* mice were sensitized i.n. to HDM on day 0 (1 μg) and challenged i.n. for 5 consecutive days (on days 7–11, 10 μg HDM) (25), and different hallmarks of asthma were evaluated 3 days after the last challenge. Analysis of bronchoalveolar lavage (BAL) revealed similar inflammatory infiltration in Batf3-deficient and WT mice at day 14 (Figure 2A). Moreover, histological analysis of lung sections showed similarly increased perivascular and peribronchial inflammation and mucus production (Figure 2B). However, while serum levels of HDM-specific IgG1 were similar in *Batf3$^{-/-}$* and WT mice (Figure 2C), HDM-exposed *Batf3$^{-/-}$* mice did not induce the production of HDM-specific IgG2a (Figure 2D), an antibody isotype associated with Th1 immunity.

To further dissect the specificity of the generated immune response, we restimulated mLNs and splenocytes from day 14 HDM–challenged WT and *Batf3 $^{-/-}$* mice with HDM extract. Tissue from Batf3-deficient mice showed a selective impairment in IFN-γ production, while production of IL-5, IL-17A, and IL-10 was similar locally (mLNs) and systemically (splenocytes) (Figure 3). We additionally restimulated lung cells with HDM and assessed IFN-γ production by CD4$^+$ and CD8$^+$ T cells. *Batf3$^{-/-}$* mice showed significantly lower production of IFN-γ by CD4$^+$ T cells compared with WT controls (Supplemental Figure 2A). Notably, we did not detect IFN-γ production by CD8$^+$ T cells in response to HDM challenge in any of the experimental groups (Supplemental Figure 2B). These results suggest that Batf3-dependent DCs are essential for induction of CD4$^+$ Th1 immunity after acute HDM challenge.

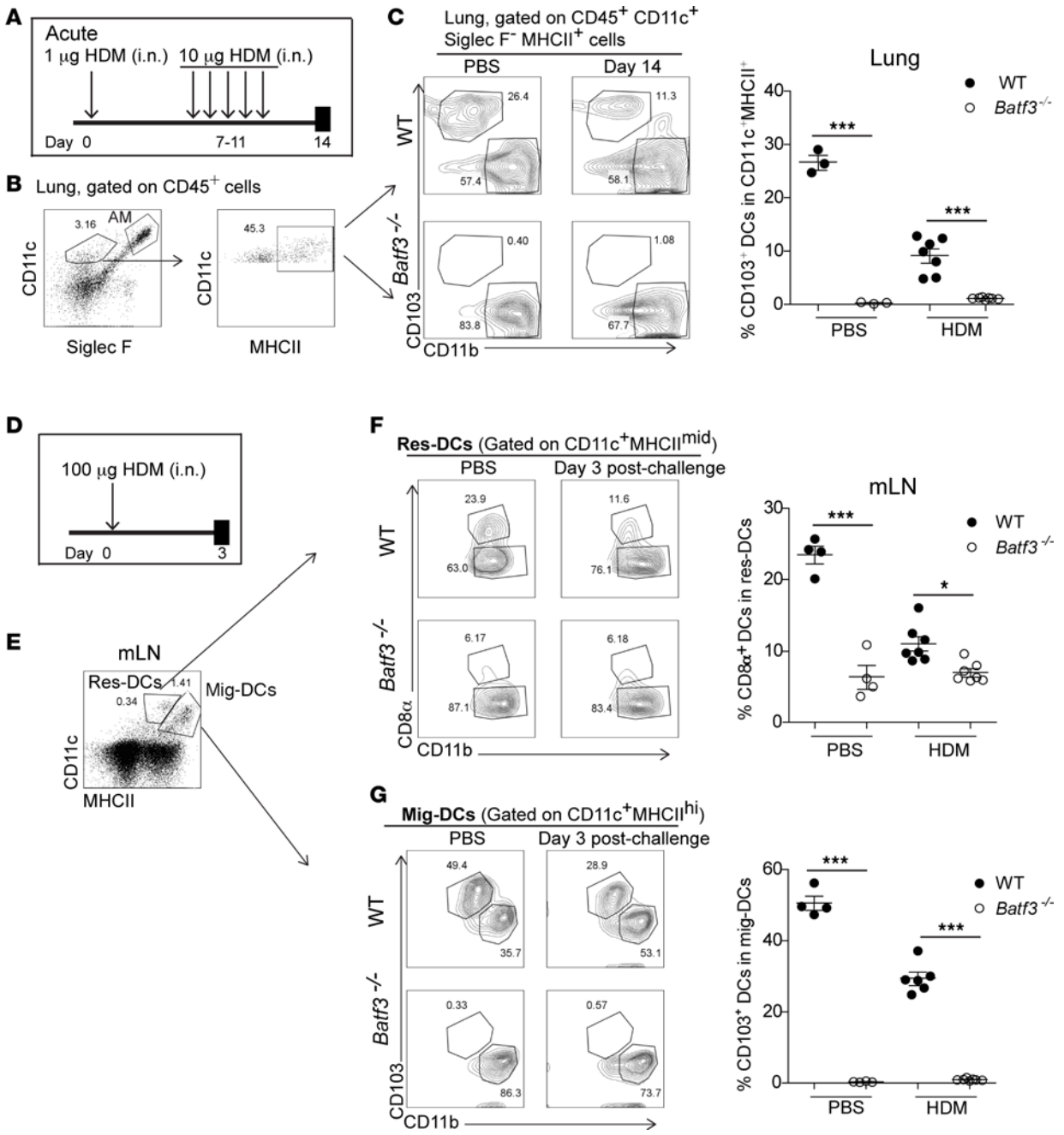

Figure 1. *Batf3*$^{-/-}$ mice lack CD103$^+$ cDC1s in the lung and mLN and show a reduced CD8α$^+$ cDC1 compartment in mLN. (**A**) HDM sensitization and challenge regime (acute protocol). (**B**) Gating strategy for lung Batf3-dependent DCs. (**C**) Staining of lung CD103$^+$ and CD11b$^+$ DCs in mice treated as indicated in **A**. Left: representative plots. Right: percentage of CD103$^+$ cDC1s in the CD11c$^+$MHCII$^+$ compartment in the lung. (**D**) HDM administration protocol to evaluate early DC response following allergen challenge. (**E**) Gating strategy to identify migratory (mig) and resident (res) DCs in mLNs. (**F** and **G**) Res-DCs (**F**) and mig-DCs (**G**) in mLNs from mice i.n. challenged with 100 μg HDM. Left: representative plots. Right: percentage of Batf3-dependent DCs in the CD11c$^+$MHCII$^+$ compartment in the mLN. (**C**, **F**, and **G**) Individual data (n = 3–4 PBS, n = 6–7 HDM) and mean ± SEM of 1 representative experiment of 3 performed. *P < 0.05, ***P < 0.001, 1-way ANOVA followed by Bonferroni's post-test. HDM, house dust mite; mLN, mediastinal lymph nodes.

Lack of Batf3 exacerbates the Th2 and Th17 response to chronic HDM airway exposure. Although asthma has classically been associated with eosinophilia and Th2 cytokines, some patients show a neutrophil-predominant disease with an absence or low Th2 cytokine profile (14). Eosinophilia usually predominates in acute inflammation, while neutrophilia and IL-17 is common in chronic disease (29). Despite the lack of Th1 immunity in *Batf3*$^{-/-}$ mice upon acute HDM challenge, we did not find differences in the typical hallmarks of asthma (Figure 2 and Figure 3). Therefore, we next investigated the role of Batf3-dependent

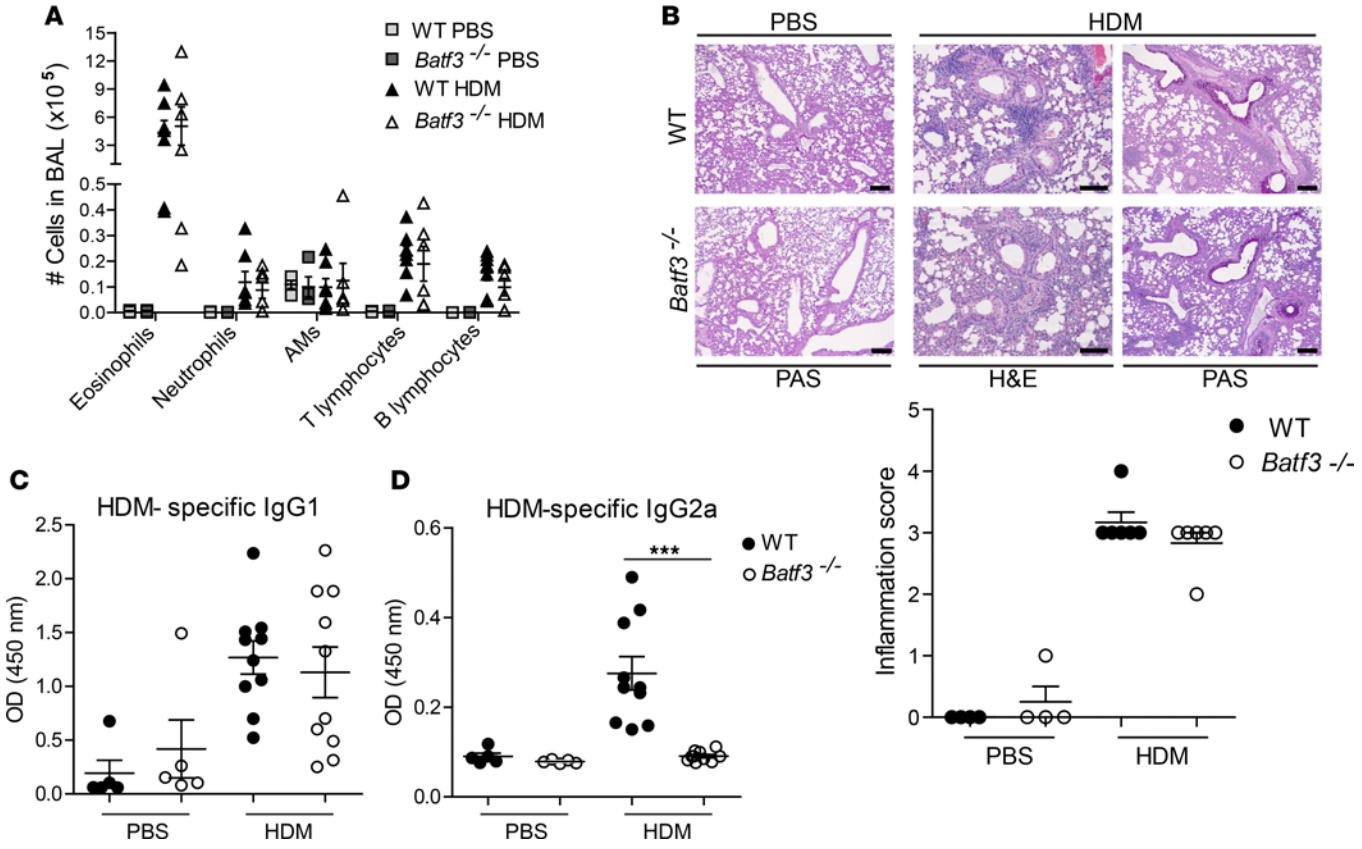

Figure 2. Batf3 deficiency does not impact on airway inflammation following acute HDM exposure. (**A**) Inflammatory infiltrates in BAL of HDM-sensitized and -challenged *Batf3*$^{-/-}$ and WT mice (n = 3 PBS, n = 7 HDM). (**B**) Top: representative H&E and PAS staining in formalin-fixed lung sections. Scale bars: 200 μm (PAS) or 100 μm (H&E). Bottom: inflammation and mucus score (n = 4 PBS, n = 6 HDM). (**C** and **D**) Serum HDM-specific IgG1 (**C**) and IgG2a (**D**) determined by ELISA (n = 5 PBS, n = 10 HDM). (**A–D**) Individual data and mean ± SEM of 1 representative experiment of at least 3 performed. ***P < 0.001, Mann Whitney U test. HDM, house dust mite; PAS, periodic acid Schiff.

DCs in a model that recapitulates clinical features of severe chronic asthma, including neutrophilia (14). We postulated that lack of Th1 immunity in *Batf3*$^{-/-}$ mice would have a detrimental effect on HDM airway exposure over the long-term, aggravating the asthmatic phenotype by disturbing the Th1/Th2/Th17 balance. After chronic airway exposure to HDM (3 × 10 μg doses per week for 6 weeks), eosinophil and neutrophil influx was dramatically increased in *Batf3*$^{-/-}$ mice compared with WT mice (Figure 4B). Histological analysis revealed enhanced inflammation, goblet cell metaplasia, mucus production, and airway collagen deposition in HDM-treated mice (Figure 4C). HDM-exposed *Batf3*$^{-/-}$ mice showed significantly increased perivascular and peribronchiolar inflammation (Figure 4C). Total serum IgE, a marker of allergic sensitization, was increased in Batf3-deficient mice (Figure 4D). Consistent with the data from the acute model, Th1-linked HDM-specific IgG2a production was impaired in chronically HDM-exposed *Batf3*$^{-/-}$ mice compared with similarly treated WT mice (Figure 4E). However, *Batf3*$^{-/-}$ and WT mice showed no differences in HDM-specific IgG1 titers after chronic HDM challenge (Figure 4F).

Restimulation of mLNs and splenocytes isolated from chronically HDM-exposed mice revealed exacerbated local and systemic IL-5 and IL-17A production in *Batf3*$^{-/-}$ mice (Figure 5, A, B, E, and F). There were no differences in IL-10 production (Figure 5, C and G). Consistent with the data from the acute model, IFN-γ production was impaired in Batf3-deficient mice (Figure 5, D and H). These data suggest that Batf3-dependent DCs, through their contribution to Th1 immunity, might restrain the allergic response and airway inflammation triggered by chronic HDM challenge.

Treg response after HDM challenge is not affected in the absence of Batf3. Previous evidence indicates that Batf3-dependent DCs in the lung contribute to airway homeostasis by inducing Tregs (16), which can suppress Th2 cell–mediated allergic reactions (30). Although IL-10 production did not differ between HDM-restimulated *Batf3*$^{-/-}$ and WT tissues from acutely or chronically exposed mice (Figure 3, C and G, and

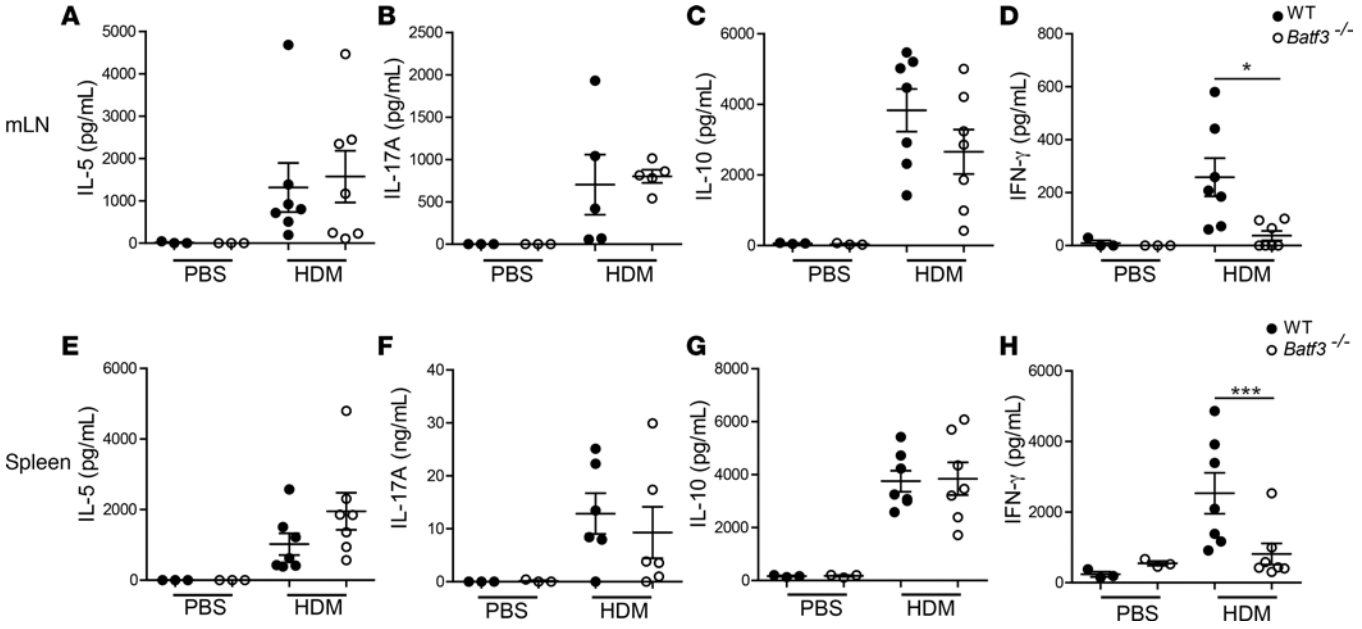

Figure 3. *Batf3*$^{-/-}$ mice show impaired local and systemic IFN-γ production upon acute HDM challenge. mLNs (**A**–**D**) and splenocytes (**E**–**H**) from HDM-sensitized and HDM-challenged mice were restimulated with HDM, and released cytokines were measured by ELISA. Individual data and mean ± SEM from a representative experiment of at least 3 performed (n = 3 PBS, n = 7 HDM); *P < 0.05, ***P < 0.001, Mann Whitney U test. HDM, house dust mite; mLN, mediastinal lymph node.

Figure 5, C and G), we analyzed whether Batf3-dependent DCs promote Treg expansion after allergen exposure. HDM sensitization and acute challenge increased Treg frequencies and total numbers in the lung (FoxP3$^+$CD25$^+$CD4$^+$ T cells) to the same extent in WT and Batf3-deficient mice (Figure 6A). HDM exposure, thus, promotes FoxP3$^+$ Treg differentiation, regardless of the presence of Batf3-dependent DCs.

The induction of adaptive FoxP3$^+$ Treg cells is essential for establishing mucosal tolerance via the respiratory and oral routes, and these cells modulate sensitization to allergens and the severity of chronic inflammation (31). Inducible Tregs (iTregs, ICOS$^+$) differentiate in the periphery as a consequence of antigen exposure (32), whereas natural Tregs (nTregs, Helios$^+$) develop in the thymus (33). Analysis of FoxP3$^+$CD4$^+$ T cells from the lungs of HDM-sensitized and -challenged mice showed a drop in the frequency of Helios$^+$ nTregs and an increased frequency of ICOS$^+$ iTregs; however, these changes were similar in WT and *Batf3*$^{-/-}$ mice, indicating that Batf3 deficiency does not selectively affect iTreg frequency after HDM challenge (Figure 6, B and C).

Although Batf3 deficiency appears not to affect Treg frequencies and numbers, it could affect Treg function. We therefore analyzed CD4$^+$ effector T cells (CD44$^+$ T cells) in the lung for the production of the anti-inflammatory cytokine IL-10, an essential immune regulator in mucosal tissues, such as lung and intestine (34). HDM exposure increased IL-10 production by CD44$^+$ T cells (Figure 6D), but the absence of Batf3 did not impair this effect. Together, these data indicate that Batf3-dependent DCs are neither required for Treg expansion nor involved in IL-10 production by effector CD4$^+$ T cells after airway exposure to HDM.

CD103$^+$ mig-cDC1s are the main DC source of IL-12 in the mLNs upon HDM exposure. The impaired HDM-induced Th1 immunity in the absence of Batf3 prompted us to investigate whether HDM induces IL-12 secretion by Batf3-dependent lung CD103$^+$ cDC1s; these DCs would migrate to the draining mLNs to produce IL-12 that promotes Th1 differentiation, providing a mechanism for dampening the Th2 and Th17 immune response (35, 36). Mice were exposed to a single 100 μg HDM dose, and 3 days later, CD11c$^+$ DCs purified from *Batf3*$^{-/-}$ mLNs showed impaired IL-12p40 mRNA expression (Figure 7A). We also examined mRNA expression of IL-6, a cytokine produced by CD103$^+$ DCs in the intestine (37) and by airway CD11b$^+$ DCs (13). The CD11c$^+$ fraction in mLNs showed no difference in IL-6 mRNA expression between WT and *Batf3*$^{-/-}$ mice (Figure 7B).

Intracellular staining in mLN mig-DCs isolated after HDM challenge confirmed enhanced IL-12p40 production in WT compared with *Batf3*$^{-/-}$ mice (Figure 7C). Among the mig-DC subsets, comprising CD11b$^+$ cDC2s and Batf3-dependent CD103$^+$ cDC1s, CD103$^+$ cDC1s were the main contributors to IL-12p40 produc-

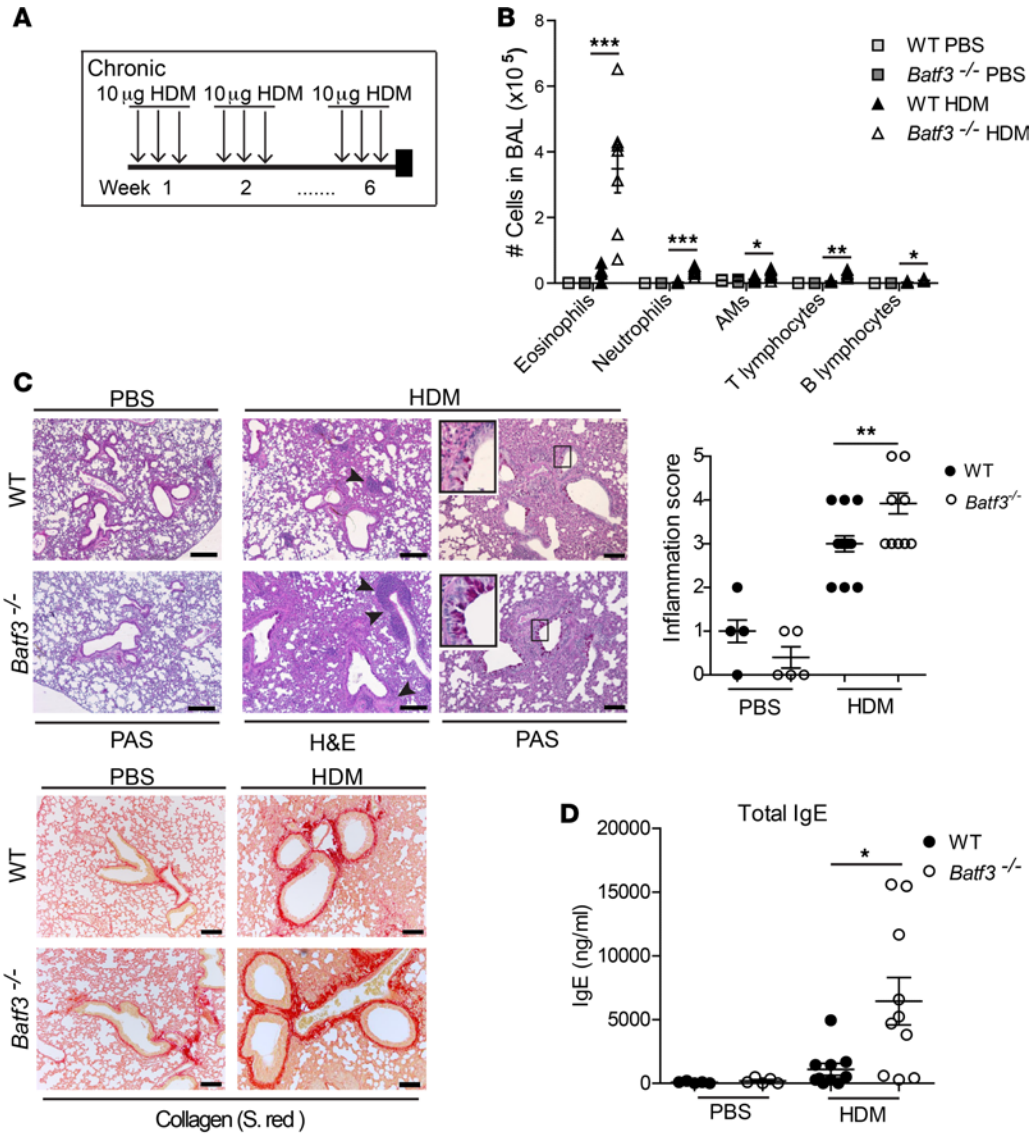

Figure 4. *Batf3*$^{-/-}$ mice develop exacerbated HDM-induced chronic airway inflammation. (**A**) Schematic regime of chronic HDM exposure. (**B**) Inflammatory infiltrates in BAL from the indicated treatments and genotypes (n = 3 PBS, n = 7 HDM). (**C**) Representative histological staining of mice of the indicated genotype chronically exposed to HDM. Top: H&E staining (arrowheads point at perivascular and peribronchial infiltration) and PAS staining (insets depict mucus-producing goblet cells). Bottom: staining for collagen (Sirius red). Scale bars: 200 μm (PAS-PBS or H&E) or 100 μm (PAS-HDM or Sirius red). Right: inflammation score (n = 5 PBS, 9 HDM). (**D–F**) Total serum IgE (**D**) and HDM-specific IgG2a (**E**) and IgG1 (**F**) (n = 5 PBS, n = 10 HDM). (**B–F**) Individual data and mean ± SEM from 1 experiment representative of at least 3 independent experiments; *P < 0.05, **P < 0.01, ***P < 0.001, Mann Whitney U test. HDM, house dust mite; PAS, periodic acid Schiff.

tion, in terms of both frequency and numbers, and both before and after HDM challenge (Figure 7, D and E). Significantly increased production of IL-12p40 by CD103$^+$ cDC1s was also found when mice were exposed to a single 10 μg HDM i.n. dose (Supplemental Figure 3A). CD11b$^+$ mig-DCs were unable to compensate for the impaired IL-12p40 production in Batf3-deficient mice. Notably, CD8α$^+$ res-cDC1s did not produce any IL-12p40 following HDM i.n. challenge in WT or *Batf3*$^{-/-}$ mice (Supplemental Figure 3, B and C). These results indicate that CD103$^+$ cDC1s are key providers of IL-12 in the DC compartment upon HDM challenge.

Exogenous IL-12 is sufficient to suppress exacerbated allergic inflammation. To evaluate whether IL-12 delivery is the essential signal provided by CD103$^+$ cDC1s to restrain an exacerbated Th2 and Th17 response, we administered IL-12 i.n. to *Batf3*$^{-/-}$ mice chronically exposed to HDM. Exogenous IL-12 provision was sufficient to drastically reduce BAL cellularity, mainly eosinophilia, to WT levels (Figure 8A). Similarly, IL-12 was sufficient to decrease total IgE titers in serum (Figure 8B). Notably, the dosing of IL-12 provided was sufficient to decrease HDM-driven IL-5 production locally (mLN) and reduce both IL-5 and IL-17

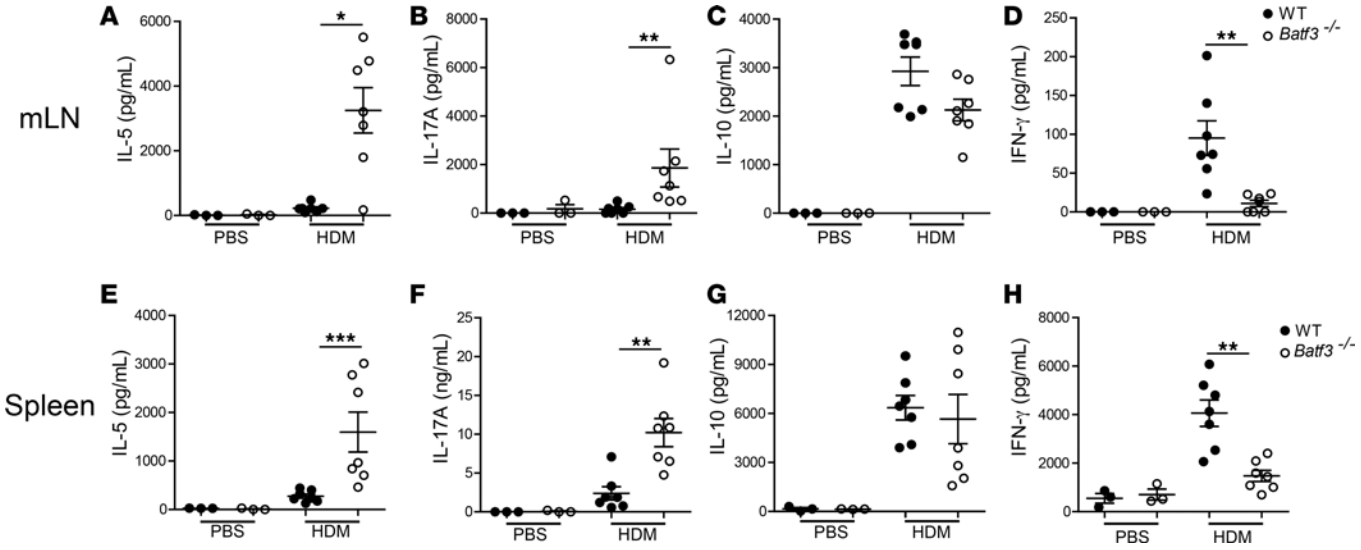

Figure 5. Enhanced Th2 and Th17 immunity upon chronic HDM challenge in *Batf3*$^{-/-}$ mice. mLNs (**A**–**D**) and splenocytes (**E**–**H**) from mice challenged over 6 weeks with HDM (3 × 10 μg doses per week) were restimulated with HDM, and released cytokines were measured by ELISA. Individual data and mean ± SEM from a representative independent experiment of at least 2 performed (n = 3 PBS, n = 7 HDM); *P < 0.05, **P < 0.01, ***P < 0.001, Mann Whitney U test. HDM, house dust mite; mLN, mediastinal lymph node.

production systemically (spleen) (Figure 8, C, D, F, and G). However, it was not sufficient to induce HDM-dependent IFN-γ production locally or systemically (Figure 8, E and H). These results suggest that IL-12 is sufficient to control Th2 and Th17 immune responses in Batf3-deficient mice independently of the induction of Th1 immunity.

Discussion

In recent years, intense research interest has focused on the division of labor among the different pulmonary DC populations and how each contributes to the initiation and maintenance of Th2 immunity and allergen tolerance (9–11). The role of lung CD103$^+$ cDC1s in this context is still debated and poorly understood, with reports suggesting opposing functions (10, 15–17). While some reports suggest that CD103$^+$ cDC1s prime Th2 responses to inhaled allergens (15), others suggest that these cells are not required for Th2 cell–mediated immune responses following exposure to HDM (10). Other findings postulate that CD103$^+$ cDC1s promote airway tolerance through the induction of FoxP3$^+$ Tregs in models of airway inflammation (16) or through a Treg-independent production of IL-10 (17). Here, we identify Batf3-dependent CD103$^+$ cDC1s as a key source of IL-12 upon HDM exposure; IL-12 is known to promote Th1 immunity, restraining Th2 and Th17 immunity and resulting in attenuated allergic airway inflammation.

Our findings are consistent with a previous report indicating that CD103$^+$ cDC1s do not induce Th2 cell–dependent eosinophilic airway inflammation (10). However, another study suggested that pulmonary CD103$^+$ cDC1s prime Th2 responses to inhaled allergens, such as cockroach antigens and HDM (15). The authors found that CD103$^+$ cDC1s prime Th1 differentiation in response to these allergens, but also found a contribution to Th2 and Th17 differentiation. The discrepancy between these findings and ours could be due to many factors, including the determination of cytokine production by coculture of DC subsets with naive CD4 T cells in the presence of anti-CD3 and anti-CD28 antibodies, contrasting with our ex vivo HDM restimulation of mLNs or splenocytes from sensitized and challenged mice. For in vivo studies, Nakano and colleagues used *Ccr7*$^{-/-}$ mice as a model of CD103$^+$ cDC1 deficiency in mLNs; however, these mice also have reduced numbers of CD11b$^+$ mig-DCs (38), which might explain their phenotype because CD11b$^+$ mig-DCs are major contributors to Th2 immunity and HDM-induced allergic airway inflammation (10). The authors also used BXH2/TykJ mice, which have a point mutation in the transcription factor *Irf8*. These mice lack CD103$^+$ cDC1s in the lung and CD8α$^+$ cDC1s in the spleen, but also present other immune and hematopoietic abnormalities that might explain their results (39). The different results might also reflect the effect on outcome of a different allergen dose, batch, and administration regime.

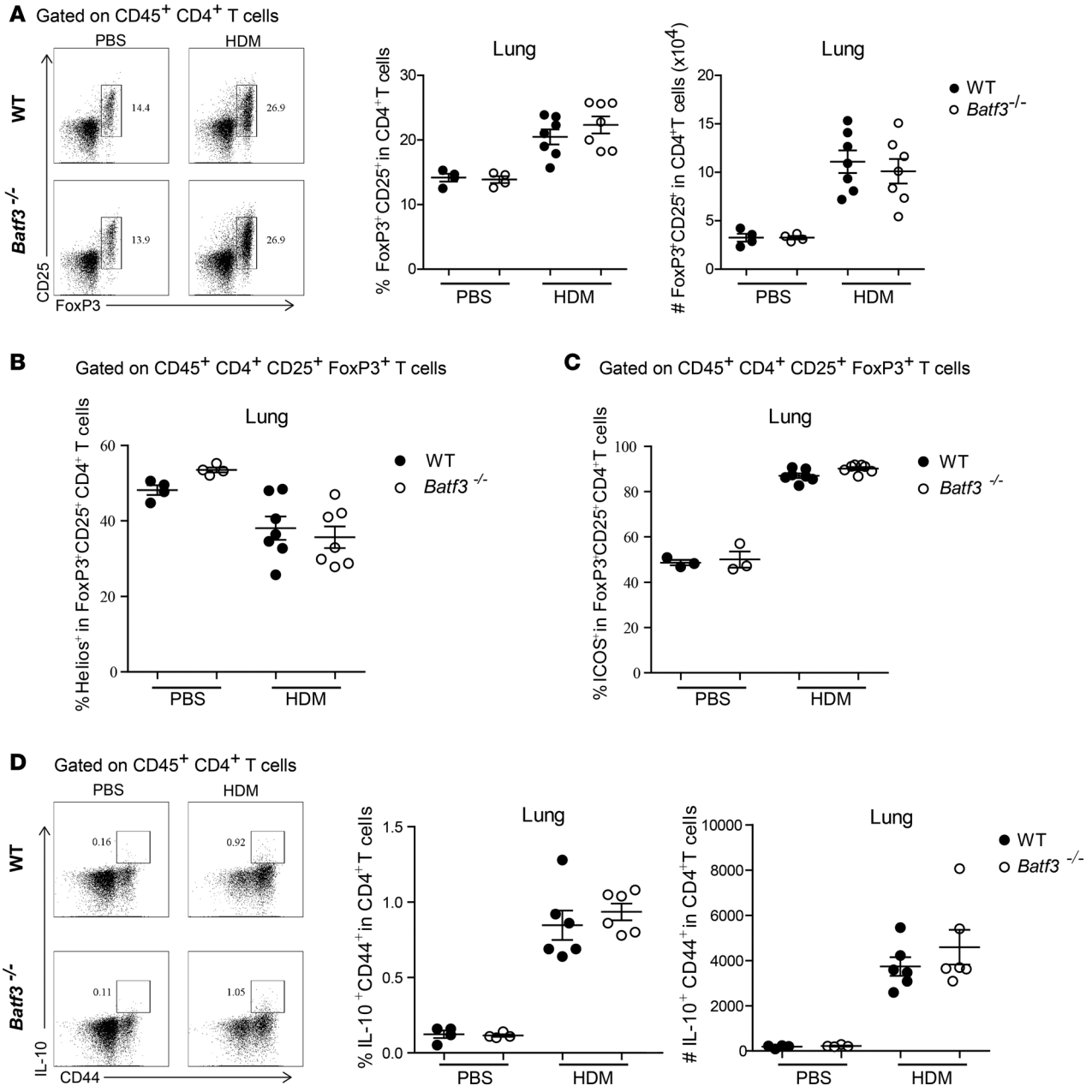

Figure 6. Batf3-dependent DCs are neither required for lung Treg expansion nor necessary for IL-10 production by lung CD4⁺ T cells, following HDM airway exposure. Lungs from WT and *Batf3* $^{-/-}$ mice sensitized and challenged with HDM were collected at day 14. (**A**) Representative staining for FoxP3⁺CD25⁺ Tregs, either depicted as plots (left) or scatter plot graphs (right), representing frequency and number of cells expressing FoxP3 and CD25 (n = 3–4 PBS, n = 6–7 HDM). (**B** and **C**) Frequencies of Tregs expressing Helios and ICOS (n = 3–4 PBS, n = 7 HDM). (**D**) Lung cells were restimulated with anti-CD3 and anti-CD28 for 6 hours in the presence of Brefeldin A for the last 4 hours, and CD4⁺CD44⁺ T cells were analyzed for intracellular IL-10; representative plots (left) and percentages and numbers (right) are shown (n = 4 PBS, n = 6 HDM). (**A–D**) Individual data and mean ± SEM from a representative independent experiment of 3 performed. HDM, house dust mite.

During the course of inflammation in allergic asthma or autoimmune diseases, antigen-specific Tregs are generated that help to limit the damage caused by Th1, Th17, and Th2 cells (40). Both nTregs and iTregs contribute to peripheral immune tolerance in models of asthma; their relative contribution is still unknown, but nonredundant roles have been described and a substantially more tolerogenic role has been postulated for iTregs in this setting (16, 31, 32). In humans, the marker CD141 defines DCs with a function similar to mouse CD103⁺ cDC1s (4, 41). CD141⁺ DCs in the skin have been shown to induce Treg differentiation; however, their role in the lung is still unknown (42). Because FoxP3⁺ Tregs appear to be generated in pulmonary LNs, and then recruited to the lung (29), we analyzed whether lack of Batf3-dependent DCs alters

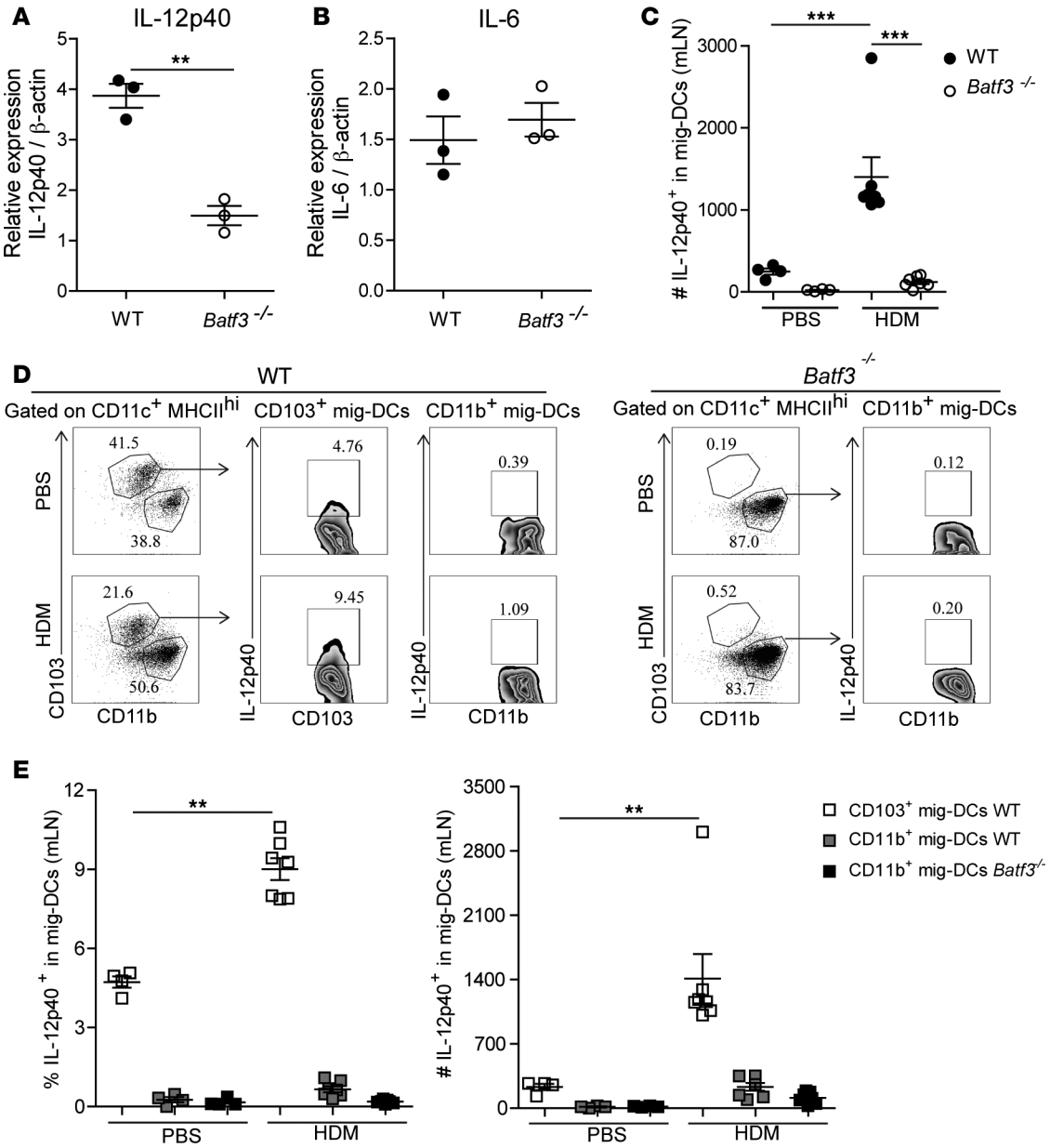

Figure 7. CD103⁺ mig-DCs are the main DC source of IL-12 in mLNs after HDM exposure. Mice were challenged with 100 μg HDM i.n., and mLNs were collected 3 days later. (**A**) IL-12p40 and (**B**) IL-6 mRNA expression was analyzed in purified CD11c⁺ cells, and mRNA was normalized against β-actin. Data shown (mean ± SEM) is a pool of 3 independent experiments; each symbol represents 1 experiment (5–10 mice pooled per experiment); $^{**}P < 0.01$, 2-tailed Student's *t* test. (**C**–**E**) mLN cells were stained for CD11c, MHC class II, CD103, CD11b, and intracellular IL-12p40. (**C**) Numbers of IL-12p40–producing cells in the mLN CD11c⁺MHCII^{hi} fraction. (**D**) Representative plots showing IL-12p40 staining in CD103⁺ and CD11b⁺ mig-DCs. (**E**) Frequencies (left) and numbers (right) of IL-12p40–producing mig-DC subsets. (**C** and **E**) Individual data and mean ± SEM from a representative independent experiment of 3 performed (*n* = 4 PBS, *n* = 7 HDM); $^{**}P < 0.01$, $^{***}P < 0.001$, 1-way ANOVA followed by Bonferroni's post-test (**C**) and Mann Whitney *U* test (**A** and **E**). HDM, house dust mite; mLN, mediastinal lymph node.

Treg induction in the lung after HDM exposure. Our results demonstrate that CD103⁺ cDC1s are not necessary for the expansion of FoxP3⁺ Tregs following i.n. sensitization and challenge with HDM. This result is consistent with findings in other organs, such as gut (19), and other settings, such as helminth infection (43). However, we cannot rule out a role for Batf3-dependent DCs in Treg generation in other settings. For instance, in models of OVA tolerance, pulmonary CD103⁺ cDC1s establish airway tolerance by promoting T cell FoxP3 expression (16). It has been proposed that nTregs primarily target autoimmune responses, whereas iTregs modulate the response to exogenous antigens (32). However, our model showed no evidence for a role of Batf3-dependent DCs in iTreg induction. We also examined the potential involvement of IL-10

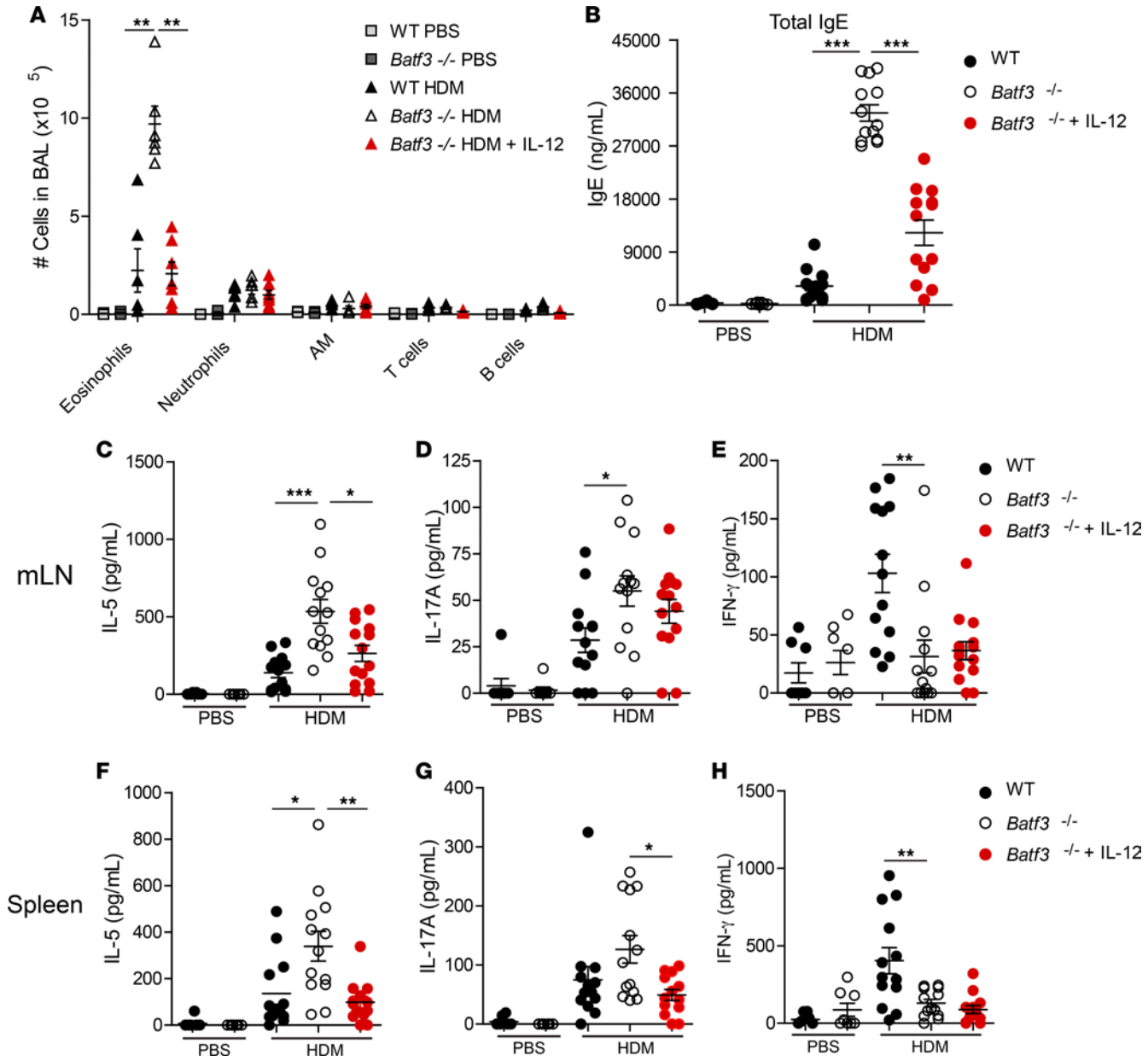

Figure 8. Intranasal administration of IL-12 dampens allergic airway inflammation in *Batf3*$^{-/-}$ mice chronically exposed to HDM. WT and *Batf3*$^{-/-}$ mice were challenged for 6 weeks with HDM, as depicted in Figure 4A. *Batf3*$^{-/-}$ mice subjected to this chronic HDM challenge were treated or not with IL-12p70 (i.n.), as indicated in Methods. (**A**) Inflammatory infiltrates in BAL (n = 4 PBS, n = 7 HDM). (**B**) Total serum IgE was determined in serum by ELISA (n = 7 PBS, n = 13 HDM). mLNs (**C–E**) and splenocytes (**F–H**) were restimulated with HDM, and the indicated released cytokines were measured by ELISA (n = 6 PBS, n = 13 HDM). One representative experiment (**A**) or a pool of 2 independent experiments (**B–H**) of 3 performed is shown. Symbols denote individual mice, and lines represent mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001, Mann Whitney U test. HDM, house dust mite; mLN, mediastinal lymph node.

because its potent immunosuppressive action is crucial not only for establishing peripheral tolerance and blocking allergic inflammation and Th17 responses, but also for protecting the host from exaggerated inflammatory responses in different contexts (29, 44, 45). However, in our model, CD103$^+$ cDC1 has no effect on IL-10 production by CD4$^+$ T cells in the lung.

The expression of IL-12 by lung CD103$^+$ mig-DCs in the steady state (46) prompted us to explore the potential involvement of this cytokine in Batf3-dependent actions during allergen-induced immune activation. Our results demonstrate that HDM-challenged CD103$^+$ cDC1s upregulate the expression and secretion of IL-12p40. This cytokine directs differentiation of naive T cells toward the Th1 effector lineage (47). Recent reports have established CD103$^+$ DCs as a major producer of IL-12, driving protective

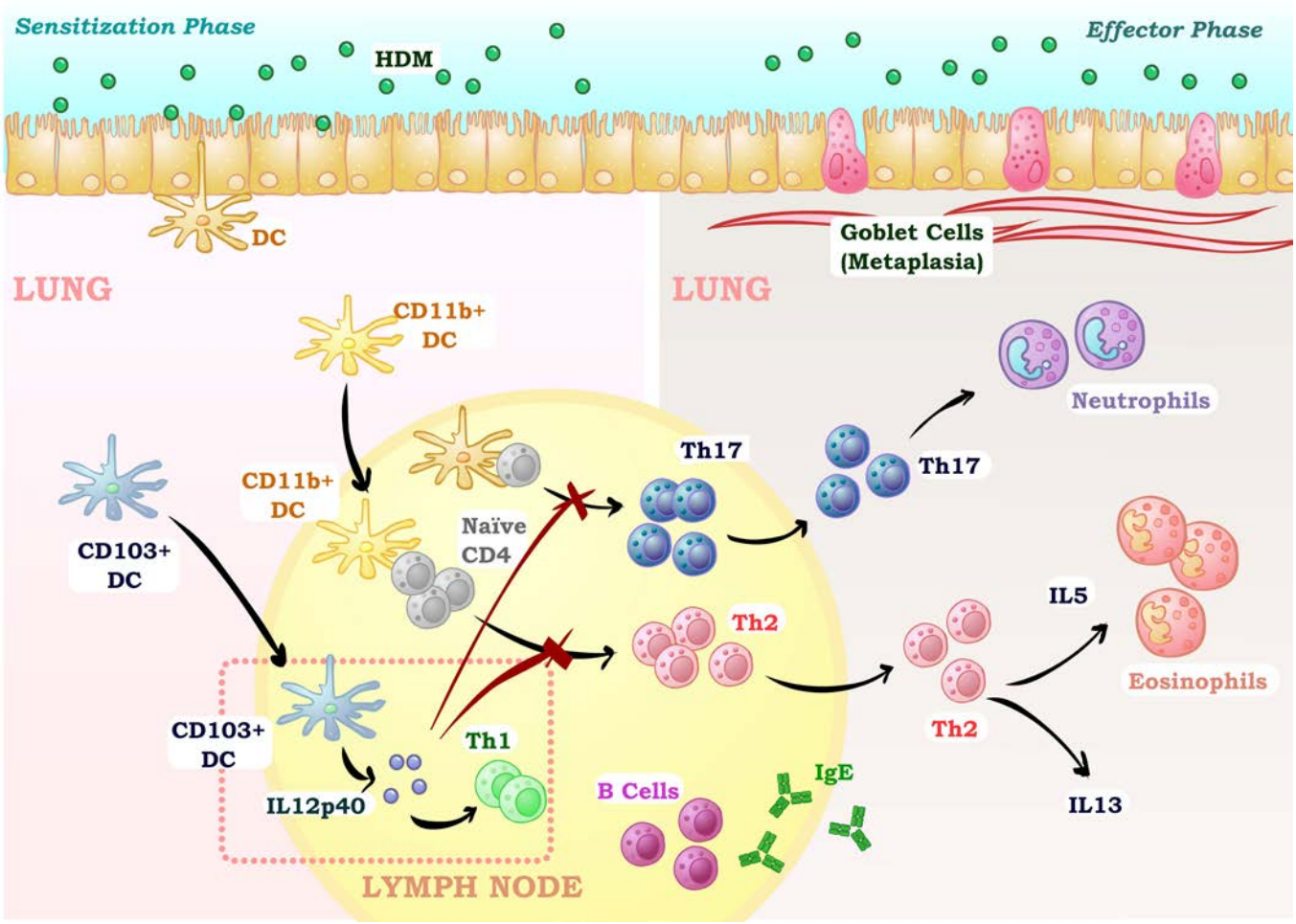

Figure 9. Proposed function of pulmonary CD103⁺ DCs in HDM-induced asthma. Upon HDM exposure, CD103⁺ cDC1s migrate from the lung to the mLNs, where they produce IL-12 that restrains Th2 and Th17 immune responses and contributes to Th1 differentiation. Batf3-dependent DCs might, thus, play a protective role in HDM-induced airway inflammation, dampening the classical features of asthma, including eosinophilia, mucus secretion, and IgE production.

Th1 immunity against several pathogens, including *Toxoplasma gondii* (48) and *Leishmania major* (49). Similarly, IL-12 production by CD103⁺ mig-DCs is important for suppressing helminth-driven Th2 immunity and, thus, controlling Th2-associated immunopathology during chronic *Schistosoma mansoni* infection (43). Our data indicate that exogenous administration of IL-12 results in restrained Th2 and Th17 immune responses, even in the absence of Th1 immunity, suggesting a protective role for CD103⁺ cDC1s in airway inflammation through IL-12 production and independently of the generation of Th1 responses. This is in agreement with previous reports showing that IL-12 exerts a direct inhibitory effect in Th2 and Th17 polarization in allergic airway inflammation (50, 51). The absence of IL-12 in *Batf3*$^{-/-}$ mice, thus, likely explains the skewed Th2 and Th17 immune response and the lung pathology developed following chronic HDM exposure. We do not find an increased allergic phenotype in the acute model in the absence of Batf3-dependent DCs, consistent with a redundant role for these cells in triggering Th2 responses (10). However, in the long-term, absence of IL-12 produced by Batf3-dependent DCs might account for exacerbated and "unbalanced" Th2 and Th17 responses, indicating the crucial role of these cells in controlling severe chronic asthma. Interestingly, exogenous IL-12 inhibits antigen-induced airway hyperresponsiveness and other features of asthma, even when delivered during the challenge phase in sensitized mice (50). Genetically engineered DCs constitutively expressing IL-12 induce Th1 responses to inhaled antigen, preventing allergic airway inflammation; however, these DCs did not prevent eosinophilic inflammation when transferred to previously sensitized mice (52). As discussed above, different functions have been attributed to cDC1s depending on the nature of the setting/insult (including pathogens, harmless particles, and homeostasis). CD103⁺ cDC1s control type 1 immune response against viruses and intracellular pathogens inducing Th1

cells. Their production of IL-12 is crucial for many of these functions (7, 43, 48, 49). However, they also induce Tregs in response to oral antigens (7) and mediate induction of Tregs in tolerized mice in an OVA model (16). This suggests that — depending on the nature of the insult, administration regime, and delivery route — CD103$^+$ cDC1s can exert different functions. This is in agreement with our finding that CD103$^+$ cDC1s, in the context of the clinically relevant allergen (HDM) that promotes IL-12 production in cDC1s, play a protective role in restraining Th2 and Th17 immunity and airway inflammation. Nevertheless, we cannot rule out a different function for these DCs in a different setting or in the presence of a different allergen that, for example, does not induce IL-12 production by cDC1s.

Translating our results into human patients, a high heterogeneity in CD4$^+$ T cell responses (Th1/Th2 and Th17) and dysregulated Th1/IFN-γ immune response in severe asthma have been reported (53, 54). However, IL-12 administration tested in clinical trials for the treatment of asthma did not improve airway responsiveness to inhaled allergens and rather resulted in adverse effects (55). Our results indicate that IL-12 is essential for a balanced immune response in the context of asthma to HDM allergen, being crucial to control exacerbated allergic airway inflammation. Notably, our results suggest that an adequate dosing of IL-12 could result in modulation of Th2 and Th17 immunity without inducing pro-inflammatory Th1 responses. Our findings establish a role for pulmonary CD103$^+$ cDC1s in the production of IL-12 and modulation of Th2 and Th17 immunity in a noninfectious setting (Figure 9). These results highlight the importance of tight regulation of the immune system in order to preserve the function and fate of the different cell populations that ultimately dictate disease outcome. Novel strategies selectively targeting CD103$^+$ cDC1s could potentially alter the balance of allergen-induced immunity subtly and pave the way toward innovative immunotherapy for allergic airway inflammation.

Methods

Supplemental Methods are available online with this article.

Mice. *Batf3*$^{-/-}$ mice backcrossed more than 10 times to the C57BL/6 background were provided by Kenneth M. Murphy (Washington University, St. Louis, Missouri, USA) (18, 19) and further backcrossed with C57BL/6 mice at the CNIC animal facility to establish WT and *Batf3*$^{-/-}$ colonies. Animals were housed and bred in specific pathogen–free conditions.

HDM extracts, asthma models, and in vivo treatment with recombinant murine IL-12. HDM (*Dermatophagoides pteronyssinus*) extracts (Greer Laboratories) were used throughout the experiments. For the acute model, mice were sensitized i.n. with 1 μg HDM on day 1 and subsequently challenged with 10 μg HDM on days 7–11 in 50 μl PBS under light anesthesia (Figure 1A) (25). On day 3 after the last i.n. challenge (day 14 after sensitization), BAL fluid, lungs, mLNs, spleens, and serum were collected. For the chronic model, mice were i.n. challenged with 10 μg HDM 3 times per week for 6 weeks, and organs were collected 3 days after the last i.n. challenge (Figure 4A). In some experiments, in parallel, *Batf3*$^{-/-}$ mice received, on the same day of HDM challenge, an i.n. administration of recombinant murine IL-12p70 (eBioscience, catalog 34-8121; 3 × 50 ng dose per week for 3 weeks, followed by 2 × 75 ng dose per week for 2 weeks). To evaluate the production of different cytokines by DCs in mLNs, mice were i.n. challenged with a high dose of HDM (100 μg), and mLNs were harvested 3 days later.

Cell isolation and restimulation. BAL was performed by 3 consecutive flushes of the lung through the trachea with 700 μl of DMEM; cells were counted by trypan blue exclusion, and the cell profile was determined by flow cytometry. Lungs, mLNs, and spleens were collected in RPMI, and lungs and mLNs were enzyme digested. mLN cells were plated at 2 × 10^5 cells/well in 96-well round-bottom plates and restimulated for 5 days with 30 μg/ml HDM extract in FBS-supplemented RPMI. Similarly, splenocytes were plated at 5 × 10^5 cells/well and restimulated for 3 days. Cytokine production was measured by ELISA in culture supernatants.

Cells for flow cytometry analysis of lung infiltrates were prepared by systemic perfusion of lungs with saline. Intracellular IL-10 in lung T cells was detected after polyclonal restimulation with plated anti-CD3 (145-2C11, 10 μg/ml, Bio X Cell) and soluble anti-CD28 (37.51, 5 μg/ml, Bio X Cell) in 96-well plates for 6 hours, the last 4 hours in the presence of Brefeldin A (5 μg/ml; Sigma-Aldrich).

Flow cytometry. Stainings were performed at 4°C with the appropriate antibody cocktail in cold PBS supplemented with 2.5% FBS, 2 mM EDTA and 0.1% sodium azide. Samples were processed with a BD FACSCanto flow cytometer using FACSDiva software, and data were analyzed with FlowJo software (Tree Star). CD16/CD32 (2.4G2, Tonbo Biosciences) was used to reduce nonspecific binding. To determine

the cellular composition of BAL, cells were stained with fluorochrome-labeled antibodies directed against CD11c (HL3), Ly6G (1A8), CD11b (M1/70), Siglec-F (E50-2440), CD3 (17A2), and CD19 (1D3), purchased from BD Bioscience, and Ly6C (HK1.4) from eBioscience. For detection of Tregs and IL-10 production in the lung, cells were stained with CD45 (30-F11) purchased from BD Bioscience; CD4 (RM4-5) and CD25 (PC61.5) from Tonbo Biosciences; CD44 (IM7), FoxP3 (FJK-16s), and IL-10 (JESS-16E3) from eBioscience; and ICOS (7E.17G9) and Helios (22F6) from BioLegend. For intracellular staining, cells were fixed for 10 minutes in 4% paraformaldehyde-PBS at room temperature, and staining was conducted for 40 minutes at 4°C in permeabilization buffer (1% BSA [Sigma-Aldrich], 0.1 % saponin [Sigma-Aldrich], and 0.2% sodium azide [Sigma-Aldrich]). FoxP3 and Helios in T cells were detected using the FoxP3 staining buffer set from eBioscience. For detection of cDCs in lung and mLNs, the following fluorochrome-labeled antibodies were used: CD45 (30-F11), Siglec-F (E50-2440), I-A/I-E (MHCII, clone 2G9), CD11c (HL3), CD11b (M1/70), and CD103 (M290), from BD Biosciences, and CD8α (53.6-7) from Tonbo Biosciences. Hoechst 33258 (0.1 μM) was used as a counterstain to exclude dead cells.

For intracellular IL-12p40 staining ex vivo, mice were i.p. injected with Brefeldin A (125 μg/mouse) 60 hours after i.n. challenge with high-dose HDM (100 μg), and mLNs were collected 12 hours later (72 hours after challenge) in RPMI containing Brefeldin A (5 μg/ml). Single-cell suspensions were prepared from mLNs as described above, and cells were stained for cDC surface markers, fixed and permeabilized, and stained with anti-IL-12p40 mAb (C17.8) from Tonbo Biosciences.

ELISA. HDM-specific IgG1 and IgG2a and total IgE were determined in serum samples from blood collected at the end of the experiments. Briefly, for HDM-specific IgG1 and IgG2a, high binding plates were coated overnight at 4°C with 5 μg/ml HDM in carbonate buffer, blocked with 10% FBS, and incubated with serial serum dilutions. HDM-specific immunoglobulins were detected with biotin rat anti-mouse IgG2a (R19-15) and biotin rat anti-mouse IgG1 (A85-1), both from BD Bioscience. Signal was developed by incubation with streptavidin-HRP (Sigma-Aldrich), standard TMB development (KPL), and optical density read at 450 nm. Total serum IgE was quantified with capture mAb (purified rat anti–mouse IgE [R35-72]) and detection mAb (biotin-conjugated anti–mouse IgE [R35-118]) from BD Biosciences. Readings were taken from a standard curve prepared with purified mouse IgE (27-74).

Cytokine production was quantified in mLN and splenocyte culture supernatants by ELISA. IL-5, IL-10, and IFN-γ were measured with OptEIA ELISA kits from BD Biosciences, according to the manufacturer's instructions. IL-17A production was quantified using capture mAb (rat anti–mouse IL-17A [TC11-18H10]) and detection mAb (biotin-conjugated IL-17A [TC11-8H4]) both from BD Pharmingen or the mouse IL-17A ELISA Ready-Set-Go! from eBioscience. The standard curve was prepared with purified mouse IL-17 (Peprotech).

qPCR. Single-cell suspensions were prepared from mLNs, and cells from 5-10 mice were pooled for each biological replicate. Cell suspensions were enriched for DCs by immunomagnetic isolation using mouse anti-CD11c microbeads (N418) (Miltenyi Biotec). mRNA was extracted with the RNeasy Plus Micro kit (Qiagen, catalog 74034). RNA concentration was determined with a Nanodrop 1000 spectrophotometer (Thermo Scientific) and RNA integrity assessed with an Agilent 2100 Bioanalyzer (Caliper Life Science). Samples with RNA integrity values > 8 were retained for further processing. RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reverse transcription PCR was carried out in a C1000 Thermal Cycler (Bio-Rad). Quantitative PCR (qPCR) was performed with the GoTaq qPCR master mix (Promega) in a 7900-FAST-384 instrument (Applied Biosystems). All reactions were performed in a 20 μl volume. The housekeeping gene *β-actin* was used to verify equal cDNA loading. qPCR for *β-actin*, *IL12p40*, and *IL6* was performed using primers from Sigma-Aldrich. Primers used for SYBR Green assays were as follows: β-actin sense, 5′ – GGCTGTATTCCCCTCCATCG – 3′, β-actin antisense, 5′ – CCAGTTGGTAACAATGCCATGT – 3′; IL-12p40 sense, 5′ – GGAAGCACGGCAGCAGAATA – 3′, IL-12p40 antisense, 5′ – AACTTGAGGGAGAAGTAGGAATGG – 3′; IL-6 sense, 5′– CCGTGTGGTTACATCTACCCT – 3′, IL-6 antisense, 5′– CGTGGTTCTGTTGATGACAGT – 3′. Relative cytokine expression was determined by comparison with *β-actin* expression.

Histology. Lungs were harvested 3 days after the final i.n. challenge with HDM and immediately fixed in 10% neutral buffered formalin. Paraffin-embedded tissues were cut into 5-μm–thick sections and stained with H&E to evaluate airway inflammation and hematoxylin-counterstained periodic acid-Schiff (PAS) to analyze mucus production. Slides were scored on a 0–5 scale for inflammation. Sections were examined with a Leica DM 2500 light microscope and images captured with a Leica DFC 420 digital camera.

Statistics. Statistical comparisons were made with Prism software v5 (GraphPad Software). Unless indicated otherwise in the figure legend, differences between 2 groups were calculated with the Mann-Whitney *U* test for unpaired data, and differences between 3 or more groups were calculated with one-way ANOVA followed by Bonferroni's Multiple comparison test. Data were expressed as mean ± SEM. Differences were considered significant at $P < 0.05$ ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$).

Study approval. All animal procedures were reviewed and approved by Animal Ethics Committee at the CNIC, Madrid Autonomous University Ethics Committee, and the Community of Madrid authority. All animal procedures conformed to EU Directive 2010/63/EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

Author contributions

LC and DS conceptualized the study; LC helped with methodology; LC, SCK, SMC, HMI, and PB helped with investigation; LC and DS helped with writing, supervision, and funding acquisition.

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