

Development of eosinophilic inflammation is independent of B–T cell interaction in a chronic house dust mite-driven asthma model

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Summary

Background Chronic exposure to environmental triggers, such as house dust mite (HDM), drives T helper 2 (Th2) cell-mediated asthma. Recent evidence has shown that B–T cell interaction, and in particular germinal centre reactions and follicular T helper (Tfh) cells are required for the development of eosinophilic airway inflammation in HDM-driven models containing a sensitization and challenge phase. Whether B–T cell interactions are essential for pulmonary eosinophilic inflammation following chronic allergen provocation remains unknown.

Aims In this study, we investigated the importance of B–T cell interaction in the development of eosinophilic airway inflammation and pulmonary remodelling in a chronic HDM-driven asthma model.

Methods We exposed C57BL/6, *Cd40l*^{−/−}, and *Mb1*^{−/−} mice to HDM three times a week for five consecutive weeks.

Results Chronic HDM exposure induced a pronounced eosinophilic allergic airway inflammation in broncho-alveolar lavage fluid (BALf) and lung tissue, associated with the formation of immunologically active inducible bronchus-associated lymphoid tissue (iBALT) in the lungs. The absence of B cells or lack of CD40L signalling did not hamper eosinophilic inflammation in the airways, although the number of Tfh and Th2 cells was substantially reduced in the lungs. Importantly, type 2 innate lymphoid cell (ILC2) numbers in BALf and lung were not affected by the absence of B cells or B–T cell interaction. Furthermore, eosinophilic airway inflammation is not sufficient to induce pulmonary remodelling and airway hyperresponsiveness.

Conclusion and Clinical Relevance From these findings, we conclude that B–T cell interaction is required for robust Tfh and Th2 cell induction, but not essential for eosinophilic airway inflammation during a chronic HDM-driven asthma model.

Keywords asthma, B cells, house dust mite, Tfh cells, Th2 cells

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Introduction

Asthma is a chronic inflammatory disease of the airways, which is initiated by exposure to environmental factors in susceptible individuals [1]. Asthma is defined by airway hyperresponsiveness (AHR) of the bronchioles to various stimuli, such as house dust mite (HDM), leading to increased mucus production and airway remodelling. T helper (Th) 2 cell cytokines produced by both Th2 cells (IL-4, IL-5, and IL-13) and type 2 innate lymphoid cells (ILC2s) (IL-5 and IL-13) are critically involved in allergic airway inflammation [2], whereas in non-allergic asthma also Th17 cells are implicated.

Th2 cytokines facilitate classical allergic responses, such as IgE class switching by B cells (IL-4), eosinophilic infiltration (IL-5), and goblet cell hyperplasia (IL-13) [2, 3]. Th17 cytokines can also contribute to asthma symptoms as they increase smooth muscle cell proliferation (IL-17A/IL-22) and collagen deposition (IL-17A) [4]. Tregs on the contrary will dampen these pro-inflammatory Th2 and Th17 cells [3].

In allergic asthma, B cells are well known for their allergen-specific IgE secretion, which exacerbates airway inflammation and induces airway narrowing through mast cell activation [5]. However, beyond their antibody secretion capacities, B cells can also present

antigens, produce cytokines, regulate T cell-mediated immune responses, and induce the formation of tertiary lymphoid structures [6, 7]. The functional roles and importance of B cells in allergic airway inflammation have been controversial. Several studies in B cell-deficient mice have shown that B cells are dispensable for eosinophilic airway inflammation [8–12]. However, it was recently shown in a HDM-driven asthma model that B cells synergize with dendritic cells (DCs) to promote the differentiation of IL-4-committed follicular T helper (Tfh) cells into memory Th2 cells during the HDM sensitization phase [13]. Tfh cells have gained a lot of interest in HDM-driven asthma models that contain a sensitization and challenge phase, as they can either develop into effector Th2 cells [13] or promote the differentiation of effector Th2 cells [14]. However, asthma patients are continuously exposed to allergens, whereby sensitization and challenge phases cannot be distinguished. Therefore, we wondered whether B cells, B–T cell interaction, and Tfh cells are similarly important in an asthma model with chronic HDM provocation.

We found that exposing mice for 5 weeks with HDM induced a prominent adaptive immune response, characterized by B cell activation, development of Tfh cells, and germinal centres (GCs) containing inducible bronchus-associated lymphoid tissue (iBALT) structures. Surprisingly, eosinophilic airway inflammation was normally present in HDM-exposed B cell-deficient *Mb1*^{−/−} mice and *Cd40l*^{−/−} mice, but unexpectedly had reduced pulmonary Tfh and Th2 cell numbers.

Materials and methods

Mice

Male and female C57/BL6, *Cd40l*^{−/−}, and *Mb1*^{−/−} mice [15, 16] were bred and housed at the animal facility of the Erasmus MC Rotterdam. Mice were housed under specific pathogen-free conditions and were ~12–16 weeks at time of analysis. All experiments were approved by the animal ethical committee of the Erasmus MC (EMC3329).

HDM-induced allergic airway inflammations

During HDM exposures, mice were anesthetized using isoflurane and sensitized intranasally with 25 µg/40 µL HDM (Greer) or 40 µL PBS as a control (GIBCO Life Technologies, Carlsbad, CA, USA) three times per week, for five consecutive weeks. Mice were killed for analysis at day 37 and day 65. Lung function was measured following increasing doses of nebulized methacholine (0.4–25 mg/mL) using a restrained whole-body plethysmograph (EMKA) under urethane sedation. Broncho-

alveolar lavage fluid (BALf) was obtained by flushing the lungs three times with 1 mL PBS containing 0.5 mM EDTA (Sigma-Aldrich, Darmstadt, Germany). Lungs were inflated with either PBS/OCT (1 : 1) solution, snap-frozen in liquid nitrogen, and kept at −80°C until further processing, or placed in 4% PFA and embedded in paraffin, or homogenized for collagen measurements.

Flow cytometry procedures

Single-cell suspensions were prepared from BALf, lungs, and mediastinal lymph node (MLN) using standard procedures. Single-cell suspensions were obtained from lungs by digesting the lungs using DNase and Liberase for 30 min at 37°C [17]. Upon digestion, the lungs were homogenized through a 100-µm cell strainer (BD Biosciences, San Jose, CA, USA). Lung red blood cells were lysed using osmotic lysis buffer (8.3% NH₄CL, 1% KHCO₃, and 0.04% NA₂EDTA in Milli-Q). Flow cytometry surface and intracellular staining procedures have been described previously [18]. Monoclonal antibodies used for flow cytometric analyses are listed in Table S1. For all experiments, dead cells were excluded using Live/Dead Aqua (Invitrogen, Maltham, MA, USA). For cytokine production measured by flow cytometry, cells were stimulated at 37°C using 10 ng/mL PMA (Sigma-Aldrich), 250 ng/mL ionomycin (Sigma-Aldrich), and GolgiStop (BD Bioscience) for 4 h. Data were acquired using a LSR II flow cytometer BD Biosciences, San Jose, CA, USA and FACSDiva™ software and analysed by FlowJo version 9 (Tree Star Inc software, Ashland, OR, USA).

Lung histology

Five-µm-thick paraffin-embedded or snap-frozen lung sections were stained with haematoxylin/eosin or periodic acid-Schiff (PAS). For lung eosinophil staining, 5-µm-thick lung sections were fixed in acetone and incubated for 1 h with SigleF-F-PE (BD, Biosciences, clone E50-2240). After washing, slides were incubated for 30 min with anti-PE-PO (Rockland). Sections were counterstained with haematoxylin. Peribronchial eosinophils were counted in 4 bronchi/slide by two independent researchers.

For confocal imaging, 10-µm-thick lung sections were fixed in acetone and incubated for 1 h with primary antibodies (Table S2). After washing, slides were incubated for 30 min with secondary antibodies (Table S2). Where necessary, slides were incubated with 10% normal rat serum or 10% normal donkey serum to prevent unspecific binding of antibodies. Slides were counterstained with DAPI and analysed on a Zeiss Meta311 confocal microscope. Images were analysed using ImageJ software.

Hydroxyproline assay

Collagen quantification was performed on the right post-caval lobe by hydroxyproline assay as previously described [19]. Hydroxyproline content may be converted to collagen (1 µg hydroxyproline corresponds to 6.94 µg collagen).

ELISA

Both total IgE and antigen-specific IgE were measured in serum and BALf (Opteia; BD Biosciences). KC levels were measured in BALf (DuoSet; R&D systems, Minneapolis, MN, USA).

Statistical analysis

Mann–Whitney *U*-tests were used for comparison between two groups, and a *P*-value of <0.05 was considered statistically significant. All analyses were performed using Prism (GraphPad Software, La Jolla, CA, USA).

Results

Chronic HDM exposure induces eosinophilic inflammation and remodelling in the airways

To determine the inflammatory kinetics in a clinically relevant chronic HDM-induced asthma model, C57BL/6 mice were treated with 25 µg HDM or PBS 3 days per week for 5 consecutive weeks and analysed 4 days (day 37) or 32 days (day 65) after the last HDM provocation (Fig. 1a). HDM exposure increased eosinophil numbers in BALf and lung tissue on day 37 compared with PBS-treated controls (Fig. 1b and f and Fig. S1). Furthermore, neutrophil, B cell, Th cell, and ILC2 numbers were also increased in the BALf on day 37 compared to PBS-treated controls (Fig. 1b–d, Fig. S1–S2). IL-4-, IL-5-, and IL-13-secreting Th cells were enhanced upon HDM treatment, whereas no increase in IL-17-secreting Th cells was observed (Fig. 1e).

Remodelling of the airways is an essential feature of asthmatic disease and involves fibrosis, goblet cell hyperplasia, and smooth muscle cell hypertrophy, resulting in an increased AHR [20]. Repetitive HDM exposures induced long-lasting airway remodelling, as demonstrated by the presence of mucus-producing goblet cells (Fig. 1h) and smooth muscle cells (Fig. 1i), AHR (Fig. 1j), and increased lung collagen content (Fig. 1k). On day 65, the type 2 inflammation was no longer detectable in the BALf (Fig. 1b and e).

Together, these data show that chronic HDM provocation induces specifically robust but transient type 2 airway inflammation, as well as airway remodelling that was maintained over time.

Chronic HDM exposure induces development of iBALT in the lungs

Given the evidence that iBALT areas are increased in asthmatic patients [21], we wondered whether repetitive HDM provocations would induce iBALT formation in our mouse model.

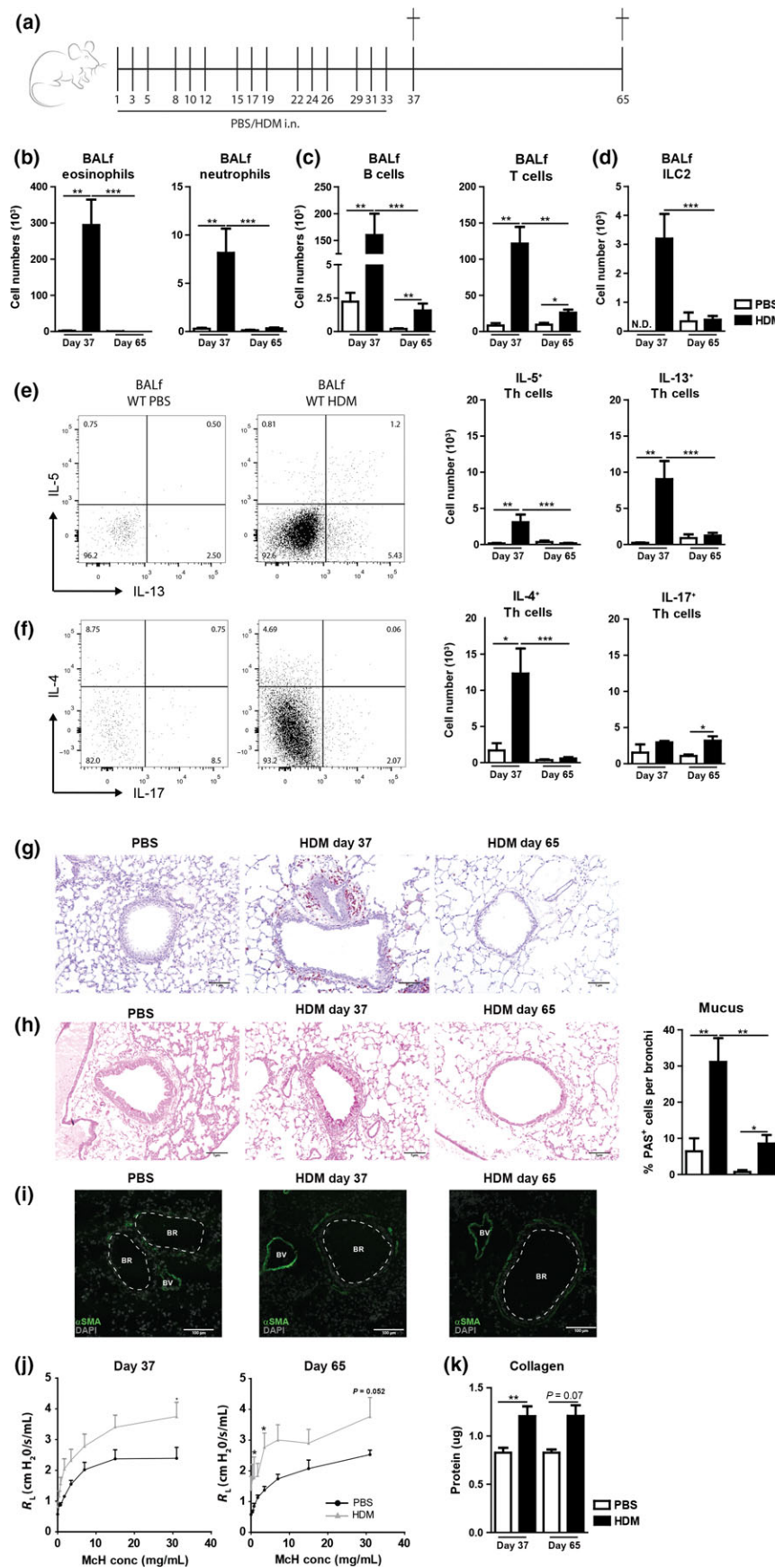
Lungs of HDM-treated mice showed dense cellular infiltrates that were associated with bronchioles and blood vessels at day 37 (Fig. 2a and b), with similarities to iBALT structures observed during influenza infection [22, 23]. These HDM-induced structures contained large B cell follicles adjacent to T cell areas and CD11c⁺ antigen-presenting cells (APCs) (Fig. 2c–d). In contrast, in HDM-driven asthma models containing a sensitization and a short challenge phase, iBALTs were not observed and B cell numbers in both BALf and lungs were 10 times lower as compared to chronic HDM treatment (Fig. S3). These findings suggest that B cells play a more prominent role in chronic than in acute HDM-driven asthma models.

iBALTs of chronic HDM-provoked mice contained GC B cells surrounded with plasma cells. IgE-expressing GC B cells and plasma cells were found in association with B cell follicles (Fig. 2e–g and Fig. S4A). Both total IgE and HDM-specific IgE were induced in BALf and serum (Fig. 2h–i). T cells were also located in HDM-induced iBALT structures and could be detected inside the B cell follicles, indicative of Tfh cells (Fig. 2c). An accordingly significant increase in differentiated CXCR5⁺PD1⁺ Tfh, GATA3⁺ Th2, RORγT⁺ Th17, and FOXP3⁺CD25⁺ Tregs was observed in the lungs of HDM-treated mice compared with control mice (Fig. 2j, Fig. S4B), whereas naïve CD62L⁺ Th cells were not increased (Fig. S5).

Taken together, these data show that chronic HDM treatment induced active iBALT structures containing both Tfh cells and GC B cells, associated with IgE plasma cell formation locally in the lungs.

Germinal centre B cells and Tfh cells are induced in the MLN upon chronic HDM exposure

GC reactions and Tfh cells were shown to be important in Th2 differentiation in acute HDM-driven asthma models [13, 14]; therefore, we investigated whether GCs and Tfh cells were also induced in the MLN of chronic HDM-exposed mice. On day 37, a strong increase in total B cells (Fig. 3a), GL7⁺CD95⁺ GC B cells (Fig. 3b and c), and CD138⁺ plasma cell numbers (Fig. 3d and e) was observed in the MLN of HDM-treated animals compared with PBS-treated mice. HDM treatment also increased the total number of Th cells (Fig. 3f), Tfh cells (Fig. 3g and h), Th2 cells, Th17 cells (Fig. 3i–k), and Treg cells (Fig. 3l and m). B and T cell inflammation



remained enhanced on day 65 in HDM-treated mice, but GC B cells and plasma cells were significantly reduced compared with day 37 of HDM-treated mice (Fig. 3a–m).

Thus, chronic HDM exposures also induce robust GC reactions in the lung-draining MLN.

Eosinophilic inflammation is not dependent on B–T cell interactions

Given that HDM provocations induced a strong GC response and iBALT formation, we wondered whether iBALT formation and B–T cell interactions within GCs are essential for the development of eosinophilic airway inflammation. Therefore, we exposed B cell-deficient mice (*Mb1*^{−/−}) and mice deficient for CD40L signalling (*Cd40l*^{−/−}) to our chronic HDM-driven asthma model. *Mb1*^{−/−} mice are deficient in the expression of Iga/CD79a molecule, an essential transducer of B cell receptor signals. As a result, B cell differentiation is abrogated in the bone marrow, and mature B cells are lacking [15]. *Cd40l*^{−/−} mice lack expression of the CD40L molecule; thus, CD40L–CD40 interaction between T and B cells is hampered, and as a consequence, these mice fail to develop GCs, and class switch recombination is absent [16, 24].

Surprisingly, both HDM-treated *Cd40l*^{−/−} and *Mb1*^{−/−} mice developed BALF eosinophilic inflammation compared to HDM-exposed wildtype controls as assessed at day 37 (Fig. 4a). Neutrophil numbers in HDM-treated *Cd40l*^{−/−} and *Mb1*^{−/−} mice were also not different from HDM-treated WT mice; *Mb1*^{−/−} mice even showed increased neutrophil numbers compared with *Cd40l*^{−/−} mice (Fig. 4a). B cells were reduced in HDM-exposed *Cd40l*^{−/−} mice and as expected completely absent in *Mb1*^{−/−} mice (Fig. 4b). BALF Th cell and ILC2 numbers were not different in HDM-treated *Cd40l*^{−/−} and *Mb1*^{−/−} mice from HDM-treated controls (Fig. 4c). Strikingly, the numbers of BALF IL-4⁺, IL-5⁺, IL-13⁺, and IL-17⁺ Th cells and ILC2s in HDM-treated *Cd40l*^{−/−} and *Mb1*^{−/−} mice were similar to WT controls (Fig. 4d and Fig S6). Eosinophilic inflammation in lung tissue in HDM-treated *Cd40l*^{−/−} and *Mb1*^{−/−} mice was also not different from HDM-treated WT mice (Fig 4e–f).

These data show that eosinophilic airway inflammation and Th2 cytokine production can develop in the

absence of B–T cell interaction upon chronic HDM exposure.

Reduced Tfh and Th2 cell numbers but normal ILC2 numbers in the absence of iBALT structures in the lung

As Th2 inflammation in the BALF was similar between WT, *Cd40l*^{−/−} and *Mb1*^{−/−} mice, we investigated whether lung inflammation was affected by the absence of B–T cell interactions or B cells. Total lung B cells of HDM-treated *Cd40l*^{−/−} mice were reduced and completely absent in HDM-treated *Mb1*^{−/−} mice as compared to HDM-treated controls. As expected, GC B cells and plasma cells were completely absent in both HDM-treated *Cd40l*^{−/−} and *Mb1*^{−/−} mice (Fig. 5a). In contrast to our findings in BALF, total pulmonary Th and Tfh cells of HDM-treated *Cd40l*^{−/−} and *Mb1*^{−/−} mice were significantly reduced compared to HDM-treated controls; Th2 cell numbers in the lungs of *Cd40l*^{−/−} mice were also reduced (Fig. 5b). Lung Th17 and Treg cell numbers of HDM-treated *Cd40l*^{−/−} and *Mb1*^{−/−} mice were similar to those in HDM-treated control mice (Fig. 5b). Surprisingly, pulmonary ILC2 numbers were similar between HDM-treated WT and *Cd40l*^{−/−} and *Mb1*^{−/−} mice (Fig. 5c).

As the inflammation in the lungs is largely reduced, we wondered whether the remaining T cells would still cluster together in the absence of B cells and B–T cell interaction. HDM-treated *Cd40l*^{−/−} and *Mb1*^{−/−} mice exhibited no iBALT structures, although T cells were located in close proximity to the bronchioles in all HDM-treated mice (Fig. 5d).

Therefore, these data show that as expected B–T cell interaction is essential for iBALT development. However, ILC2 numbers are not affected by the absence of iBALT structures.

Tfh development in MLN in the absence of CD40L signalling upon chronic HDM treatment

As eosinophilic airway inflammation developed normally in the absence of B–T cell interaction, we wondered whether Tfh and Th2 cell differentiation would be hampered in the MLN in the absence of CD40L signalling and B cells upon chronic HDM exposure.

Fig. 1. Chronic house dust mite (HDM) exposure induces eosinophilic inflammation and remodelling in the airways. (a) Chronic HDM model. Mice were treated with PBS or HDM at indicated time-points. Analysis was performed at day 37 and day 65. (b–d) Quantification of broncho-alveolar lavage fluid (BALF) eosinophils, neutrophils, B cells, Th cells, and ILC2s by flow cytometry. (e–f) Flowcytometric gating and quantification of IL-4⁺, IL-5⁺, IL-13⁺, and IL-17⁺ Th cells in BALF by flow cytometry. (g) Histologic analysis of Siglec-F⁺ eosinophils in lung tissue. (h) Histologic analysis and quantification of mucus-producing cells in 3 bronchi per lung in paraffin-embedded lung tissue using a periodic acid-Schiff (PAS) staining. (i) Immunofluorescent staining of α SMA (green) in lungs at day 37 and day 65. (j) Airway hyperresponsiveness was measured upon increasing doses of inhaled methacholine. (k) Lung collagen content was determined by hydroxyproline assay. Results are presented as mean $\pm$ SEM of $n = 5$ –7 per group and representative of two or more independent experiments with 5–7 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

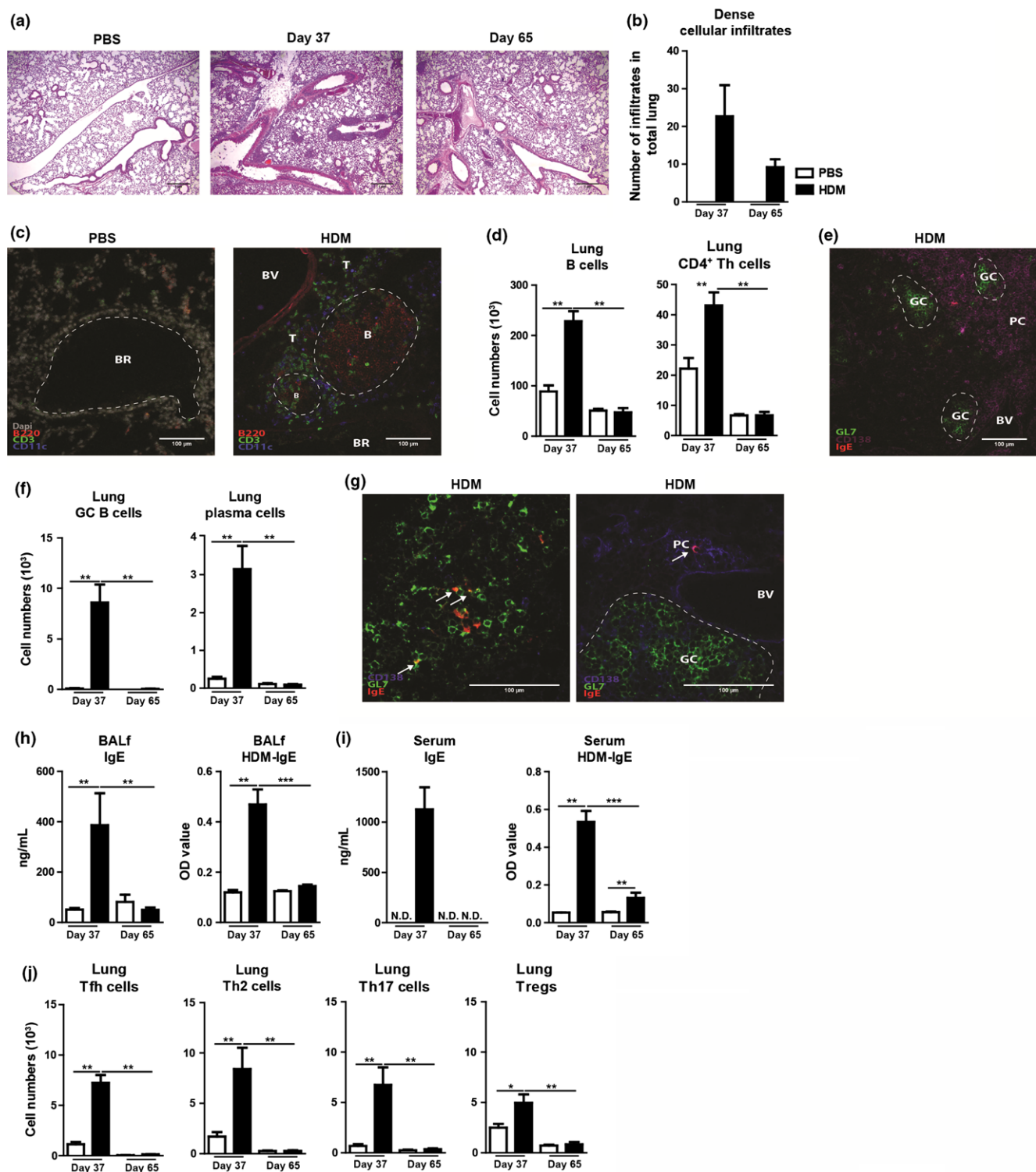


Fig. 2. Chronic house dust mite (HDM) exposure induces development of iBALT in the lungs. (a) Histological overview images showing dense cellular infiltrates in the lungs, and bars represent 100 μ m. (b) Number of dense cellular infiltrates in total lungs counted double-blinded. (c) Immunofluorescent staining of CD3⁺ T cells (green), B220⁺ B cells (red), and CD11c⁺ APCs (blue) in lungs at day 37. (d) B and Th cells in lung tissue. (e) Immunofluorescent staining of GL7⁺ GC B cells (green), CD138⁺ plasma cells (blue), and IgE⁺ cells (red) in lungs at day 37. (f) Quantification of GC B cells and plasma cells in lung tissue. (g) Immunofluorescent staining of IgE⁺ GC B cells (yellow) and IgE⁺ plasma cells (purple) in lung tissue at day 37. (h) IgE and HDM-IgE in broncho-alveolar lavage fluid (BALf). (i) IgE and HDM-IgE in serum. (j) Quantification of Tfh (CXCR5⁺, PD1⁺), Th2 (GATA3⁺), Th17 (ROR γ T⁺), and Treg (CD25⁺FoxP3⁺) cells in lung tissue. Results are presented as mean $\pm$ SEM of $n = 5-7$ per group representative of two or more independent experiments with 5-7 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

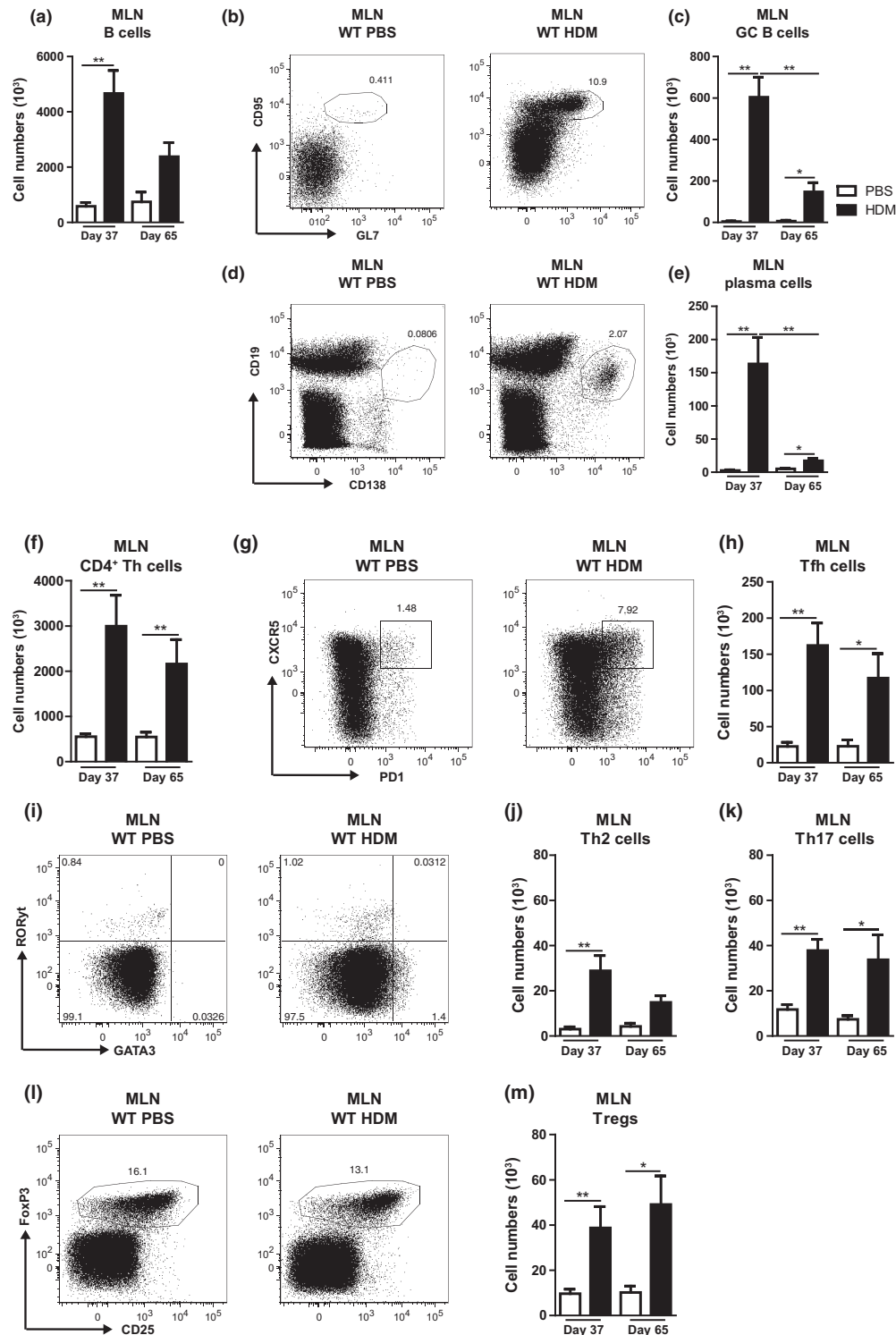


Fig. 3. Germinal centre B cells and Tfh cells are induced in the MLN upon chronic house dust mite (HDM) exposure (a) Quantification of B cells by flow cytometry. (b) Flow cytometric gating of GC B cells from CD19⁺B220⁺ B cells in MLN at day 37. (c) Enumeration of GC B cells by flow cytometry. (d) Flow cytometric gating of plasma cells from live cells at day 37. (e) Enumeration of plasma cells by flow cytometry. (f) Enumeration of Th cells by flow cytometry in MLN. (g) Flow cytometric gating of Tfh cells from CD4⁺ T cells in MLN at day 37. (h) Quantification of Tfh cells by flow cytometry. (i) Flow cytometric gating of RORγt⁺ Th17 and GATA3⁺ Th2 cells from CD4⁺ T cells in MLN at day 37. (j, k) Enumeration of Th2 and Th17 cells in MLN by flow cytometry. (l) Flow cytometric gating of FoxP3⁺CD25⁺ Tregs cells from CD4⁺ T cells in MLN at day 37. (m) Quantification of Treg cell numbers in MLN by flow cytometry. Results are presented as mean ± SEM of *n* = 5–7 per group and representative of two or more independent experiments with 5–7 mice per group. **P* < 0.05, ***P* < 0.01.

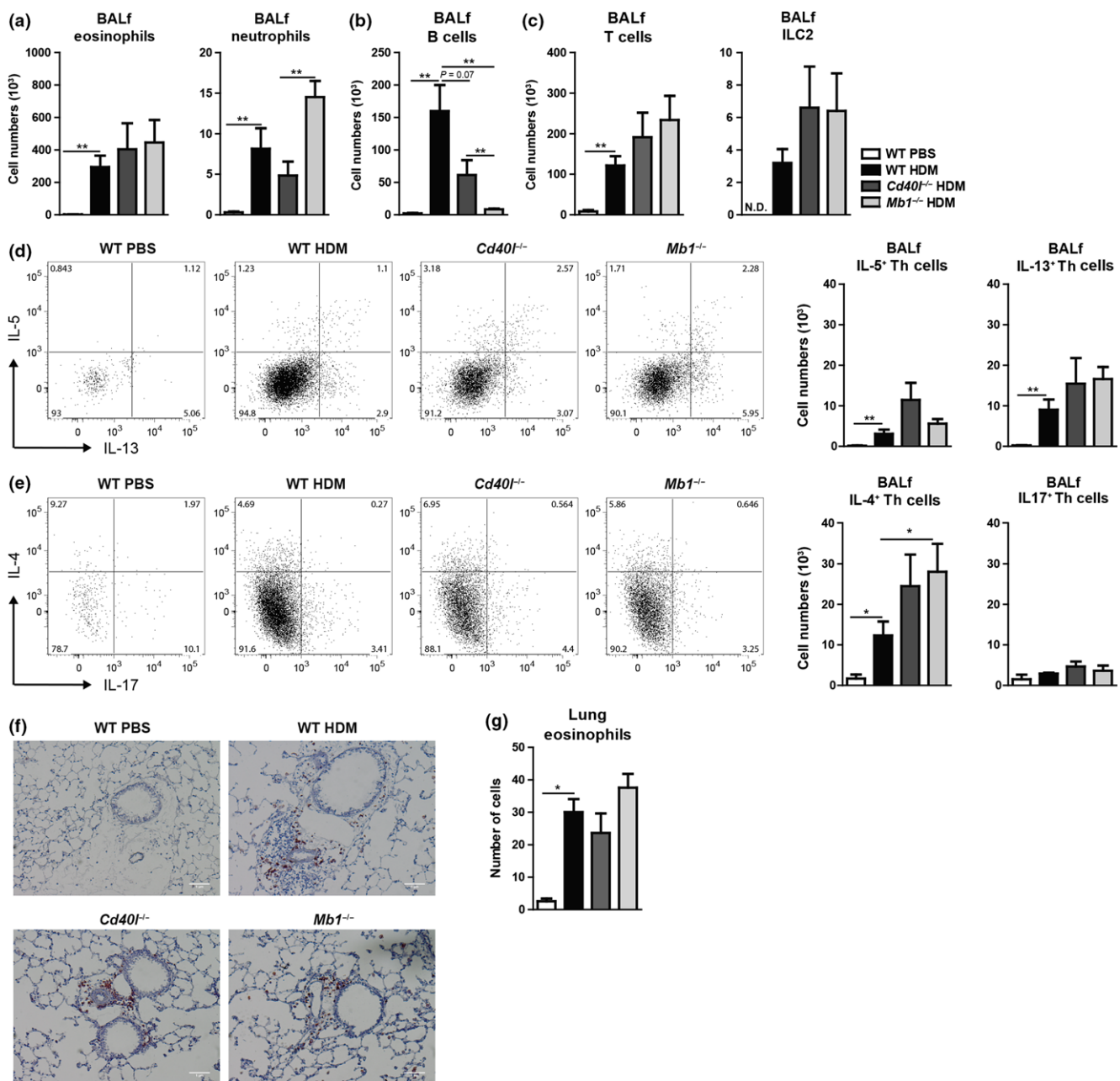


Fig. 4. Eosinophilic inflammation is not dependent on B-T cell interactions. (a-c) Quantification of eosinophil, neutrophil, B cell, Th cell, and ILC2 cell numbers in broncho-alveolar lavage fluid (BALf) by flow cytometry. (d, e) Flow cytometric gating and quantification of IL-4⁺, IL-5⁺, IL-13⁺, and IL-17⁺-producing Th cells in BALf by flow cytometry. (f) Histologic analysis of Siglec-F⁺ eosinophils in lung tissue. (g) Number of eosinophils in the lung tissue. Results are presented as mean $\pm$ SEM ($n = 4$ and 7 in PBS and house dust mite (HDM), respectively). * $P < 0.05$, ** $P < 0.01$.

As expected, MLN from *Cd40l*^{-/-} mice had reduced B and T cell numbers, compared with HDM-treated WT mice (Fig. 6a and b), whereas B cells were absent in *Mb1*^{-/-} mice (Fig. 6a). GC B cells and plasma cells were completely absent in HDM-treated *Cd40l*^{-/-} mice, indicating that GC B cell formation is dependent on CD40L signalling in a chronic HDM-induced asthma model (Fig. 6c). Surprisingly, Tfh cell numbers were not significantly reduced in *Cd40l*^{-/-} mice; Tfh differentiation was

lacking in *Mb1*^{-/-} mice (Fig. 6d and e). In PBS-treated *Cd40l*^{-/-} mice, Tfh cells were reduced compared with WT mice, as reported before (Fig. S7) [25]. Tfh cells are characterized by the expression of CXCR5, a chemokine receptor that enables the migration towards the GC [26], and PD1, a potent inhibitory receptor inhibiting excessive Th cell proliferation [27]. PD1 expression was reduced on Tfh cells of both HDM-treated *Cd40l*^{-/-} and *Mb1*^{-/-} mice, compared with HDM-treated WT Tfh cells.

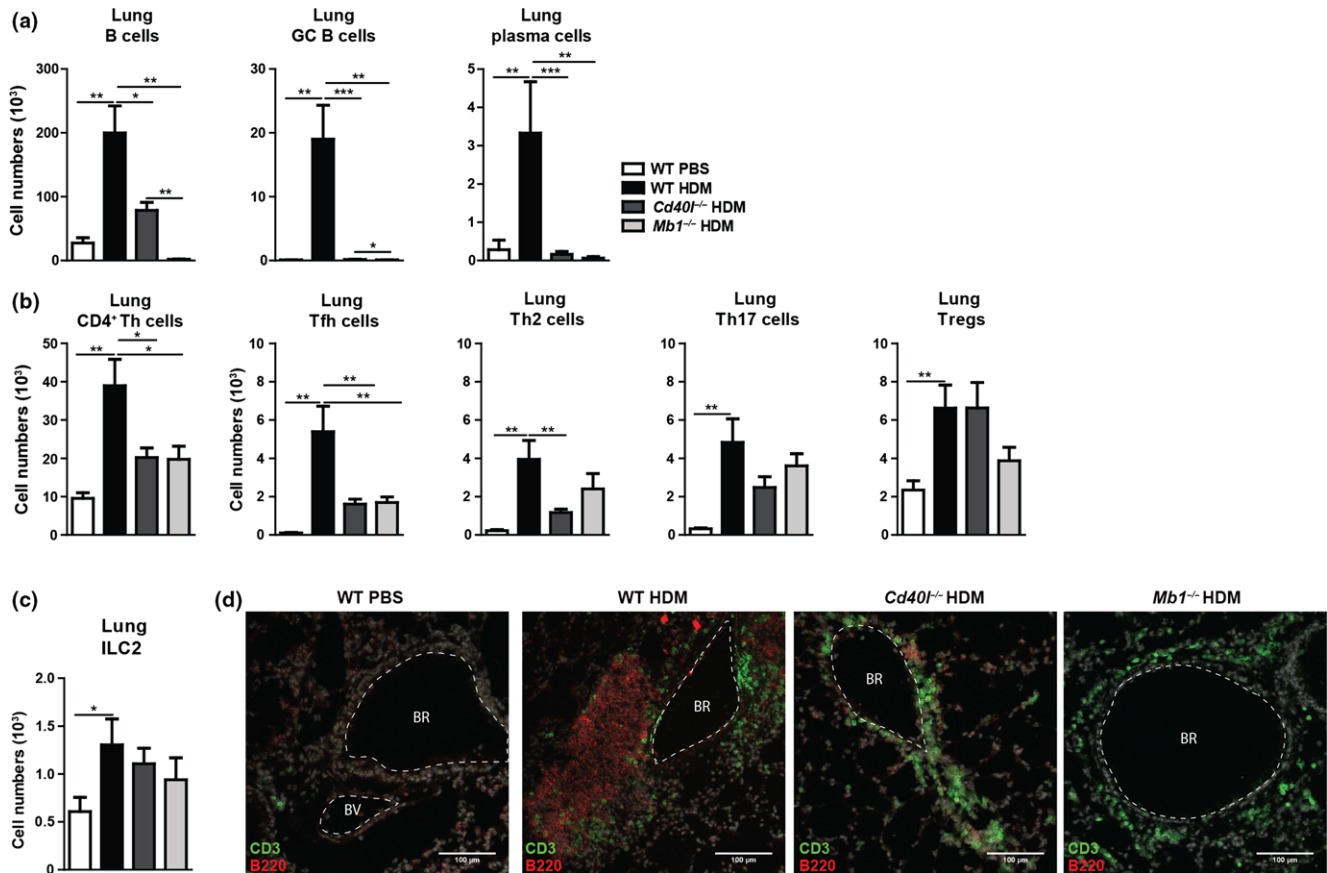


Fig. 5. Reduced Tfh and Th2 cell numbers but normal ILC2 numbers in the absence of iBALT structures in the lung. (a) Quantification of B cell, GC B cell, and plasma cell numbers in lung tissue by flow cytometry. (b) Quantification of Th, Tfh, Th2, Th17, and Treg cell numbers in lung tissue by flow cytometry. (c) Immunofluorescent staining of CD3⁺ T cells (green) and B220⁺ B cells (red) in lungs. (d) Results are presented as mean $\pm$ SEM ($n = 4$ and 7 in PBS and house dust mite (HDM), respectively). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Nevertheless, PD1 expression on Tfh cells of HDM-treated *Cd40l*^{-/-} mice was still elevated, compared with PBS-treated WT Tfh cells. CXCR5 expression was only reduced in Tfh cells of *Mb1*^{-/-} mice (Fig. 6d, quantified in Fig. 6f). In contrast to Tfh cells, Th2 cells were strongly reduced in MLN of both HDM-treated *Cd40l*^{-/-} and *Mb1*^{-/-} mice, compared with HDM-treated WT mice. Tregs were reduced in both HDM-treated *Cd40l*^{-/-} and *Mb1*^{-/-} mice, and Th17 cell numbers were only significantly reduced in *Mb1*^{-/-} mice (Fig. 6g).

In summary, these data confirm that GC B cell formation in the MLN is dependent on CD40L signalling. Importantly, in the MLN, Tfh cell induction during chronic HDM exposure does not require CD40L, but is dependent on B cells. In contrast, Th2 cell development in the MLN is dependent on both CD40L signalling and B cells.

B cells and B–T cell interaction are essential for pulmonary remodelling

Both cytokine production by Th2 cells and IgE secretion by B cells are known to contribute to pulmonary

remodelling [28]; therefore, we wondered whether remodelling is dependent on the presence of B cells or B–T cell interaction. HDM-treated *Cd40l*^{-/-} and *Mb1*^{-/-} mice had a reduced area of α -smooth muscle actin (α SMA) surrounding the bronchioles and mucus-producing goblet cells compared to HDM-treated WT mice (Fig. 7a–d). A trend towards reduced AHR was observed at lower methacholine concentrations in both HDM-treated *Cd40l*^{-/-} and *Mb1*^{-/-} mice, compared to HDM-treated WT mice (Fig. 7c).

These data demonstrate that the presence of B cells and CD40L signalling is essential for airway remodelling that accompanies chronic airway inflammation.

Discussion

B cells are essential for the maintenance and final differentiation of Tfh cells [29]. Tfh cells have recently been reported to contribute to Th2-mediated inflammation in asthma, either by the differentiation into effector Th2 cells or by promoting Th2 cell differentiation [13, 14]. However, whether B cells and

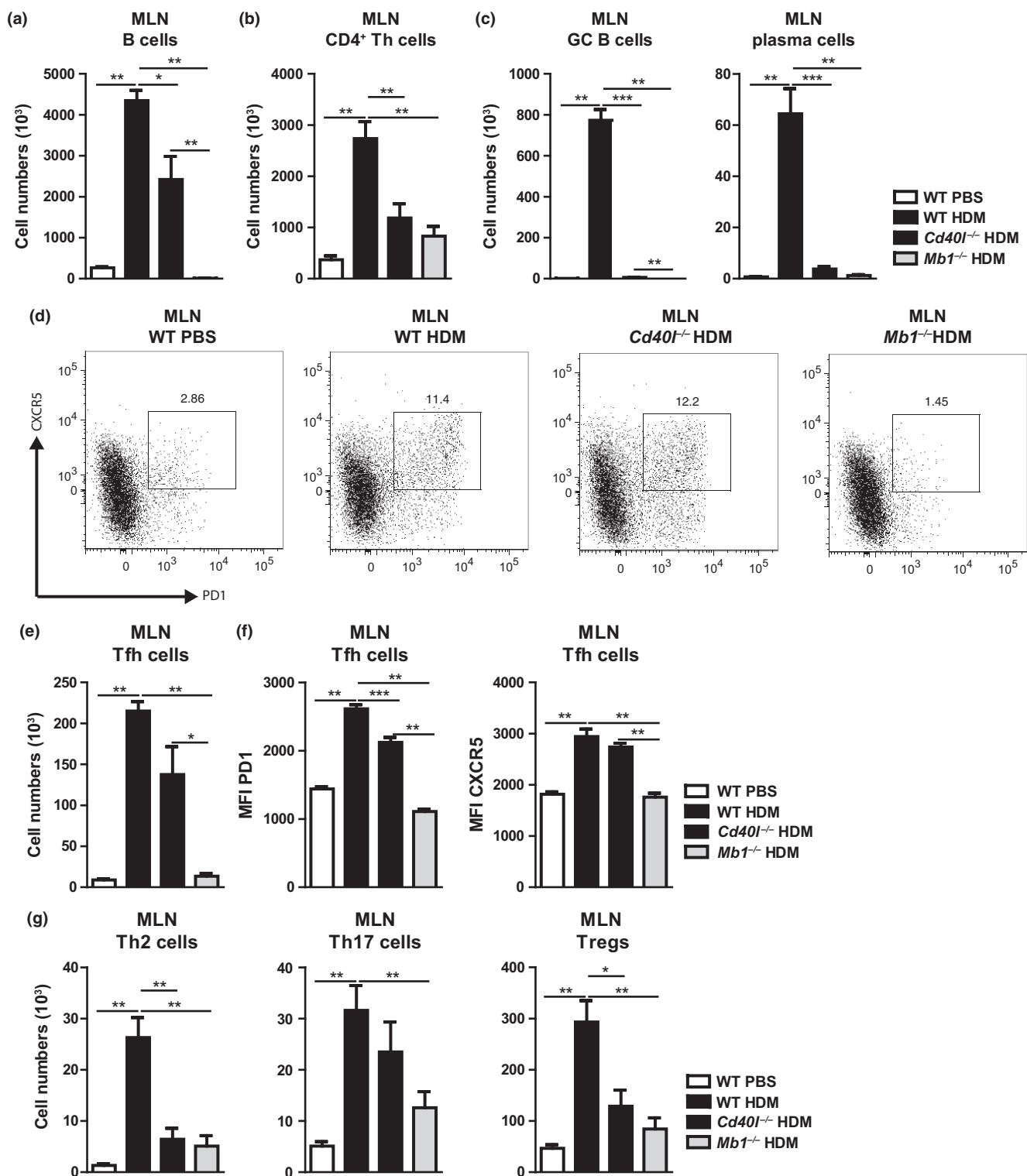


Fig. 6. Tfh development in MLN requires B cells but not CD40L signalling upon chronic house dust mite (HDM) treatment. (a–c) Quantification of B cell, Th cell, GC B cell, and plasma cell numbers in MLN by flow cytometry. (d) Flow cytometric gating of Tfh cells. (e) Quantification of Tfh cells in MLN by flow cytometry. (f) MFI of PD1 and CXCR5 of Tfh cells by flow cytometry. (g) Quantification of Th2, Th17, and Treg cells by flow cytometry. Results are presented as mean $\pm$ SEM of $n = 5$ –7 per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

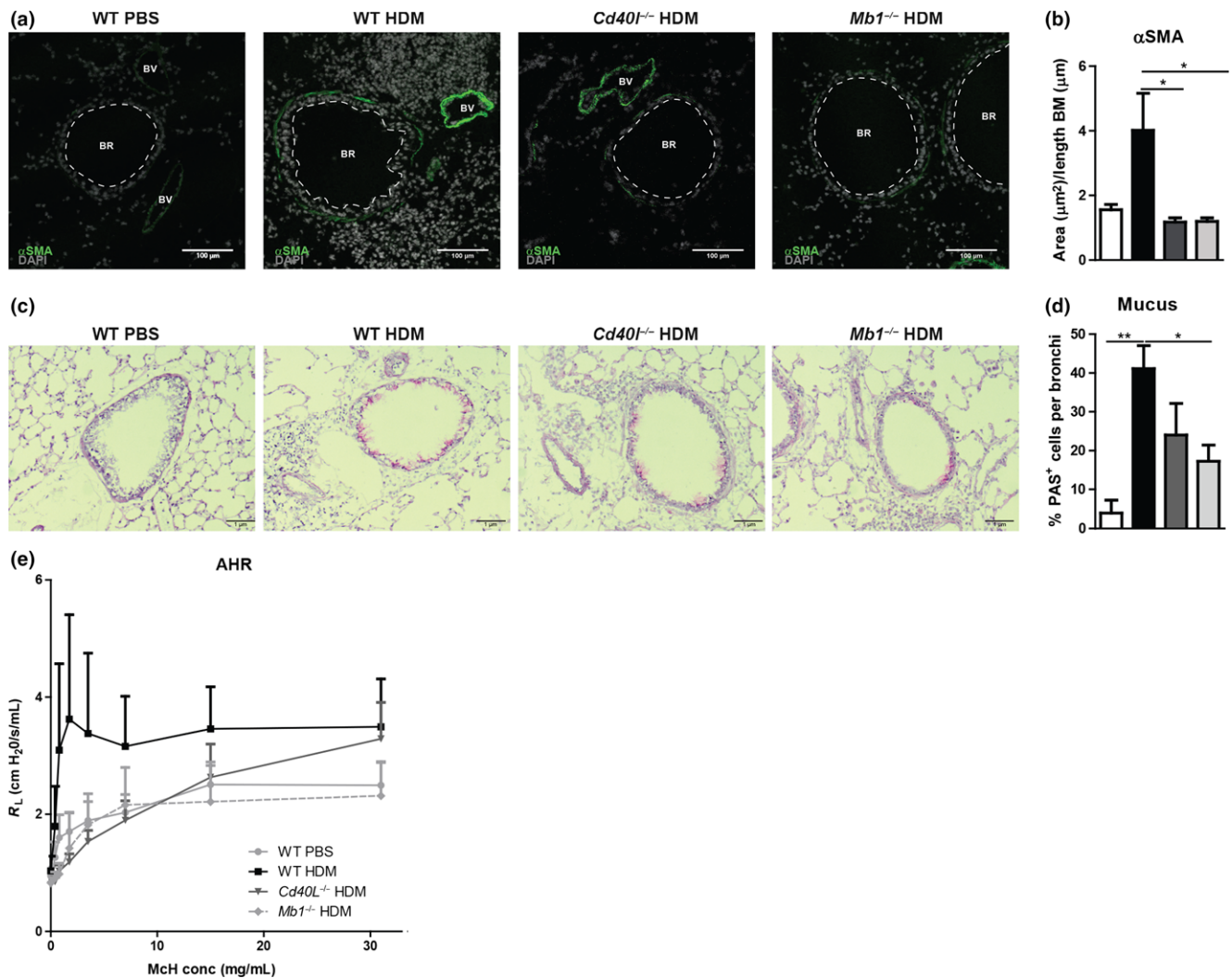


Fig. 7. B cells and CD40L are essential for the development of pulmonary remodelling. (a) Immunofluorescent staining of αSMA (green) in lungs at day 37. (b) Quantification of the area αSMA per basal membrane of 3 bronchioles per mice. (c–d) Histologic analysis and quantification of mucus-producing cells in snap-frozen lung tissue periodic acid-Schiff (PAS) staining. (e) Airway hyperresponsiveness was measured upon increasing doses of methacholine at day 37. Results are presented as mean ± SEM ($n = 4$ and 7 in PBS and house dust mite (HDM), respectively). * $P < 0.05$.

B–T cell interaction are also important for eosinophilic airway inflammation in a clinically relevant chronic asthma model characterized by prolonged allergen exposure remains unknown. Chronic HDM exposures induced transient type 2 airway inflammation, whereas structural changes such as airway remodelling and airway hyperresponsiveness sustained over time. Our study demonstrates that in a chronic HDM-mediated asthma model, eosinophilic inflammation in the BALf and lung tissue can develop in the absence of B–T cell interaction and iBALT structures. The accumulation of ILC2s in BALf and lung tissue was not affected by the absence of iBALT tissue. In particular, we have found that the absence of B cells precluded the formation of Tfh cells in the MLN upon

chronic HDM exposure, but nevertheless eosinophilic inflammation was induced. Thus, in contrast to published findings in acute HDM models characterized by sensitization and challenge phases [13, 14], our findings in a chronic HDM asthma model demonstrate that pulmonary eosinophilic inflammation is independent of B–T cell communication and iBALT formation. Nevertheless, our chronic HDM model revealed that pulmonary Th2 inflammation and airway remodelling require B cells and B–T cell interaction.

Although CD40L is mostly studied because of its prominent role on activated T cells in the context of B–T cell interaction, CD40L is also expressed on several other cell types, including mast cells, eosinophils, monocytes, B cells, and DCs [30]. It is therefore

conceivable that additional immunological responses may be hampered in *Cd40l*^{-/-} mice, next to B–T cell interaction and communication. For instance, CD40–CD40L signalling in DCs is also involved in Th2 cell differentiation [31]. Furthermore, upon CD40–CD40L interaction, OX-40L, a Th2 cell-promoting costimulatory molecule, is induced on DCs [32]. However, it is not likely that decreased Th2 differentiation in the MLN of *Cd40l*^{-/-} mice is mainly a result of insufficient DC–T cell interaction, rather than abrogated B–T cell communication, because *Mb1*^{-/-} mice – in which CD40–CD40L signalling between DC and T cells is still intact – also showed a similar reduction in the numbers of Th2 cells in MLN as *Cd40l*^{-/-} mice.

Eosinophils were also reported to be important for airway remodelling in OVA-induced asthma models [33]. In this context, however, our data indicate that a reduction in airway remodelling is observed, although similar eosinophilic inflammation is present in *Mb1*^{-/-}, *Cd40l*^{-/-}, and WT mice. This is consistent with other reports that investigated the role of eosinophils in airway remodelling upon HDM-driven asthma models [34]. Recently, eosinophil-derived IL-4 was also described to induce both proliferation and IL-5 and IL-13 secretion by ILC2s, suggesting a positive feedback loop between ILC2s and eosinophils [35]. This would complement our data, as we found normal levels of both eosinophils and ILC2s in the airways of mice deficient of B cells or CD40L signalling.

B cells can contribute to various aspects of asthma pathology through the secretion of cytokines, antigen presentation, and their production of antigen-specific IgE [36]. Our data demonstrates that during chronic HDM exposure, B cells are dispensable for pulmonary eosinophilic influx, but are essential for the development of airway remodelling. Other studies have also found similar numbers of eosinophilic inflammation in B cell-deficient mice [8, 11, 37], whereas especially allergen-specific IgE secretion by plasma cells was shown to be sufficient to induce AHR [38, 39], smooth muscle cell proliferation, and collagen deposition [40, 41]. In contrast, Drake et al. [42] found that B cells are important for Th2-mediated asthmatic inflammation; however, in contrast to our study, mice were exposed to different allergens such as ovalbumin, *Alternaria*, *Aspergillus*, and HDM.

It has been reported in a model containing a sensitization and challenge phase that HDM-specific CD4⁺ T cells are first primed by lung-migratory DCs at the B cell follicle border where they proliferate and acquire a Tfh-like signature [13]. IL-4-committed pre-Tfh cells subsequently encounter allergen-bearing B cells that acquired allergen from lung-migratory DCs. These allergen-presenting activated B cells replace DCs as the primary APCs in the B cell follicle and provide Th cell

survival and differentiation signals [13]. We also found significantly lower numbers of Th2 and Tfh cells in the MLN and lung, whereas the numbers of Th17 and Tregs were similar in the absence of B cells. However, similar numbers of Th cells were found in association with the bronchioles. This suggests that the decrease in both total Th cells and Th2 cells in the lung is likely caused by the absence of iBALT structures. This is in contrast to the BALF, where we found normal Th2 levels in both HDM-treated *Mb1*^{-/-} and *Cd40l*^{-/-} mice as compared to WT mice, indicating that the lungs could contain two separately controlled Th2 compartments.

It is well possible that the observed role for both B cells and Tfh cells is dependent on the experimental model, allergen type, and kinetics of exposure. Clearly, our data demonstrate that in a chronic HDM-driven asthma model, normal lung eosinophilic inflammation can be initiated in the absence of B–T cell interactions and iBALT structures. This finding indicates that chronic HDM exposure elicits a very robust and/or long-lasting pulmonary inflammatory response that overcomes the need for Tfh cells during Th2 cell differentiation that was observed in acute HDM models [12, 13]. Unlike in acute models, chronic allergen exposure could result in prolonged activation of ILC2s that may support or induce eosinophilic inflammation, independently of Tfh cells or GC formation in the MLN and lungs.

Collectively, our data show that upon chronic allergen exposure, B–T cell interactions are required for robust Tfh and Th2 cell induction and however are not essential for eosinophilic airway inflammation. Eosinophilic airway inflammation alone is not sufficient to induce pulmonary remodelling and airway hyperresponsiveness, which is likely dependent on the outcome of B–T cell interactions.

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Author contributions

HV, BL, RH, and MK designed the experiments. HV, IB, BL, JvH, ML, and DU performed experiments and analysed data. HV, RH, and MK wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Flow cytometric gating of eosinophils, neutrophils, B cells, and T cells in BALf. Eosinophils were gated as Siglec F⁺, GR-1⁺, Neutrophils were gated as GR1⁺, Siglec F^{int}, T cells were gated as CD3⁺MHCII⁺, and B cells were gated as CD19⁺MHCII⁺. Due to the limited amount of fluorochromes available, CD19 and CD3 were stained with the same fluorochrome, enabling the distinction between B and T cells based on the expression of MHCII.

Figure S2. Flow cytometric gating of ILCs in BALf. ILC2s were gated as Lineage negative (CD3, CD5, CD8 α , CD11b, CD11c, CD19, B220, NK1.1, GR-1, Ter-119, Fc ϵ RI α , and CD14) and CD45⁺, Sca-1 (Ly6A/E)⁺, T1ST2⁺, and CD127⁺.

Figure S3. B cell inflammation is less prominent in the BAL and lungs of mice exposed to an acute HDM-driven asthma model. (A) Scheme for acute intranasal HDM treatment of C57BL/6 mice to induce airway inflammation. Mice were sensitized with PBS or 1 μ g HDM at day 1, and challenged with 10 μ g of HDM at day 8–12. Analysis was performed at day 13. (B) Quantification of eosinophil and neutrophil cell numbers in BALf by flow cytometry. (C) Quantification of B and T cell numbers in BALf by flow cytometry. (D) Quantification of B and T cell numbers in lung by flow

cytometry. Results are presented as mean $\pm$ SEM ($n = 6$ in PBS and HDM). * $P < 0.05$, ** $P < 0.01$.

Figure S4. Flow cytometric gating of B and Th cell subsets. (A) Flow cytometric gating of total B cells, germinal center B cells, and plasma cells. (B) Flow cytometric gating of total Th cells, Tfh, Th2, Th17, and Treg cells.

Figure S5. Naïve T cell numbers in the lung are not increased upon chronic HDM-treatment. Quantification of naïve CD62L⁺ Th cell numbers in the lung by flow cytometry. Results are presented as mean $\pm$ SEM of $n = 5–7$ per group and all panels are representative of two or more independent experiments with 5–7 mice per group. * $P < 0.05$.

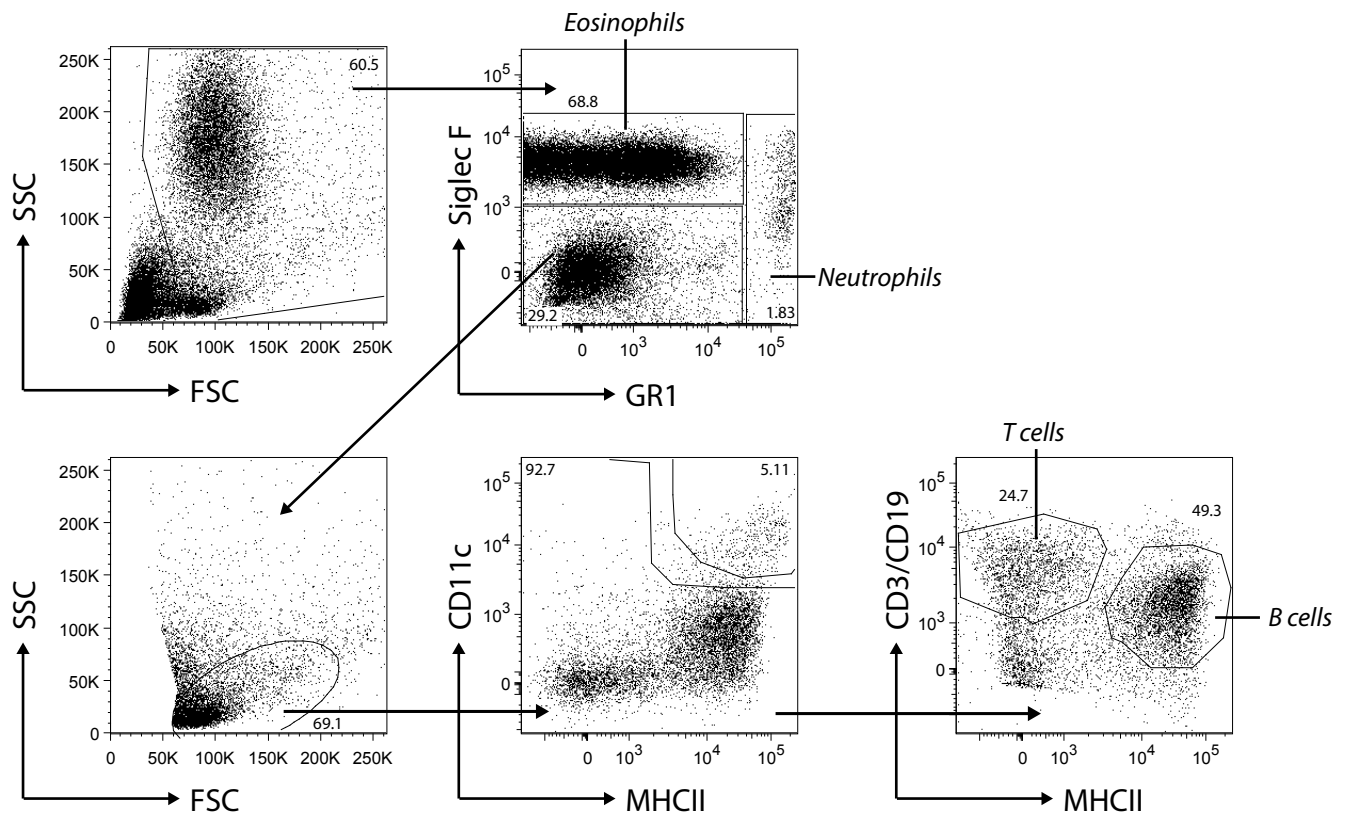
Figure S6. Cytokine production by ILC2 in the BALf of HDM-treated *Cd40l*^{−/−} and *Mb1*^{−/−} mice is similar to HDM-treated WT mice. Flow cytometric gating of IL-5 and IL-13 producing ILC2 cells in the BALf of HDM-treated WT, *Cd40l*^{−/−} and *Mb1*^{−/−} mice at day 37.

Figure S7. Total CD4⁺ T and Tfh cell numbers are reduced in MLN of *Cd40l*^{−/−} mice. (A) Quantification of Th cell numbers in the lung by flow cytometry. (B) Flow cytometric gating of Tfh cells in MLN. (C) Quantification of Tfh cells in the MLN by flow cytometry. Results are presented as mean $\pm$ SEM of $n = 5–7$ per group. * $P < 0.05$, ** $P < 0.01$.

Table S1. Antibodies used for flow cytometry.

Table S2. Primary and secondary antibodies used for confocal microscopy.

Figure S1



Supplementary Figure 2: ILC2 gating

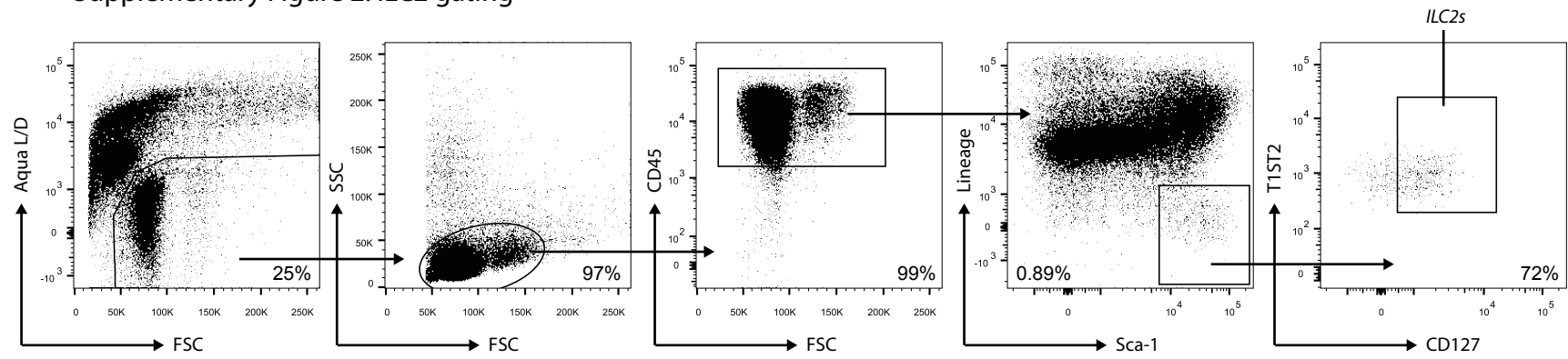
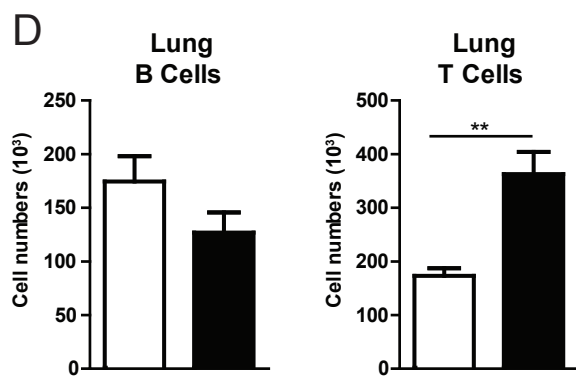
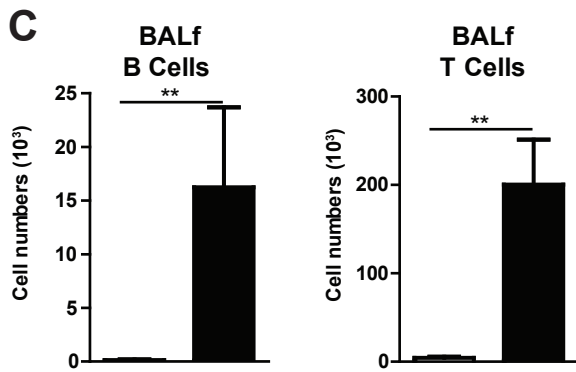
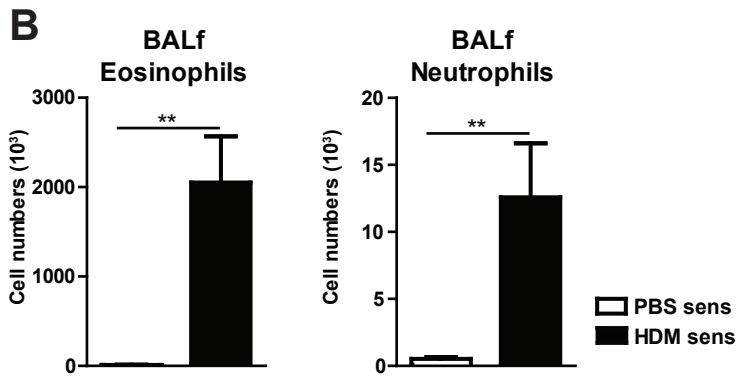
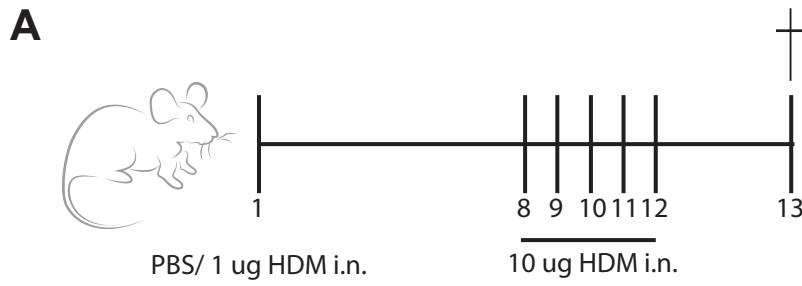


Figure S3



Supplementary Figure 4: B and Th cell gating

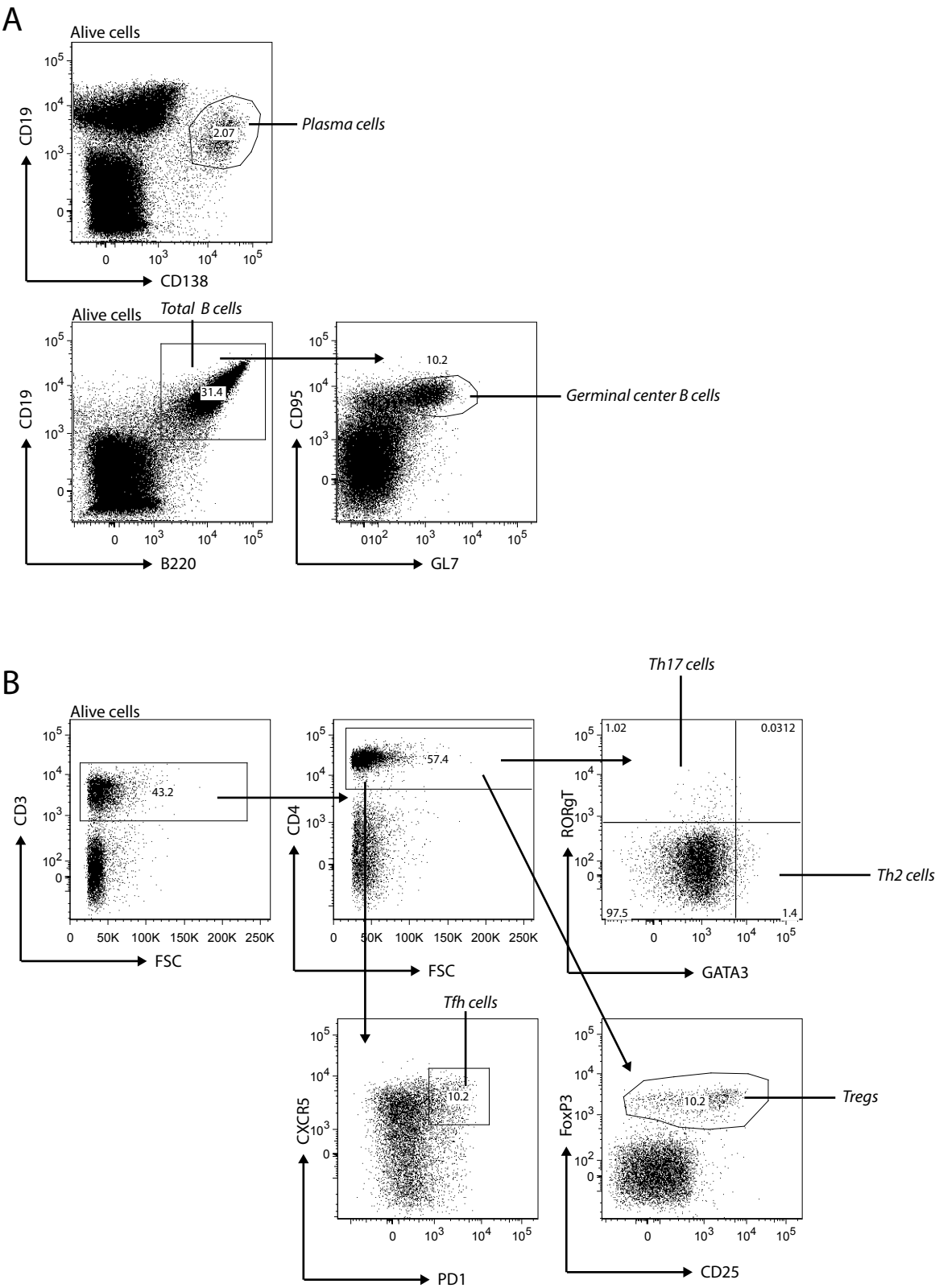


Figure S5

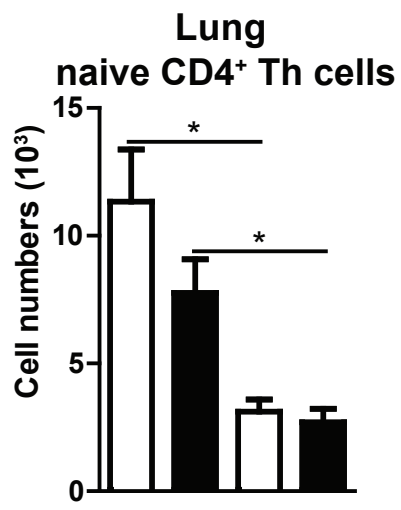


Figure S6: Cytokine production by ILC2 in the BALf of HDM-treated *Cd40l*^{-/-} and *Mb1*^{-/-} mice is similar to HDM-treated WT mice

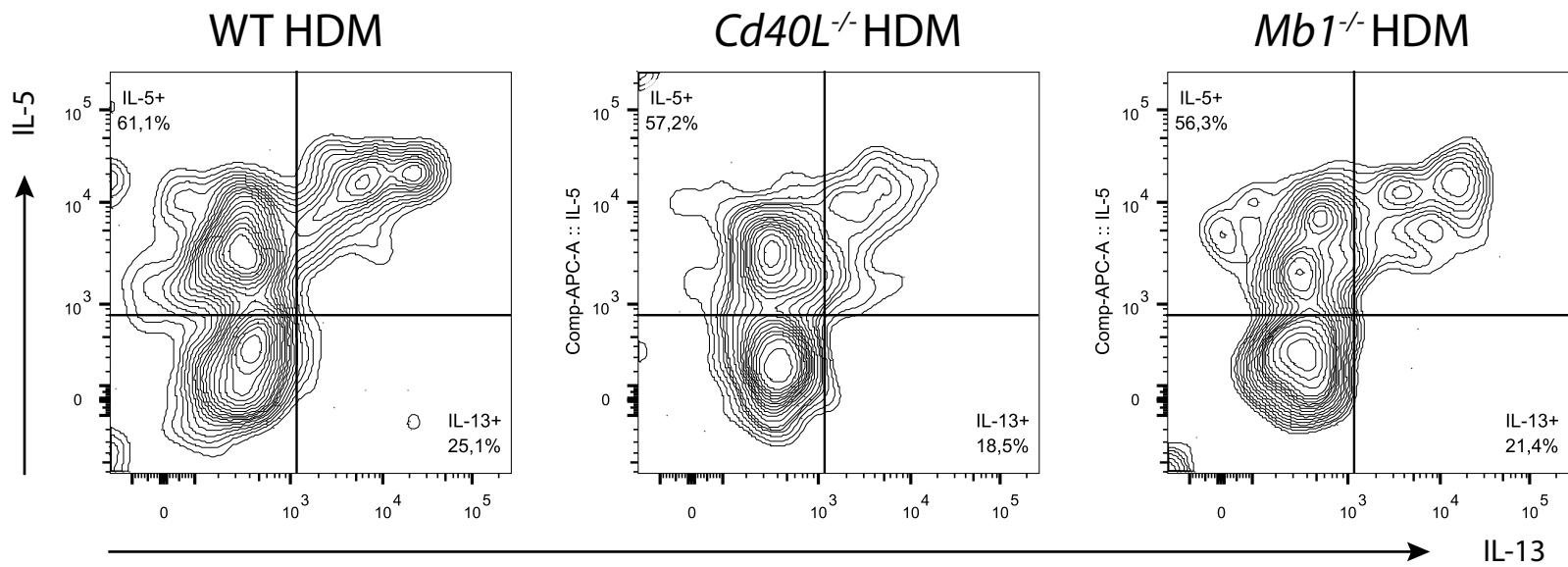
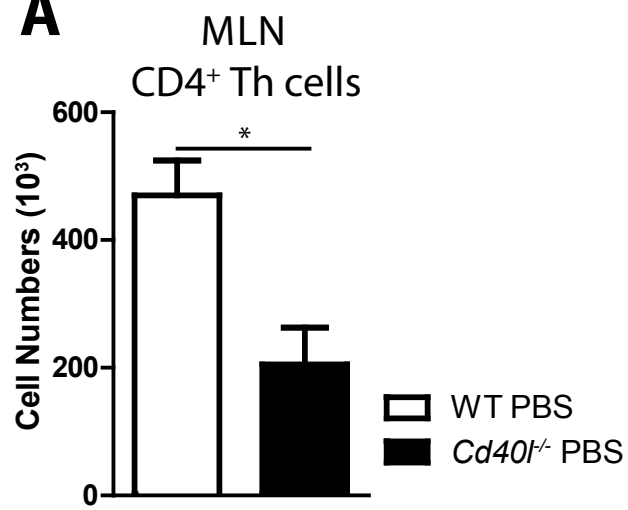
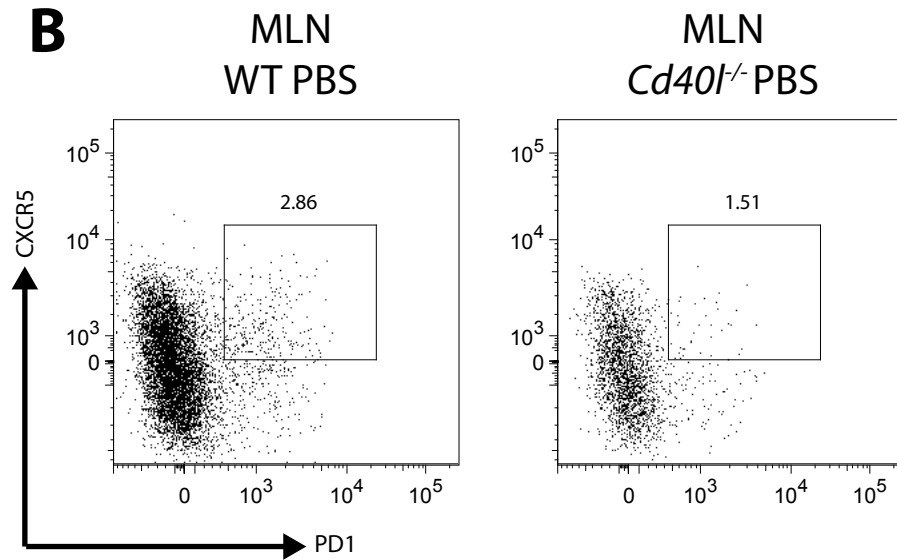


Figure S7

A



B



C

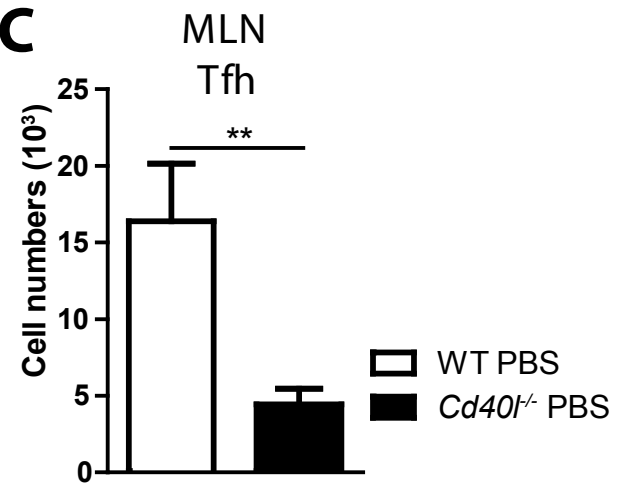


Table S1 Antibodies used for flow cytometry

Antibody	Conjugate	Clone	Company
B220	PE	RA3-6B2	eBiosciences
CD11b	PE	M1/70	eBiosciences
CD11c	PE	N418	eBiosciences
CD127	PE-Cy7	SB/14	BD Biosciences
CD138	APC	281-2	eBiosciences
CD19	APC-eFluor 780	ID3	eBiosciences
CD19	PE	ID3	BD Biosciences
CD25	PerCP-Cy5.5	PC61	BD Biosciences
CD3	PE-CF594	145-2C11	BD Biosciences
CD3	APC-eF780	17A2	eBiosciences
CD3	PE	145-2C11	eBiosciences
CD4	Brilliant Violet 605	RM4-5	BD Biosciences
CD45	Pe-CF594	I3/2.3	Abcam
CD5	PE	53-7.3	eBiosciences
CD8a	PE	53-6.7	eBiosciences
CD62L	APC-eFluor 780	MEL-14	eBiosciences
CD95	PE-CF594	Jo2	BD Biosciences
CXCR5	Biotin	2G8	BD Biosciences
FcεR1a	PE	MAR-1	eBiosciences
FoxP3	AF488	FJK-16s	eBiosciences
GATA3	eFluor 660	TWAJ-14	eBiosciences
GL7	FITC	GL7	BD Biosciences
Gr-1	PE	RB6-8C5	eBiosciences
IL-13	eFluor 450	eBio13A	eBiosciences
IL-4	Brilliant Violet 711	11B11	BD Biosciences
IL-5	APC	TRFK-5	BD Biosciences
Ly6A/E	Brilliant Violet 786	D7	BD Biosciences
Ly6C/Ly6G	Pe-Cy7	RB6-8C5	eBiosciences
MHCII	Alexa Fluor 700	M5/114.15.2	eBiosciences
NK1.1	PE	PK136	eBiosciences
PD1	Brilliant Violet 421	J43	BD Biosciences
RORyt	PE	Q31-378	BD Biosciences
Siglec-F	PE	E50-2440	BD Biosciences
Streptavidin	Brilliant Violet 786		BD Biosciences
T1ST2	FITC	DJ8	mdbioproducts
Ter-119	PE	TER119	eBiosciences

Table S2 Primary and secondary antibodies used for confocal microscopy

Primary antibodies	Host IgG	Producer	Cat#
a-SMA	Mouse IgG2a	R&D systems	IC1420G
B220	Rat IgG2a	eBioscience	56-0452-82
CD11c	Armenian Hamster IgG1	eBioscience	12-0114-83
CD138	Rat IgG2a	BD biosciences	553713
CD3	Armenian Hamster IgG1	BD biosciences	553062
collagen	Rabbit IgG	Merck Millipore	ABT123
GL7	Rat IgM	BD biosciences	553666
IgE	Goat IgG	Novus Biologicals	NB7530
Secondary antibodies	Fluorochrome	Producer	
donkey anti-goat IgG	Cy-3	Jackson ImmunoResearch	
donkey anti-rabbit IgG	Cy-3	Jackson ImmunoResearch	
goat anti-armenian hamster IgG	Cy-3	Jackson ImmunoResearch	
donkey anti-rat IgG	Cy-5	Jackson ImmunoResearch	
mouse anti-FITC	AF488	Jackson ImmunoResearch	