

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

LAPCs contribute to the pathogenesis of allergen-induced allergic airway inflammation in mice

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Abstract

Background: The inflammatory immune response associated with allergic airway inflammation in asthma involves T helper type 2 (Th2) immunity. Given the data that a newly described late activator antigen-presenting cell (LAPC) population promotes Th2 immunity in viral infections, we undertook studies to investigate whether LAPCs have a pathogenic role in allergic airway inflammation.

Methods: We employed acute ovalbumin (OVA) and house dust mite (HDM) sensitization and challenge models to establish allergic airway inflammation in mice, followed by the analysis of lungs and draining lymph node (DLN) cell infiltrates, immunoglobulin E (IgE) production, and airway hyper-responsiveness (AHR). We tested whether adoptive transfer of LAPCs isolated from mice with established allergic airway inflammation augments the development of sensitization in naïve mice.

Results: We provide evidence that in both OVA and HDM mouse models of allergic inflammation, LAPCs accumulate in the lungs and draining lymph nodes (DLNs), concomitant with the onset of lung pathology, allergen-specific IgE production, eosinophilia, and Th2 cytokine production. Adoptive transfer experiments using OVA-activated LAPCs reveal exacerbation of disease pathology with an increase in lung inflammatory cells, eosinophilia, circulating IgE, Th2 cytokine production, and a worsening of AHR. OVA-activated LAPCs preferentially increased GATA-3 induction in naïve CD4⁺ T cells.

Conclusions: Together, these data suggest an important role for LAPCs in polarizing the Th2 response in mouse models of allergic airway inflammation.

The inflammatory immune response associated with asthma develops as a consequence of allergen inhalation, invoking interactions between immune cells. Antigen-presenting cells (APCs) are recruited to the lungs, then process allergens for the presentation to T cells, and in the case of allergic airway inflammation, drive Th2 differentiation. IL-4 is implicated in inducing Th2 differentiation by activating STAT6 and GATA-3 transcription factor-mediated signaling pathways. Th2 cells then produce and secrete Th2 cytokines: IL-3, IL-4, IL-5, IL-9, IL-13, and GM-CSF, which target a variety of lymphocytes, resulting in the inflammatory asthmatic response (1). IL-4 and IL-13 along with co-stimulatory molecules induce B cells to undergo class switching to produce allergen-specific IgE.

We previously identified a novel mouse APC, designated late activator APC (LAPC: mPDCA-1⁺CD11c⁺TCRβ⁺B22⁺CD38⁺CD44^{int}CD45⁺Gr1⁺) (2, 3), and demonstrated that after pulmonary influenza H1N1 virus infection, LAPCs enter the lungs, capture viral antigen, and migrate to the DLNs and spleen. In the DLN, virus-activated LAPCs present antigen and selectively induce Th2 effector cell polarization by cell–cell contact-mediated modulation of GATA-3 expression. In adoptive transfer experiments, virus-activated LAPCs augmented Th2 effector T-cell responses in the DLN, increased production of circulating anti-influenza immunoglobulin, and increased levels of Th2 cytokines in bronchoalveolar lavage (BAL) fluid in recipient virus-infected mice. LAPC recipient mice exhibited exacerbated pulmonary

pathology, with delayed viral clearance and enhanced pulmonary eosinophilia. Moreover, LAPCs promote follicular helper T-cell differentiation of antigen-primed CD4⁺ T cells during influenza virus respiratory infection (4). Collectively, these results highlight the importance of LAPCs as immunomodulators of type 2/Th2 (T2) immunity during influenza virus infection. Therefore, we undertook studies to investigate the potential contribution of LAPCs to the pathogenesis of allergic airway inflammation and provide evidence for their involvement in two different mouse models of acute allergic airway inflammation.

Methods

Female Balb/c (Jackson Laboratories) and DO11.10 (Taconic) mice 6–8 weeks old were housed in accordance with Animal Care Committee guidelines of the UHN. Two different protocols were employed to induce allergic airway inflammation: i.p. OVA sensitization and i.n. challenge; and i.n. instillation with purified *Dermatophagoides pteronyssinus* extract.

Serum, BAL, lungs, and DLNs were harvested from mice at different times relative to disease induction. BAL cytokine levels were analyzed using a mouse Th1/Th2/Th17/Th22 multiplex kit (eBiosciences, San Diego, CA, USA), and serum anti-OVA IgE and total IgE levels were measured (Chondrex kits, Redmond, WA, USA). Three-micrometer lung paraffin sections were stained with H&E or periodic acid–Schiff (PAS) reagent.

Disaggregated cells were stained with antibodies against CD3, CD8 α , CD11c, B220/CD45R, TCR β , Siglec-F, CD4, CD45, PDCA-1, CXCR3, icosL, and TSLPR. For intracellular cytokine analyses, cells were stained with antibodies against IL-4 and IFN- γ . Stained cells were analyzed on a FACSCalibur or LSRII flow cytometer (BD). Data were analyzed using FlowJo software (Treestar, Inc., Ashland, OR, USA).

In vivo LAPC and DC migration assays were conducted using i.n. CFSE instillation and by examining the extent of CFSE⁺ cells by FACS analysis of lungs and mLNs.

Adoptive transfer of LAPCs and DCs into recipient mice was performed using FACS-sorted cells from the lungs and mLNs of OVA-sensitized and OVA-challenged mice and i.v. injection (2×10^6 cells).

Methacholine dose–response studies were conducted using a flexiVent[®] system (Scireq Inc., Montréal, QC) to measure AHR.

In vitro co-cultures of naïve splenic DO11.10 T cells with FACS-sorted LAPCs or DCs from the lungs and DLNs of OVA-sensitized and OVA-challenged mice were set up to examine T-cell polarization, staining for intracellular GATA-3 and T-bet.

Details of the methods and statistical analyses used in these studies are provided in the Supporting Information.

Results

LAPCs are present in the lungs and DLNs of mice with OVA-induced allergic airway inflammation

We employed an OVA sensitization and challenge model to investigate the role of LAPCs in the development of allergic

airway inflammation in Balb/c mice. As anticipated, we observed an increase in inflammatory cell infiltrates and mucus production in the lungs of OVA-challenged mice compared with control mice (OVA-sensitized, but challenged with PBS), an increase in BAL and lung cell infiltrates, and an increase in circulating OVA-specific IgE (Figs S1 and S2). BAL from OVA-challenged mice exhibits a Th2 cytokine profile typical of an allergic response, with increases in IL-4, IL-5, IL-13, IL-6, IL-21, IL-17, and IL-22, but not in IFN- γ , IL-1 α , IL-2, IL-10, IL-27, IL-1 β , or TNF- α (Fig. S3). Importantly, LAPCs were evident in the lung tissues, BAL, and DLNs from the affected mice up to 9 days postchallenge (Fig. 1A–C). When compared to DCs, LAPCs in the lungs of OVA-challenged mice expressed higher levels of the cell surface receptor CXCR3, implicated in LAPC migration to DLNs (4), and higher expression of ICOSL and TSLPR, linked to promoting T2 immune responses (4) (Fig. 1D).

LAPCs accumulate in the lungs and traffic to the DLNs

To investigate the migration kinetics of LAPCs, we performed an *in vivo* migration assay, employing carboxyfluorescein diacetate succinimidyl ester (CFSE) (Fig. 2A). CFSE was administered i.n. 24 h after the final i.n. OVA challenge, allowing for the staining of all lung cell infiltrates, including LAPCs. We observed CFSE-positive LAPCs in the lungs of challenged mice at the earliest time point measured (6 h post-CFSE infusion), with CFSE-labeled LAPCs present in the lungs for a further 72 h (96 h post-OVA challenge), followed by their migration to the DLNs (Fig. 2B). This was confirmed by the analysis of absolute numbers of CFSE-positive LAPCs in the DLNs (Fig. 2Ci). Notably, when we considered LAPC accumulation in the DLNs, both CFSE stained and unstained, we identified a population of LAPCs that were apparently resident in the DLNs, after i.p. OVA sensitization (Fig. 2Cii). This is consistent with our observation of LAPCs in the DLNs at 24 h after the final OVA challenge, as shown in Fig. 1. Our earlier studies provided the evidence for resident LAPCs in lymphoid tissues of different strains of naïve mice, including LNs of Balb/c mice (2). One explanation is that LAPCs in the DLNs migrate to the lungs following the final i.n. OVA challenge, where they are then labeled with CFSE (Fig. 2Ciii). In comparison, pDCs exit the lungs, consistent with their accumulation in the DLNs (Fig. 2D). These data suggest that LAPCs and DCs exhibit different kinetics of lung and DLN accumulation.

LAPCs are present in the lungs of mice in an HDM model of allergic airway inflammation

Next, we considered a more biologically relevant allergen model of allergic airway inflammation that mimics the human allergic response. House dust mites (HDMs) contain various components that are allergenic and activate the immune system to initiate an inflammatory response (5, 6). Figure 3A outlines the sensitization and challenge protocol of i.n. instillation of HDM extract employed. We observed

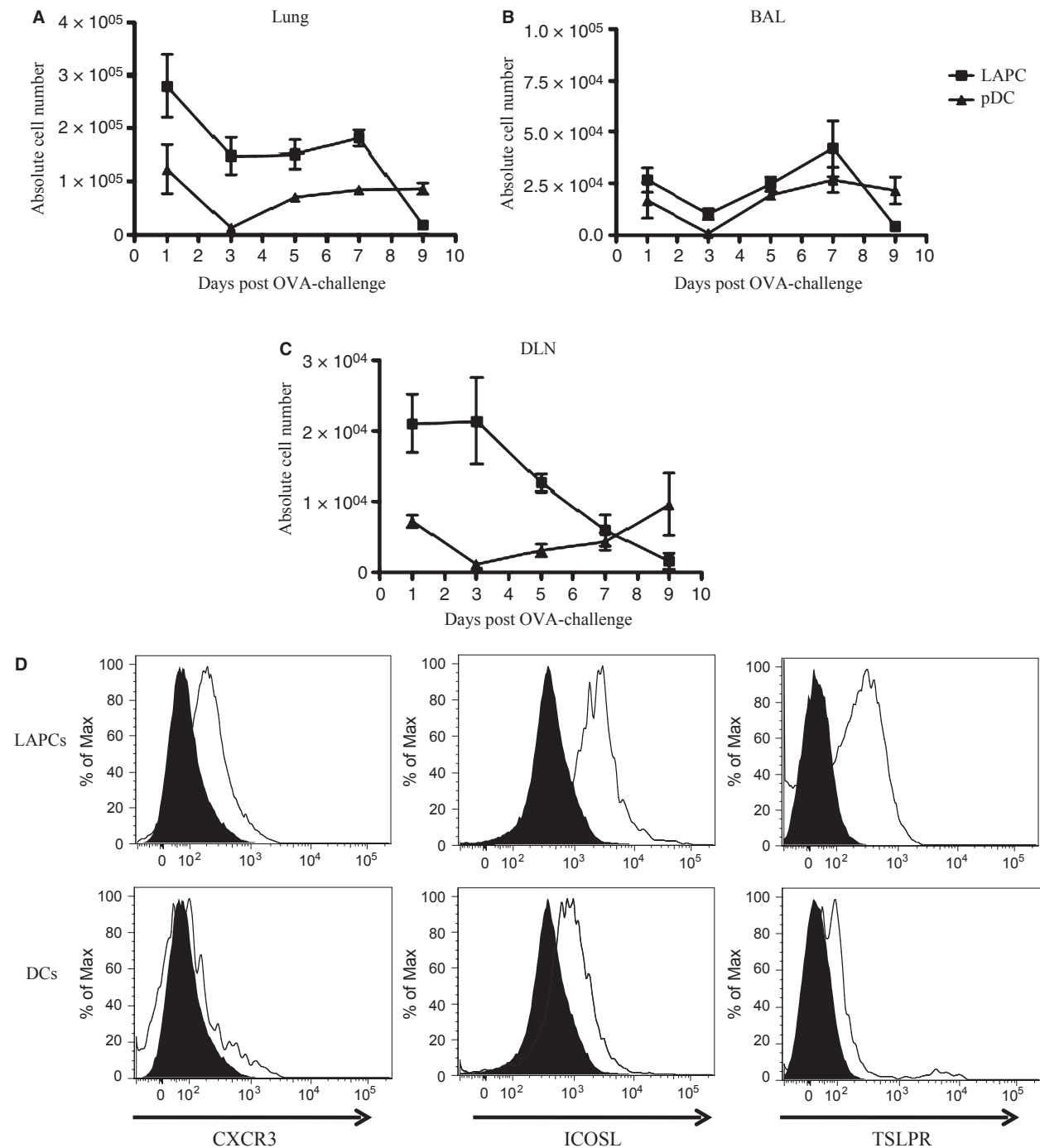


Figure 1 LAPCs are present in the lungs and DLNs of ovalbumin (OVA)-challenged mice. Mice were sensitized and challenged with OVA according to the protocol in Fig. 1A. (A) (B) (C) Mice ($n = 5$ per group) were killed at the indicated time points, and BAL fluid, lung tissue, and the mediastinal lymph nodes (DLN) were harvested. Cells were stained with the appropriate fluorochrome-conjugated mAbs for LAPCs (mPDCA-1⁺CD11c⁻TcR β ⁻B220⁻) and pDCs (mPDCA-1⁺CD11c^{int}). (D) In separate

experiments, 24 h following the final OVA challenge, mice ($n = 5$ per group) were killed, and BAL fluid, lung tissue, and DLNs were harvested. LAPCs (mPDCA-1⁺CD11c⁻TcR β ⁻B220⁻) and DCs (CD11c⁺) were stained with the relevant fluorochrome-conjugated antibodies to detect CXCR3, ICOSL, and TSLPR (open histograms). Isotype controls are included (black histograms). Data representative of two independent experiments are shown as means $\pm$ SEM.

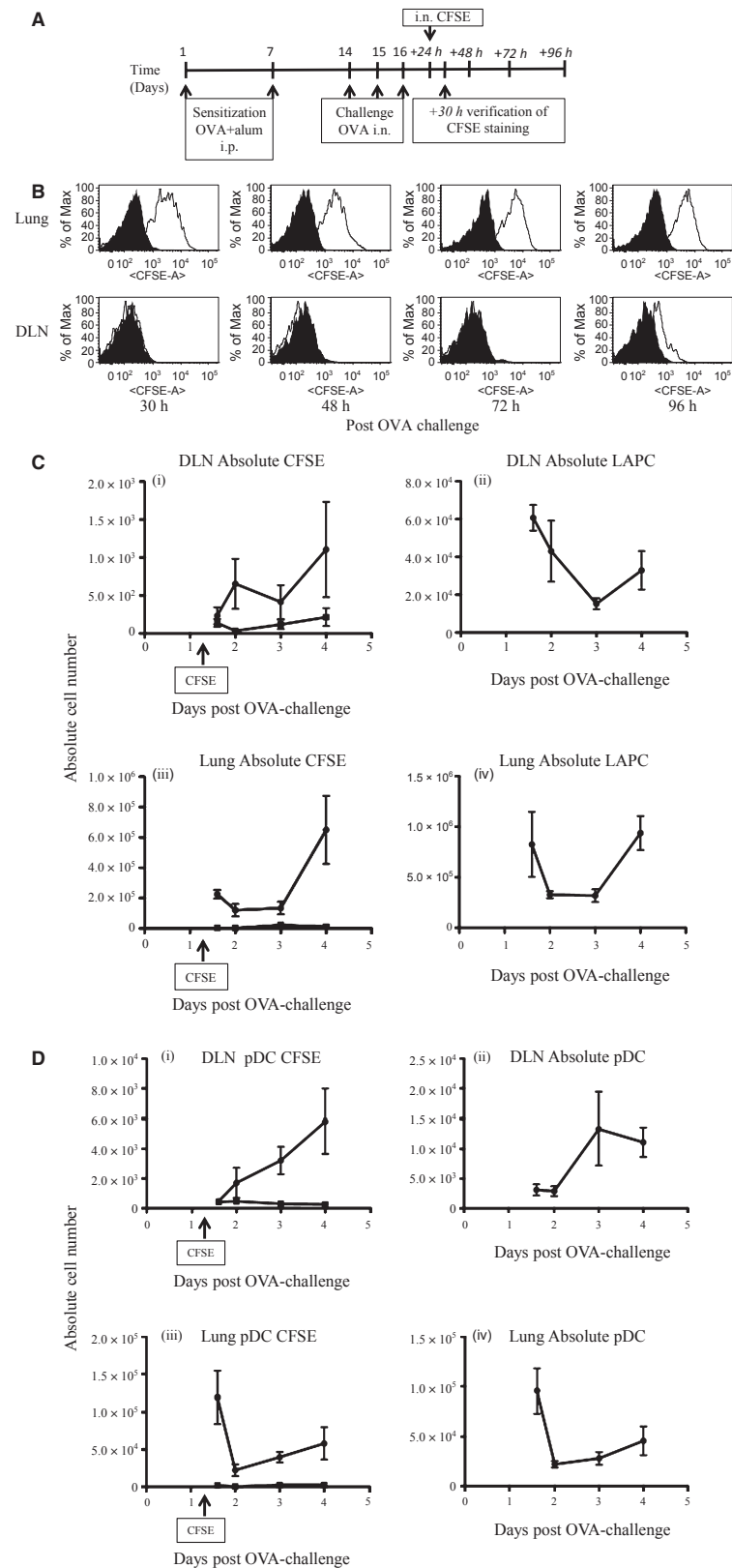


Figure 2 LAPCs accumulate in the lungs and traffic to the DLNs in a mouse model of allergic airway inflammation. (A) Schematic for murine model of allergic airway inflammation including CFSE instillation to track cell migration. Mice received an i.n. instillation of CFSE at the indicated time point post-ovalbumin (OVA) challenge. Control mice received i.n. instillation of the carrier medium, IMDM. (B) Following CFSE instillation, mice ($n = 3$ per group) were killed at the indicated time points, and lungs and DLNs were harvested. Tissues were processed and cells stained with the appropriate fluo-

rochrome-conjugated mAbs for LAPCs (mPDCA-1⁺CD11c⁺TcR β -B220⁺). (C) (i) CFSE-labeled LAPCs in the DLN, (ii) CFSE-labeled and CFSE-unlabeled LAPCs in the DLN, (iii) CFSE-labeled LAPCs in the lung, and (iv) CFSE-labeled and CFSE-unlabeled LAPCs in the lung. (D) (i) CFSE-labeled pDCs in the DLN, (ii) CFSE-labeled and CFSE-unlabeled pDCs in the DLN, (iii) CFSE-labeled pDCs in the lung, and (iv) CFSE-labeled and CFSE-unlabeled pDCs in the lung. Data representative of two independent experiments are shown as means $\pm$ SEM.

an influx of inflammatory cells in the lungs of HDM-challenged mice compared with PBS controls (Fig. 3B). HDM-challenged mice exhibited an increase in total circulating IgE levels (Fig. 3C), an increase in lung eosinophilia (Fig. 3D), and enhanced LAPC numbers in their lungs (Fig. 3E). *In vivo* methacholine challenges were conducted in anesthetized mice to assess the methacholine-induced changes in total respiratory resistance (R_{rs}) in the mice with allergic airway inflammation (Fig. 3F, G). We observed modest increases in total respiratory resistance that are methacholine dose-dependent (Fig. 3F) and a greater maximum resistance in response to methacholine in the HDM recipient mice compared with PBS controls (Fig. 3G).

OVA-activated LAPCs modulate T2 immunity *in vivo*

We next examined the contribution of LAPCs to the Th2 response by *in vivo* adoptive transfer experiments (Fig. 4A). Mice were induced to develop allergic airway inflammation, and 24 h following the final OVA challenge, the mice were euthanized, their lungs and DLNs were harvested, and LAPCs and DCs were FACS-sorted. LAPCs or DCs were then injected into the tail vein of OVA-sensitized recipient mice, 2 h following their first OVA challenge (at the same time point as in the initial model). Recipient mice then underwent two more days of OVA challenge and were euthanized 24 h after the final OVA challenge. We observed a greater influx of inflammatory cells and mucus production in LAPC recipient mice compared with mice that received either DCs or PBS (Fig. 4B). Similarly, LAPC recipient mice developed enhanced eosinophilia (Fig. 4C) and increased levels of circulating OVA-specific IgE (Fig. 4D) compared with DC or PBS recipients. Importantly, examination of T-cell types in both the DLNs and lungs of recipient mice revealed a greater skewing toward Th2 cells in LAPC recipients compared with the DC and PBS recipients, with no changes in Th1 cell numbers (Fig. 4E). The BAL of LAPC recipient mice exhibited an increase in T2 cytokines, namely IL-4, IL-5, IL-13, IL-6, IL-21, IL-17, and IL-22, compared with DC and PBS recipients (Fig. 4F).

Methacholine dose-response studies were conducted to evaluate changes in total respiratory resistance (R_{rs}) (Fig. 4G, H). There were no significant differences in respiratory resistance changes in the OVA-sensitized and OVA-challenged mice that received either PBS vehicle or DCs. However, in the LAPC recipient mice, we observed significant increases in

both the methacholine dose-response relationships (Fig. 4G) and the maximal total respiratory resistance to methacholine (Fig. 4H).

LAPCs induce Th2 effector cell polarization *ex vivo*

In a final series of experiments, we examined whether LAPCs from OVA-challenged mice would induce polarization of T cells *ex vivo*. LAPCs and DCs were harvested from the lungs and DLNs of mice 24 h after their final OVA challenge. Naive CD4⁺ T cells were obtained from spleens of DO11.10 mice. DO11.10 mice carry an MHC class II-restricted T-cell receptor that reacts with OVA and should therefore be restricted to activation by LAPCs or DCs presenting OVA on MHC class II. LAPCs or DCs were cultured with the CD4⁺ transgenic T cells for 96 h. CD4⁺ T cells were subsequently stained for intracellular GATA-3 (Fig. 5A) and T-bet (Fig. 5B). Co-culture with LAPCs resulted in enhanced GATA-3 expression, but not T-bet expression, compared with co-culture with DCs.

Discussion

In this study, we provide evidence for a role of LAPCs in modulating a T2 immune response in the lungs of mice with allergic airway inflammation. Previous studies have provided evidence that APCs, particularly DCs, promote allergic inflammation in mice (7, 8). Characteristically, mouse lungs have resident cDCs and pDCs. cDCs may be CD11c⁺CD103⁺ and CD11c⁺CD11b⁺. CD103⁺ DCs are closely associated with respiratory epithelial cells, while CD11b⁺ DCs are found within the lung parenchyma (9, 10). While both subsets are capable of priming and re-stimulating lung CD4⁺ T cells, it remains unclear which subset of DCs is responsible for allergic airway inflammation. Recent evidence suggests that it is the CD103⁺ DCs that are responsible for priming Th2 responses, while CD11b^{hi} DCs prime Th1 responses (11). Other data suggest that it is resident CD11b⁺Ly6⁺ lung DCs that are required for the induction of allergic airway inflammation (12). Most recently, studies have suggested that conventional CD11b⁺ DCs and not CD103⁺ cDCs drive Th2 immunity and that there is a role for monocyte-derived CD11b⁺ in promoting allergic inflammation in the lungs of HDM-treated mice (13). In contrast, pDCs are involved in generating tolerance and protection against inhaled antigen in mice, which is

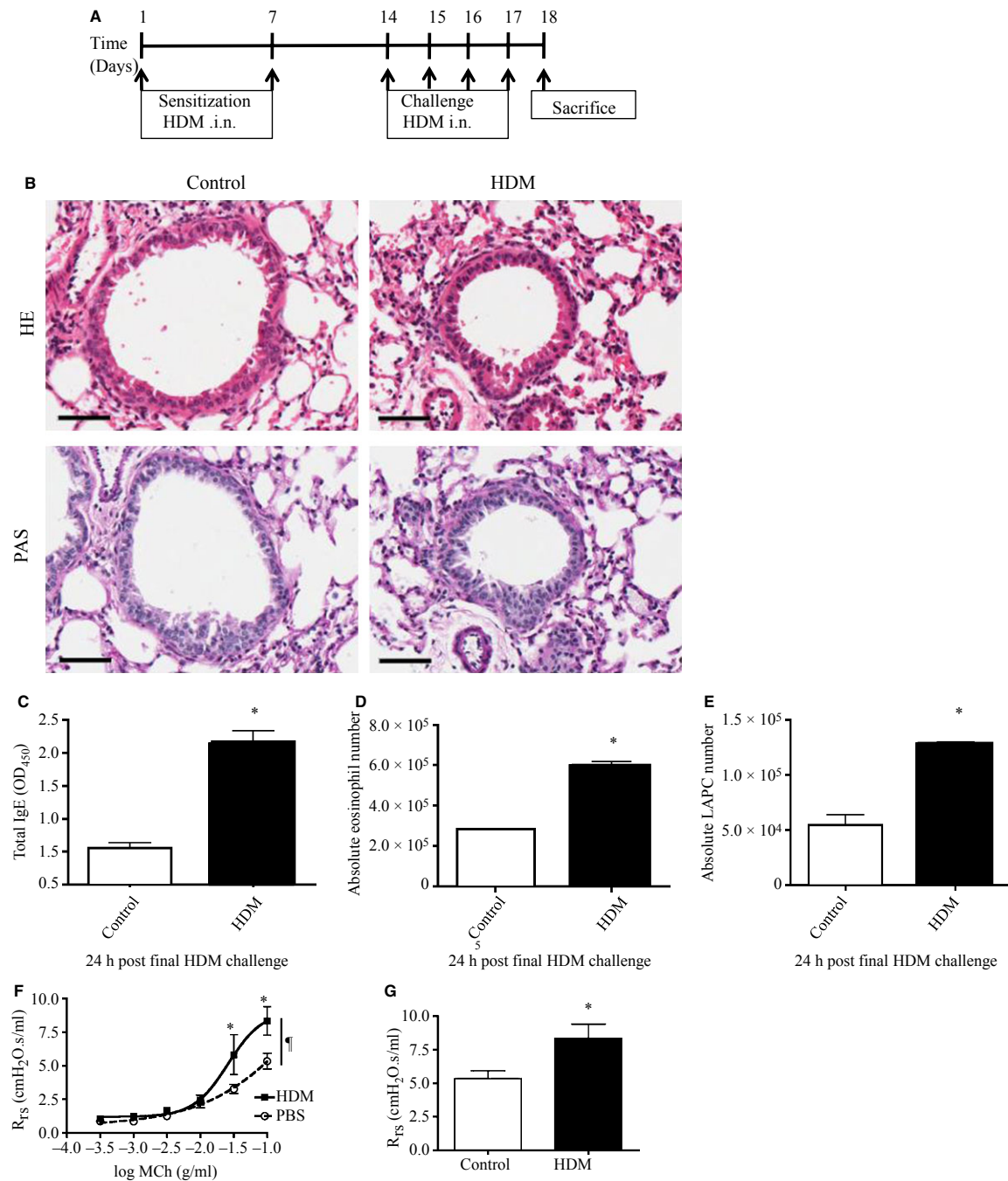


Figure 3 LAPCs are present in the lungs of mice in an HDM model of allergic airway inflammation. (A) Schematic for HDM model of allergic airway inflammation. Mice received i.n. instillations of 2.5 mg/ml of HDM extract Der p in 20 μ l of PBS on days 1 and 7, followed by i.n. challenge of 10 μ l of the same extract on days 14, 15, 16, and 17. Control mice were sensitized with HDM extract and challenged with i.n. PBS. (B) 24 h following the final HDM challenge, lungs were harvested, thin sections prepared and fixed in formalin, then stained with HE or PAS. 20 $\times$ magnification. Size bar = 60 μ m. Images are representative of two independent experiments. (C) At the indicated time points ($n = 3$ per group), serum

was collected via cardiac puncture and analyzed for total IgE by ELISA; (D) eosinophil and (E) LAPC infiltrates were assessed by staining of cells with the appropriate fluorochrome-conjugated mAbs: eosinophils (SiglecF⁺CD11c⁻) and LAPCs (mPDCA-1⁺CD11c⁻TcR β -B220⁻). (F) (G) 24 h following the final HDM challenge, methacholine dose-response studies were conducted (Methods) to examine changes in total respiratory resistance (R_{rs}). Data representative of two independent experiments are shown as means $\pm$ SEM and were analyzed using Student's t test. * $P < 0.05$.

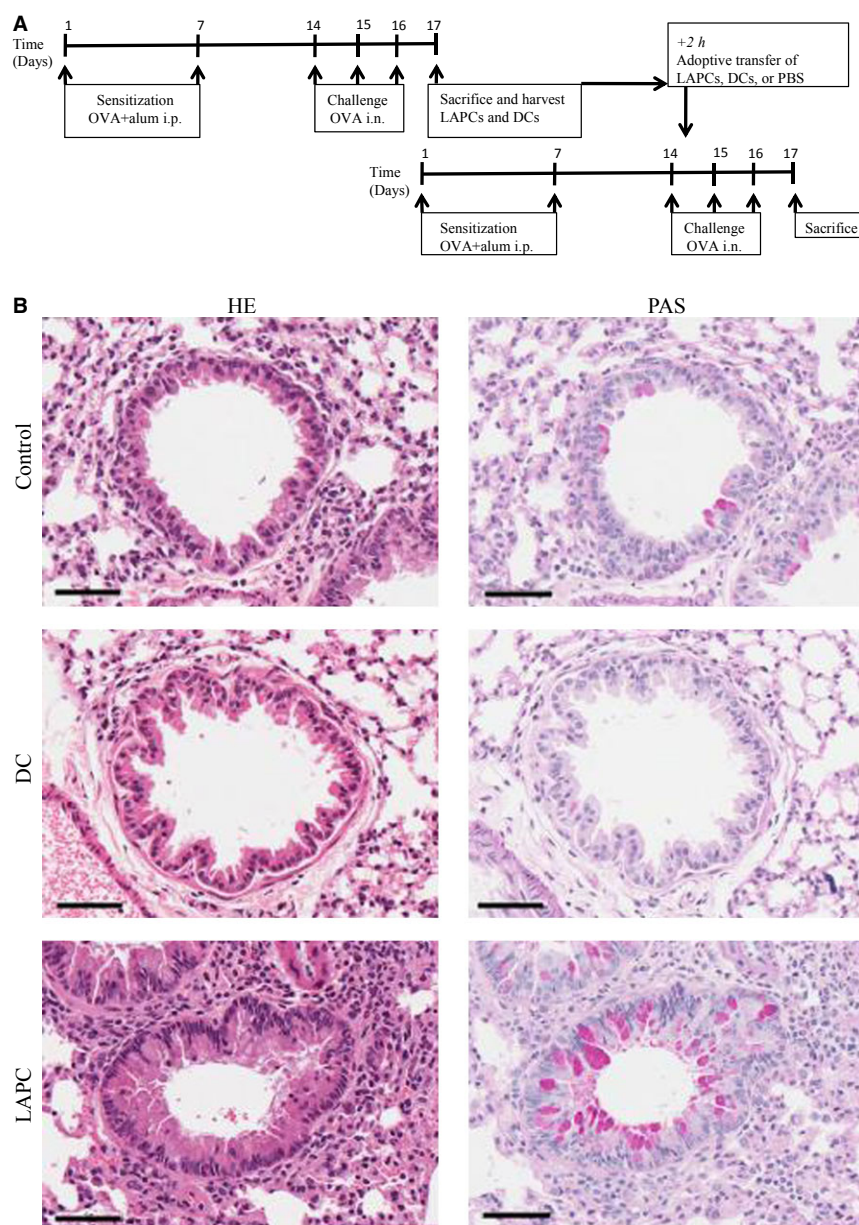
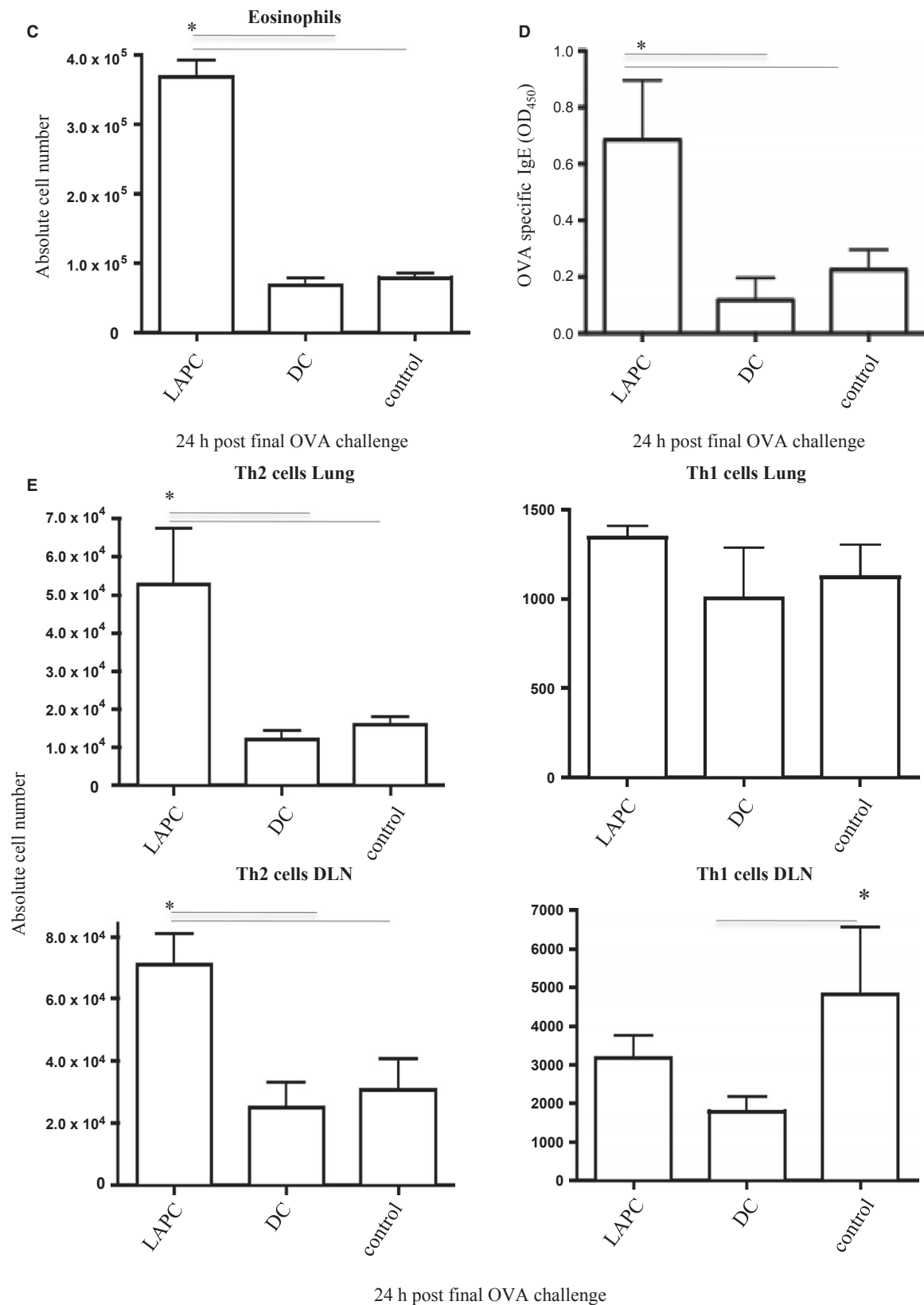


Figure 4 Ovalbumin-activated LAPCs modulate T2 immunity *in vivo*. (A) Schematic for LACP adoptive transfer experiments. Mice ($n = 50$) were killed at the indicated time points, and lungs and DLNs were harvested. Cells were stained with the appropriate fluorochrome-conjugated mAbs for LAPCs (mPDCA-1⁺CD11c⁺TcR β ⁺B220⁺) and cDCs (CD11c⁺B220⁺) and were then FACS-sorted. Two hours after the first ovalbumin (OVA) challenge, LAPCs or DCs (2.0×10^6 cells/mouse) or PBS (carrier) were adoptively transferred into the recipient mice by tail vein injection. (B) Twenty-four hours after the final OVA challenge, lungs were harvested, thin sections were prepared, fixed in formalin, and then stained with HE or PAS. 20 $\times$ magnification. Size bar = 60 μ m. Images are representative of two independent experiments. (C) Mice ($n = 5$ per group) were killed, and eosinophil infiltrates in the BAL and lung tissue

were assessed by staining of cells with the appropriate fluorochrome-conjugated mAbs (SiglecF⁺CD11c⁺). (D) Twenty-four hours after the final OVA challenge, cardiac punctures were performed and the mice were killed ($n = 5$ per group). OVA-specific IgE was assessed by ELISA. (E) Tissues were processed, and cells stained with the appropriate fluorochrome-conjugated mAbs for Th2 (CD3⁺CD4⁺IL-4⁺) and Th1 (CD3⁺CD4⁺IFN- γ ⁺). (F) 24 h following the final OVA challenge, mice ($n = 3$ per group) were killed, and BAL assessed for cytokine production by FlowCytomix. (G) (H) Twenty-four hours after the final OVA challenge, methacholine dose-response studies were conducted to examine changes in total respiratory resistance (R_{rs}). Data representative of two independent experiments are shown as means $\pm$ SEM and were analyzed using Student's t test. * $P < 0.05$.

**Figure 4** (Continued)

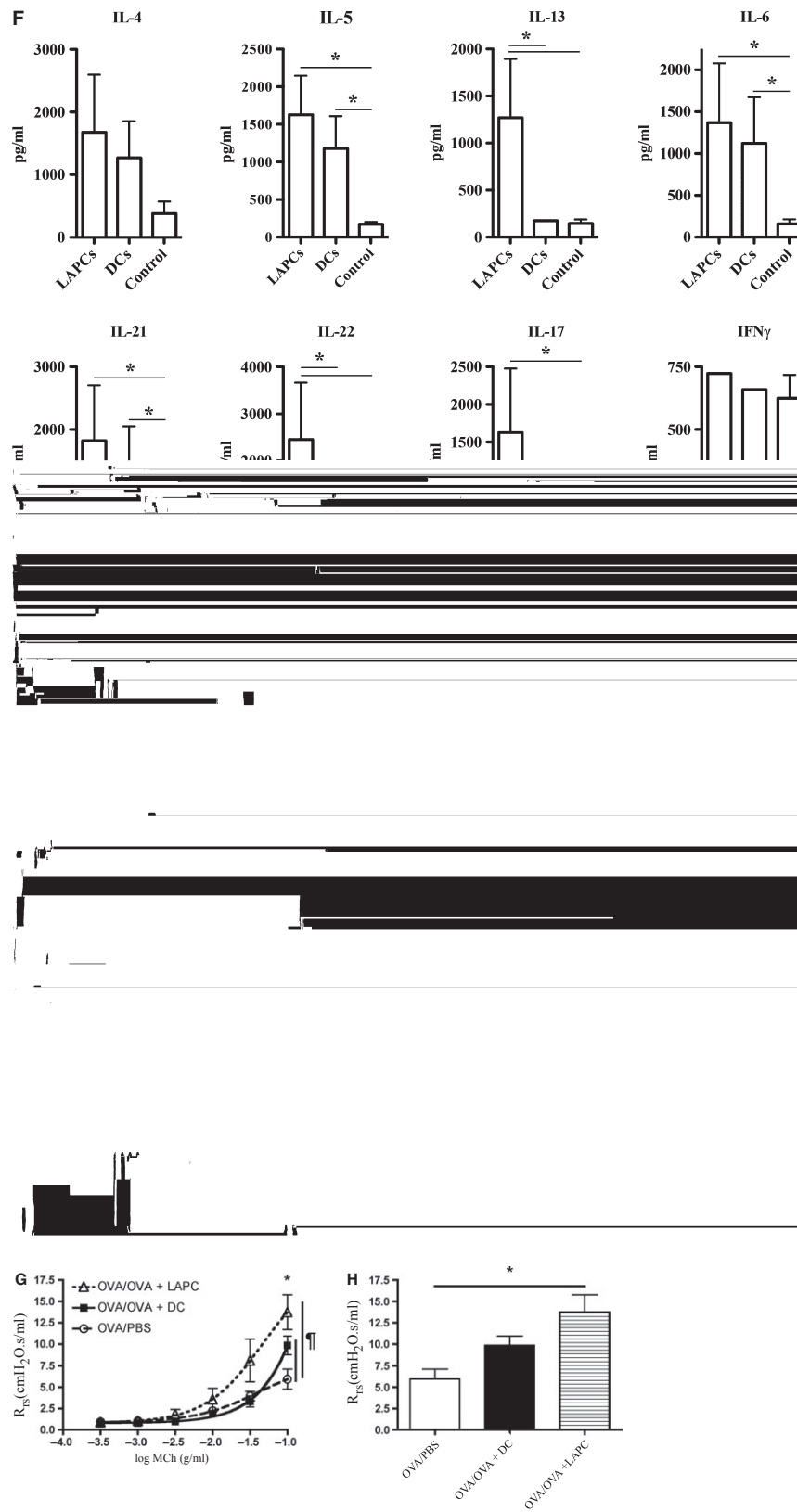


Figure 4 (Continued)

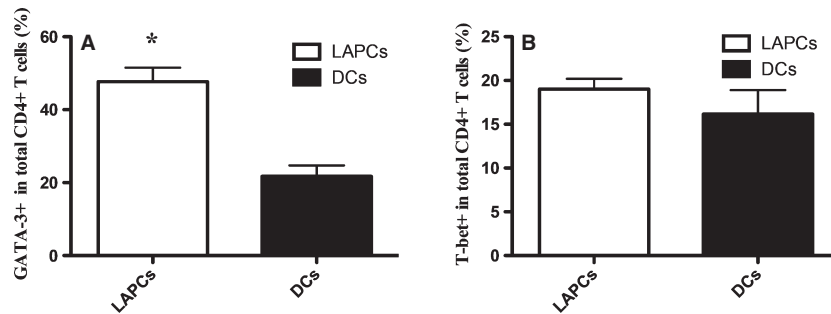


Figure 5 LAPCs promote Th2 differentiation of naïve CD4⁺ T cells. Mice were sensitized and challenged with ovalbumin (OVA) according to the protocol in Fig. 1A. Mice ($n = 10$ per group) were killed at 24 h following the final OVA challenge, and lungs and DLNs were harvested. Cells were stained with the appropriate fluorochrome-conjugated mAbs for LAPCs (mPDCA-1⁺CD11c⁺TcRβ⁺B220⁺) and cDCs (CD11c⁺B220⁺) and FACS-sorted. Splenocytes were harvested from naïve DO11.10 mice, and CD4⁺ T cells

sorted using MACS CD4⁺ microbeads (Methods). LAPCs or DCs from OVA-challenged mice were co-cultured with naïve CD4⁺ T cells from DO11.10 transgenic mice for 96 h. (A) GATA-3 and (B) T-bet expression was analyzed by intracellular staining of cells with the appropriate fluorochrome-conjugated mAbs (GATA-3, T-bet, and CD4). Data representative of two independent experiments are shown as means $\pm$ SEM and were analyzed using Student's *t* test. * $P < 0.05$.

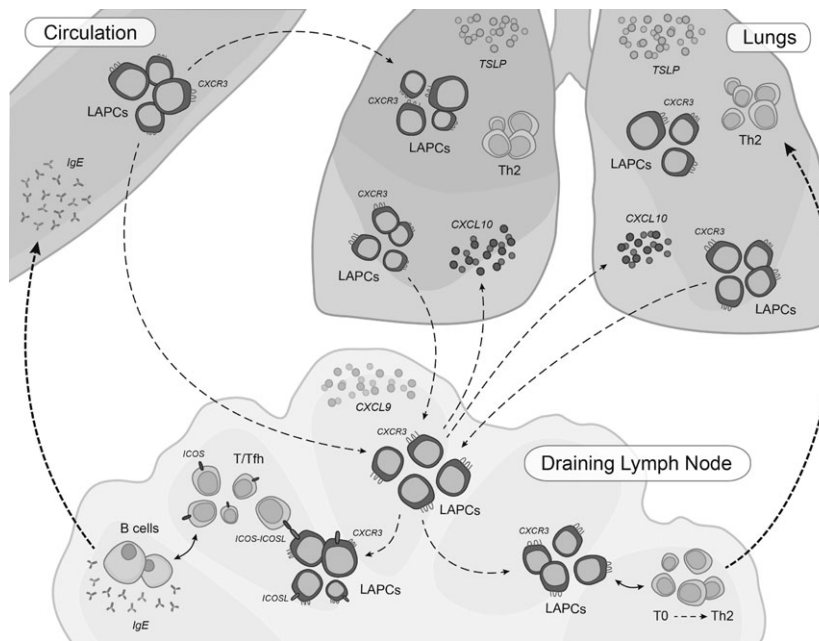


Figure 6 Scheme outlining mechanisms whereby LAPCs may promote T2 immunity. Allergen-activated LAPCs capture antigen in the lungs and migrate to the DLNs, mediated by CXCL9–CXCR3 interactions, where they encounter T and B cells. T cells are polarized to Th2 lineage commitment, exit the DLN and traffic to the lungs where TSLP is a co-factor in Th2 polarization. LAPCs may also promote T follicular helper (Tfh) cell differentiation in the DLN, mediated by ICOS–ICOSL-dependent signaling. Tfh cells

promote B-cell class switching to IgE. In the case of the ovalbumin (OVA) sensitization and challenge protocol for allergen-induced airway inflammation, OVA sensitization by i.p. injection results in OVA-activated LAPCs in the circulation that traffic either to the lungs, mediated by CXCR3–CXCL10 interactions, or to the DLNs, mediated by CXCR3–CXCL9 interactions. Subsequent i.n. OVA challenge would trigger LAPC emigration from the DLN to the lungs.

likely linked to their capacity to secrete large amounts of type I IFN and drive regulatory T-cell polarization (14–16). In addition to these DC populations, there may also be minor roles for CD8α⁺ DCs and basophils as APCs involved in airway inflammation, although their numbers are rare in the lung and possibly dispensable for airway

inflammation (17–19). It is clear that there are a number of potentially different APC populations present in the lung that may have different potencies in priming airway inflammation. Adding to this heterogeneity, we provide evidence that in both OVA sensitization and HDM models of mouse allergic airway inflammation, in comparable numbers to

DCs, there is a novel APC population, LAPCs, in the lungs and DLNs of affected mice.

We have shown that LAPCs accumulate in the lungs of OVA-sensitized and OVA-challenged mice and have the capacity to migrate to the DLN during the course of an asthma-like allergic airways inflammatory response (Fig. 6). Our data also suggest that LAPCs that accumulate in the DLN following allergen challenge may acquire the ability to migrate to the lung, although we cannot rule out the possibility that this late influx of LAPCs into the lung may be due to delayed migration of LAPCs out of the circulation. Several lines of evidence suggest that it is the interaction between chemokines CCL19 and CCL21, and their cognate receptor, CCR7, expressed on DCs that is responsible for the migration of DCs from the lung to the DLNs during allergic inflammation in the lung (8, 20, 21). In addition, a role for CD47 and its ligand, signal regulatory protein α (SIRP- α), in rendering DCs competent to migrate from the lungs to DLNs during allergic airway inflammation, has been suggested (22). Our earlier studies with LAPCs in a mouse model of influenza A virus infection identified that LAPCs express the chemokine receptor CXCR3, enabling their initial trafficking out of the circulation into infected lungs that produce CXCL10, mediated by CXCR3–CXCL10 interactions, followed by migration into the DLN via CXCR3–CXCL9 interactions (2, 4). A similar mechanism may be responsible for the migration of LAPCs into the lungs and DLNs during allergic airway inflammation, given the cell surface CXCR3 expression in OVA-activated LAPCs (Fig. 1). Notably, in mouse models of allergic airway inflammation, pDCs migrate between the lungs and DLNs orchestrated by similar chemokine–chemokine receptor interactions (23, 24).

Adoptive transfer of OVA-activated LAPCs into recipient mice led to exacerbation of T2 immunity, as demonstrated by increased eosinophilia, increased IgE production, increased Th2 cell numbers, and a worsening of AHR. Following sensitization with HDM, lung epithelial cells produce TSLP, IL-25, and IL-33, driving DCs to preferentially promote Th2 cell lineage commitment (25). Mediated by OX40, Notch ligand, and Jagged 2, these DCs polarize a Th2 response (26). Having demonstrated that LAPCs express the receptor for TSLP, we speculate that LAPCs may promote Th2 polarization using mechanisms similar to DCs. GATA-3 is the master regulator of Th2 commitment (27, 28). We have shown that LAPCs taken from mice sensitized and challenged with OVA upregulate GATA-3 in naïve OT-II-specific T cells.

Recent studies identified a role for LAPCs in promoting T follicular helper (Tfh) cell differentiation, mediated by ICOS–ICOSL-dependent signaling (4). We provide evidence for ICOSL cell surface expression on LAPCs 24 h after the final OVA challenge. LAPCs may have a role in priming an IgE

response mediated by Tfh cells, as adoptive transfer of LAPCs resulted in increased circulating OVA-specific IgE in the OVA mouse. While it has been suggested that IL-21, which we demonstrate to be present in the BAL of mice with allergic airway inflammation, is a cytokine produced by Tfh cells that inhibits IgE class switching, data indicate that other factors may override this IL-21-mediated inhibition (29–31). IgE class switching can occur outside the germinal center, and somatic hypermutation to high-affinity IgE B cells occurs via switching from IgG1 B cells outside the lymph node (32, 33). In other studies, class switching to IgE occurred within the germinal center, independent of IgG1 B cells, evident in mice lacking the gamma 1 switch (34, 35).

Altogether, our studies suggest a role for LAPCs in the development of allergic inflammation in the lungs of allergen-challenged mice. Our data are supportive of a role for LAPCs in promoting the polarization of a Th2 response that results in exacerbation of pulmonary pathology.

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Author contributions

CH and ENF contributed to the conception and design of the study, and ENF supervised the project. CH performed the experiments. JAS and CWC assisted with the flexiVent system studies and corresponding data analysis. CH and ENF conducted the other data analysis and interpretation of results. CH and ENF wrote the manuscript, and CWC and JAS reviewed and edited the article.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. OVA challenged mice exhibit lung pathology consistent with asthma.

Figure S2. OVA challenged mice exhibit eosinophilia and OVA-specific IgE.

Figure S3. OVA challenged mice exhibit a T2 cytokine profile.

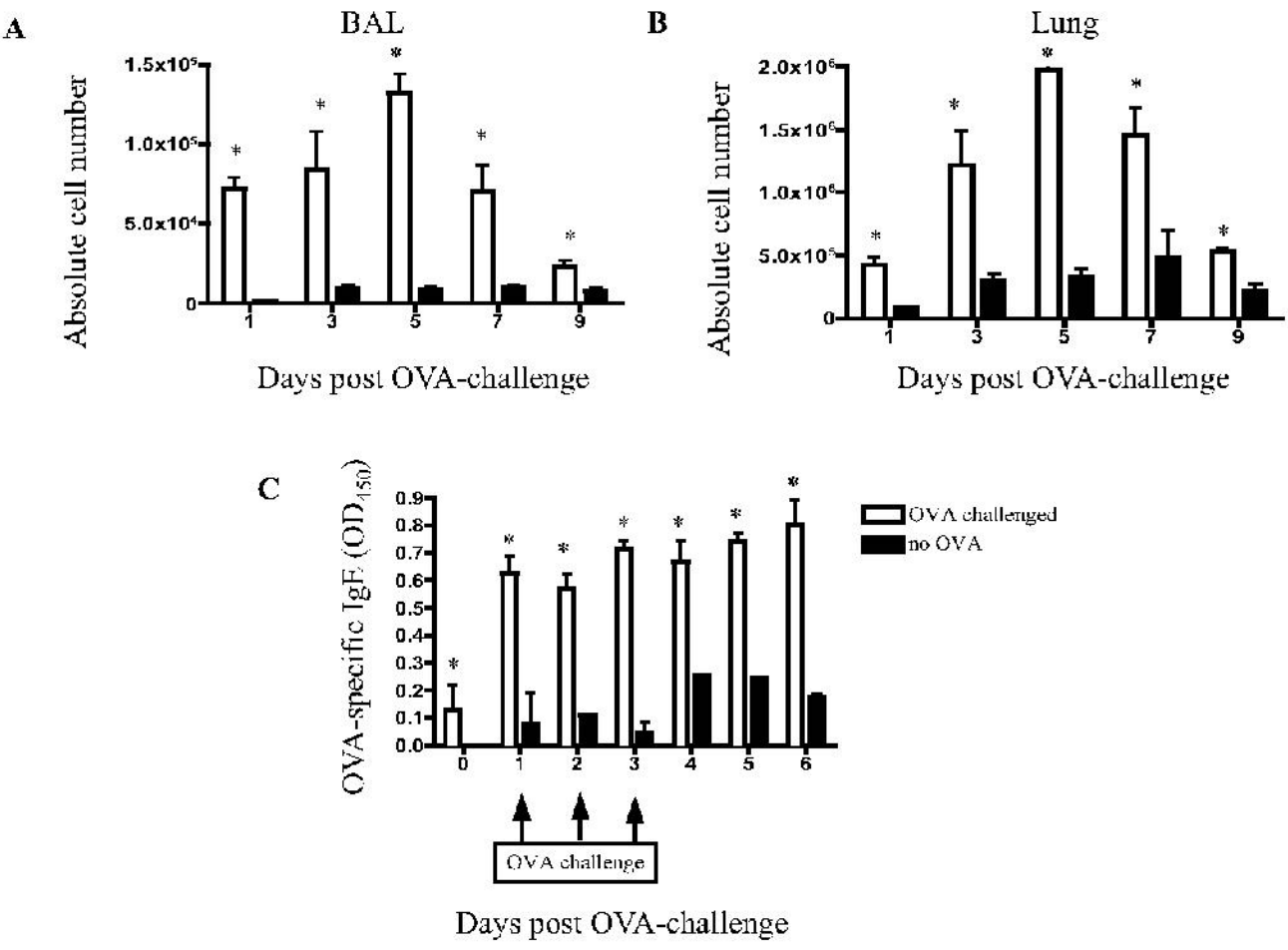
Data S1. Supplementary Information.

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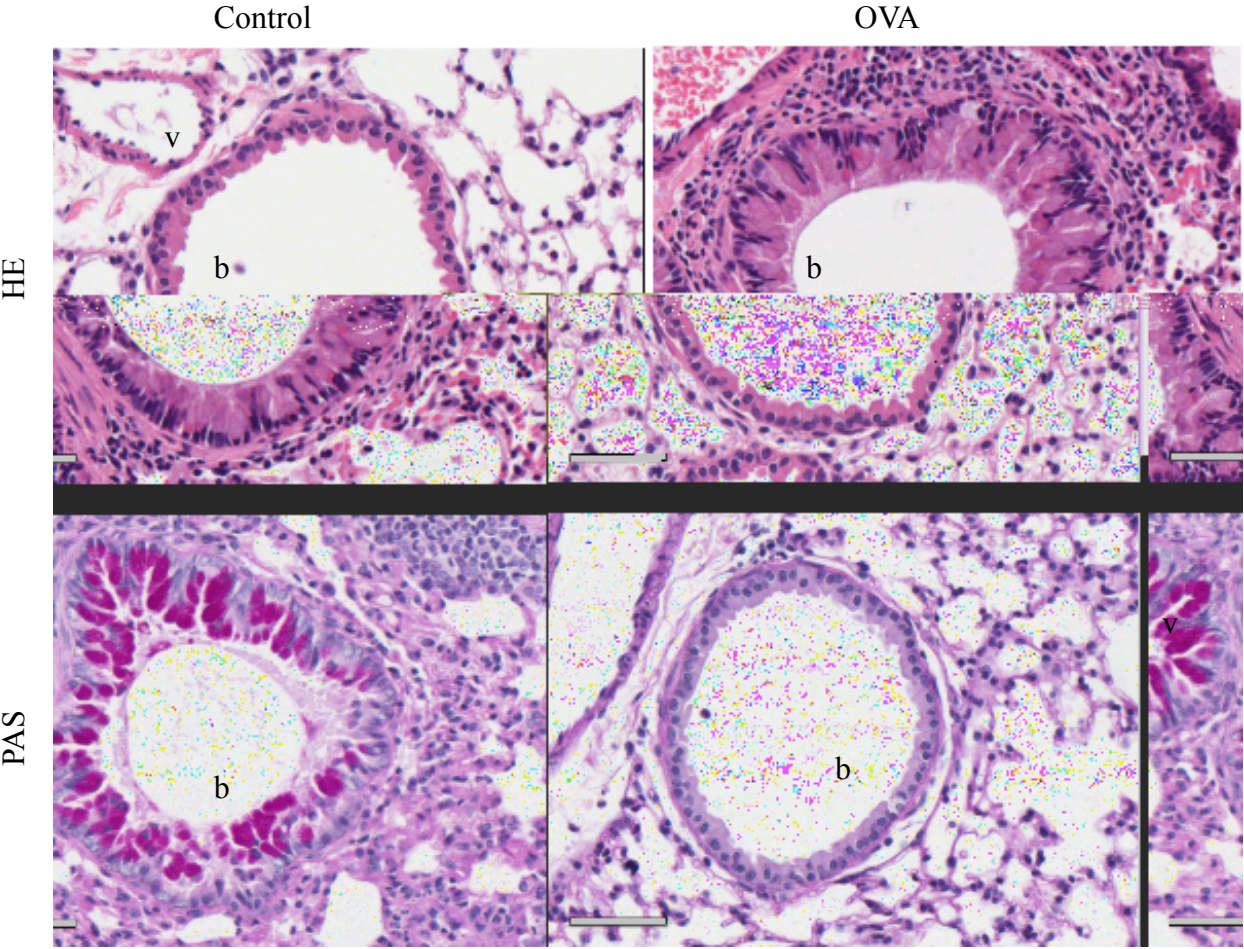
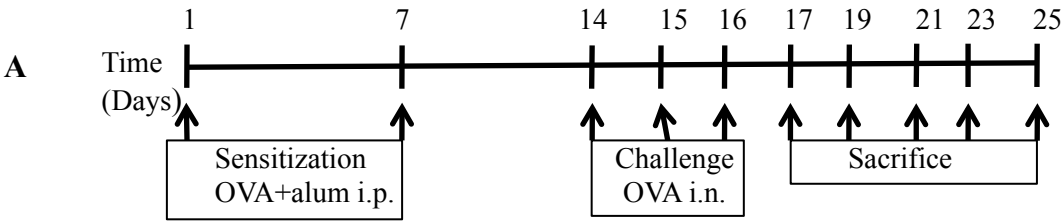
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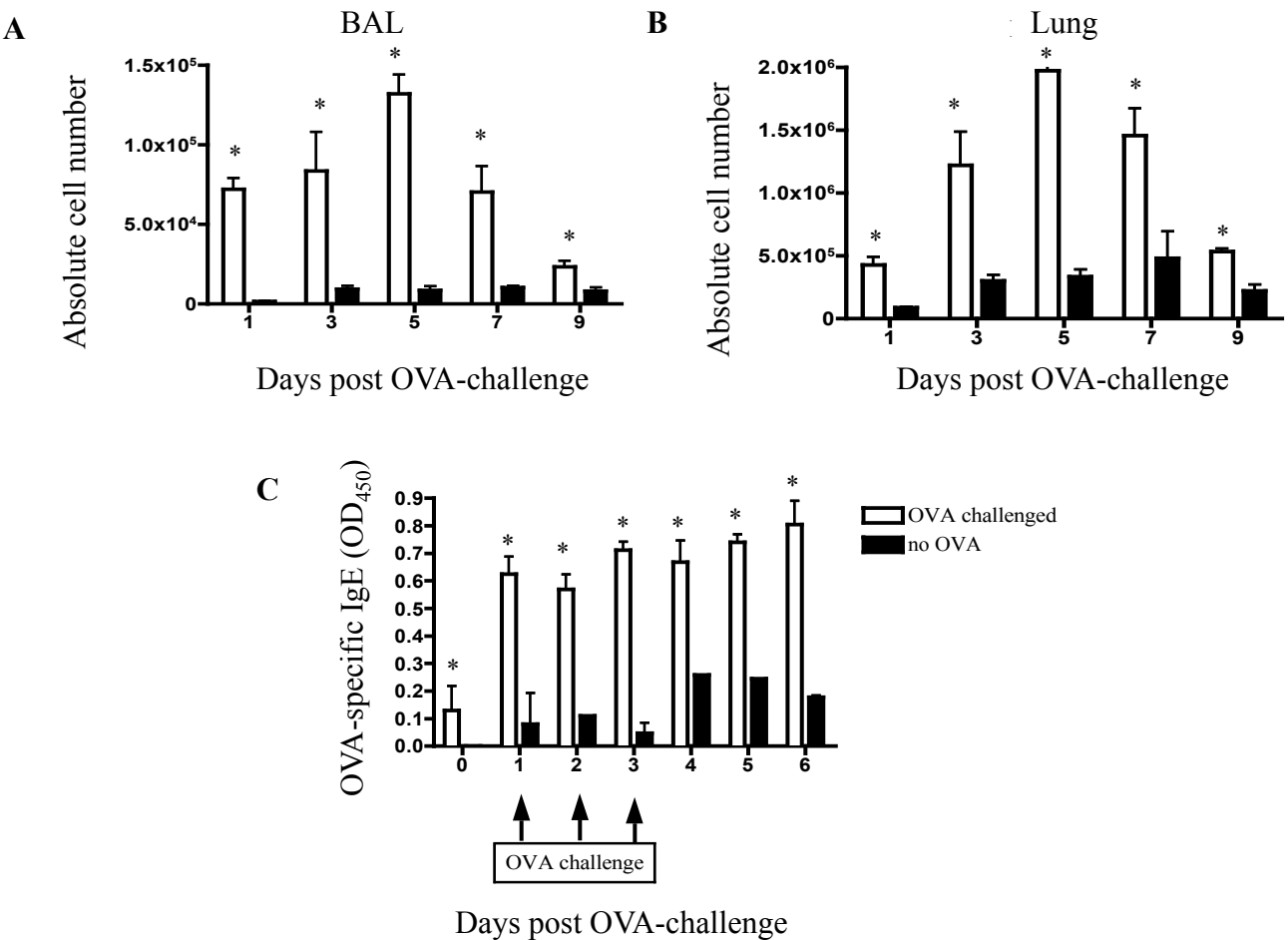
Supplementary Figure 2



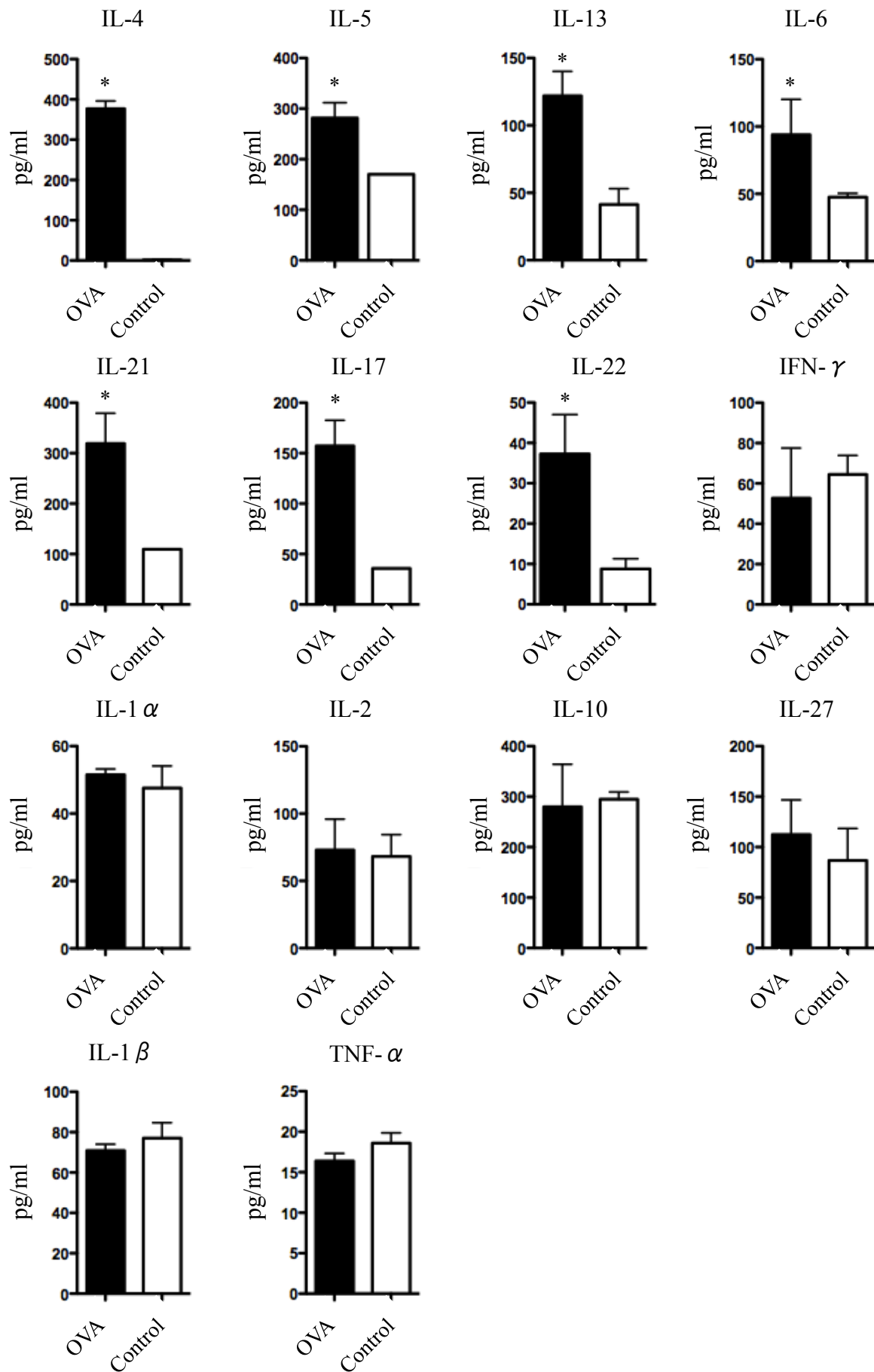
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



Supplementary Figure 1. OVA challenged mice exhibit lung pathology consistent with asthma.

(A) Mice (n=15) received 2 intraperitoneal (i.p.) instillations of 50µg of OVA absorbed in 2 mg of alum, followed by i.n. (i.n.) instillation of 50µg of OVA on three consecutive days. Control mice (n=15) were sensitized with OVA and challenged with i.n. PBS. **(B)** Twenty-four hours following the final OVA challenge, lungs were harvested from 3 OVA challenged and 3 control mice and thin sections were prepared and fixed in formalin, then stained with HE or PAS. b~bronchus, v~vessel. 20 x magnification. Size bar = 60 µm. Representative images from 2 independent experiments.

Supplementary Figure 2. OVA challenged mice exhibit eosinophilia and OVA-specific IgE.

Mice were sensitized and challenged with OVA according to the protocol in Figure 1A. At the indicated time, mice (n= 3 per group) were sacrificed and eosinophil infiltrates in the **(A)** BAL and **(B)** lung tissue, were assessed by staining with the appropriate fluorochrome-conjugated mAbs (SiglecF⁺CD11c⁻). **(C)** At the indicated time points, serum was collected via cardiac puncture (n=3 mice per group) and analyzed for OVA-specific IgE by ELISA. Data representative of two independent experiments are shown as means ± SEM and were analyzed using a Student's t test. *, p<0.05.

Supplementary Figure 3. OVA challenged mice exhibit a T2 cytokine profile.

Mice were sensitized and challenged with OVA according to the protocol in Figure 1A. 24 hours following the final OVA challenge mice were sacrificed (n=3 per group) and cytokine levels in the BAL were analyzed using FlowCytomix.

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Supplementary Information

Induction of allergic airways inflammation

We employed an OVA sensitization and challenge sub-acute model of allergic airways inflammation: Mice were sensitized by intraperitoneal (i.p.) injection with 50 μ g of OVA (Grade V, Sigma-Aldrich) absorbed to 2 mg of Imject® Alum (Thermo Scientific) on days 0 and 7. On days 14, 15 and 16, mice were anesthetized by i.p injection of ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively) and challenged by intranasal (i.n.) administration with 50 μ l PBS containing 50 μ g of OVA. Control mice received PBS only. At the indicated times mice were sacrificed by cervical dislocation and their lungs and mediastinal lymph nodes were harvested and processed.

Additionally, a house dust mite (HDM) model of allergic airways inflammation was employed: Whole body *Dermatophagoides pteronyssinus*, crushed by mortar and pestle (Greer Laboratories) was dissolved in sterile PBS at 2.5 mg total weight/ml. For HDM extract sensitizations, mice were anesthetized as described above and 20 μ l of HDM extract was introduced by i.n. instillation on days 0 and 7, followed by four consecutive days of i.n. challenge from day 14 with 10 μ l of the same extract. Control mice received an i.n. challenge of PBS. Mice were sacrificed 24 h after the final challenge and the relevant tissues harvested.

Cell fractionation

Mice were euthanized by cervical dislocation and their lungs and mediastinal lymph nodes (mLN) were harvested, mechanically disrupted, and enzymatically digested with 1 mg/ml collagenase D and 0.3 mg/ml of DNase I (Roche) in cold PBS supplemented with 1mM MgCl₂ and 1.8 mM CaCl₂ at 37°C for 30 min. The digestion reaction was stopped by the addition of 1mM EDTA for 10 min at room temperature. A single-cell suspension was prepared by filtering the tissue through a 70 µm nylon mesh. RBCs were removed using ACK lysis buffer. Cell numbers were determined by manual counting using a hemocytometer.

To obtain bronchoalveolar lavage (BAL) samples the lungs were harvested with the trachea *en bloc* and washed 3 times with 1 ml PBS. Cells were pelleted by centrifugation and the supernatants were collected and stored at -80°C until analyzed.

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BAL supernatants were collected as described and cytokine levels were analyzed using a mouse Th1/Th2/Th17/Th22 multiplex kit FlowCytomix (eBiosciences):

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Adoptive transfer of LAPCs and DCs

24 h after the final OVA challenge mice were sacrificed and their lungs and mLNs were harvested. LAPCs and DCs were collected by FACS sorting using either a FACS Aria II (BD Biosciences) or a MoFlo (Beckman Coulter). Isolated cells were subjected to FACS analysis to confirm purity ($\geq 98\%$) (5). LAPCs or DCs (2×10^6 cells in 200 μ l of sterile PBS) were injected i.v. into recipient mice 2h after their first OVA challenge; control mice received PBS alone. Recipient mice were sacrificed 24 h following the final OVA challenge and their lungs and mLNs harvested for the indicated analyses.

In vivo responsiveness to methacholine

Mice were anaesthetised (ketamine/xylazine) and underwent intratracheal cannulation with a sterile 18G stainless steel cannula (18G BD Intramedic™ Luer-Stub Adapter, Fisher Scientific, Mississauga ON) for pulmonary function testing on a flexiVent® system (Scireq Inc., Montréal QC), as described previously (8-10). Mice were ventilated at 150 breaths per minute with a tidal volume of 10 mL/kg. After stable baseline resistance measures were obtained, methacholine (0.3-100 mg/mL in phosphate buffered saline) was administered directly into the ventilatory circuit using a Standard Volume AeroNebLab nebulizer (Scireq Inc.; median diameter: 4.0-6.0 μ m). The maximum total respiratory resistance (R_{rs}) was taken at each dose; only values with a Coefficient of Determination >0.95 were accepted for subsequent statistical analyses.

Coculture of naïve T cells with LAPCs

Mice were OVA sensitized and challenged as described above. Twenty-four hours following the final OVA challenge mice were sacrificed and their lungs and mLNs harvested. LAPCs and DCs were separated by FACS sorting using a FACS Aria II (BD) and a MoFlo (Beckman Coulter) (10). Naive CD4⁺ T cells were prepared from spleens of DO11.10 mice by a magnetic bead-based cell sorting system MACS (Miltenyi, Germany) using antibodies for anti-mouse CD4. LAPCs or DCs (4×10^4 per well) were cultured with the CD4⁺ transgenic T cells (2×10^5 per well) in 200 μ L RPMI 1640 containing 10% FCS, 50 μ M β -mercaptoethanol and 1% penicillin and streptomycin, in individual wells of a 96-well U-bottom plate, for 96h. CD4 T cells were subsequently stained for intracellular GATA-3 and T-bet.

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Supplementary Figure 1. OVA challenged mice exhibit lung pathology consistent with asthma. (A) Mice (n=15) received 2 intraperitoneal (i.p.) instillations of 50µg of OVA absorbed in 2 mg of alum, followed by i.n. (i.n.) instillation of 50µg of OVA on three consecutive days. Control mice (n=15) were sensitized with OVA and challenged with i.n. PBS. **(B)** Twenty-four hours following the final OVA challenge, lungs were harvested from 3 OVA challenged and 3 control mice and thin sections were prepared and fixed in formalin, then stained with HE or PAS. b~bronchus, v~vessel. 20 x magnification. Size bar = 60 µm. Representative images from 2 independent experiments.

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