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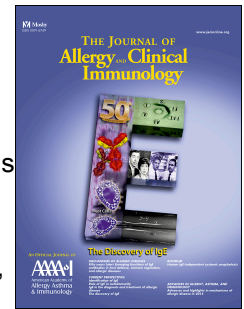
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Pathogenic Th17 inflammation is sustained in the lungs by conventional dendritic cells and TLR4 signaling

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ABSTRACT

Background: Mechanisms that elicit mucosal Th17 cell responses have been described, yet how these cells are sustained in chronically inflamed tissues remains unclear.

Objective: We sought to understand whether the maintenance of lung Th17 inflammation requires environmental agents in addition to antigen, and to identify the lung antigen-presenting cell types (APCs) that sustain the self-renewal of Th17 cells.

Methods: Animals were repeatedly exposed to aspiration of ovalbumin alone, or together with environmental adjuvants, including extracts of common house dust (HDE), to test their role in maintaining lung inflammation. Alternatively, antigen-specific effector/memory Th17 cells, generated in culture using CD4⁺ T cells from *Il17a* fate-mapping mice, were adoptively transferred to assess their persistence in genetically modified animals lacking distinct lung APC subsets or cell-specific TLR4 signaling. Th17 cells were also co-cultured with lung APC subsets to determine which of these could revive their expansion and activation.

Results: Th17 cells and consequent neutrophilic inflammation were poorly sustained by inhaled antigen alone, but were augmented by inhalation of antigen together with HDE. This was associated with weight loss and changes in lung physiology consistent with interstitial lung disease. The effect of HDE required TLR4 signaling predominantly in lung hematopoietic cells, including CD11c⁺ cells. CD103⁺ and CD11b⁺ conventional dendritic cells (cDCs) directly interacted with Th17 cells *in situ* and revived the clonal expansion of Th17 cells *ex vivo* and *in vivo*, whereas lung macrophages and B cells could not.

Conclusion: Th17-dependent inflammation in the lungs can be sustained by persistent TLR4-mediated activation of lung cDCs.

Key Words: Th17 cells; dendritic cells; chronic inflammation; house dust extracts; TLR4

Capsule Summary:

Th17 cell fate-mapping indicates that inhaled house dust extracts sustain clonal expansion of Th17 cells by conventional dendritic cells through a TLR4-dependent mechanism.

Clinical Implications:

TLR4 antagonism might be an effective treatment for chronic lung conditions, such as asthma and interstitial lung disease, that are characterized by Th17 cell and neutrophilic inflammation.

Abbreviations: DC, dendritic cell; HDE, house dust extract; APC, antigen presenting cell; BAL, bronchoalveolar lavage; PCLS, precision cut lung slices; OVA, ovalbumin; Asp, *Aspergillus* protease extract; Flt3L, FMS-like tyrosine kinase 3 ligand; MFI, mean fluorescence intensity

INTRODUCTION

Inhaled compounds in the environment that stimulate innate immune pathways are critical for the priming of antigen-specific T cell responses in the lungs. Yet it is unclear how such responses are sustained in chronically inflamed tissues after the onset of inflammation. IL-17 family member-producing T helper (Th)17 cells have been implicated in numerous chronic inflammatory diseases, including respiratory diseases such as chronic obstructive pulmonary disease¹, hypersensitivity pneumonitis², cystic fibrosis³,⁴ and asthma^{5, 6}. Th17 cells produce IL-17A and IL-17F, both of which signal through a heterodimeric receptor comprising IL-17 receptor A (IL-17RA) and IL-17RC. In the lung, IL-17A binding to its receptor on airway epithelial cells induces neutrophil-attracting chemokines, such as IL-8 in humans and CXCL1 and CXCL5 in mice⁷. In addition, IL-17A can also act directly on airway smooth muscle⁸, epithelial cells⁹, fibrocytes¹⁰ and fibroblasts^{11, 12} to elicit the synthesis of pro-inflammatory and pro-fibrotic mediators. Consequently, IL-17A can promote pathologies such as airway hyperresponsiveness, fibrosis and remodeling. Unlike Th2 cells, which are associated with predominantly eosinophilic asthma, Th17 cells are resistant to glucocorticoids¹³ and there is an unmet need for new therapeutic strategies that target Th17-dependent inflammation. An improved understanding of how Th17 cells are maintained and reactivated in the diseased lung would help to achieve this goal.

The development of antigen-specific Th17 responses depends on the activation of lung antigen-presenting cells (APCs) by inhaled environmental adjuvants, including microbial products such as LPS, which signals through TLR4¹⁴. Inhalation of house dust extracts (HDEs) can also promote Th17 development, and this activity is largely dependent on TLR4. Studies suggest that TLR4 signaling specifically in hematopoietic cells of the lungs is important for Th17 cell development^{15, 16}. However, once Th17 cells occupy the lungs, it is unclear whether they require continued stimulation by adjuvants, or if antigen alone is sufficient to maintain their self-renewal.

It is also unknown which MHC class II-displaying APCs in the lungs are responsible for sustaining tissue-resident Th17 cells. Conventional dendritic cells (cDCs) in the lungs are characterized by their dependence on the cytokine Flt3L and can be divided into two distinct populations, the transcription factor BATF3-dependent CD103⁺CD11b⁻ cDCs and the IRF4-dependent CD11b⁺CD103⁻ cDCs. Interstitial macrophages, which are sometimes also referred to as monocyte-derived DCs, constitute another subset of lung CD11b^{hi} APCs. Finally, alveolar macrophages and B cells in the lungs may also be capable of presenting antigen to and sustaining Th17 cells. cDCs, monocytic cells and B cells have all been reported to contribute to Th17 responses in various tissues, such as the lung, gastrointestinal tract, skin, or the central nervous system¹⁷⁻²⁴.

In the present report, we show that chronic antigen challenges alone, after the cessation of adjuvant exposures, poorly sustain lung Th17-dependent inflammation. Th17 adoptive transfer and fate-mapping experiments reveal that HDE sustains the self-renewal of antigen-specific Th17 cells, promoting severe neutrophilic inflammation, morbidity and impaired lung function, reminiscent of chronic hypersensitivity pneumonitis. This effect is dependent on TLR4 signaling in multiple cell types, though primarily in hematopoietic and CD11c-expressing cells. Conventional DCs are the only APCs capable of reviving Th17 responses *ex vivo* and both subsets sustain Th17 responses in the lungs.

METHODS

Animals. Mice were bred and housed in specific pathogen-free conditions at the NIEHS, and used between 6 and 12 weeks of age in accordance with guidelines provided by the Institutional Animal Care and Use Committees. The following mouse strains were purchased from Jackson Laboratory, Bar Harbor, ME: C57BL/6J, OT-II (B6.Cg-Tg[Tcr α Tcr β]425Cbn/J), B6.Cg-Gt(ROSA)^{tm9(CAG-tdTomato)Hze}/J, *Nur77*^{GFP} (C57BL/6-Tg(Nr4a1-EGFP/cre)820Khog/J), *Cd11c-cre* (B6.Cg-Tg(Itgax-cre)1-1Reiz/J), *Tlr4*^{fl/fl} (B6(Cg)-Tlr4^{tm1.1Karp}/J), *Irf4*^{fl/fl} (B6.129S1-Irf4^{tm1Rdf}/J), *Batf3*^{-/-} (B6.129S(C)-Batf3^{tm1Kmm}/J), *Il12p35*^{-/-} (B6.129S1-*Il12a*^{tm1Jm}/J) and *Il12p40*^{-/-} (B6.129S1-*Il12btm1Jm/J). *Il17ra*^{-/-} mice (B6.129-*Il17ra*^{tm1Koll}) were obtained from Taconic Farms, Inc, Germantown, NY, and used with permission from Amgen Inc., Thousand Oaks, CA. *Flt3l*^{-/-} mice (C57BL/6-*flt3l*^{tm1Imx}) were also purchased from Taconic Farms. *Il17a/f*^{-/-} (C57BL/6-*Il17a/Il17f*^{tm1.1Impr})²⁵ and *Tlr4*^{-/-} (B6.129P2-*Tlr4*^{tm1Aki}) mice were provided by Immo Prinz (Hannover, Germany) and Shizuo Akira (Osaka University, Japan), respectively.*

We generated *Il17a* fate mapping mice in which DNA encoding a Cre recombinase/yellow fluorescent protein (Cre/YFP) fusion protein was inserted into the 3' untranslated region of *Il17* downstream of an engineered, internal ribosome entry site (*Il17-cre*) mice (**Supplementary Figure 1A**). These animals were crossed to mice having a Cre-inducible Tomato transgene at the *Rosa26* locus (**Supplementary Figure 1B**). The resulting animals (**Supplementary Figure 1C**) were in turn crossed to OT-II mice to generate OVA-specific Th17 fate mapping mice whose *Il17*-expressing cells (and their progeny) permanently acquire Tomato fluorescence (**Supplementary Figure 1D**). Fluorescence resulting from the Cre/ YFP fusion gene was undetectable and therefore not utilized in the current study.

Bone marrow chimeric mice. Reciprocal bone marrow chimeric mice were generated using C57BL/6J or *Tlr4*^{-/-} mice, as described in the Supplementary Methods. Prior to their use in experiments, chimeric mice were rested for 12-16 weeks to allow for hematopoietic reconstitution. Mice selectively lacking *Tlr4* in lung epithelial cells were generated by crossing *Tlr4*^{fl/fl} mice to *Sftpc-cre* mice, provided by Brigid Hogan (Duke University). Mice lacking TLR4 in *Cd11c*-expressing cells were generated by crossing *Tlr4*^{fl/fl} mice to *Cd11c-cre* mice.

House dust extracts (HDEs). Sterile HDEs were prepared from dust collected from North Carolinian homes as described previously²⁶. Allergens in the extracts were evaluated using a multiplex array for indoor allergens (MARIA) (Indoor Biotechnologies, Charlottesville, VA), and endotoxin concentration determined to be 10⁻¹ μ g LPS/ 20 μ L HDE, as determined by a Limulus Amebocyte Lysate (LAL) assay (Lonza, Karlsruhe, Germany).

Chronic antigen exposure. Naïve CD4⁺ cells were isolated from the spleens and skin-draining lymph nodes of OT-II mice using antibody-labeled magnetic bead-mediated negative selection (AutoMACS,

Miltenyi Biotec, Germany). 1×10^5 cells were transferred by retro-orbital injection to naïve C57BL/6J animals, which were sensitized on days 0 and 7 by oropharyngeal (o.p.) instillations of 60 μ l sterile PBS containing 10 μ g LPS-free OVA (Worthington Biomedical, CA), either alone or together with an adjuvant. The adjuvants tested included 100 ng LPS from *E. coli* 0111:B4 (Sigma-Aldrich, St. Louis, MO), 20 μ g protease from *Aspergillus oryzae* (Sigma-Aldrich, U.S.A.), or 10 μ l of HDE. Similar exposures were repeated on days 14-16 ('Acute challenge') to establish airway inflammation. This was followed by a 6 week period of chronic challenges, during which the animals were given either OVA alone, or OVA together with the same adjuvant to which they had previously been exposed, three times per week. Mice were harvested three days after the final challenge (**Figure 1A**). Bronchoalveolar lavage (BAL) was performed and cells were analyzed by the Diff-Quik® method (Medical Diagnostics, Düringen, Germany). Left lungs were excised and incubated 24 h at 37 °C in complete Iscove's modified Dulbecco's medium (cIMDM) containing 10 μ g/ml OVA to evaluate the antigen-stimulated lung explant cytokine response *ex vivo*.

Analysis of cytokines in BALF, lung explants and culture supernatants. IL-17A and other mediators were measured by ELISA (Biolegend, San Diego, U.S.A.) or multiplex fluorescent bead-based immunoassay, according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

OVA-specific Ig. OVA-specific IgG1 and IgG2a from serum were evaluated using commercial ELISA kits purchased from Fisher Scientific, and Invitrogen BD Biosciences, San Diego CA, respectively. Briefly, plates were coated overnight at room temperature with 10 μ g/mL OVA Grade V (Sigma) in coating buffer containing 1.94g/L NaHCO₃ and 3.52 g/L Na₂CO₃ in deionized H₂O to 1 L (pH 9.6). The plates were washed 3 times with 0.05% Tween20 in PBS, blocked with 200 μ l PBS containing 1% BSA (Gemini Biosciences) for 1.5 h, and then washed three more times. 100 μ l of serum at 1:20 dilutions for IgG2a and 1:100 dilutions for IgG1 were added and incubated for 2 hours. The remaining steps of the protocol were carried out exactly as described by the manufacturers.

OVA-specific Th17 differentiation. Naïve CD4⁺ T cells were isolated by negative selection from the spleens and skin-draining lymph nodes of OT-II *IL17a* fate mapping mice, as described in the Supplementary Methods. DCs were isolated from the enzymatically digested lungs of WT C57BL/6J mice and were enriched by positive selection using anti-CD11c antibodies and the AutoMACS system (Miltenyi Biotec, Germany). 1×10^6 naïve CD4⁺ T cells were cultured together with 0.5×10^6 splenic DCs in 2 ml of cIMDM containing 10 μ g/ml OVA³²³⁻³³⁹ peptide. To generate Th17 cells, rhTGF- β (3 ng/ml), rIL-1 α (10 ng/ml), rIL-6 (10 ng/ml), anti-IL-2 mAb (10 μ g/ml), anti-IL-4 mAb (10 μ g/ml), anti-IL-12 mAb and anti-IFN γ mAb (5% concentration of respective hybridoma supernatants) were added to

the culture. Th17 cells were split on day 3 of culture by adding the same cocktail of cytokines and antibodies (minus OVA peptide), and with the addition of rIL-23 (10 ng/ml). The cells were split again on day 5 using cIMDM containing IL-6 and IL-23 only (10 ng/ml).

Fate mapping of antigen-specific Th17 cells. After 7 days of Th17 cell-promoting culture, 2.5×10^5 OT-II *Il17a* fate mapping cells were adoptively transferred to recipient mice by retro-orbital injection. Within 2 h of transfer, recipient mice were instilled with 10 μ g OVA together with 10 μ l HDE (OVA/HDE) in PBS. The animals continued to receive further challenges, every other day, of OVA alone, HDE alone, or OVA/HDE up to a total of 5 exposures (day 8). Airway inflammation was assessed by BAL 24 h after the final challenge (day 9), and Tomato⁺ cells were analyzed by flow cytometry.

Live confocal imaging of inflammatory cells in lungs. Fluorescent imaging of cells in freshly harvested, unfixed lung tissue slices was performed as previously described²⁷ following adoptive transfer of *in vitro*-differentiated Th17 cells and OVA/HDE challenge. Precision cut lung slices (PCLS) were prepared from the right superior lobe of agarose-inflated lungs using a Compressstome™ VF-300 (Precisionary Instruments, Inc., Greenville, NC). The lung slice was stained with a blocking solution containing fluorochrome-conjugated antibodies against CD324/E-cadherin, CD11c, CD88, CD103/ α E integrin, or CD172/Sirp1 α . A LSM-880 Airyscan confocal microscope (20x objective lens) was used to generate a z-stack across the entire lung slice. The images were then superimposed and processed with Zen Black 2012 software (Carl Zeiss AG, Chesterfield, Virginia) to yield a 2D image of Tomato fluorescent Th17 cells and lung APCs within the whole lung slice. Alternatively, a 63x oil immersion lens was used for high resolution imaging of lung cells interacting with Tomato⁺ Th17 cells *in situ*. Imaris software version 8.4 (Bitplane AG, Zurich, Switzerland) was used to generate 3D surface renderings of these cell-cell interactions.

Lung function testing. Animals were anesthetized (Urethane, i.p. 2 g/kg, Sigma-Aldrich, U.S.A.) and mechanically ventilated using a FlexiVent FX small animal ventilator (Scireq Scientific Respiratory Equipment Inc., Montreal, Canada). Paralysis was induced with pancuronium bromide (Sigma-Aldrich, U.S.A.), i.p. 2 mg/kg, to eliminate interference from spontaneous breathing efforts and the animals were then inflated to a pressure of 30 cmH₂O over 3 seconds and held at that pressure for another 3 seconds in order to normalize baseline lung volumes. Quasi-static pressure-volume curves were generated using a stepwise, pressure-controlled perturbation (PVs-P). Resulting PV data were then fit to the Salazar-Knowles equation by the operating software (flexiWare v7.5.3), from which the K parameter (concavity/curvature of the deflation limb of the PV loops) and compliance (slope) were extracted²⁸.

Analysis and isolation of lung antigen-presenting cells. Lungs were perfused and enzymatically digested as described earlier²⁹. Low density cells were stained with fluorochrome-conjugated monoclonal

antibodies against CD88, Siglec-F, CD11c, I-A^b, Ly6G, Ly6C, CD11b and CD103. Cell viability was determined using 7-AAD or APC-eFluor 780 fixable viability dye. Conventional DCs were identified as non-autofluorescent, Siglec F⁻CD88⁻CD11c⁺I-A^b ^{hi} cells ³⁰, as shown in **Supplementary Figure 2**. Stained cells were acquired on an LSRII flow cytometer using Diva software (BD Biosciences), or sorted using a BD FACS Aria II (purity of the sorted populations was >95%). Lung B cells were analyzed from the lymphocyte fraction using fluorochrome-conjugated monoclonal antibodies against CD19, B220 and I-A^b, or were sorted by negative selection using a MACS LD column (purity >75%). The costimulatory molecules, CD40 and CD86, were also analyzed in lung CD11c-expressing APCs and B cells.

Co-culture of lung antigen-presenting cell subsets with mature Th17 cells. To determine which lung APC subsets may be responsible for maintaining Th17 responses, lung APCs (alveolar macrophages, interstitial macrophages, CD103⁺ cDCs, CD11b⁺ cDCs or B cells) were sorted by flow cytometry 16 h after the last of 3 OVA/HDE exposures and were co-cultured at a ratio of 1:6 with mature Th17 cells, i.e. Tomato⁺ cells sorted on day 7 from *in vitro* OT-II Th17 cultures (10,000 APC: 60,000 Tomato⁺ Th17 cells). 72 h later, Tomato⁺ cell frequency was analyzed in each culture condition, as well as IL-17A levels in the culture supernatants.

Statistical Analysis. Data were analyzed using GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, Calif.) and are presented as means ± SEM. Differences between groups of three or more were identified by analysis of variance (ANOVA) and Newman-Keuls' multiple comparison tests. Data were log-transformed prior to statistical analysis when not normally distributed and a *P* value of less than 0.05 was considered statistically significant.

RESULTS

Chronic exposure to environmental adjuvants is necessary to sustain lung inflammation

We first tested whether continued exposure to adjuvants becomes dispensable for sustaining allergic pulmonary inflammation after it has developed in the lungs. To do this, we transferred naïve T cells from OT-II mice (which bear a transgene encoding an OVA-specific TCR) into C57BL/6J recipient mice, then sensitized them twice through the airways, one week apart, with OVA mixed with an adjuvant; LPS, HDE or a protease extract from *Aspergillus oryzae* (Asp) (**Figure 1A**). The animals were then acutely challenged in the same manner on three additional occasions, on days 14–16, to activate antigen-specific T cells and establish allergic airway inflammation. Experimental groups then continued to receive either chronic OVA exposures with the same adjuvant, or just OVA alone. As expected, mice that inhaled OVA without an adjuvant failed to become sensitized and did not develop allergic inflammation after subsequent OVA challenge, in agreement with previous results¹⁴. Surprisingly, however, mice receiving OVA together with an adjuvant during sensitization and acute challenge, but challenged with OVA alone for the remainder of the protocol, also displayed relatively little inflammation (**Figure 1B**). By contrast, mice chronically challenged with OVA together with an adjuvant displayed robust pulmonary inflammation, the nature of which depended on which adjuvant had been inhaled. Thus, mice challenged with OVA together with Asp (OVA/Asp) displayed predominantly eosinophilic inflammation, whereas mice challenged with OVA together with either LPS (OVA/LPS) or HDE (OVA/HDE) displayed neutrophilic inflammation with few eosinophils. Notably, neutrophils were even higher in mice challenged with OVA/HDE than in those challenged with OVA/LPS. OVA-specific levels of the Th1/Th17-associated immunoglobulin isotype, IgG2a, were highest in mice chronically challenged with OVA/LPS, but were also elevated in mice challenged with OVA/HDE (**Figure 1C**). This isotype was relatively low in mice challenged with OVA/Asp. By contrast, the nature of the adjuvant during sensitization had little effect on levels of OVA-specific IgG1, which is associated with type 2 responses.

Pathogenic Th17 responses in the lung are sustained by the inhalation of adjuvants that activate TLR4

To assess the impact of the chronic adjuvant exposures on antigen-induced cytokine production, lungs were harvested from the animals and incubated overnight in the presence of OVA. Lung explant supernatants from mice challenged chronically with OVA/Asp had higher amounts of IL-4 than the other groups (**Figure 1D**), although levels of this cytokine were low, and there were no significant differences among the groups in IL-13 (data not shown). Mice challenged with OVA/HDE had elevated levels of IL-17A. This suggested that inhalation of HDE, together with OVA, may sustain the antigen-specific Th17

response in the lungs. To directly test this, we crossed OT-II mice to *Il17a* fate mapping mice in which a Cre recombinase gene that is expressed in conjunction with *Il17a* permanently activates expression of a gene encoding a tandem dimer Tomato fluorescent protein (**Supplementary Figure 1**). We were thus able to differentiate OVA-specific pathogenic Th17 cells from these animals *in vitro* and use their fluorescence to follow the fate of these cells (and their progeny) following adoptive transfer. Approximately half of the *in vitro*-differentiated T cells were positive for Tomato and virtually all expressed the effector-memory T cell marker, CD44 (**Figure 2A**). Further analysis of these cells indicated that they were capable of producing IL-17A, IL-17F, IL-6 and TNF- α , but produced little IL-4 and no IFN- γ (data not shown), and thus acquired a characteristic pathogenic Th17 phenotype described previously³¹. The Th17 fate mapping cells were adoptively transferred into recipient mice lacking both IL-17A and IL-17F (*Il17a/f^{-/-}*) to ensure that the Tomato⁺ Th17 cells would be the sole source of these cytokines. All animals received a first challenge with OVA/HDE to establish airway inflammation and were then divided into groups that were challenged every second day with OVA alone, HDE alone, or OVA/HDE (**Figure 2B**). Animals challenged repeatedly with OVA/HDE displayed significantly elevated total BAL leukocyte counts (**Figure 2C**) and, importantly, Tomato⁺ cell numbers (**Figure 2D**), indicating a higher number of Th17 cells in the airways originating from the adoptively transferred population. Consistent with the increase in Th17 cells, OVA/HDE also augmented BAL neutrophil numbers (**Figure 2C**), as well as BAL and lung explant IL-17A cytokine levels in the *Il17a/f^{-/-}* mice (**Figure 2E**). A small number of eosinophils was also observed, possibly from an innate immune response triggered by HDE, or by small numbers of Th2 cells.

We next explored whether inhaled HDE may affect the pathogenicity of the adoptively transferred Th17 cells, i.e. their production of IL-17 cytokine on a per cell basis, in addition to increasing their numbers. We performed conventional intracellular staining, as well as a flow cytometry-based, cytokine capture assay to evaluate IL-17 being actively secreted by stimulated Tomato⁺ cells. Neither approach revealed differences between OVA- and OVA/HDE-treated mice in the proportion of Tomato⁺ cells expressing IL-17A or F, or in the median fluorescence intensity (MFI) of those cytokines (**Supplementary Figure 3**). However, lungs of OVA/HDE-challenged mice had higher numbers of IL-17A-secreting Tomato⁺ cells compared to mice that were challenged with OVA alone (**Supplementary Figure 3C**), consistent with the differences observed in the BALF. The total numbers of Tomato⁺ Th17 cells were far greater than the number of adoptively transferred cells, indicating that HDE augmented the self-renewal of these Th17 cells. Examination of precision cut lung slices confirmed that Tomato⁺ cells in the lung increased after multiple OVA/HDE challenges and further revealed that these cells initially localized around the airways, but were diffusely distributed within the lungs after additional challenges (**Figure 2F**). Collectively, these experiments demonstrated that HDE augmented the Th17 response

deriving from the adoptively transferred Th17 cells, and thus, the recall Th17 response to an unrelated antigen that is concomitantly inhaled.

OVA/HDE challenge also caused significant weight loss in Th17 recipient animals, whereas challenge with OVA or HDE alone did not (**Figure 2G**). Weight loss is a comorbidity of chronic lung diseases, such as COPD³² and hypersensitivity pneumonitis³³, likely due to a combination of compromised air exchange, reduced cardiac function and reduced airflow. Analysis of lung function in Th17 recipients further revealed that OVA/HDE challenged animals also had flatter pressure-volume curves, as indicated by the significantly lower K value extracted from these curves (**Figure 2H**). Moreover, these animals also showed a trend towards lower static compliance of the lungs (**Figure 2H**) and inspiratory capacity (data not shown). Overall, these changes are consistent with an increased stiffness of the lungs, a common clinical feature of interstitial lung diseases, such as pulmonary fibrosis and chronic hypersensitivity pneumonitis³⁴. Using *Il17a*^{f/f} mice and *Il17ra*^{-/-} mice as recipients, we confirmed that IL-17 production by the transferred Th17 cells was responsible for the observed phenotype. (**Supplementary Figure 4A - C**). Thus, the outcomes observed after Th17 adoptive transfer and repeated OVA/HDE exposure were specifically caused by the sustained expansion and activation of the transferred cells rather than a *de novo* Th17 response in the treated animals.

HDEs are crude extracts containing numerous allergens, microbial products, enzymes, and a variety of other compounds. To determine whether an enzyme, or other heat-labile molecule is responsible for the ability of HDE to sustain Th17 responses, we heat-inactivated the extract at 90 °C for 30 min. Surprisingly, there was no difference between intact and heat-inactivated HDE upon the Th17 response or other outcomes (**Figure 3A-C**), indicating that a heat-stable agent within HDE is responsible for the maintenance of Th17 cells. Endotoxin is one such component, and we have previously reported that the induction of lung Th17 responses by HDE is mediated via TLR4¹⁵. Therefore, we investigated whether HDE also sustains lung Th17 responses via TLR4 signaling. Remarkably, despite its rich composition, the effect of HDE in boosting OVA-specific Th17 cells was entirely TLR4-dependent; *Tlr4*^{-/-} recipients had significantly lower numbers of BAL neutrophils and Tomato⁺ cells, as well as lower lung explant IL-17A cytokine levels (**Figure 3A-C**). *Tlr4*^{-/-} mice also lost significantly less weight than did WT mice (**Figure 3D**). Unlike neutrophils, eosinophils were increased in the *Tlr4*^{-/-} recipients, in agreement with a previous report that TLR4 signaling can suppress HDE-mediated eosinophilic inflammation³⁵. Together, these data suggest that endotoxin is a major component in HDE that selectively sustains Th17-mediated disease.

TLR4 expression in multiple cell types contributes to the sustained expansion of Th17 cells

Tlr4 is expressed in a wide variety of cell types, including both radio-resistant and -sensitive cells. To gain a better understanding of which *Tlr4*-expressing cells are required for maintaining Th17 responses, we adoptively transferred the fate mapping Th17 cells to reciprocal *Tlr4* bone marrow chimeric mice and challenged them with OVA alone or OVA/HDE. As expected, irradiated *Tlr4*^{-/-} mice receiving bone marrow from *Tlr4*^{-/-} animals had lower numbers and percentages of neutrophils and Tomato⁺ cells compared with WT mice receiving WT marrow (**Figure 4A**). Interestingly, WT mice that received marrow from *Tlr4*^{-/-} mice also had markedly fewer neutrophils and Tomato⁺ Th17 cells than WT control mice, indicating that TLR4 signaling in hematopoietic cells is critical for the maintenance of lung Th17 inflammation. Radio-resistant, *Tlr4*-expressing cells also contributed to neutrophilic inflammation because *Tlr4*^{-/-} mice that received WT marrow also had reduced inflammation.

To determine more specifically in which lung cell types TLR4 is engaged by HDE, we transferred Th17 cells to conditional *Tlr4* knockouts, namely Surfactant protein C (*Sftpc*)-cre *Tlr4*^{fl/fl} or *Cd11c*-cre *Tlr4*^{fl/fl} mice, in which *Tlr4* is deleted in lung epithelial cells and in CD11c⁺ DCs and macrophages, respectively. Although *Sftpc* is not expressed in all mature epithelial cells in the lung, it is expressed in their precursors, and recombination events that occur in these cells are carried forward to mature epithelial cells³⁶. After receiving Th17 cells and challenge with OVA/HDE, *Cd11c*-Cre *Tlr4*^{fl/fl} and *Sftpc*-Cre *Tlr4*^{fl/fl} mice each had reduced neutrophil and Tomato⁺ cell numbers compared with (WT) *Tlr4*^{fl/fl} mice (**Figure 4B**). Lungs from *Cd11c*-Cre *Tlr4*^{fl/fl} and *Sftpc*-Cre *Tlr4*^{fl/fl} mice also produced less IL-17A after restimulation *ex vivo* with OVA compared with lungs from WT *Tlr4*^{fl/fl} mice (**Figure 4C**). These results confirm that TLR4 signaling in *Cd11c*-expressing cells is critical for the maintenance of lung Th17 inflammation and suggest that TLR4 activation in airway epithelial cells also contributes to the persistence of Th17 responses in the lung.

cDCs are the only APCs capable of interacting with lung-resident Th17 cells and reviving Th17 expansion

Our finding that both antigen (OVA) and HDE are required for the amplification of OVA-specific Th17 responses (**Figure 2B**) suggested that enhanced antigen presentation is a key component of the continued clonal expansion of Th17 cells. We therefore performed flow cytometric analysis following repeated OVA or OVA/HDE exposures to identify HDE-induced changes in lung antigen-presenting cell types, including CD103⁺ cDCs, CD11b⁺ cDCs, alveolar macrophages (identified as Siglec-F⁺CD88⁺CD11c⁺I-A^{b+} cells), and interstitial macrophages (Siglec-F⁻CD88⁺CD11c⁺ CD11b⁺I-A^{b+} cells). We observed that exposure of mice to OVA/HDE increased numbers of CD11b⁺ cDCs and interstitial macrophages compared with mice exposed to OVA alone, and that these increases were dependent on

TLR4 (**Figure 5A**), whereas insignificant changes were seen in lung CD103⁺ cDCs and alveolar macrophage numbers. Analysis of co-stimulatory molecules indicated that OVA/HDE only moderately increased CD86 MFI on cDCs, but strongly increased CD40 MFI on both CD103⁺ cDCs and CD11b⁺ cDCs (**Figure 5B and C**). Consequently, the number of CD103⁺ cDCs, CD11b⁺ cDCs and interstitial macrophages displaying CD40 was increased by HDE exposures. The observed changes in co-stimulatory molecules were also dependent on TLR4 because they were greatly reduced in *Tlr4*^{-/-} mice (**Figure 5B and C**). These data indicated that multiple lung APC subsets are activated by HDE. We further found that each of these APC subsets can take up fluorescently-labeled OVA (OVA-AF647) following its inhalation with HDE (**Figure 5D**), raising the question of which of these subsets is primarily responsible for T cell activation. To address this, we sorted lung cDCs and interstitial macs from OVA/HDE-exposed mice and co-cultured each cell type with naïve OVA-specific T cells bearing a fluorescent reporter of TCR activation (*Nur77*-GFP). Whereas T cells cultured alone displayed negligible GFP expression, both subsets of cDCs, as well as interstitial macrophages, induced TCR activation (**Figure 5E**). This suggested that multiple APCs might also be capable of interacting with and stimulating lung Th17 cells.

To examine which APCs, CD103⁺ cDCs, CD11b⁺ cDCs, macrophages, or B cells might interact with Th17 cells in the lungs, we performed live fluorescent imaging. This corroborated our flow cytometric data (**Figure 5B**) showing increases in CD11c⁺ cells in the lungs after Th17 adoptive transfer and successive OVA/HDE challenges (**Figure 6A**). Higher power microscopy revealed areas in which Tomato⁺ Th17 cells (red) and CD11c⁺ cells (green) co-localized (yellow) (**Figure 6B**), suggesting potential interaction of these cells *in situ*. To confirm this, we stained lung slices with antibodies against CD103, Sirp1 α and CD88. The latter two markers were used to distinguish CD11b⁺ cDCs, which are Sirp1 α ⁺CD88⁻, from CD88⁺ macrophages or neutrophils³⁰. Using 3D rendering of high power images, we confirmed that both CD103⁺ and CD11b⁺ cDCs physically interact with Tomato⁺ Th17 cells in the lungs (**Figure 6C and Supplementary Videos**). By contrast, we did not observe clear interactions of Tomato⁺ Th17 cells with macrophages or B cells (data not shown). We next examined whether such interactions can support Th17 responses *ex vivo*. APC subsets sorted from the lungs of OVA/HDE-exposed animals were individually co-cultured with *in vitro*-differentiated, mature Tomato⁺ Th17 cells. The vast majority of Tomato⁺ cells died when they were cultured with B cells or without any APCs. Although alveolar and interstitial macrophages stabilized Th17 cells in the co-cultures by supporting their survival, these APCs did not significantly increase the numbers of Tomato⁺ cells beyond those that were initially cultured, whereas Tomato⁺ cell numbers were significantly increased after culture with either CD103⁺ cDCs or CD11b⁺ cDCs (**Figure 6D**). The latter were particularly potent in this regard, and IL-17A cytokine levels in the culture supernatants were also highest for Th17 cells cultured with CD11b⁺ cDCs. To determine if high amounts of Th17-promoting cytokines in the two cDC subsets could explain their ability to drive

Th17 expansion and production of IL-17 *ex vivo*, we compared expression of *Il23p19*, *Il1a*, *Il1b* and *Il6* in various lung APCs in a microarray database (<http://www.ncbi.nlm.nih.gov/geo/info/linking.html>; accession no. GSE64896)³⁰. CD11b⁺ cDCs expressed the highest amounts of *Il1b* RNA (**Figure 6E**). However, interstitial macrophages produced more RNA encoding the other Th17-promoting cytokines than did either CD11b⁺ cDCs or CD103⁺ cDCs. Analysis of Th17 cells after their culture with CD103⁺ and CD11b⁺ cDCs did not reveal clear differences in cytokine production (**Supplementary Figure 5**), suggesting that these different cDCs do not promote a unique phenotypic signature in the Th17 cells.

IL-23 is reported to maintain Th17 cells in some settings^{31, 37, 38}. Therefore, even though both cDC subsets contained less *Il23p19*-specific mRNA than did interstitial macrophages, we nonetheless tested the requirement of IL-23 by comparing the Th17 expansion in *Il12p40*-deficient mice (which lack both IL23 and IL12) and *Il12p35*-deficient mice (which lack IL-12, but retain IL-23). *Il12p40*-deficient mice had fewer Tomato⁺ Th17 cells (**Figure 6F**) and IL-17A in BALF and lung explants (**Figure 6G**) than did WT mice or *Il12p35*-deficient mice. These data suggest that in this model, IL-23 contributes to the sustained clonal expansion of memory Th17 cells and their production of IL-17A.

CD103⁺ cDCs and CD11b⁺ cDCs independently sustain lung Th17 inflammation

We next investigated the individual requirements of CD103⁺ cDCs and CD11b⁺ cDCs in maintaining Th17 responses *in vivo*. The transcription factor BATF3 is required for development of CD103⁺ cDCs, and these cells are therefore absent in *Batf3*^{-/-} mice³⁹. A different transcription factor, IRF4, is required for the development of CD11b⁺ cDCs, and mice bearing a floxed allele of *Irf4* (*Irf4*^{fl/fl}) as well as a *Cre recombinase* gene under control of a *Cd11c* promoter (*Cd11c-Cre Irf4*^{fl/fl} mice) have markedly reduced numbers of CD11b⁺ cDCs⁴⁰. Surprisingly, after Th17 adoptive transfer and OVA/HDE challenges, *Batf3*^{-/-} mice, *Cd11c-Cre Irf4*^{fl/fl} mice, and WT mice all had similar levels of airway inflammation, Tomato⁺ Th17 cell numbers and IL-17 cytokine levels (**Figure 7A-D**). This result suggested two possibilities: that neither of these cDC subsets promoted Th17 expansion and survival *in vivo*, or that they have redundant activities in this regard. To distinguish between these alternatives, we adoptively transferred Tomato⁺ Th17 cells to mice lacking Flt3L (*Flt3l*^{-/-}), which are deficient in both CD103⁺ cDCs and CD11b⁺ cDCs, but have normal numbers of monocyte-derived DCs (interstitial macs) and alveolar macs⁴¹. Following OVA/HDE challenge of the recipient animals, *Flt3l*^{-/-} mice had dramatically fewer neutrophils, Tomato⁺ Th17 cells, and IL-17A compared with similarly challenged WT recipients (**Figure 7E and F**). Thus, CD103⁺ cDCs and CD11b⁺ cDCs can each respond to HDE and independently sustain the self-renewal of antigen-specific Th17 cells, thereby maintaining lung Th17-dependent inflammation and airway disease caused by inhaled antigens.

DISCUSSION

Elucidating the mechanisms by which Th17 responses are sustained in the lungs is critical for an improved understanding of chronic pulmonary diseases. In addressing this question, we found that repeated inhalation of environmentally relevant HDE with antigen sustains Th17 inflammation in the lungs, and that absent such environmental signals, these responses gradually diminish. Pathogenic Th17 cells expressing the effector/ memory T cell marker CD44, were revived and sustained in the lungs specifically by TLR4 signaling and cDCs. These findings shed light on how lung-resident memory Th17 cells may be reactivated and suggest that Th17-dependent lung disease may be ameliorated by targeting these pathways.

HDE is a complex mixture of dead skin cells, allergens, microbial components, proteases, and protease inhibitors. Surprisingly, the ability of HDE to maintain or expand Th17 cells in the lungs was entirely dependent on TLR4 signaling. It is known that LPS is a potent adjuvant for inducing Th17 responses to inhaled allergens¹⁴, and our current findings suggest that LPS is also required to maintain Th17 inflammation of the lungs. Interestingly, chronic exposures of mice to OVA/HDE promoted even greater neutrophilia and more IL-17 production than did OVA/LPS, although the amounts of endotoxin were similar, suggesting that additional components of HDE may be involved in TLR4 activation. Heat-treatment of HDE did not modify its ability to expand and activate the adoptively transferred cells, suggesting that a heat-stable component of HDE or an endogenous TLR4 ligand released by tissue damage, such as the glycosaminoglycan hyaluronan, may be involved. Further studies will be necessary to confirm whether TLR4 ligands in the environment other than endotoxin can sustain lung Th17 inflammation.

The expansion and activation of Th17 cells by concomitant antigen and HDE exposures was accompanied by severe neutrophilic inflammation and apparent stiffening of the lungs, as well as cachexia. These pathologies share common features with chronic hypersensitivity pneumonitis, an interstitial lung disease caused by unresolved inflammation and tissue damage, commonly triggered by organic dust exposures as well as other environmental agents⁴². Our work indicates that TLR4 may play a major role in forms of this disease that are characterized by severe Th17 and neutrophilic inflammation. Moreover, our study may also be relevant to exacerbations of lung diseases, such as asthma. Higher numbers of neutrophils and Th17 cytokines were recently reported in the bronchial/ nasal mucosa of severe asthmatics compared to mild asthmatics, and particularly in severe asthmatics who suffer frequent exacerbations^{43, 44}. The presence of endotoxin in house dust in urban environments has been associated

with asthma symptoms, use of asthma medications and wheezing⁴⁵. It will be informative to learn whether endotoxin exposures also correlate with severe Th17-associated asthma.

Our observation that sustained expansion of Tomato⁺ Th17 cells and consequent disease required inhalation of both HDE and OVA indicates that the pro-inflammatory stimuli in HDE are, on their own, insufficient for disease development, but can promote Th17 cell expansion in an antigen-dependent manner. This suggested that HDE might enhance the ability of APCs to promote the self-renewal of antigen-specific Th17 cells in the lung. Indeed, the effect of HDE on Th17 cells and the associated disease was completely abolished in *Flt3l*^{-/-} mice, which lack cDCs but retain macrophages, indicating that cDCs are essential for sustaining the expansion of Th17 cells. Prior studies have suggested that CD11b⁺ cDCs are critical for the priming of lung Th17 responses induced by fungal infection²², while CD103⁺ cDCs also contribute to and control the pathogenicity of this response⁴⁶ and, in fact, prime more robust Th17 responses in the context of bacterial infection²³. T cells are also capable of interacting sequentially with CD103⁺ and CD11b⁺ cDCs⁴⁷. Our analysis of individual APC subsets that had acquired OVA *in vivo* demonstrated that both CD103⁺ and CD11b⁺ cDCs can revive the expansion and activation of mature Th17 cells in *ex vivo* cultures, and do not distinctly modulate the phenotype of the self-renewing Th17 cells. CD103⁺ cDCs were slightly less potent in reviving Th17 cells *ex vivo*, which may be due to their propensity to secrete IL-2, a cytokine that restrains Th17 responses⁴⁶. However, we observed that the Th17 and associated neutrophil response in WT mice were equivalent to the responses seen in *Batf3*^{-/-} and *Cd11c-Cre Irf4*^{fl/fl} mice. Collectively, these data indicate that the two subsets of cDCs independently sustain Th17 expansion and suggest that these cells may be able to compensate for each other in this regard.

Lung macrophages and B cells were unable to revive the proliferation of co-cultured Th17 cells, indicating that they may lack molecules required for this purpose. It is unclear why interstitial macrophages, which express co-stimulatory molecules such as CD40 and are capable of taking up OVA in the lungs and triggering TCR activation *ex vivo* cannot sustain Th17 responses on their own. Using high resolution fluorescent imaging of unfixed lung tissue, we observed CD103⁺ cDCs and CD11b⁺ cDCs physically interacting with Tomato⁺ Th17 cells, but we did not observe interactions of alveolar or interstitial macrophages with Tomato⁺ Th17 cells. Thus, on their own, lung macrophages might be incapable of promoting Th17 expansion because they do not form sufficiently strong interactions with Th17 cells. However, macrophages expressed more *Il23p19*, *Il1a* and *Il6* than either cDC subset, and were able to promote the survival of co-cultured Th17 cells. IL-23 contributes to the pathogenicity, stability and expansion of Th17 cells^{31, 37, 38}, and our experiments with *Il12p35*^{-/-} and *Il12p40*^{-/-} mice suggested that IL-23 does contribute to Th17 maintenance in this model. Together, these data suggest that

by providing cytokines, macrophages might cooperate with cDCs to maintain or expand antigen-specific Th17 cells in the lung. Whether Th17 cells produce cytokines that can alter the function of macrophages and DCs remains unclear.

TLR4 is expressed by several cell types in the lung, and recent studies have examined how cell-specific TLR4 signaling affects lung inflammation. *Tlr4* expression in hematopoietic cells was shown to be necessary for IL-17A production and consequent neutrophilic inflammation in an acute model of asthma¹⁶, but that study did not specifically examine Th17 cells or the role of TLR4 in sustaining inflammation. Our current findings that expansion of adoptively transferred Th17 cells is attenuated in animals lacking cDCs, and also in mice selectively lacking *Tlr4* in *Cd11c*-expressing cells, suggests that cDCs are likely activated directly by TLR4 ligands to sustain lung Th17 inflammation. This does not exclude a role for TLR4 signaling in other cell types, such as lung epithelial cells, and it will be important to gain a deeper understanding of how communication among different cell types coordinate responses to inhaled adjuvants and allergens. The requirement of TLR4 for Th17 cell maintenance in the lung suggests that targeting this pathway might be an effective therapeutic approach in established Th17-dependent diseases of the lung.

SUPPLEMENTARY MATERIALS

Supplementary Materials include additional methods, 5 figures, and two videos depicting the 3-dimensional renderings of lung CD103⁺ cDCs or CD11b⁺ cDCs interacting with Tomato⁺ Th17 cells *in situ*.

AUTHOR CONTRIBUTIONS

KHS and DNC conceptualized the study; KHS, HN, SYT and DNC designed experiments; IP provided *Il17a/f^{-/-}* DKO mice; KHS, MLC, HN, SYT and GSW performed all experiments and analyzed data; KHS and DNC wrote the manuscript and all authors approved the final version.

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Figure Legends

Figure 1. Adjuvants are required during chronic antigen challenge to sustain airway inflammation.

(A) Timeline of chronic challenge experiment. (B) Numbers of inflammatory cells in BAL of mice that underwent sensitization and acute challenge by instillations of OVA together with the indicated adjuvant, or no adjuvant (–), followed by chronic challenge with either OVA alone (white rectangles) or together with the same adjuvant used during sensitization (black rectangles). (C) OVA-specific IgG2a and IgG1 in serum. (D) Cytokines in supernatants of lung explants stimulated *ex vivo* with OVA. Data shown are from 4 independent experiments (n= 8 per group; *p < 0.05, ** p < 0.01, **** p < 0.0001, OVA only challenge vs. OVA/adjuvant challenge).

Figure 2. Repeated inhalation of HDE augments antigen-specific Th17 responses.

(A) Naïve CD4⁺ cells (left) from OT-II IL-17 fate-mapping mice were differentiated to mature, Tomato-expressing Th17 cells (right). (B) Timeline of adoptive transfer of Th17 cells to *Il17a/f*^{–/–} DKO mice and subsequent exposures to OVA ± HDE. Shown also are inflammatory cells in the BAL (C), percentages and numbers of Tomato⁺ Th17 cells (D), and amounts of IL-17A in lung explant cultures (E) from *Il17a/f*^{–/–} mice. Data are from 2 independent experiments (n=10 animals per group). (F) Live cell imaging of precision cut lung slices from WT recipients of Th17 cells after multiple OVA/HDE exposures; Tomato⁺ Th17 cells (red) and E-cadherin stained epithelia (blue). Zoomed-in images (top) are of the area denoted by the white rectangular inset (below). (G) Weight loss and (H) lung function measurements, including baseline pressure-volume (PV) curves (left), and the PV curve-derived K parameter (middle) and compliance (right), following adoptive transfer of Th17 cells to WT mice and repeated challenge with OVA ± HDE (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

Figure 3. HDE sustains Th17 cells through TLR4.

WT or *Tlr4*^{–/–} animals received 0.25 x 10⁶ Th17 cells and were subject to repeated exposures of OVA/HDE or OVA/heat-inactivated HDE. Shown are (A) BAL inflammatory cell counts, (B) Tomato⁺ cell frequencies and numbers, (C) IL-17A in lung explant supernatants, and (D) weight loss after the indicated number of days post-adoptive transfer and first challenge. Data are combined from two independent experiments (n= 5-8 animals per group; *p < 0.05, ** p < 0.01, **** p < 0.0001).

Figure 4. Requirement of TLR4 in hematopoietic cells for HDE-dependent maintenance of Th17 inflammation.

In vitro-differentiated Th17 cells were adoptively transferred into *Tlr4* reciprocal bone marrow chimeric mice (A) or into mice in which *Tlr4* was deleted in either *Cd11c-cre*-expressing cells or *Sftpc-cre*-expressing lung epithelial cells (B). Shown are BAL inflammatory cell numbers and frequencies (A and B), as well as IL-17A in BAL and lung explants (A and C). Data are from 2-3 independent

experiments. (A) n=6-12 animals per group; (B) n=2-7 animals per OVA alone-treated groups and n=8-16 animals per OVA/HDE-treated groups (*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

Figure 5. OVA/HDE exposure increases the number and activation of CD11b⁺ cDCs and interstitial macrophages. Naïve WT or *Tlr4*^{-/-} mice received 3 instillations of OVA or OVA/HDE, every other day, and lungs were harvested 24 h after the final exposure. (A) Numbers of the indicated APCs in lungs of mice challenged with OVA alone or OVA/HDE. (B,C) Representative flow plots (left) and bar histograms (right) depicting CD86 (B) and CD40 (C) display on the indicated APCs. Pooled data are from two independent experiments, n=6 animals/ group (*p < 0.05, ** p < 0.01, *** p < 0.001). (D) Uptake of OVA-AF647 by different lung APC subsets following its administration together with HDE. (E) Induction of TCR signaling by APCs sorted from lungs of OVA/HDE-treated mice. APCs were co-cultured with naive CD4 T cells from OT-II mice crossed with *Nur77-gfp* reporter mice, and GFP signals in the T cells were analyzed 20 h later by confocal microscopy and flow cytometry.

Figure 6. Activation of Th17 cells by lung APCs. (A,B) Live fluorescent imaging of CD11c⁺ cells and Tomato⁺ Th17 cells in unfixed lung slices. (A) Layered image of a slice from an entire upper right lobe after either 3 or 5 OVA/HDE challenges. Shown are 20x magnification images (upper), and a zoomed-in image of the area demarcated by the white rectangular inset (lower). (B) Zoomed in area depicting CD11c⁺ cells (green), Tomato⁺ Th17 cells (red), and co-localization of the two (yellow), indicated by white arrows. (C) High resolution images (left) and 3D renderings (right) of CD103⁺ or Sirp1α⁺CD88⁻ DCs (CD11b⁺ cDCs) interacting with Tomato⁺ Th17 cells *in situ*. (D) Activation of sorted memory Tomato⁺ Th17 cells. The indicated lung APC subsets were sorted from lungs after three OVA/HDE exposures and were co-cultured with *in vitro*-differentiated Tomato⁺ Th17 cells. Data shown were pooled from two independent experiments (n=6 samples/ condition; 'Medium only' group (n=2) was not included in the statistical analysis; # denotes significant difference of B cells compared to all other groups. (E) Expression of Th17-promoting genes in the indicated APCs, as determined from a single microarray experiment with three samples per APC group. (F,G) Inflammation of the airway (F) and IL-17A in BALF and supernatants of lung explants (G) from WT, *Il12p35*^{-/-} and *Il12p40*^{-/-} mice that received adoptive transfer of *in vitro*-differentiated Th17 cells and were challenged with OVA alone (white rectangles) or OVA/HDE (black rectangles). *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

Figure 7. Lung CD103⁺ and CD11b⁺ cDC subsets independently sustain Th17 responses. *In vitro*-differentiated Th17 cells were adoptively transferred to WT and *Batf3*^{-/-} mice deficient in CD103⁺ cDCs (A,B), WT and *Cd11c-cre Irf4*^{fl/fl} mice lacking CD11b⁺ cDCs (C,D), or WT and *Flt3l*^{-/-} mice lacking both

cDC subsets (**E,F**). Shown are BAL inflammatory cells (**A,C,E**), as well as IL-17A in BALF and in lung explants (**B,D,F**). Data are from two independent experiments for each genotype with (n=1-5) animals for mice challenged with OVA alone (white rectangles) and n=8-23 mice challenged with OVA/HDE (black rectangles); *p < 0.05, ** p < 0.01, **** p < 0.0001).

Figure 1

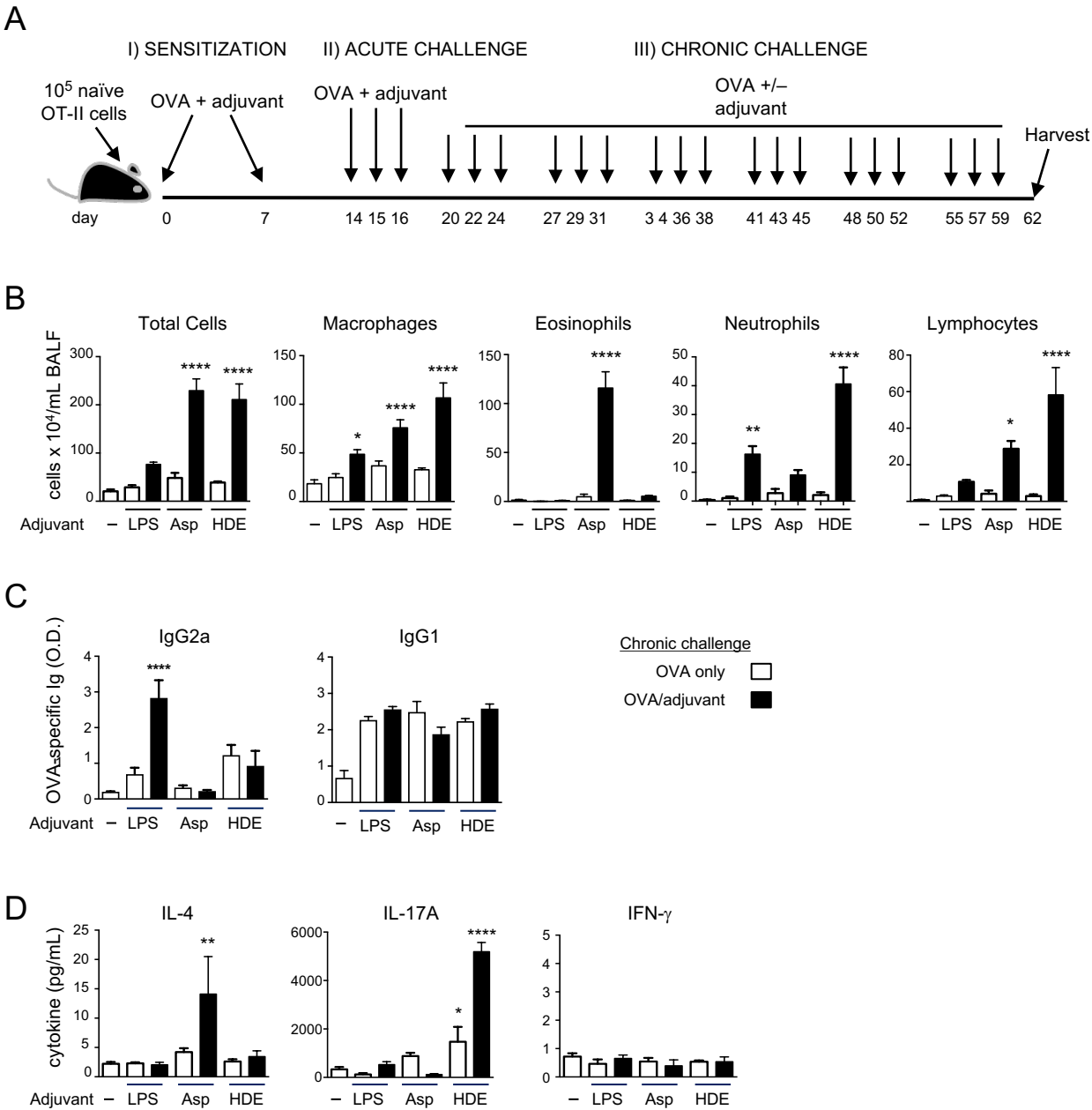


Figure 2

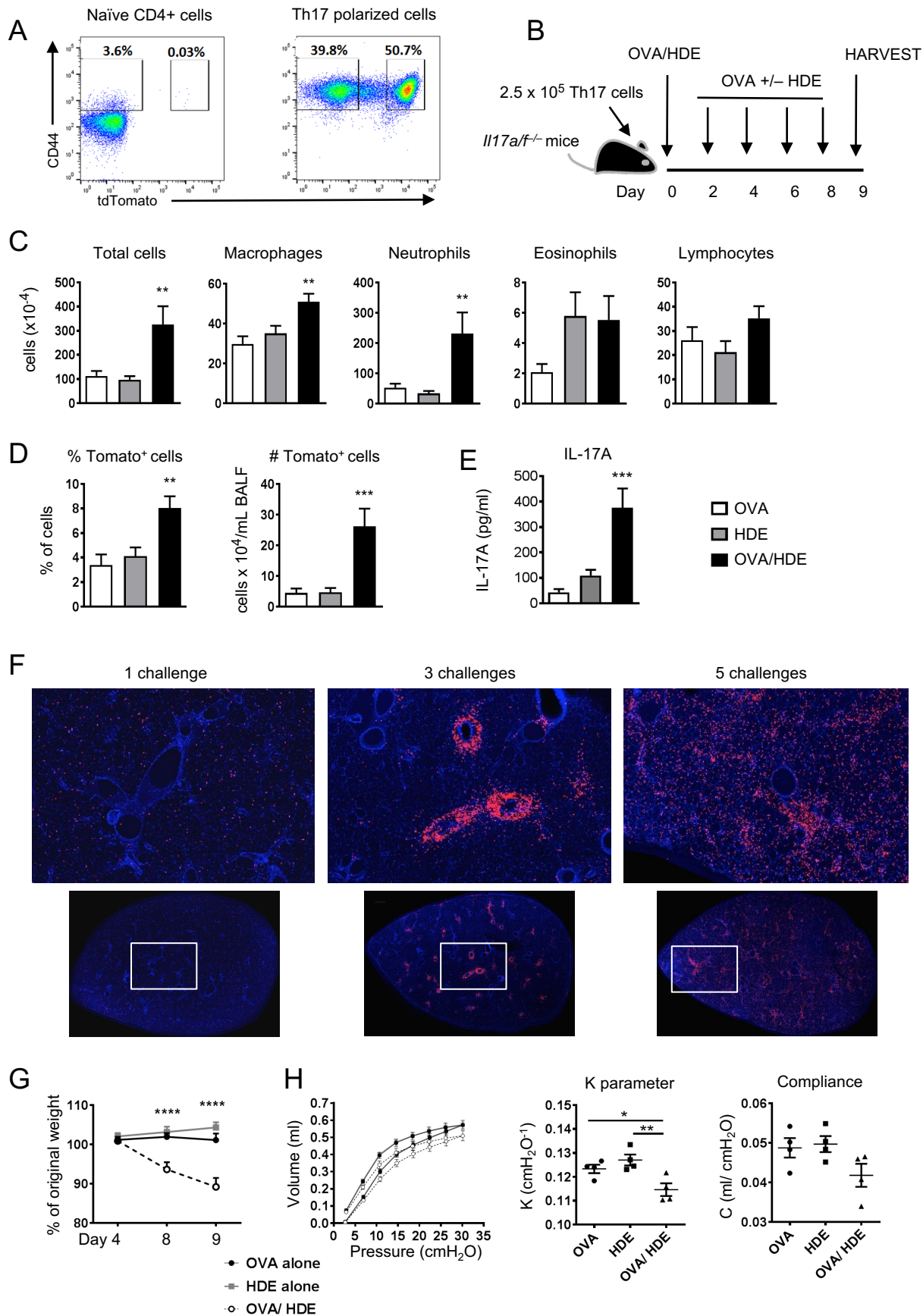
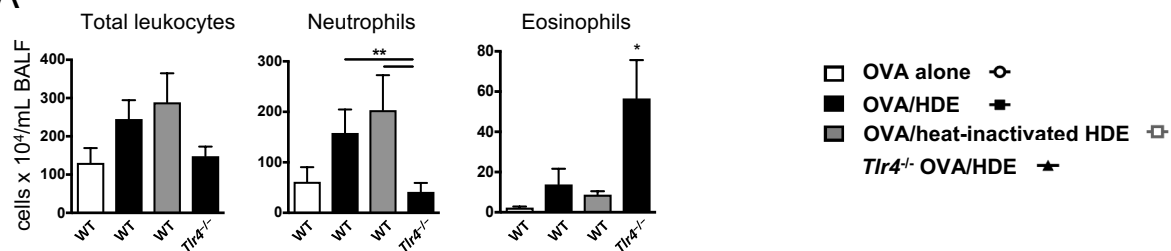
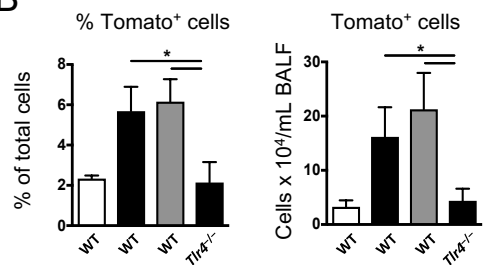


Figure 3

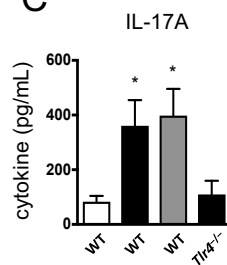
A



B



C



D

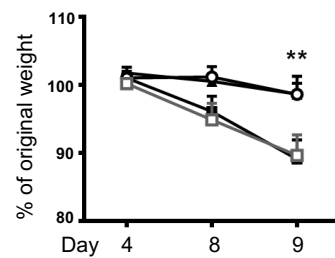
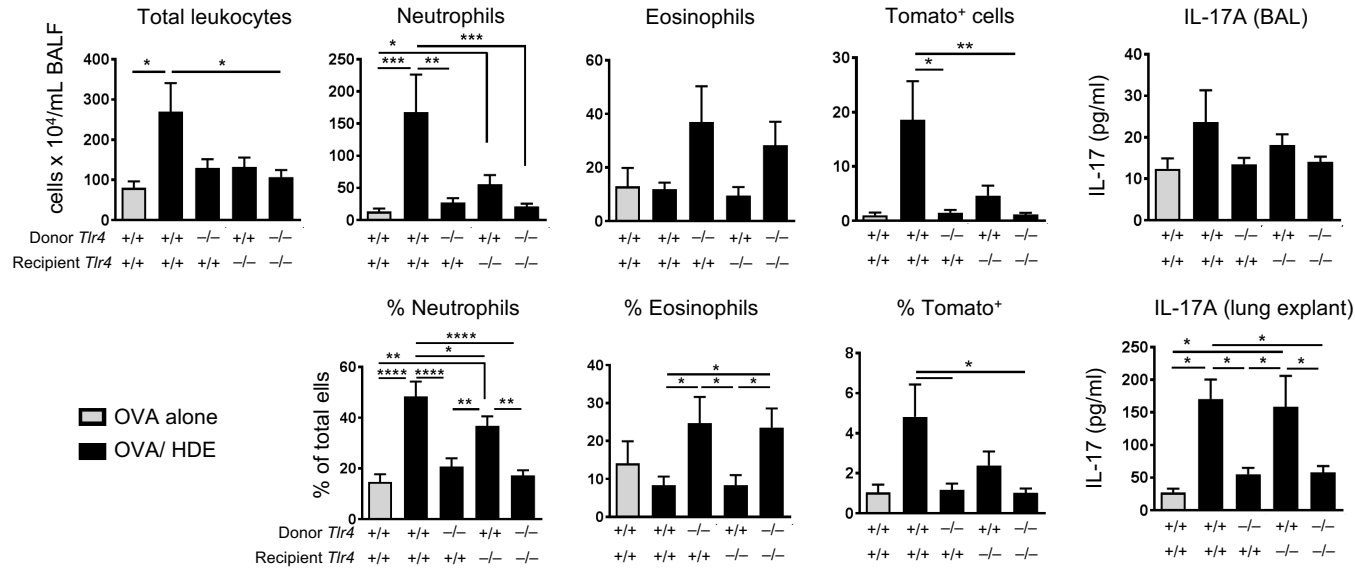
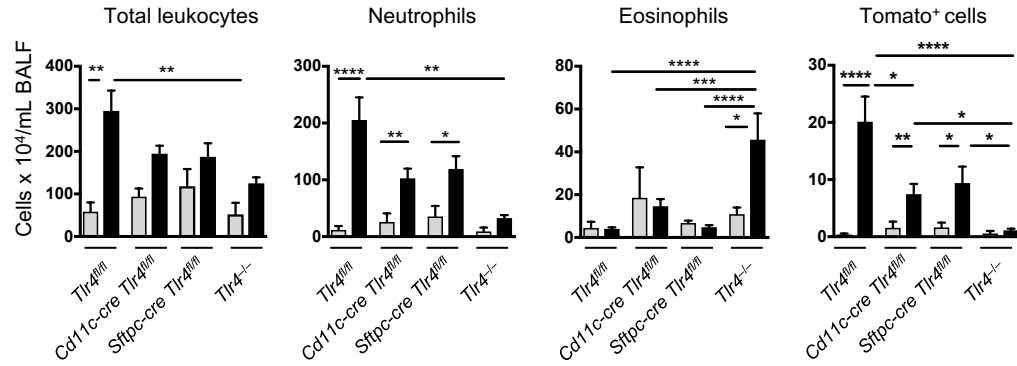


Figure 4

A



B



C

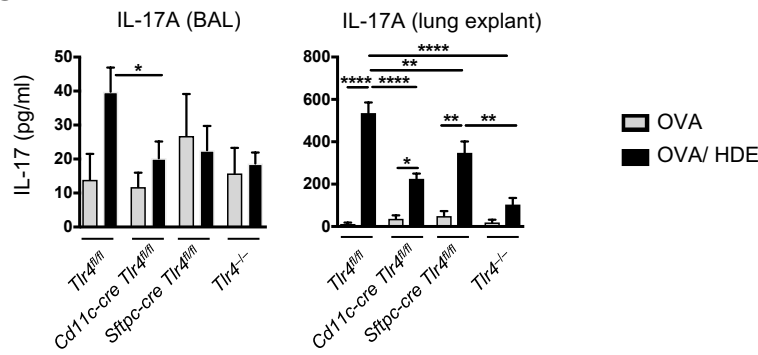


Figure 5

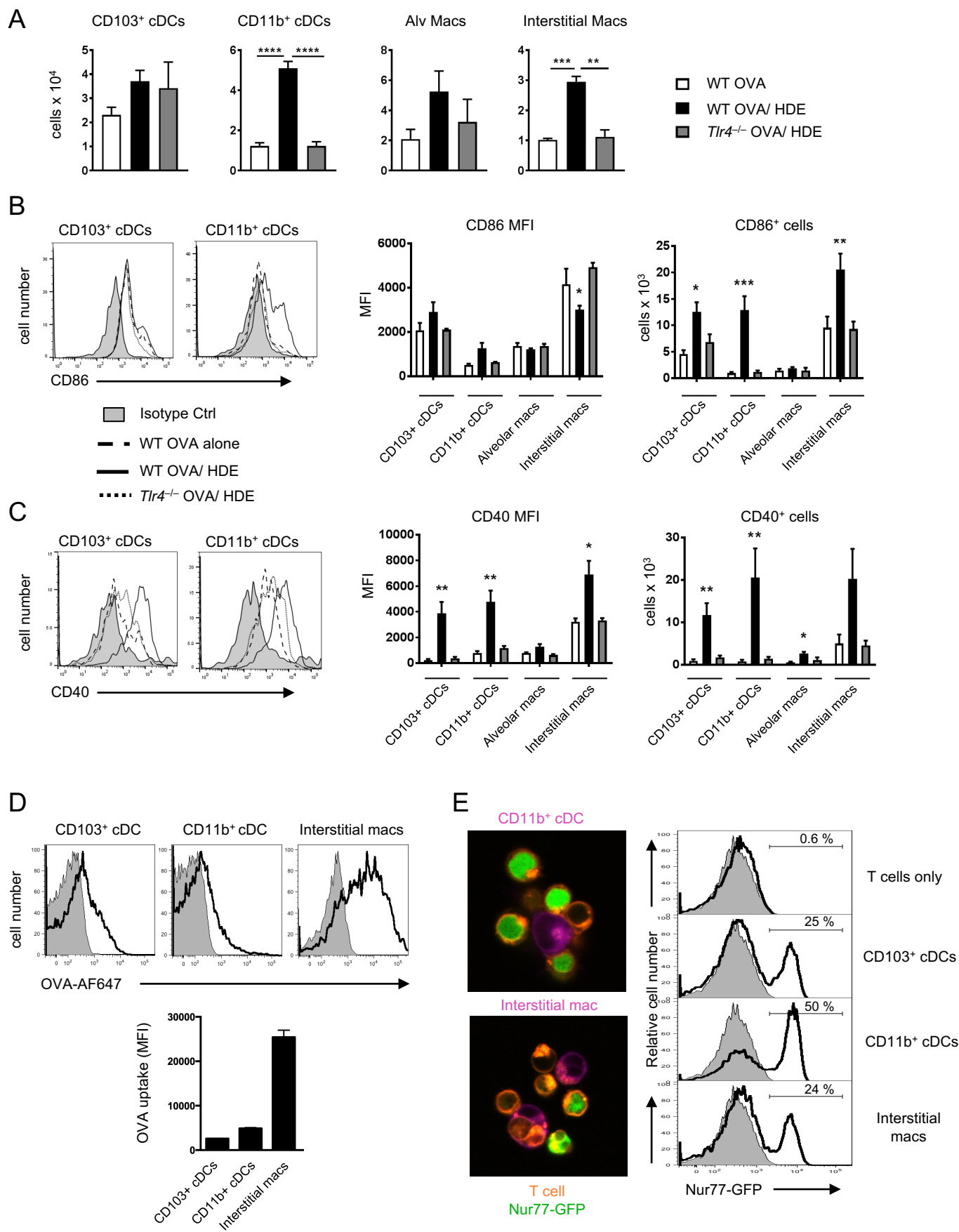


Figure 6

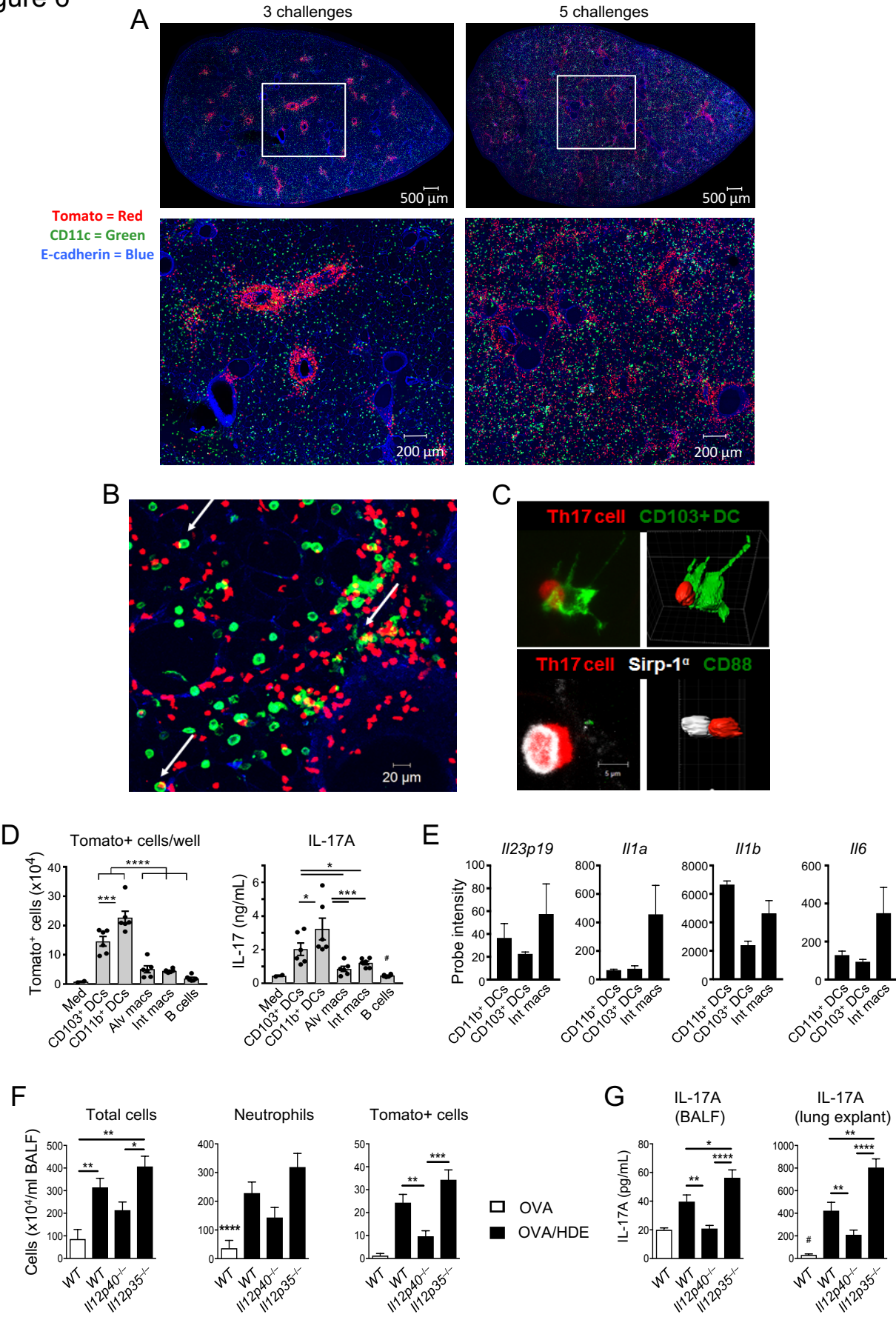
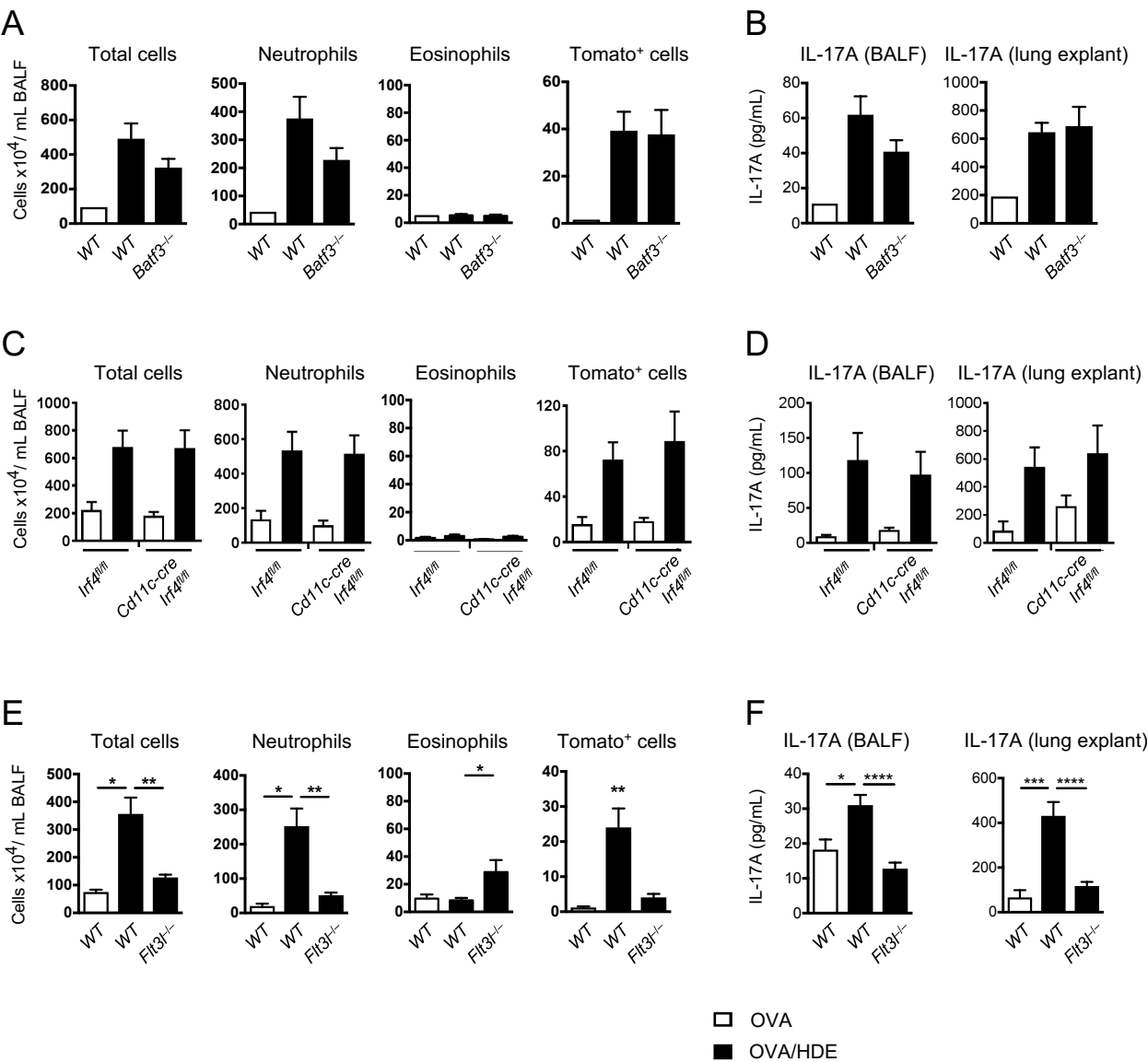


Figure 7



Supplementary Figure Legends:

Figure S1; related to Experimental Procedures and Figure 2. Breeding strategy to generate OVA-specific, IL17 fate mapping mice expressing Tomato.

Figure S2; related to Figures 5 & 6. Gating strategy for the flow cytometric analysis and sorting of lung APC subsets. Alveolar macrophages (P4) were distinguished from interstitial macrophages based on Siglec-F expression and from eosinophils based on CD11c, MHC-II and CD88 expression. Interstitial macrophages (P3; Siglec-FCD88+) were distinguished from neutrophils based on CD11c and MHC-II expression. Conventional dendritic cells - Siglec-F-CD88- (P5), non-autofluorescent (P6), CD11c+MHC-II+ (P7), Ly6C- (P8) – were separated into two distinct populations based on CD103 and CD11b expression.

Figure S3; related to Figure 2. IL-17A production in adoptively transferred Th17 cells. (A,B) Intracellular staining for IL-17A in CD4+ T cells following adoptive transfer of 10⁶ in vitro-differentiated Th17 cells into C57Bl/6J mice and three challenges with OVA alone or OVA/HDE. Lungs were harvested 48 h after the last challenge. Shown are (A) representative cytograms for IL-17A staining and (B) histograms showing MFI for intracellular staining of IL-17A within Tomato+ cells (left), number of Tomato+IL-17A+ double positive cells (middle) and Tomato+IL-17F+ double positive cells (right). (C) MFI of IL-17A staining (left) and number of Tomato+IL-17A+ cells (right), as measured with a cell surface capture assay of PMA/Ionomycin-stimulated (Stim) or unstimulated (Unstim) cells from OVA/HDE-treated mice

Figure S4; related to Figure 2. Morbidity and inflammation in recipient mice is dependent on IL-17 production by transferred Th17 cells. (A,B) Weight loss in WT and IL17a/f-/- DKO mice (A) and in WT and IL17ra-/- mice (B) at the indicated number of days post-adoptive transfer of Th17 cells and the initial challenge. (C) Shown are inflammation (top) and percent and number of Tomato+ cells (bottom), as well as IL-17A in lung explants in the indicated strains of mice. (n=3-5 animals/ group; *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

Figure S5; related to Figure 6D. CD103+ and CD11b+ cDCs revive mature Th17 cells but do not induce distinct phenotypic signatures. (A) CD103+ cDCs (top two rows) or CD11b+ cDCs (bottom two rows) were isolated and pooled from the lungs of OVA/HDE-exposed mice and co-cultured with Tomato+ Th17 cells. Following 72 h of co-culture, cells were restimulated with PMA/ Ionomycin and analyzed by mass cytometry. The multidimensional data were visualized using viSNE plots. Without stimulation, cells did not produce significant amounts of any cytokine, whereas stimulation of the cells induced heterogeneous expression of IL- 17A and TNF- α . The cells also produced IL-13, but not IFN- γ or IL-10.

Figure S1

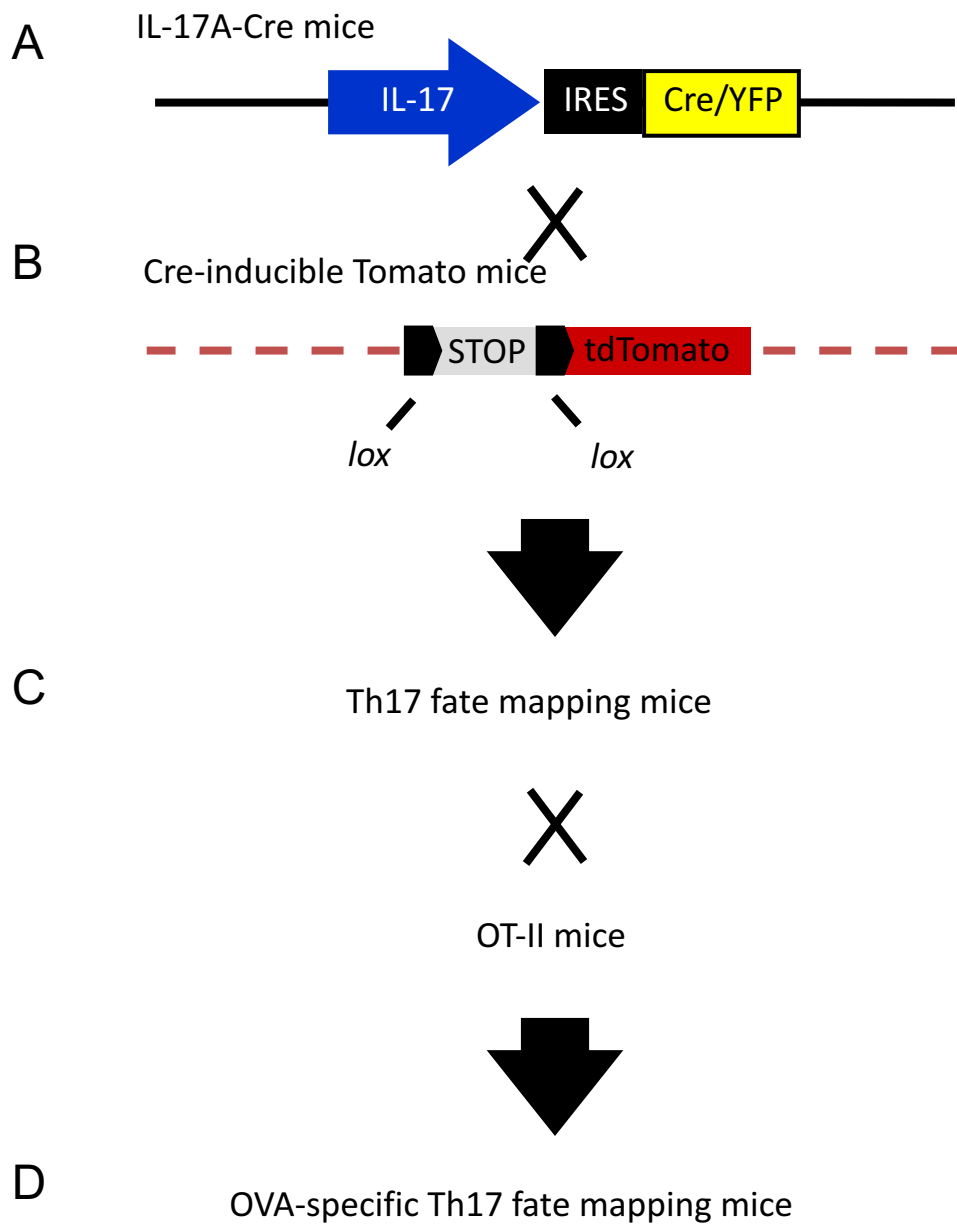


Figure S1; related to Experimental Procedures and Figure 2. Breeding strategy to generate OVA-specific, IL17 fate mapping mice expressing Tomato.

Figure S2

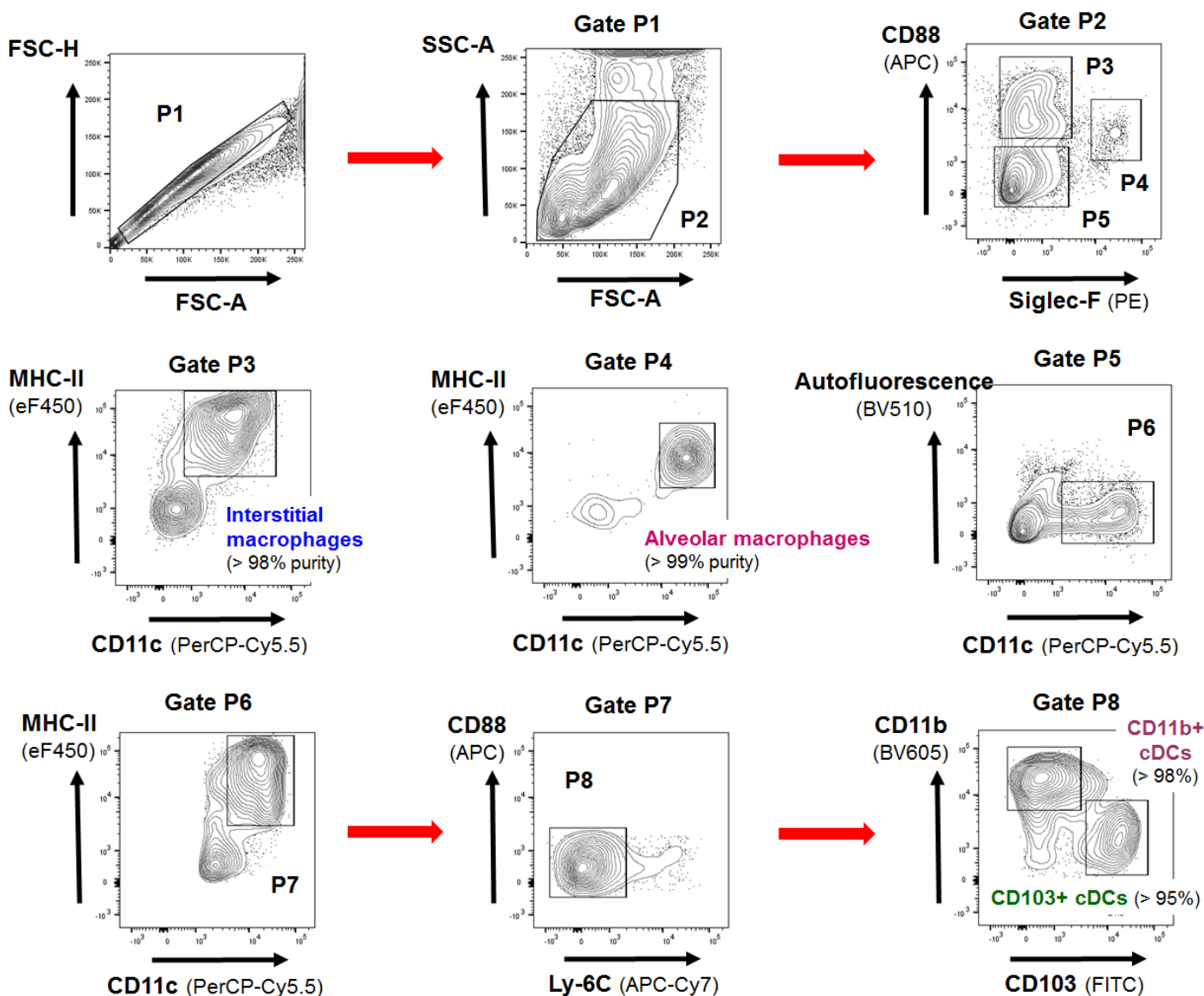


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Figure S3

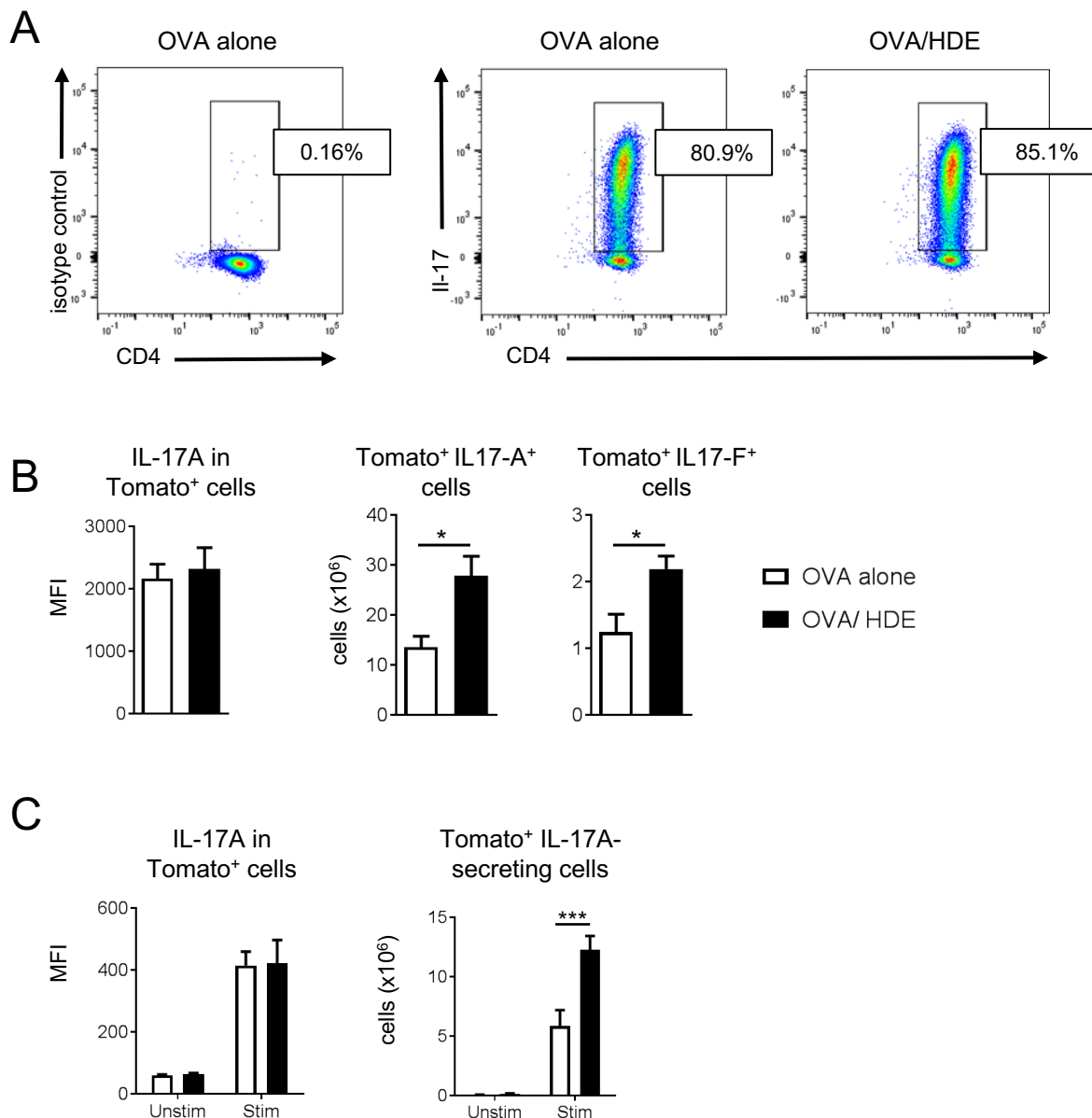


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Figure S4

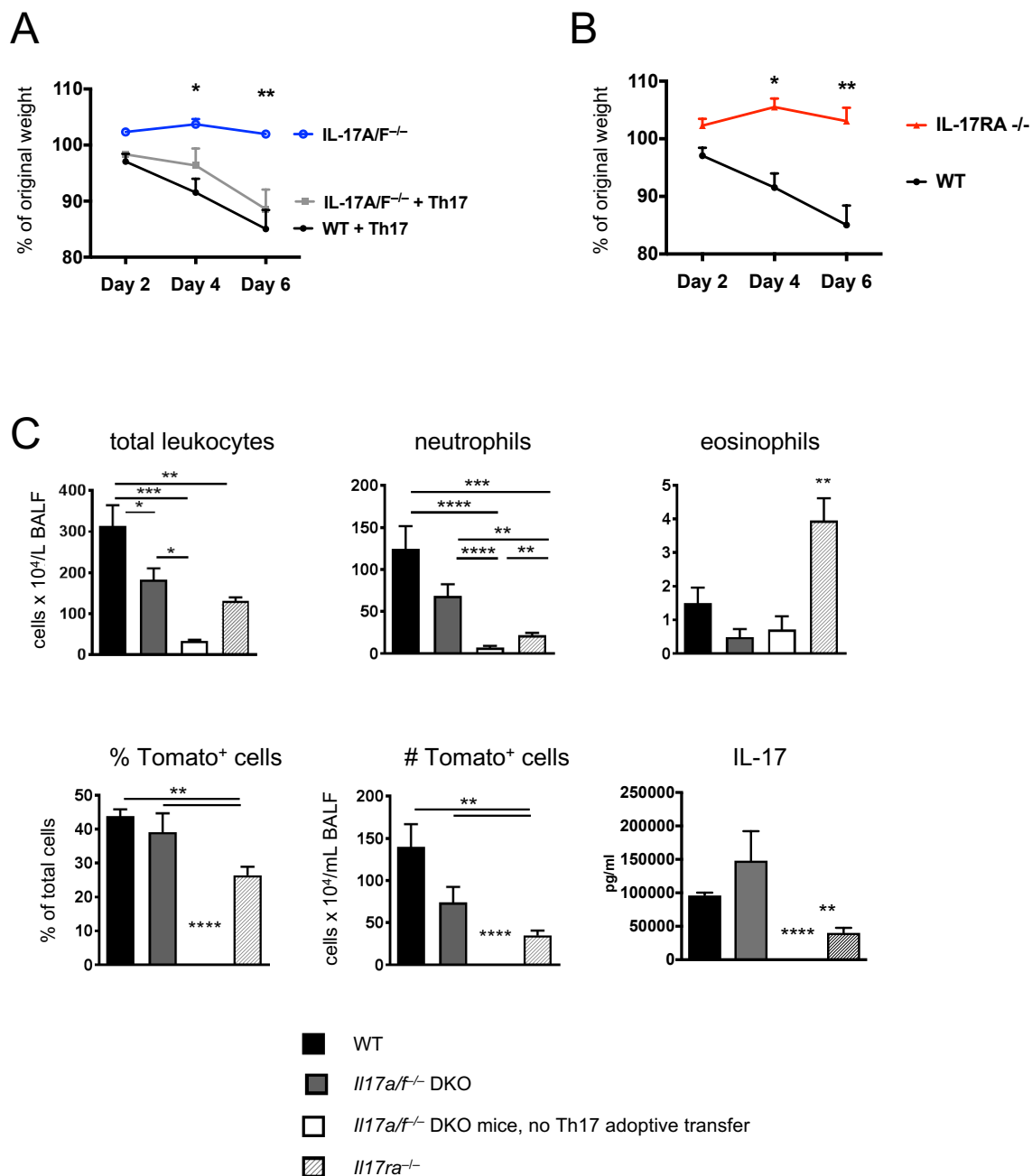


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Figure S5

unstimulated

stimulated

CD103⁺ DC

CD11b⁺ DC

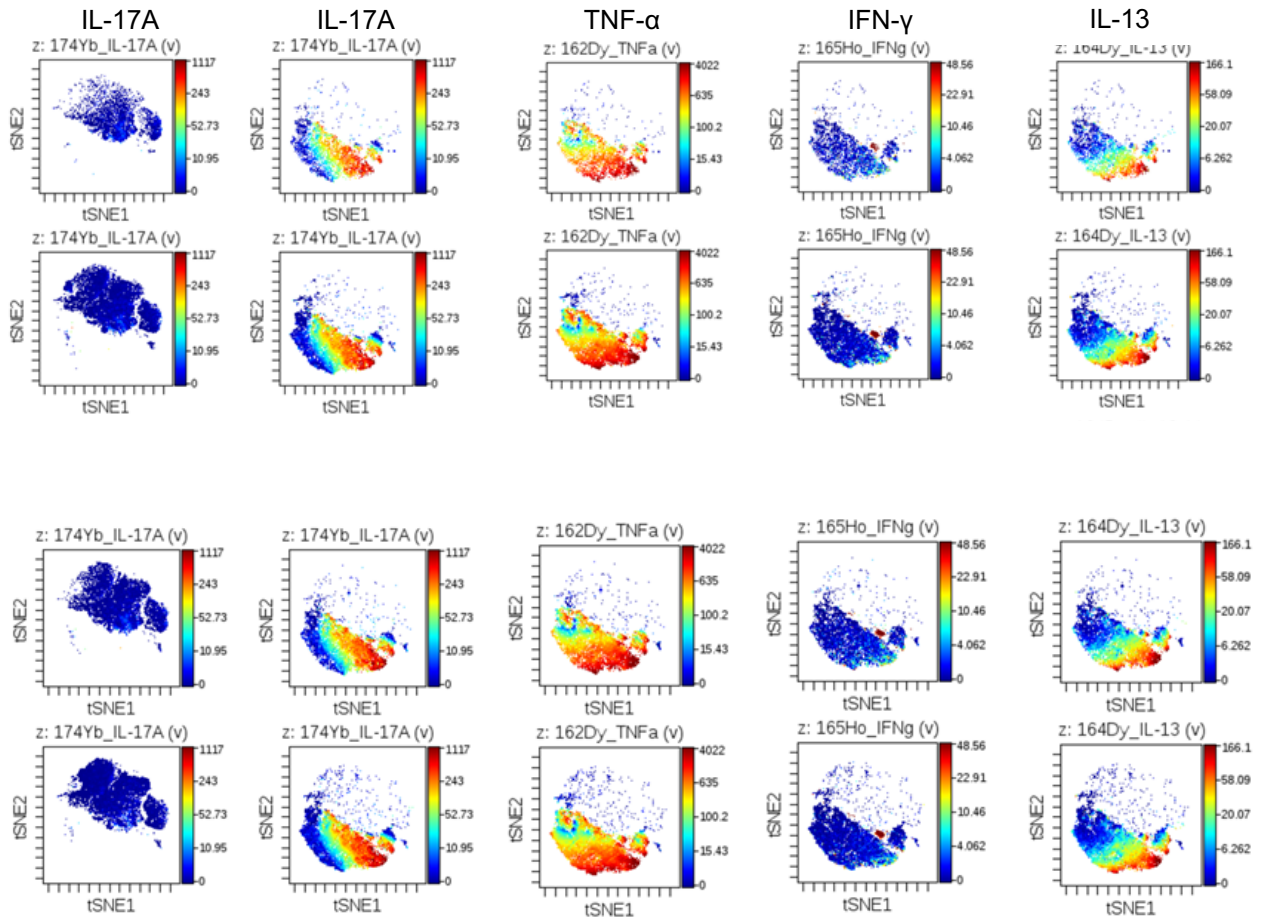


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