



Boost the immune response with
High-quality PRR ligands



This information is current as
of December 12, 2016.

Allergen-Induced CD4⁺ T Cell Cytokine Production within Airway Mucosal Dendritic Cell –T Cell Clusters Drives the Local Recruitment of Myeloid Effector Cells

Tibor Z. Veres, Tamás Kopcsányi, Nicholas van Panhuys,
Michael Y. Gerner, Zhiduo Liu, Pia Rantakari, Johannes
Dunkel, Masayuki Miyasaka, Marko Salmi, Sirpa Jalkanen
and Ronald N. Germain

J Immunol published online 30 November 2016
<http://www.jimmunol.org/content/early/2016/11/29/jimmunol.1601448>

-
- Supplementary Material** <http://www.jimmunol.org/content/suppl/2016/11/29/jimmunol.1601448.DCSupplemental.html>
- Subscriptions** Information about subscribing to *The Journal of Immunology* is online at:
<http://jimmunol.org/subscriptions>
- Permissions** Submit copyright permission requests at:
<http://www.aai.org/ji/copyright.html>
- Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
<http://jimmunol.org/cgi/alerts/etoc>



Allergen-Induced CD4⁺ T Cell Cytokine Production within Airway Mucosal Dendritic Cell–T Cell Clusters Drives the Local Recruitment of Myeloid Effector Cells

Tibor Z. Veres,^{*,†} Tamás Kopcsányi,^{*} Nicholas van Panhuys,^{†,‡} Michael Y. Gerner,[†] Zhiduo Liu,[†] Pia Rantakari,^{*} Johannes Dunkel,^{*} Masayuki Miyasaka,^{*,§} Marko Salmi,^{*,¶} Sirpa Jalkanen,^{*,¶} and Ronald N. Germain[†]

Allergic asthma develops in the mucosal tissue of small bronchi. At these sites, local cytokine production by Th2/Th17 cells is believed to be critical for the development of tissue eosinophilia/neutrophilia. Using the mouse trachea as a relevant model of human small airways, we performed advanced in vivo dynamic and in situ static imaging to visualize individual cytokine-producing T cells in the airway mucosa and to define their immediate cellular environment. Upon allergen sensitization, newly recruited CD4⁺ T cells formed discrete Ag-driven clusters with dendritic cells (DCs). Within T cell–DC clusters, a small fraction of CD4⁺ T cells produced IL-13 or IL-17 following prolonged Ag-specific interactions with DCs. As a result of local Th2 cytokine signaling, eosinophils were recruited into these clusters. Neutrophils also infiltrated these clusters in a T cell–dependent manner, but their mucosal distribution was more diffuse. Our findings reveal the focal nature of allergen-driven responses in the airways and define multiple steps with potential for interference with the progression of asthmatic pathology. *The Journal of Immunology*, 2017, 198: 000–000.

Allergic asthma primarily affects the mucosal tissue layers of conducting airways. The morphological and functional changes leading to pathological broncho-obstruction in asthmatics occur in a well-defined microanatomical compartment of small-diameter bronchi. Classically, cytokine production (e.g., IL-4, IL-5, and IL-13) by effector Th2 cells was considered to be

primarily responsible for the pathological changes in asthma, such as airway hyperreactivity, airway remodeling, and increased mucus production, as well as the infiltration of the airway mucosa with eosinophils, basophils, and mast cells. However, recent studies also suggest the involvement of type 2 innate lymphoid cells (ILC2s) as a source of effector cytokines (1, 2). Additionally, some severe forms of asthma are dominated by a Th17-type response with IL-17–producing CD4⁺ T cells, whose activation in the tissue leads to neutrophilia (3). Indeed, it is becoming increasingly clear that, especially in chronic inflammation, several Th cell subsets (Th1, Th2, Th17, T follicular helper, and regulatory T cells) simultaneously participate in the inflammatory allergic response (4, 5).

Although the key cellular players and mediators of asthma are well defined (6, 7), we have only limited knowledge about the micro-anatomical organization of the various mediator-producing cells and the specific cellular targets of each mediator within the airway mucosal compartment. This is mainly due to technical difficulties in accessing the airway mucosa of mice for microscopic studies, as well as visualizing signaling molecules in histological samples. It is also difficult to analyze inflammation in the airway mucosa separately from the lung parenchyma; commonly measured immunological parameters (e.g., inflammatory cells and cytokines in the bronchoalveolar lavage fluid) reflect changes at the level of the whole bronchoalveolar space that might not be representative of the bronchial mucosa. Furthermore, there is a pronounced difference between the mouse and human intrapulmonary airways: in contrast to human bronchi, mouse intrapulmonary airways have no cartilage and lack several layers of the mucosa that are present in humans. Moreover, mice do not have bronchial arteries, and their blood supply relies on the pulmonary circulation (8, 9).

Overcoming these problems would allow us to better understand how each cell type finds its “designated” microanatomical position in the airway wall and how specific interactions among different cell types (e.g., dendritic cells [DCs] and effector CD4⁺ T cells) result in cellular activation, mediator production, and local inflammatory

^{*}MediCity Research Laboratory, University of Turku, 20520 Turku, Finland;

[†]Lymphocyte Biology Section, Laboratory of Systems Biology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; [‡]Sidra Medical and Research Center, Doha, Qatar; [§]World Premier International Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan; and [¶]Department of Medical Microbiology and Immunology, University of Turku, 20520 Turku, Finland

ORCID: 0000-0002-6980-8900 (T.Z.V.); 0000-0003-1638-5075 (P.R.); 0000-0001-6122-5201 (M.M.).

Received for publication August 24, 2016. Accepted for publication November 7, 2016.

This work was supported by the Academy of Finland, the Sigrid Juselius Foundation, the University of Turku, and the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

T.Z.V. and R.N.G. conceived the study, planned all experiments, and wrote the manuscript; T.Z.V. performed most experiments and analyzed data with T.K.; N.v.P. and Z.L. planned and performed specific experiments; M.Y.G. provided feedback on experimental design; P.R. provided edits; J.D. performed image analysis and provided feedback and edits; M.M., M.S., and S.J. helped with funding acquisition and provided feedback and edits; and T.Z.V. visualized microscopic data and prepared all data presentation.

Address correspondence and reprint requests to Dr. Tibor Z. Veres or Dr. Ronald N. Germain, MediCity Research Laboratory, University of Turku, Tykistökatu 6A, 20520 Turku, Finland (T.Z.V.) or National Institute of Allergy and Infectious Diseases, National Institutes of Health, 9000 Rockville Pike, Building 4, Room 126A, MSC 0421, Bethesda, MD 20892. (R.N.G.). E-mail addresses: tibor.veres@utu.fi (T.Z.V.) or rgermain@nih.gov (R.N.G.).

The online version of this article contains supplemental material.

Abbreviations used in this article: cDC, conventional DC; CT, cholera toxin; DC, dendritic cell; HDM, house dust mite; ILC, innate lymphoid cell; ILC2, type 2 innate lymphoid cell; MP-IVM, multiphoton intravital microscopy; o.p.h., oropharyngeal(ly); P/I, PMA/ionomycin; PLP, periodate-lysine-paraformaldehyde.

Copyright © 2016 by The American Association of Immunologists, Inc. 0022-1767/16/\$30.00

cell (e.g., eosinophil) recruitment. Such information would also provide a markedly improved understanding of the cellular and molecular events underlying asthma pathogenesis.

Insight into the spatiotemporal aspects of immune effector function in a tissue context has been gained by the use of dynamic multiphoton intravital microscopy (MP-IVM) (10), multiplex immunohistochemistry (11, 12), and their combinations with functional read-outs (13–16). For example, such analyses in infected liver and Ag-challenged ear skin showed that, during Th1-type responses, effector cytokine production by CD4⁺ cells depends on specific Ag recognition and TCR signaling that results in T cell migration arrest and localized cytokine production (14, 17–19). In the context of asthma, the respective question is whether such a tight spatiotemporal control of T cell cytokine production by local Ag presentation also applies to Th2- or Th17-type responses of the airway mucosa.

In this study, we developed a new approach to address the role of effector CD4⁺ T cells in organizing the allergic response of the airway mucosa. We combined *in situ* cytokine staining and confocal microscopy with intravital microscopy to accurately localize cytokine-producing CD4⁺ T cells and then studied the dynamics of their interaction with airway mucosal DCs. Furthermore, we examined the local consequences of effector cytokine production, ultimately leading to tissue eosinophilia and neutrophilia. An important feature of our approach was imaging the mouse trachea because its structure and cellular composition more closely resemble human small airways involved in asthma than do mouse small airways (20). Our detailed spatiotemporal analyses reveal that, upon allergen challenge, there is a very localized low-frequency activation of Ag-specific T cells to cytokine production in DC–T cell clusters and subsequent focal recruitment of myeloid effectors, such as eosinophils, to these focal sites of T cell activation. These findings suggest that the inflammation driving asthmatic remodeling may occur in an episodic manner in restricted locations that, over time, comes to involve larger tissue areas, with possible therapeutic implications.

Materials and Methods

Mice

C57BL/6J, CAG-ECFP, CAG-DsRed, and TCR $\alpha^{-/-}$ mice were purchased from the Jackson Laboratory. OT-II Rag-1 $^{-/-}$ mice were purchased from Taconic. CD11c-EYFP mice were a kind gift from Dr. Michel C. Nussenzweig (The Rockefeller University) (21). NJ.1638 mice were obtained from the laboratories of Drs. Nancy and Jamie Lee (Mayo Clinic) (22), and IL-13 DsRed mice were kindly provided by Dr. William Paul (National Institute of Allergy and Infectious Diseases, National Institutes of Health) (23). Male and female mice were used at 6–12 wk of age; mice in different experimental groups were matched for age and sex. Animals were maintained at the Central Animal Laboratory of the University of Turku or at an Association for Assessment and Accreditation of Laboratory Animal Care–accredited animal facility at the National Institute of Allergy and Infectious Diseases. All animal procedures were approved by the Ethical Committee for Animal Experimentation in Finland and/or the Animal Care and Use Committee, National Institute of Allergy and Infectious Diseases, National Institutes of Health. They were done in adherence with the rules and regulations of the Finnish Act on Animal Experimentation (62/2006) and were performed according to 3R principles (animal license number 5588/04.10.07/2014).

Allergen sensitization and challenge

For sensitization, mice were treated with 100 μ g (dry weight) of house dust mite (HDM; *Dermatophagoides pteronyssinus*; Greer Labs) and 1 μ g of cholera toxin (CT; Sigma-Aldrich) in a volume of 20 μ l via the oropharyngeal (o.p.) route under isoflurane anesthesia (HDM/CT-model). In contrast to the commonly used volume of 50 μ l, this smaller volume ensured that only a small amount reaches the lungs, whereas the rest is evenly distributed along the tracheal wall/mainstem bronchi. In some experiments, mice were challenged with 100 μ g of HDM o.p. 1 wk later. Alternatively, mice were sensitized with 100 μ g of HDM on day 0 and challenged o.p. between days 7 and 11 with 10 μ g of HDM daily, all in a volume of 20 μ l, as described earlier (24) (repetitive HDM-model). Mice were analyzed on day 14.

Cell isolation and adoptive-transfer experiments

For the generation of polyclonal HDM-primed T cells, CAG-ECFP or CAG-DsRed mice were sensitized o.p. on day 0 with HDM/CT. On day 6, the lung-draining bronchial lymph nodes were harvested, and CD4⁺ T cells were immunomagnetically isolated using negative selection (CD4⁺ T Cell Isolation Kit, mouse; Miltenyi Biotec). Typically, the purity of isolated cells was >97%. After washing twice with PBS, cells were injected i.v. into the tail vein of recipient mice. For the generation of OT-II effector cells, OT-II Rag-1 $^{-/-}$ mice were sensitized o.p. with HDM/CT also containing 100 μ g of Endograde OVA (Hyglos) in a volume of 30 μ l. On day 6, a single-cell suspension of tracheas and lungs was prepared, and leukocytes were enriched by density gradient centrifugation with Lympholyte M (CEDARLANE). CD4⁺ T cells were isolated via positive selection using CD4 (L3T4) MicroBeads (Miltenyi Biotec), yielding a purity of CD4⁺ effector T cells typically >95%. T cells were subsequently labeled with 0.5 μ M CellTracker Deep Red Dye (Life Technologies) in HBSS at 37°C for 10 min. Labeled cells were washed twice with complete RPMI and twice with PBS. Finally, 1–2 $\times 10^7$ cells were injected i.v. into the tail vein of recipient mice 12–16 h before *in vivo* imaging.

Eosinophils were immunomagnetically isolated from the spleens of NJ.1638 mice via negative selection. After incubation with CD90.2 and CD45R/B220 MicroBeads, splenocytes were passed through a CS column (all from Miltenyi Biotec), resulting in Siglec-F⁺ SSC^{high} cells with a typical purity > 90%. Eosinophils were labeled with 1 μ M CellTracker Deep Red (or 2 μ M TAMRA), and 2–5 $\times 10^7$ cells were injected via a tail vein catheter just before imaging.

Tissue collection and flow cytometry

Mice were injected i.p. with an overdose of pentobarbital sodium; once under deep anesthesia, 15 μ g of CD45-allophycocyanin-Cy7 Ab (30-F11; BD Biosciences) was injected i.v. into the retro-orbital plexus to label intravascular leukocytes. Single-cell suspensions of tracheas and lung were prepared using Liberase TM, DNase I, Collagenase XI, and hyaluronidase, as adapted from a previous protocol (25). The tissue digest was passed through a 100- μ m cell strainer and washed twice with FACS buffer (PBS, 2% FCS, 0.01% Na₂S₂O₅). Samples were blocked in the presence of anti-CD16/32 (clone 2.4 G2) for 10 min, followed by labeling for 20 min with various conjugates of the following Abs: CD3 (17A2, 1:100), CD4 (RM4-5, 1:400), CD11c (HL3, 1:200), CD44 (IM7, 1:200), CD127 (SB/199, 1:100), KLRG-1 (2F1, 1:100), Ly-6G (1A8, 1:200), Siglec-F (E50-2440, 1:200), TCR- β (H57-597, 1:200), and TCR $\gamma\delta$ (GL3, 1:200) (all from BD Biosciences or BioLegend). Lineage mixture contained CD3 (17A2, 1:100), CD5 (53-7.3, 1:400), CD8 (53-6.7, 1:200), CD11b (M1/70, 1:200), CD11c (HL3, 1:200), CD19 (1D3, 1:200), B220 (RA3-6B2, 1:200), NK1.1 (PK136, 1:100), TER-119 (1:200), and Gr-1 (RB6-8C5, 1:200) (all from BD Biosciences, eBioscience, or BioLegend). PE-labeled CD1d Dextramer was obtained from IMMDEX. Just before analysis, a viability dye (7-aminoactinomycin D, 1:40; eBioscience) was added to each sample. Samples were analyzed using a BD Fortessa flow cytometer running FACSDiva 8.0.1, which was also used for data analysis. Dead cells and intravascular leukocytes were excluded from the final analysis.

Analysis of intracellular cytokines by flow cytometry

Direct *ex vivo* analysis of cytokines was performed as described previously (18). *In vitro* stimulation with Ag or PMA/ionomycin (P/I) was done using a published protocol (26). After staining for surface Ags, samples were washed twice with FACS buffer and fixed with 4% paraformaldehyde for 20 min at 4°C. After two wash steps with FACS buffer, intracellular cytokine staining with Abs against IL-13 (eBio13A, 1:50; eBioscience) and IL-17A (TC11-18H10, 1:50; BD Biosciences) or respective isotype controls was done using BD Perm/Wash buffer, according to the manufacturer's instructions.

Tissue fixation and immunofluorescent staining

For the collection of tracheas, mice were injected i.p. with an overdose of pentobarbital sodium; once under deep anesthesia, they were perfused with 10 ml of ice-cold periodate-lysine-paraformaldehyde (PLP) buffer. In other mice, lungs were inflated with PLP buffer via a tracheal cannula, and lung tissues were collected. Tracheas were tethered to a small piece of silicone (SYLGARD; Dow Corning) with two insect pins and fixed overnight in PLP buffer. Thereafter, tracheas were washed and cut open on the ventral side while still pinned to SYLGARD.

Whole-mount immunostaining and mounting for microscopy were performed as described earlier (27). Tracheas (or lungs) for frozen-section immunostaining were immersed in 30% sucrose in phosphate buffer overnight before snap-freezing in OCT (Tissue-Tek; Sakura). Sections (20 μ m thick) were air-dried

and fixed with ice-cold acetone for 10 min. Immunostaining was performed using Shandon Sequenza immunostaining racks and coverplates (Thermo Fisher). Sections were blocked with 5% mouse serum (before primary Abs) or 5% donkey serum (before secondary Abs) in PBS/0.3% Triton-X-100 (blocking buffer) for 15 min, followed by incubation with directly conjugated or primary/secondary Abs (diluted in blocking buffer), each for 1 h at room temperature. Washes were performed using PBS/Triton X-100.

For indirect immunostaining, primary Abs used included chicken anti-GFP (polyclonal, 1:500; Abcam), rabbit anti-RFP (polyclonal, 1:200; Rockland), rabbit anti-phospho-STAT6 (pTyr641) (polyclonal, 1:200; Sigma-Aldrich), and rat anti-MHC class II (M5/114.15.2; BD Pharmingen). Secondary Abs were species-selective F(ab')₂ fragments raised in donkey and conjugated with Alexa Fluor 488, tetramethylrhodamine, or Alexa Fluor 647 (all from Jackson ImmunoResearch). For in situ cytokine staining, Alexa Fluor 488 conjugates of Abs for IL-13 or IL-17A (the same as those used for flow cytometry) were used as primary Abs at 1:50 dilution. These were followed by rabbit anti-Alexa Fluor 488 (polyclonal, 1:400; Life Technologies) as a second-step label. In the third step, samples were labeled with a donkey anti-rabbit Alexa Fluor 488 F(ab')₂ fragment.

Confocal microscopy, data visualization, and image processing

Multichannel XYZ image acquisition was performed on a Zeiss LSM 780 confocal microscope using 20× (air) and 40× (water immersion) objectives. Z-projections or combined XY, XZ, and YZ representations were prepared using Imaris 8.1.2 (Bitplane). Confocal data involving p-STAT6 immunostains were processed as shown in Supplemental Fig. 1.

Intravital two-photon microscopy and motility analysis

Mice were anesthetized with an i.p. injection of 75 mg/kg ketamine and 1 mg/kg medetomidine. After shaving the ventral neck area, a 30G cannula, connected to a piece of PE 10 polyethylene tubing (Becton Dickinson), was inserted into the tail vein for the i.v. administration of reagents during imaging. Mice were placed on a custom-built microscope stage in a supine position (Supplemental Fig. 2A). The head was immobilized via slight pulling of the front teeth with an adjustable device. The trachea was surgically exposed, and a custom-modified steel intubation cannula (Harvard Apparatus) was inserted orotracheally (with the exception of the experiment shown in Supplemental Video 2, which was performed without intubation). The cannula was secured in the trachea with an adjustable holding device. Finally, the exposed area was sealed with a custom-made imaging window (Supplemental Fig. 2B, 2C). This window was carefully positioned using a UM-3C coarse manipulator (Narishige) so that the coverslip mounted on it would touch the trachea, allowing the observation of two or three cartilage-free areas. A ring of vacuum grease was used to seal the gap between the skin and window. Importantly, in vivo imaging was performed on spontaneously breathing mice, and the tracheal wall was mechanically stabilized for imaging by holding it between the intubation cannula and the coverslip of the imaging window. Just before microscopic analysis, a drop of Immersol W immersion medium (Carl Zeiss) was applied on the window.

Multidimensional (XYZT) images were acquired using a Leica TCS SP5 MP two-photon microscope equipped with a Chameleon Vision II and a Chameleon Ultra II femtosecond laser (Coherent), using excitation at 865 nm (for ECFP and CellTracker Deep Red) and 920 nm (for EYFP and tdTomato). An HCX IRAPO L 25×/0.95 W objective with WD = 2.5 mm was used to bridge the >2-mm difference in height between the skin surface and the tracheal wall. For the simultaneous detection of ECFP, EYFP, DsRed/tdTomato, and CellTracker Deep Red, 483/32, 535/30, 585/40, and 650/50 band-pass filters were used. Image processing for visualization (axial projections) and quantitative analysis were done using Imaris 8.1.2. Quantitation of cell motility was done using automated spot detection and track analysis; the result of the automated detection was verified manually and corrected. Arrest coefficient was defined as the fraction of time points in which instantaneous velocity was <2 μm/min, as described earlier (18). Exported movies were annotated with text and drawing elements using ImageJ Fiji.

Statistical analysis

Prism 6 (GraphPad Software) was used for the visualization and statistical analysis of quantitative data. The statistical significance of differences between the mean values of two groups was determined using the Mann-Whitney U test.

Results

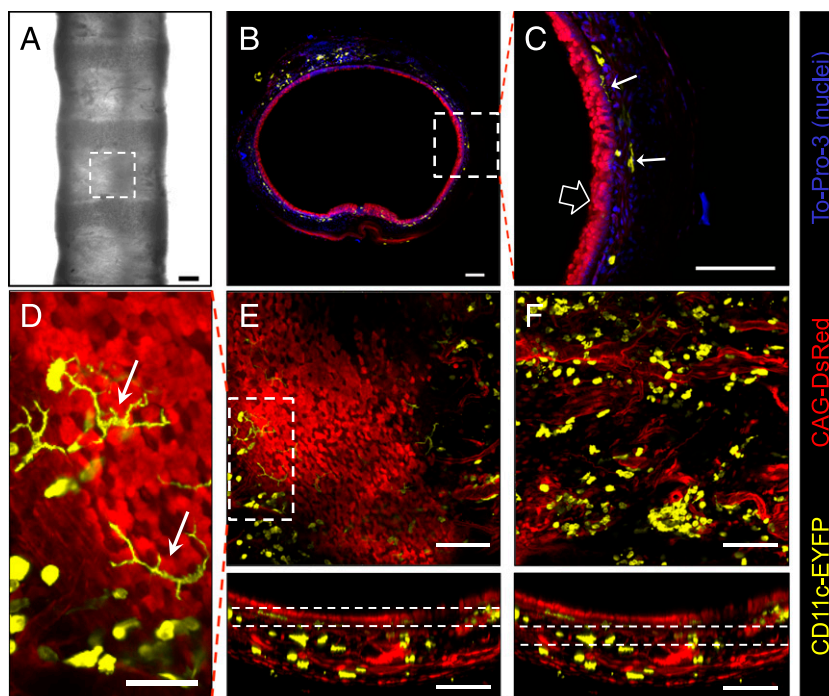
The mouse trachea as a more relevant model to study allergic immune responses of the airway mucosa

It was reported earlier that, unlike the mouse intrapulmonary airways, the mouse trachea shares microanatomical and physi-

ological similarities with human small bronchi, the anatomical sites primarily affected by asthma (28). Although the trachea of mice is clearly very different from human small peripheral airways [e.g., the mouse trachea has cartilage rings (Fig. 1A), whereas human bronchi have cartilage plates/islands], there are several similarities, such as a comparable internal diameter (~1.2 mm), a complex pseudostratified epithelium (Fig. 1B, 1C) with basal cells, submucosal glands (both of which are lacking in mouse bronchi), and real arterial circulation (9, 20, 28). The trachea was used earlier to visualize DC and mast cell dynamics in response to allergen challenge ex vivo (29), and it was demonstrated that this is also achievable in vivo (30). Therefore, we established a combined histological and intravital microscopic approach to study immune responses at this anatomical site. Using MP-IVM, we typically observed the anterior wall between two cartilage rings (Fig. 1A, 1D–F, Supplemental Video 1), allowing us to visualize, in living anesthetized mice, such events as the dynamic probing movement of DCs within and beneath the epithelium (Supplemental Video 2), as well as blood flow in mucosal postcapillary venules (Supplemental Video 3).

With this imaging capacity in hand, we searched for an appropriate model of airway mucosal inflammation in which exposure to a clinically relevant allergen would produce a similar type of cellular infiltrate in the tracheal mucosa as is observed in humans (i.e., one dominated by CD4⁺ T cells and eosinophils/neutrophils). HDM has been an increasingly well-characterized model allergen because of its clinical relevance and the properties that it shares with several other environmental allergens, such as protease activity (31), agonist activity for pattern recognition receptors (32), and an ability to provoke a positive skin-prick test in asthmatics (33). In prior studies using HDM as a model allergen, multiple exposures were necessary to produce an allergic phenotype (5, 24); however, repetitive exposures blur the line between primary sensitization and challenge (34). Although such models can be used to induce an asthma-like inflammatory response, they do not allow study of the temporal sequence of cellular recruitment and activation as it occurs after single allergen encounter. To overcome this problem, we developed a system that is based on the single-bolus administration of HDM in combination with the mucosal adjuvant CT, which was used in earlier studies to induce an airway mucosal Th2-type response (35). This approach allowed us to perform a kinetic study on cellular recruitment to the trachea and select optimal time points for imaging analysis. Sensitization of C57BL/6 mice with HDM/CT via the o.p.h. route induced a tracheal (and lung) T cell response that developed from day 4 postsensitization and was followed by an eosinophil response (Fig. 2A). By day 8, we observed tracheal mucosal T cell clusters, infiltrated with eosinophils (Fig. 2B). A careful analysis of tracheas (and lungs) showed that CD4⁺ cells dominated the CD3⁺ T cell response (Fig. 2C, 2D), without the presence of CD8⁺ cells. However, a few γδ T cells and some NKT cells were present in the CD3⁺CD4[−] population (data not shown). We also observed that the eosinophilia (Fig. 2E, 2F) coemerged with a DC and neutrophil response (Fig. 2E, 2G, 2H). The key components of the tracheal and lung response at day 8 postsensitization with HDM/CT (e.g., the recruitment and coclustering of DCs, CD4⁺ T cells, and eosinophils) were similar to the response that developed in a more standard, adjuvant-free model after repetitive HDM administration, although neutrophilia did not develop in that standard model (Supplemental Fig. 3A–F). However, in contrast to the standard model, the new model also enabled us to capture the early phases of discrete inflammatory cluster formation in the tracheal mucosa, which was necessary for establishing the setup shown below.

FIGURE 1. The mouse trachea as a model to study immune responses of the airway mucosa. **(A)** Stereomicroscopic image of an explanted trachea. The box indicates a region between cartilage rings typically accessed by intravital microscopy. Scale bar, 200 μ m. **(B and C)** Confocal microscopic images of a tracheal cross-section of a CAG-DsRed $\times$ CD11c-EYFP mouse showing the epithelium (outlined arrow) and underlying DCs (white arrows). Nuclei are visualized by TO-PRO-3 staining. Scale bars, 100 μ m. **(D–F)** Two-photon microscopic Z-stack. Epithelial (E) and subepithelial (F) tissue. Cross-sectional views (lower panels). Regions between the dashed lines are shown as Z-projections (upper panels). Scale bars, 100 μ m. **(D)** Enlargement of the boxed region shown in (E). Arrows indicate epithelial DCs. Tracheas from two mice were used in a single experiment. Scale bar, 40 μ m.



Visualization of effector $CD4^+$ T cell cytokine responses

Classically, the asthmatic response is dominated by Th2-type $CD4^+$ cells and tissue eosinophilia. However, recent evidence suggests that more severe, corticosteroid-resistant forms of asthma are associated with a Th17 response and neutrophils (3). Our model system had components of both disease types: we observed a mixed eosinophil/neutrophil response in the mouse airways, and the effector $CD4^+$ T cells in the tracheal mucosa showed a mixed Th2/Th17 phenotype, as indicated by a prominent IL-13 or IL-17 response of these cells upon *in vitro* restimulation (Fig. 3A). Compared with the standard, adjuvant-free model, we found a similar IL-13 response but an increased IL-17 response, together with lower IFN- γ production (Supplemental Fig. 3G). Although both models showed a mixed Th1/Th2/Th17 response, HDM/CT sensitization resulted in a decreased Th1 component and an increased Th17 component, possibly due to the effect of CT (36). This increased Th17 response was presumably responsible for the development of neutrophilia, which allowed us to compare the mucosal positioning of neutrophils and eosinophils during cluster formation (see below). Although a Th1 response was present in both models, previous research suggested a key role for Th2 and Th17 cells in asthma; therefore, we focused on IL-13 and IL-17 to localize individual cytokine-producing $CD4^+$ T cells in tissue.

Initially, we visualized cytokine-producing cells using frozen tracheal and lung sections from HDM/CT-sensitized and HDM-challenged mice. A secondary allergen challenge was performed to ensure that Ag is abundantly available at the time of analysis. Staining for IL-17 and IL-13 revealed several clearly identifiable T cells in the subepithelial lamina propria of the trachea, with the presence of these cytokines within delimited cytoplasmic structures (Fig. 3B). In the lungs, we found such prominent cytokine-positive cells in the peribronchial areas, as well as in the lung parenchyma (data not shown).

Next, we performed a detailed phenotypic analysis of *in situ* cytokine-producing cells. It was reported that, in an HDM-induced pulmonary response, ILC2s significantly contribute to Th2 cytokine production (37). Because ILCs appear as $CD3^-$ cytokine $^+$ cells, we first measured the frequency of such cells among IL-13 $^+$ or IL-17 $^+$ cells in tissue sections. The vast majority of cytokine-

positive cells were $CD3^+$; the frequency of non-T cell cytokine producers ranged between 0 and 2% in the trachea and lung (Table I). We also performed a more detailed flow cytometric analysis to compare the overall numbers and intracellular cytokine levels of $CD4^+$ T cells and ILC2s in the trachea and lung (Supplemental Fig. 4A–D). Our findings suggest that, in this experimental model, the major producers of IL-13 and IL-17 are $CD3^+$ T cells, whereas the *in vivo* contribution of ILCs to cytokine production is quite limited. Although conventional $CD4^+$ T cells are the most likely cytokine producers within the $CD3^+$ population, earlier reports also suggest an important role for IL-13-producing NKT cells (38) and IL-17-producing $\gamma\delta$ T cells (39) during the asthmatic response. We found that the frequency of NKT cells and $\gamma\delta$ T cells was very low compared with conventional $CD4^+$ T cells (data not shown), and the contribution of CD1d-restricted invariant NKT cells to the IL-13 response was minimal (Supplemental Fig. 4E–G). A detailed analysis of prominent IL-13 $^+$ and IL-17 $^+$ cells in tracheal sections confirmed that these cells were primarily conventional $CD4^+$ $\alpha\beta$ T cells (Supplemental Fig. 4H, 4I). We verified this finding using the adjuvant-free repetitive HDM model. Although we could not visualize IL-17 $^+$ cells in that model, the vast majority of IL-13 $^+$ cells were $CD3^+CD4^+$ conventional T cells (Supplemental Fig. 3H).

It is important to note that the frequency of cytokine-positive cells among all T cells was very low (typically 1–2%) in the trachea and lung. These numbers are clearly below the values commonly measured when T cell IL-13 and IL-17 responses are studied using *in vitro* restimulation (typically between 10 and 30%) (40, 41). They suggest that, during an airway mucosal effector $CD4^+$ T cell response to inhaled allergen, only a few percentage of all effector cells produce cytokines at a given time. This same phenomenon was reported in liver and lung mycobacterial infection models (18, 42). To address this issue in more detail, we performed flow cytometric analysis of tracheal T cell effector cytokine production (Fig. 3C–F). Direct *ex vivo* analysis at day 8 after initial HDM/CT sensitization confirmed the low *in vivo* frequency of cytokine-producing cells in the trachea and lung. In response to a secondary *in vivo* HDM challenge, as well as after *in vitro* restimulation with HDM, we measured an $\sim$ 3-fold increase in the frequency of IL-17 $^+$ cells,

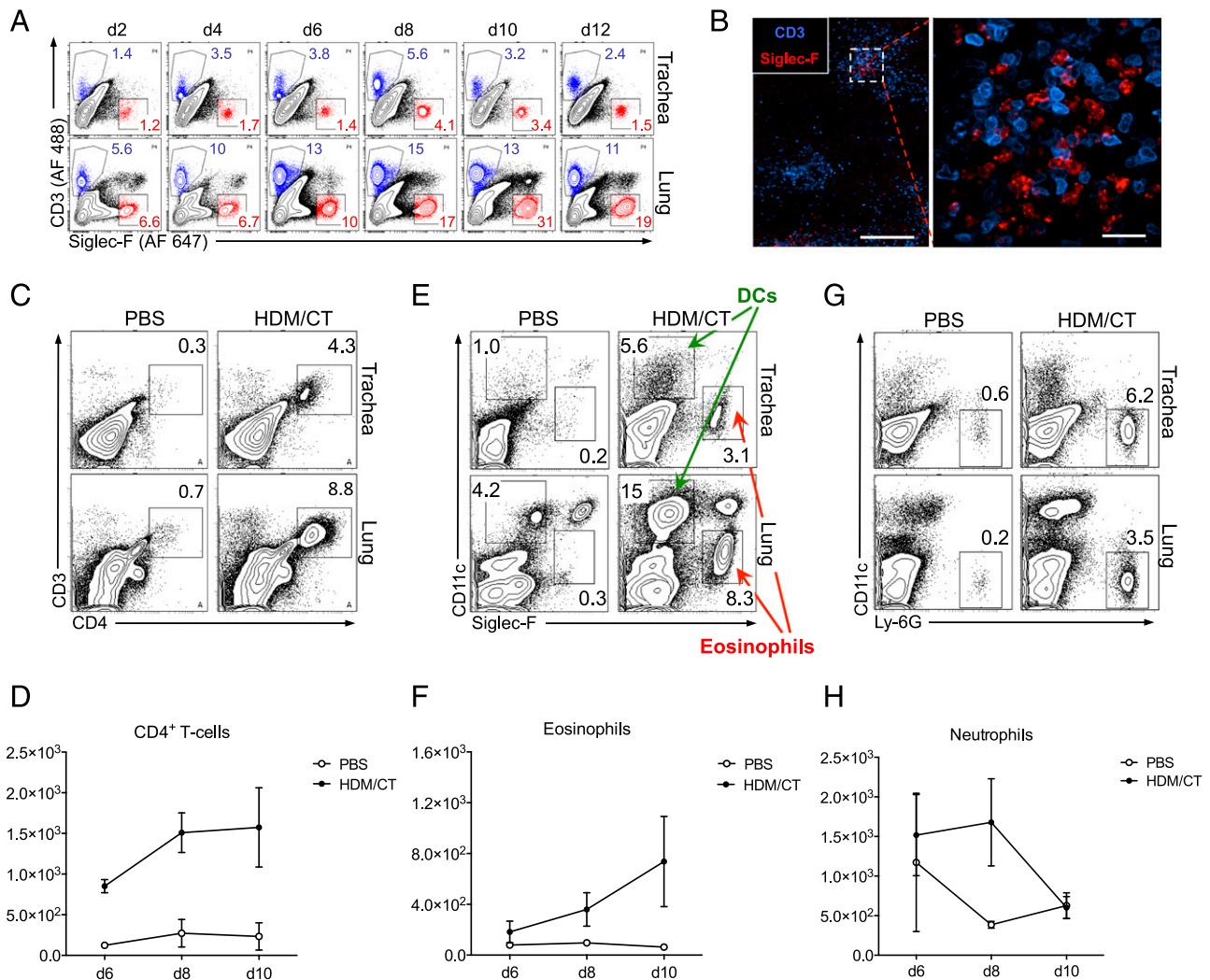


FIGURE 2. Induction of a tracheal mucosal inflammatory response to HDM allergen. **(A)** Percentages of T cells (blue) and eosinophils (red) from tracheal and lung tissue digests on days 2–12 following a single o.p.h. treatment of C57BL/6 mice with HDM/CT. Gated on live extravascular cells. Lung eosinophils were distinguished from alveolar macrophages based on their low autofluorescence in the green (Alexa Fluor 488 [AF 488]) channel. **(B)** Confocal images showing T cell clusters and associated eosinophils in a tracheal whole-mount at day 9 after HDM/CT. Boxed region in left panel (scale bar, 200 μ m) is enlarged in right panel (scale bar, 20 μ m). **(C, E, and G)** Comparison of lung and tracheal tissue digests from HDM/CT- and PBS-treated mice. Gates indicate the CD4⁺ T cell response at day 8 (C), the eosinophil and DC response at day 10 (E), and the neutrophil response at day 8 (G). Numbers are the percentages from the total live extravascular population. **(D, F, and H)** Analysis of the tracheal inflammatory response developing between days 6 and 10. Total numbers of CD4⁺ T cells (D), eosinophils (F), and neutrophils (H) per trachea were determined using the gates shown in (C), (E) and (G). Graphs show mean $\pm$ SEM. Two or three mice per time point (in a single experiment) were analyzed.

whereas the increase in IL-13⁺ cells was less pronounced. However, all of these values were clearly below those measured after a maximal stimulus, represented by P/I restimulation. Altogether, these data suggest that, in our model, the allergic response to HDM is mainly driven by conventional CD4⁺ T cells, with only a small fraction of them being activated to produce cytokines at any given time.

Cytokine-producing CD4⁺ T cells form conjugates with APCs in the airway mucosa

Previous studies on Th1 effector CD4⁺ responses in the liver and ear skin using a TCR-transgenic system suggest that cytokine production by CD4⁺ cells depends on prolonged interaction with APCs (18, 19). Therefore, we asked whether the production of IL-17 and IL-13 by endogenous polyclonal effector T cells in the tracheal mucosa also depends on local Ag presentation. Whole-mount immunostains for IL-17 enabled us to carefully localize IL-17-producing T cells within T cell clusters within the tracheal mucosa (Fig. 4A). In contrast, IL-13 staining only worked well on

thick frozen sections; therefore, we could only study the local environment of individual cells, without a proper view of the larger tissue context.

During an HDM/CT-induced tracheal mucosal response, CD3⁺ T cell clusters appeared together with APC clusters. Within these cellular clusters, a few IL-17⁺ T cells were localized in the immediate proximity of MHC-II⁺ cells (Fig. 4A, 4B). Similarly, IL-13⁺ T cells appeared in close contact with MHC-II⁺ structures, as seen on tracheal cross-sections (Fig. 4C, 4D), an observation that we confirmed in the adjuvant-free repetitive HDM model (Supplemental Fig. 3I, 3J). Quantitatively, we found that the vast majority of IL-17⁺ and IL-13⁺ T cells were in direct contact with MHC-II⁺ cells (Fig. 4E, 4F). However, we also identified a small number of cytokine-producing cells outside of APC clusters, clearly without contact with MHC-II⁺ cells (data not shown). These results suggest that direct cell–cell contact with local MHC class II-bearing cells of the airway mucosa is largely responsible for the elicitation of effector cytokine production by Th2 and Th17 cells.

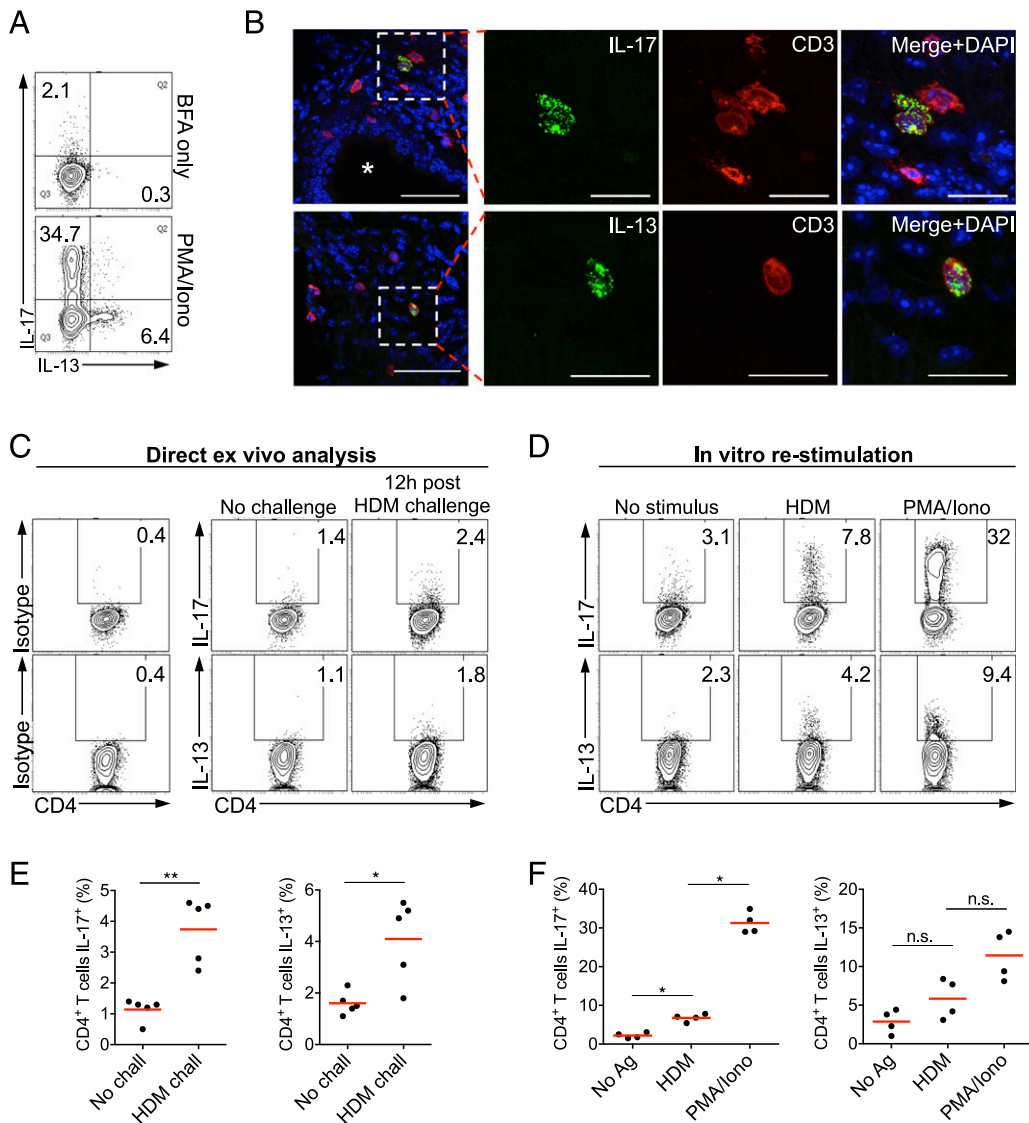


FIGURE 3. Visualization of effector cytokine production by CD4⁺ T cells in the trachea. **(A)** C57BL/6 mice were treated with HDM/CT o.p.h. Eight days later, effector cytokine production by CD4⁺ T cells from digested tracheas was analyzed after restimulation with PI or treatment with brefeldin A (BFA) only. Numbers indicate percentages. **(B)** C57BL/6 mice were treated with HDM/CT o.p.h. on day 0 and received an HDM challenge (o.p.h.) on day 7. Tracheas were analyzed on day 9. Confocal microscopic analysis of cytokine-producing cells in frozen tracheal sections. Boxed regions in left panels (scale bars, 50 μ m) are enlarged to show individual cytokine-positive cells (scale bars, 20 μ m). Asterisk denotes the tracheal lumen. Data are representative of at least two independent experiments. Analysis of T cell cytokine production directly after tissue digest **(C)** or after in vitro restimulation **(D)**. Three tracheas were pooled for each measurement; numbers indicate percentages (isotype control versus specific anti-cytokine staining was done using the same digest). Plots were gated on extravascular CD4⁺ CD44^{hi} cells. Quantitative representation of T cell cytokine production measured directly ex vivo 12 h after HDM challenge **(E)** or after in vitro restimulation **(F)**. Data were pooled from two independent experiments and each dot indicates a single measurement. $n = 5$ measurements in (E). $n = 4$ measurements in (F). Three tracheas were pooled and used for each measurement. $*p < 0.05$, $**p < 0.01$, Mann–Whitney U test. n.s., not significant.

CD11b⁺ DCs represent the major DC population serving as APCs to induce local effector cytokine production in airway mucosal CD4⁺ T cells

Like in most nonlymphoid organs, myeloid conventional DCs (cDCs) of the airway mucosa belong to one of the two major DC subtypes: CD11b⁺CD103[−] DCs or CD11b[−]CD103⁺ DCs (referred

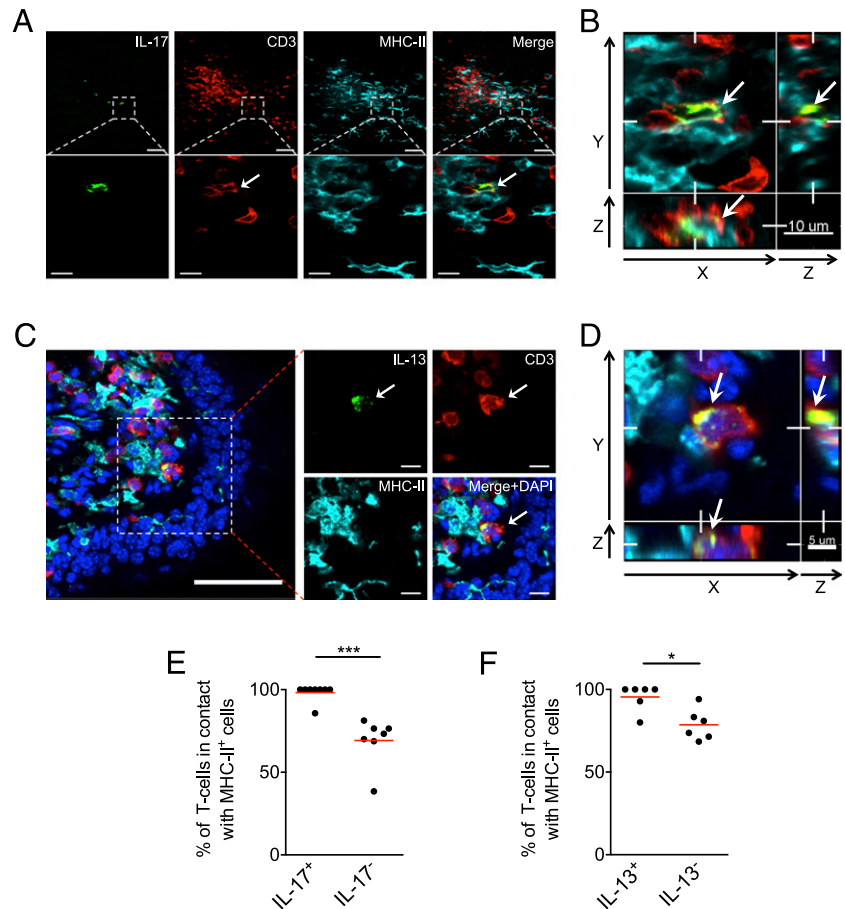
to as CD11b⁺ and CD11b[−] cDCs, respectively) (43). In HDM-induced allergic responses, large numbers of CD11b⁺ monocyte-derived DCs are recruited to the airways (44). Previous work revealed that CD11b⁺ cDCs and monocyte-derived DCs are primarily involved in the initiation and maintenance, respectively, of Th2 immunity to HDM allergen (44). Therefore, we tried to determine

Table I. Quantitative analysis of CD3⁺ and CD3[−] cytokine-producing cells in tracheal and lung tissue sections

$n = 3$ mice	IL-13 ⁺ Cells (of total CD3 ⁺ T cells) (%) (mean $\pm$ SEM)	IL-17 ⁺ Cells (of total CD3 ⁺ T cells) (%) (mean $\pm$ SEM)	No. of CD3 [−] Cells/Total IL-13 ⁺ Cells (%)	No. of CD3 [−] Cells/Total IL-17 ⁺ Cells (%)
Trachea	1.59 $\pm$ 0.11	1.06 $\pm$ 0.24	1/53 (1.89)	0/48 (0)
Lung	0.98 $\pm$ 0.30	1.93 $\pm$ 0.51	4/285 (1.4)	8/565 (1.42)

C57BL/6 mice were treated with HDM/CT (o.p.h.) on day 0 and received an HDM challenge (o.p.h.) on day 7. Tracheas and lungs were analyzed on day 9. IL-17⁺ or IL-13⁺ cells were identified manually, and costaining for CD3 was determined. Tissue samples from three mice were analyzed.

FIGURE 4. Cytokine-producing T cells are engaged in contact with APCs. C57BL/6 mice were treated with HDM/CT on day 0 and rechallenged with HDM on day 7. On day 9, perfusion-fixed tracheas were collected for confocal microscopic analysis. **(A and B)** Effector T cell–DC cluster in a tracheal whole mount. Boxed regions in **(A)** (upper panels; scale bars, 50 μ m) are enlarged (lower panels; scale bars, 10 μ m) and displayed as XY, XZ, and YZ representations **(B)** to show an IL-17⁺ T cell in direct contact with an APC (arrows). **(C and D)** Effector T cell–DC cluster in a frozen cross-section of the tracheal mucosa. Boxed region in **(C)** (left panel; scale bar, 50 μ m) is shown as separate channels (right panels; scale bars, 10 μ m) and displayed as XY, XZ, and YZ representations **(D)** to indicate an IL-13⁺ T cell in direct contact with an APC (arrows). Single slices are shown in **(B)** and **(D)**; the position of slices is indicated by tick marks. **(E and F)** Quantitative analysis showing the percentage of cytokine-positive versus cytokine-negative cells in contact with an APC. Between 74 and 104 cytokine-positive or randomly selected cytokine-negative tracheal T cells were analyzed. $n = 7$ –8 mice **(E)**. $n = 6$ mice **(F)**. Results of a single experiment are shown. * $p < 0.05$, *** $p < 0.001$, Mann–Whitney U test.



which DC subtypes are involved in local Ag presentation (defined as direct contact with cytokine-positive T cells). Although we were unable to differentiate histologically between CD11b⁺ cDCs and CD11b⁺ monocyte-derived DCs, we could clearly separate CD11b⁺ DCs from CD103⁺ DCs by whole-mount immunostaining for CD11b in CD11c-EYFP mice. We observed that, within large subepithelial DC clusters that are formed during the inflammatory response, the majority of CD11c-EYFP⁺ DCs contained for CD11b. Cytokine-producing T cells were interlaced within clusters of amoeboid DCs possessing a few dendritic extensions, in direct contact with CD11b⁺ CD11c-EYFP⁺ cells (Fig. 5A, 5B). However, mainly within the epithelium, we also observed individual CD11b⁻ (and presumably CD103⁺) CD11c-EYFP⁺ DCs with a dendritic morphology in contact with cytokine-positive T cells (Fig. 5C, 5D). Quantitatively, we found that the majority of IL-17⁺ and IL-13⁺ T cells formed contacts with CD11b⁺ DCs (Fig. 5E). However, this probably reflects the increased frequency of this DC subtype in the inflamed tracheal mucosa (Fig. 5F), rather than their increased propensity to activate T cells. Therefore, our histological findings are in accordance with earlier reports suggesting that CD11b⁺ cDCs and monocyte-derived DCs are the major Ag-presenting DC subtypes in the airways (44).

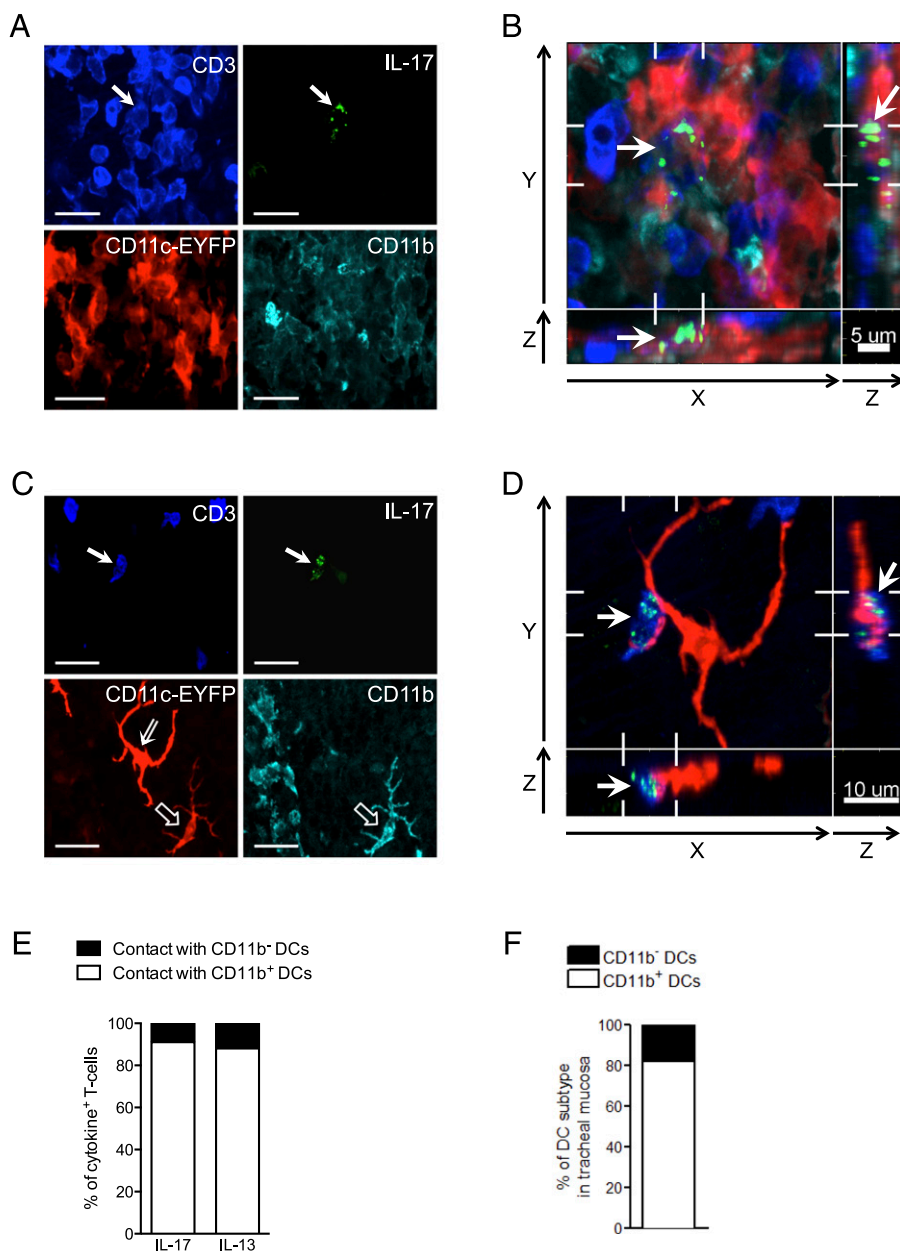
Effector CD4⁺ T cells form stable contact with DCs in the airway mucosa

Given the previously demonstrated link among Ag recognition, motility, and cytokine production by TCR-transgenic cells during Th1 responses in the liver and skin (18, 19), we sought to determine whether prolonged T cell–DC interactions also precede cytokine production in the airway mucosa in the course of a polyclonal Th2/Th17 response to HDM allergen.

To visualize the polyclonal effector CD4⁺ T cell response in the trachea in vivo, we transferred bronchial lymph node polyclonal CD4⁺ cells from HDM/CT-sensitized ubiquitously DsRed- or ECFP-expressing mice into CD11c-EYFP Rag-1^{-/-} recipients (Fig. 6A). Treatment of recipient mice with HDM/CT resulted in the recruitment of the transferred cells to the tracheal mucosa (Fig. 6B). At day 7 postsensitization, another HDM challenge ensured abundant Ag availability. Using MP-IVM 2 d later, we observed cellular interactions within DC–T cell clusters in real time (Supplemental Video 4). Within these clusters, DCs formed stable interactions with the transferred HDM-primed polyclonal effector T cells (Supplemental Video 5).

Next, we examined whether these stable T cell–DC interactions form in an Ag-specific manner. To this end, we injected in vivo-generated OT-II Rag-1^{-/-} CD4⁺ effector cells i.v. the day before analysis (Fig. 6A). These cells served as a control population with known specificity to an irrelevant Ag (OVA). It was reported that effector T cells enter inflamed tissues, even in the absence of specific Ag (18). Similar to previous observations in the liver and skin, injected OT-II effector cells were recruited to the inflamed tracheal mucosa and showed a pattern of random migration (Fig. 6C, Supplemental Video 6). However, in the absence of OVA, these cells did not stop on DCs and showed a greater mean velocity, as well as a smaller arrest coefficient, compared with the polyclonal HDM-primed population (Fig. 6D). In situ cytokine staining of tracheas fixed after MP-IVM demonstrated that polyclonal cells, but not OT-II, produced effector cytokines (Fig. 6E). These results suggest that the stable interactions of polyclonal effector T cells with airway mucosal DCs and local cytokine production, which were observed in vivo, are the result of specific Ag recognition and not cytokine-induced cytokine production by bystander lymphocytes.

FIGURE 5. CD11b⁺ and CD11b⁻ DCs form contact with cytokine-producing effector T cells in the tracheal mucosa. CD11c-EYFP mice were treated with HDM/CT on day 0 and rechallenged with HDM on day 7. On day 9, perfusion-fixed tracheas were collected for whole-mount immunostaining. Confocal microscopic analysis of interactions between IL-17⁺ T cells and CD11b⁺ (A and B) or CD11b⁻ (C and D) DCs. Scale bars, 20 μ m. Arrows indicate IL-17⁺ T cells in contact with DCs. In (C), double arrow shows a CD11b⁻ DC, outlined arrows show a CD11b⁺ DC. Separate channels are shown in (A) and (C). (B) and (D) show the respective merged images as XY, XZ, and YZ representations. A range of slices is shown in (B) and (D), as indicated by tick marks. (E) Quantitative analysis showing the contribution of CD11b⁺ and CD11b⁻ DCs to conjugate formation with cytokine-positive T cells in the tracheal mucosa. A total of 68 IL-17⁺ T cells in contact with a CD11c-EYFP⁺ DC in tracheal whole mounts of five mice was analyzed. A total of 49 IL-13⁺ T cells in contact with a DC in frozen tracheal sections of five mice were analyzed. (F) Relative frequency of CD11b⁻ versus CD11b⁺ DCs in the tracheal mucosa. Tracheal whole mounts of five mice were analyzed. Results of a single experiment are shown.



Th2 cytokine signaling and eosinophil recruitment are localized to effector CD4⁺ T cell clusters

Because the majority of T cell–DC interactions leading to effector cytokine production occur within discrete clusters, our data suggest that such allergic lesions might function as hot spots driving the allergic response. As a next step, we asked to what extent are the local effects of Th2 (and Th17) cytokines restricted to these cell clusters and whether the recruitment of myeloid effector cells (eosinophils and neutrophils) associated with a Th2/Th17 response is locally restricted to these clusters. To address these issues, we used the previously described adoptive cell transfer approach (Fig. 6A) that involves the transfer of polyclonal HDM-primed CD4⁺ T cells into Rag1^{-/-} mice. In this setting, tracheal (and lung) eosinophilia was completely dependent on the presence of the HDM-primed polyclonal T cell population (Supplemental Fig. 5A, 5B). Neutrophilia also greatly depended on T cells; however (at least in the trachea), neutrophils were present in low numbers, even in the absence of T cells. We obtained similar results when TCR α ^{-/-} mice were used as recipients instead of Rag1^{-/-} mice (results not shown).

Therefore, in the following experiments, we transferred DsRed⁺ HDM-primed polyclonal CD4⁺ T cells into TCR α ^{-/-} mice to visualize the local consequences of effector T cell cytokine production.

IL-13 (together with IL-4) is known to induce STAT6 phosphorylation in target cells (45), so we first performed immunostaining for STAT6 phosphorylated on Y641 (p-STAT6) to reveal locally responding cells. In response to HDM/CT, DsRed⁺ polyclonal T cells formed discrete small clusters in the tracheal mucosa on days 2–4 postsensitization. By day 7, the numbers of recruited cells increased, and cell clusters became larger, with scattered cells distributed between these clusters. To determine whether the effect of IL-13 is locally restricted, we visualized early small effector DsRed⁺ CD4⁺ clusters together with p-STAT6 staining (Fig. 7A–C). We observed that the DsRed⁺ effector cells were among the brightest p-STAT6⁺ cells; therefore, via image processing, we removed all p-STAT6 signals that originated from DsRed⁺ cells (Supplemental Fig. 1). We found that clusters of bright p-STAT6⁺ cells completely overlapped with DsRed⁺ effector clusters (Fig. 7A). However, in control tracheas from HDM/CT-sensitized TCR α ^{-/-} animals

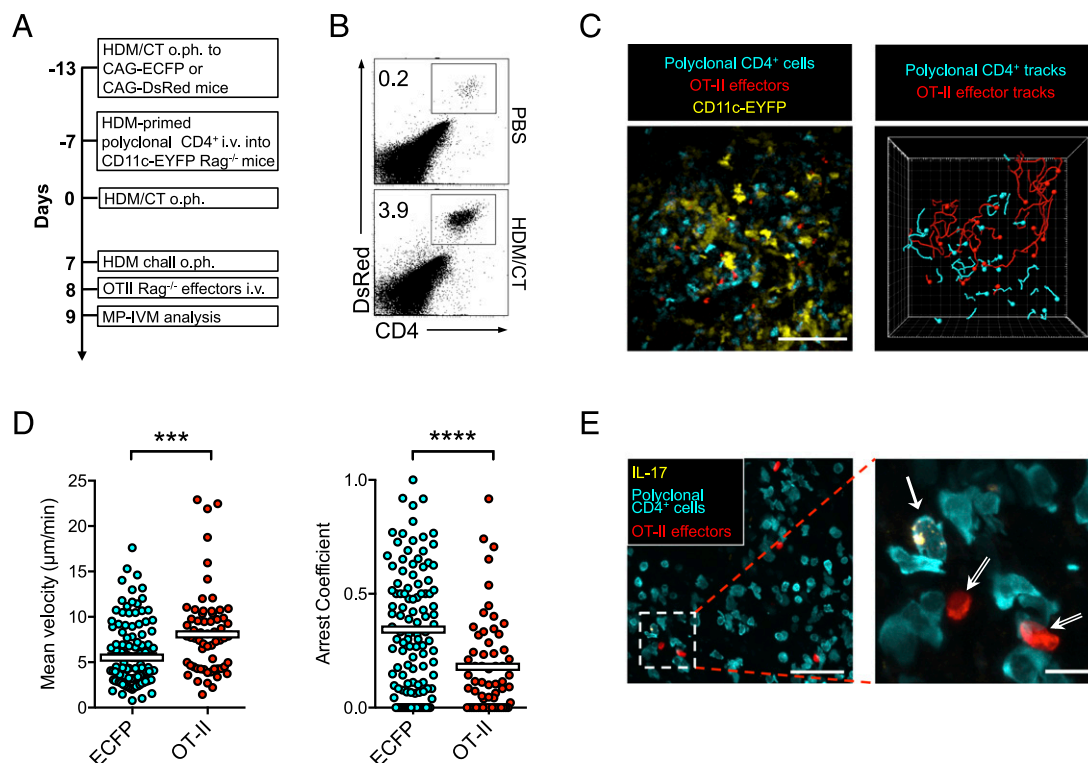


FIGURE 6. Polyclonal HDM-primed T cells, but not OT-II cells, arrest on DCs and produce effector cytokine. **(A)** Adoptive transfer and sensitization protocol. **(B)** Flow cytometric analysis of tracheal tissue digests to compare the tracheal polyclonal CD4⁺ response in HDM/CT- sensitized versus control (PBS-treated) mice. Analysis of recipient mice was performed at day 8 postsensitization. Numbers indicate percentages. **(C)** Snapshot from Supplemental Video 6 (left panel; scale bar, 100 μm) and representation of corresponding cell motility tracks (right panel). **(D)** Quantitative comparison of mean velocity and arrest coefficient of HDM-primed polyclonal versus OT-II effector cells. Circles indicate individual cell tracks; horizontal bars show mean values. Data were pooled from the analysis of three mice, which were performed as three independent experiments. **(E)** Whole-mount IL-17 staining of tracheas collected after MP-IVM analysis. Boxed region in left panel (scale bar, 50 μm) is enlarged (right panel; scale bar, 10 μm) to indicate IL-17 production by polyclonal T cells (arrow) but not by OT-II cells (double arrows). Twenty IL-17⁺ cells were analyzed in the tracheas of two mice (from two independent experiments). ****p* < 0.001, *****p* < 0.0001, Mann–Whitney *U* test.

that did not receive polyclonal T cells, only very few dim p-STAT6⁺ cells were present, and these did not form clusters (Fig. 7B). A closer examination of DsRed⁺ effector clusters revealed individual bright p-STAT6⁺ locally responding cells within and around subepithelial T cell clusters (Fig. 7C).

Because our results suggested that the effect of CD4⁺ T cell cytokines is concentrated within the clusters they form, we next asked whether the T cell-dependent recruitment of eosinophils and neutrophils occurs in a local manner. We observed earlier that eosinophilia develops at later time points (typically days 8–12 after HDM/CT). To ensure the detection of small, separate clusters at these late time points, we initially injected a smaller number of CD4⁺ polyclonal effectors (1×10^5 per mouse instead of $0.5\text{--}1 \times 10^6$ that was used for analysis at early time points). In line with our expectations, eosinophil recruitment was locally restricted to these late effector clusters (Fig. 7D). We further verified this finding by injecting a bolus of fluorescently labeled eosinophils and visualizing their tracheal localization on the next day. We found that the injected cells also localized into early (day 3, Supplemental Fig. 5C) and late (day 9, Supplemental Fig. 5D, Supplemental Video 7) effector clusters.

In contrast to the local accumulation of eosinophils, neutrophils were distributed more evenly in the tracheal mucosa and did not show such prominent clustering with T cells (Fig. 7E, 7F). However, CD4⁺ effector clusters also contained a large number of neutrophils, which suggests that Th2 and Th17 cells participate in their formation.

Discussion

In asthmatics, allergic inflammation mainly develops within the mucosal layer of small-diameter bronchi. Upon repeated allergen challenge, the prolonged activation of Th2 and Th17 cells causes, via the release of effector cytokines, tissue eosinophilia and neutrophilia and ultimately leads to tissue damage, airway remodeling, and bronchial obstruction. To explore how this complex sequence of cellular activation and migration events is organized spatially and dynamically, we established an *in vivo* dynamic/*ex vivo* static imaging-based model of small-airway allergic disease. The key features of our disease-relevant approach include focusing on the mouse trachea, because of its similarities to small human bronchi (from which mouse small airways are very different) and characterizing a polyclonal (and not TCR-transgenic) T cell response to a clinically relevant allergen (HDM). These features represent an important advance and distinguish our work from prior studies exploring T cell–DC interactions in the peripheral lung/intrapulmonary airways of mice by using *ex vivo* slice preparations with TCR-transgenic OT-II cells in response to OVA (46).

The notion that effector CD4⁺ responses to inhaled Ag occur in discrete cellular foci was suggested earlier (47). Our results clearly demonstrate that the initial recruitment of effector T cells and the formation of clusters in the airway mucosa are accompanied by the local accumulation of Ag-presenting DCs (Fig. 4A). At later time points, we also observed the local recruitment of eosinophils and neutrophils, which complete the loop of a typical adaptive Th2/Th17 immune response. The formation of clusters

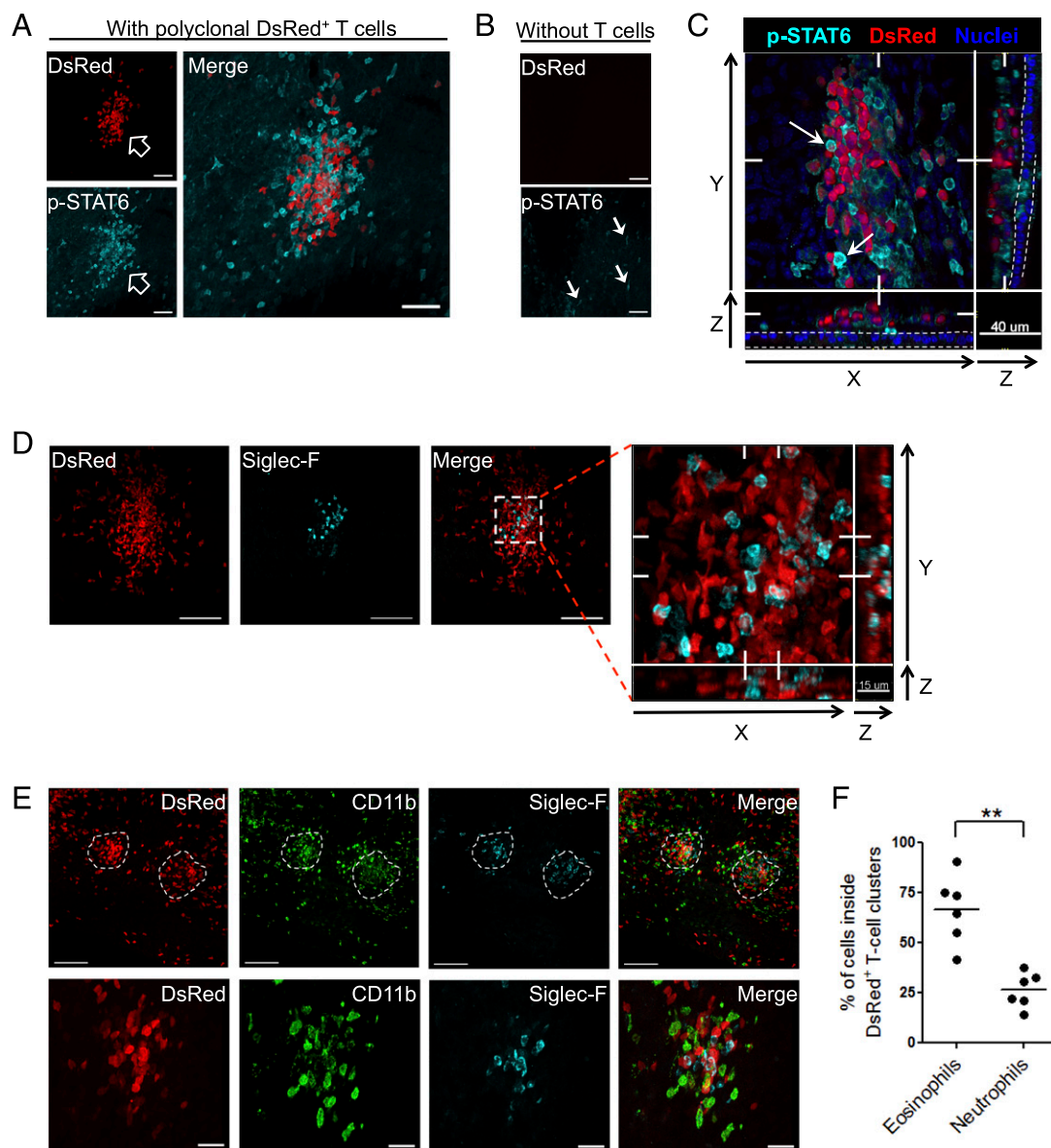


FIGURE 7. STAT6 phosphorylation and myeloid effector cell recruitment in the tracheal mucosa are associated with CD4⁺ T cell clusters. HDM-primed polyclonal CD4⁺ T cells, isolated from the bronchial lymph node of CAG-DsRed mice, were adoptively transferred into TCR $\alpha^{-/-}$ mice on day -7. At day 0, recipients received HDM/CT o.p.h., and perfusion-fixed tracheas were collected at different time points for whole-mount confocal analysis. (A-C) Analysis of p-STAT6 distribution at day 3. (A) Colocalization of a p-STAT6⁺ cell cluster with a cluster of DsRed⁺ polyclonal effector T cells (arrows indicate the clusters). (B) Lack of bright p-STAT6⁺ cells in mice without transferred T cells (arrows indicate a few dim p-STAT6⁺ cells). Images in (A) and (B) were acquired using the same laser intensity and detector settings (scale bars, 50 μ m). (C) XY, XZ, and YZ representations of a subepithelial effector cluster (slice positions are indicated by tick marks). The tracheal epithelium is shown between the dashed lines. Arrows show bright p-STAT6⁺ cells. DsRed⁺ p-STAT6⁺ cells were excluded from the final images. (D-F) Mice were challenged on day 7, and perfusion-fixed tracheas were collected for whole-mount confocal analysis of eosinophil and neutrophil accumulation on day 9. (D) Localization of Siglec-F⁺ eosinophils in a DsRed⁺ CD4⁺ effector cluster. Boxed region is enlarged (far right panel) and shown as XY, XZ, and YZ representations (a range of slices is shown, as indicated by tick marks). Scale bars, 100 μ m. (E) Lower-magnification images of two DsRed⁺ CD4⁺ effector clusters (dashed lines) showing the partial or complete colocalization of CD11b⁺ neutrophils and Siglec-F⁺ eosinophils, respectively (upper panels; scale bars, 100 μ m). Higher-magnification images of a DsRed⁺ CD4⁺ effector cluster showing eosinophils and neutrophils within the clusters (lower panels; scale bars, 20 μ m). (F) Quantitation of eosinophil or neutrophil localization within T cell clusters. Six clusters ($n = 6$) were analyzed in three tracheas. All images are representative of at least two independent experiments. ** $p < 0.01$, Mann-Whitney U test.

that contain all key cellular players of an allergic response might represent a general feature of the immune system that has evolved to localize the cellular response for the containment of helminths and parasites. Similarly, Th1 responses may also develop in the form of discrete cellular clusters, as observed, for example, in models of mycobacterial infection (granuloma formation) (17, 18).

The relatively low (1–2%) in vivo frequency of cytokine-positive T cells (as detected by immunohistology and flow cytometry)

reflects the rate at which effector T cells normally become activated in the course of a polyclonal effector CD4⁺ Th response. This is in apparent contrast to values that have been commonly measured in Th cells ex vivo, typically after restimulation with P/I, anti-CD3, or Ag (frequencies up to 30–40%). Although the fraction of cytokine-positive cells increased after secondary Ag administration in vivo and in vitro, it remained clearly below those values detected following P/I. This could be explained by the asynchronous activation of polyclonal effector T cells in vivo,

which is subject to the availability of specific Ag, the previously described process of rapid desensitization following activation (19), and other tissue factors yet to be explored.

In contrast, Ag nonspecific stimuli (e.g., with P/I) induce a synchronized activation of all cells, which does not necessarily reflect the rate at which this happens *in vivo*. The importance of such direct *in situ/ex vivo* analyses of intracellular cytokine content becomes obvious, especially in the accurate identification of the major cellular source of effector cytokines. It was reported that ILC2s represent a major source of IL-13–producing cells in an HDM-induced allergic response, as measured after P/I stimulation (37); however, their contribution to IL-13 production in our model was minimal. This was also supported by the finding that eosinophilia developed in a T cell–dependent manner, as observed in this study, as well as by other investigators (48).

Our finding that CD4⁺ T cells are the major source of local cytokine production, with only a minor involvement of ILC2s, differs slightly from previous observations made in a papain model of asthma (49, 50) or another study using repetitive HDM exposure (51), both of which suggest an important role for ILC2s in terms of cytokine production and the initiation of a Th2-type adaptive response. In this respect, our study might not accurately model how HDM normally induces initial sensitization (i.e., via the epithelial induction of IL-33, IL-25, or TSLP, which induces ILC2 cytokine production and promotes adaptive Th2 responses). Instead, we might bypass this epithelium–ILC2 pathway by the use of CT (as discussed later) and also induce a strong Th17 (and Th1) adaptive response, for which ILC2s are dispensable (51). Due to this limitation, our study is not directly comparable with previous work on how protease allergens elicit an allergic response; instead, we provide an alternative imaging-based approach to visualize the effector function of polyclonal mixed Th2/Th17 CD4⁺ T cells in the complex airway mucosal tissue in response to HDM challenge, using a protocol that was developed and adjusted for our specific imaging purposes.

The vast majority of cytokine⁺ T cells were engaged in direct cell–cell contact with MHC-II⁺ cells, which suggests that cytokine gene activation and cytokine production in effector T cells are triggered by the recognition of specific Ag presented by local DCs, mainly of the CD103[−]CD11b⁺ subtype. This notion is also supported by our *in vivo* imaging experiments showing prolonged interactions between HDM-primed polyclonal T cells with DCs, whereas control OVA-specific OT-II effector cells that were initially primed under the same conditions as the polyclonal effectors failed to form stable interactions with DCs or produce cytokine. In light of the previous observations made in liver (18) and skin (19) models of Th1 effector responses, our results suggest that the requirement of specific Ag recognition and prolonged interaction with Ag-presenting DCs for local cytokine production also applies to polyclonal Th2/Th17 responses of the airway mucosa. After dissociation from DCs, increasing T cell motility correlates with an immediate decrease in cytokine production (19). We also observed a very low number of cytokine⁺ T cells without direct contact with DCs; however, these were always within a few tens of micrometers from a neighboring MHC-II⁺ cell (data not shown), suggesting that, for a limited time period, T cells are capable of producing cytokines even after dissociating from a DC.

Lately, it has been a matter of intense debate whether effector cytokines are secreted locally or are widely distributed in complex tissues. Within lymph nodes, locally produced Th1 and Th2 cytokines were reported to reach the majority of cells (52). However, in peripheral tissues, cytokine effects might be more locally restricted. Previous studies showing an inverse correlation between motility and cytokine production (18, 19) clearly favor the model

according to which the radius of action for T cell cytokines is limited to ~80–100 μ m (14, 53), thereby reaching only neighboring cells. Our model did not allow an exact measurement of the distances at which locally released Th2 cytokines exert their effect (detected as STAT-6 phosphorylation), because there were always a few scattered CD4⁺ cells, as well as p-STAT6⁺ cells, present in areas between the prominent clusters. However, local accumulations of p-STAT6⁺ cells perfectly overlapped effector clusters, suggesting that the locally restricted model of cytokine effects also applies to airway mucosal Th2 responses.

IL-13 is a key mediator of allergic asthma, and its effects through the IL-13/IL-4/STAT-6 signaling pathway have been well characterized (54). One of the main functions of IL-13 is the induction of tissue eosinophilia via induction of eotaxin production, which promotes the recruitment of eosinophils from the bloodstream (55). In our model, eosinophil accumulation followed the pattern of STAT-6 phosphorylation (i.e., the initial clusters of newly arriving eosinophils preferentially localized to CD4⁺ effector clusters). This finding suggests that the effector arm of the allergic response is concentrated around the site of initial Ag encounter and T cell activation.

It is important to note that CT was extremely useful as an adjuvant in our imaging analyses; it induced a robust primary Th2/Th17 CD4⁺ (but not CD8⁺) response to HDM, revealing the kinetics of cluster formation and allowing the side-by-side analysis of eosinophil and neutrophil positioning in the tracheal mucosa, which occurred in a T cell–dependent manner. However, CT is known to engage relatively unique pathways (56), potentially activate CD11b⁺ DCs (36), and skew the immune response to HDM toward Th17 cells (57). Although we confirmed some of the main findings (e.g., T cell–DC cluster formation with local eosinophil recruitment and low-frequency cytokine production by T cells while in contact with DCs) also using repetitive HDM administration without CT (Supplemental Fig. 3), both models reflect changes during a very acute primary or secondary response to allergen. However, because asthma is a chronic disorder, it would be useful to focus future imaging studies on the chronic allergic response involving airway remodeling induced by long-term repetitive allergen administration. Our combined approach of *in vivo* tracheal imaging and *in situ* staining for signaling events provides an ideal platform for such future studies as well.

In conclusion, our results provide a deeper insight into the spatial and functional organization of an airway mucosal Th2/Th17 allergic response. Key features of this response are the formation of discrete cellular clusters, Ag-specific T cell–DC interactions within these clusters leading to local T cell activation and cytokine production, and the resulting recruitment of myeloid effector cells into these clusters, possibly in response to local cytokine effects.

These observations also imply that the episodic formation of inflammatory cell clusters might represent a mechanism by which acute inflammatory foci produce diffuse bronchial disease over time, which could be equivalent to the allergen-induced acute exacerbations of chronic inflammation, as observed in clinical settings. This underlines the importance of the early detection and appropriate specific therapeutic targeting of acute inflammatory episodes to preclude the development of chronic airway disease.

Acknowledgments

We thank H. F. Rosenberg for helpful discussions on eosinophil biology, T. R. Mempel for advice on MP-IVM, M. Pohjansalo for technical assistance, and I. Iagar and P. Saarenmaa for building custom-made imaging equipment.

Disclosures

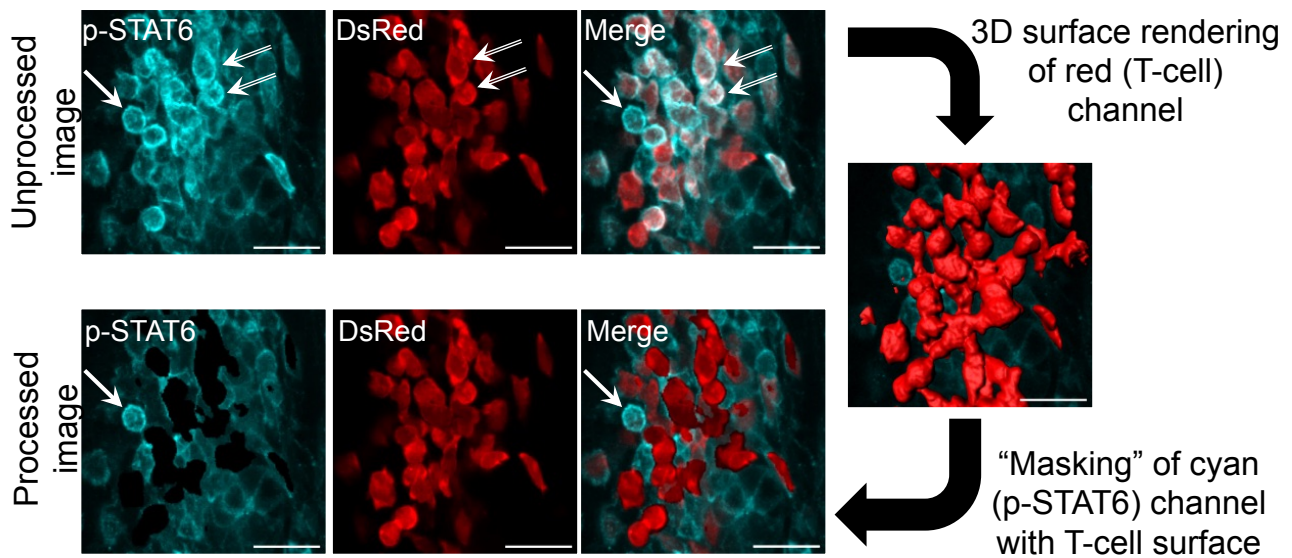
The authors have no financial conflicts of interest.

References

- Lloyd, C. M., and E. M. Hessel. 2010. Functions of T cells in asthma: more than just TH2 cells. *Nat. Rev. Immunol.* 10: 838–848.
- Licon-Limón, P., L. K. Kim, N. W. Palm, and R. A. Flavell. 2013. TH2, allergy and group 2 innate lymphoid cells. *Nat. Immunol.* 14: 536–542.
- Newcomb, D. C., and R. S. Peebles, Jr. 2013. Th17-mediated inflammation in asthma. *Curr. Opin. Immunol.* 25: 755–760.
- Islam, S. A., and A. D. Luster. 2012. T cell homing to epithelial barriers in allergic disease. *Nat. Med.* 18: 705–715.
- Coquet, J. M., M. J. Schuijs, M. J. Smyth, K. Deswarte, R. Beyaert, H. Braun, L. Boon, G. B. Karlsson Hedestam, S. L. Nutt, H. Hammad, and B. N. Lambrecht. 2015. Interleukin-21-producing CD4(+) T cells promote type 2 immunity to house dust mites. *Immunity* 43: 318–330.
- Schuijs, M. J., M. A. Willart, H. Hammad, and B. N. Lambrecht. 2013. Cytokine targets in airway inflammation. *Curr. Opin. Pharmacol.* 13: 351–361.
- Holgate, S. T. 2012. Innate and adaptive immune responses in asthma. *Nat. Med.* 18: 673–683.
- Verloop, M. C. 1949. On the arteriae bronchiales and their anastomosing with the arteria pulmonalis in some rodents; a micro-anatomical study. *Acta Anat. (Basel)* 7: 1–32.
- Mitzner, W., W. Lee, D. Georgakopoulos, and E. Wagner. 2000. Angiogenesis in the mouse lung. *Am. J. Pathol.* 157: 93–101.
- Germain, R. N., E. A. Robey, and M. D. Cahalan. 2012. A decade of imaging cellular motility and interaction dynamics in the immune system. *Science* 336: 1676–1681.
- Gerner, M. Y., W. Kastanmüller, I. Ifrim, J. Kabat, and R. N. Germain. 2012. Histo-cytometry: a method for highly multiplex quantitative tissue imaging analysis applied to dendritic cell subset microanatomy in lymph nodes. *Immunity* 37: 364–376.
- Gerner, M. Y., P. Torabi-Parizi, and R. N. Germain. 2015. Strategically localized dendritic cells promote rapid T cell responses to lymph-borne particulate antigens. *Immunity* 42: 172–185.
- Marangoni, F., T. T. Murooka, T. Manzo, E. Y. Kim, E. Carrizosa, N. M. Elpek, and T. R. Mempel. 2013. The transcription factor NFAT exhibits signal memory during serial T cell interactions with antigen-presenting cells. *Immunity* 38: 237–249.
- Müller, A. J., O. Filipe-Santos, G. Eberl, T. Aebischer, G. F. Späth, and P. Bousso. 2012. CD4+ T cells rely on a cytokine gradient to control intracellular pathogens beyond sites of antigen presentation. *Immunity* 37: 147–157.
- Bousso, P., and H. D. Moreau. 2012. Functional immunomaging: the revolution continues. *Nat. Rev. Immunol.* 12: 858–864.
- Moreau, H. D., F. Lemaître, E. Terriac, G. Azar, M. Piel, A.-M. Lennon-Dumenil, and P. Bousso. 2012. Dynamic in situ cytometry uncovers T cell receptor signaling during immunological synapses and kinapses in vivo. *Immunity* 37: 351–363.
- Egen, J. G., A. G. Rothfuchs, C. G. Feng, N. Winter, A. Sher, and R. N. Germain. 2008. Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. *Immunity* 28: 271–284.
- Egen, J. G., A. G. Rothfuchs, C. G. Feng, M. A. Horwitz, A. Sher, and R. N. Germain. 2011. Intravital imaging reveals limited antigen presentation and T cell effector function in mycobacterial granulomas. *Immunity* 34: 807–819.
- Honda, T., J. G. Egen, T. Lämmermann, W. Kastanmüller, P. Torabi-Parizi, and R. N. Germain. 2014. Tuning of antigen sensitivity by T cell receptor-dependent negative feedback controls T cell effector function in inflamed tissues. *Immunity* 40: 235–247.
- Kim, J. K., V. Vinarsky, J. Wain, R. Zhao, K. Jung, J. Choi, A. Lam, A. Pardo-Saganta, S. Breton, J. Rajagopal, and S. H. Yun. 2012. In vivo imaging of tracheal epithelial cells in mice during airway regeneration. *Am. J. Respir. Cell Mol. Biol.* 47: 864–868.
- Lindquist, R. L., G. Shakh, D. Dudziak, H. Wardemann, T. Eisenreich, M. L. Dustin, and M. C. Nussenzweig. 2004. Visualizing dendritic cell networks in vivo. *Nat. Immunol.* 5: 1243–1250.
- Lee, N. A., M. P. McGarry, K. A. Larson, M. A. Horton, A. B. Kristensen, and J. J. Lee. 1997. Expression of IL-5 in thymocytes/T cells leads to the development of a massive eosinophilia, extramedullary eosinophilopoiesis, and unique histopathologies. *J. Immunol.* 158: 1332–1344.
- Junttila, I. S., C. Watson, L. Kummola, X. Chen, J. Hu-Li, L. Guo, R. Yagi, and W. E. Paul. 2013. Efficient cytokine-induced IL-13 production by mast cells requires both IL-33 and IL-3. *J. Allergy Clin. Immunol.* 132: 704–712.e10.
- Hammad, H., M. Plantinga, K. Deswarte, P. Pouliot, M. A. M. Willart, M. Kool, F. Muskens, and B. N. Lambrecht. 2010. Inflammatory dendritic cells—not basophils—are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J. Exp. Med.* 207: 2097–2111.
- Nakano, H., M. E. Free, G. S. Whitehead, S. Maruoka, R. H. Wilson, K. Nakano, and D. N. Cook. 2012. Pulmonary CD103(+) dendritic cells prime Th2 responses to inhaled allergens. *Mucosal Immunol.* 5: 53–65.
- Foster, B., C. Prussin, F. Liu, J. K. Whitmore, and J. L. Whitton. 2007. Detection of intracellular cytokines by flow cytometry. *Curr. Protoc. Immunol.* Chapter 6: Unit 6.24.
- Veres, T. Z., S. Rochlitz, M. Shevchenko, B. Fuchs, F. Prenzler, C. Nassenstein, A. Fischer, L. Welker, O. Holz, M. Müller, et al. 2007. Spatial interactions between dendritic cells and sensory nerves in allergic airway inflammation. *Am. J. Respir. Cell Mol. Biol.* 37: 553–561.
- Rock, J. R., S. H. Randell, and B. L. M. Hogan. 2010. Airway basal stem cells: a perspective on their roles in epithelial homeostasis and remodeling. *Dis. Model. Mech.* 3: 545–556.
- Bose, O., P. Baluk, M. R. Looney, L. E. Cheng, D. M. McDonald, G. H. Caughey, and M. F. Krummel. 2015. Mast cells present protrusions into blood vessels upon tracheal allergen challenge in mice. *PLoS One* 10: e0118513.
- Veres, T. Z., S. Voedisch, E. Spies, T. Tschernig, and A. Braun. 2011. Spatio-temporal and functional behavior of airway dendritic cells visualized by two-photon microscopy. [Published erratum appears in 2011 *Am. J. Pathol.* 179: 2674]. *Am. J. Pathol.* 179: 603–609.
- Reed, C. E., and H. Kita. 2004. The role of protease activation of inflammation in allergic respiratory diseases. *J. Allergy Clin. Immunol.* 114: 997–1008, quiz 1009.
- Trompette, A., S. Divanovic, A. Visintin, C. Blanchard, R. S. Hegde, R. Madan, P. S. Thorne, M. Wills-Karp, T. L. Gioannini, J. P. Weiss, and C. L. Karp. 2009. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* 457: 585–588.
- Calderón, M. A., J. Kleine-Tebbe, A. Linneberg, F. De Blay, D. Hernandez Fernandez de Rojas, J. C. Virchow, and P. Demoly. 2015. House dust mite respiratory allergy: an overview of current therapeutic strategies. *J. Allergy Clin. Immunol. Pract.* 3: 843–855.
- Birrell, M. A., A. J. Van Oosterhout, and M. G. Belvisi. 2010. Do the current house dust mite-driven models really mimic allergic asthma? *Eur. Respir. J.* 36: 1220–1221.
- Wikstrom, M. E., E. Batanero, M. Smith, J. A. Thomas, C. von Garnier, P. G. Holt, and P. A. Stumbles. 2006. Influence of mucosal adjuvants on antigen passage and CD4+ T cell activation during the primary response to airborne allergen. *J. Immunol.* 177: 913–924.
- Mattsson, J., K. Schön, L. Ekman, L. Fahlén-Yrild, U. Yrild, and N. Y. Lycke. 2015. Cholera toxin adjuvant promotes a balanced Th1/Th2/Th17 response independently of IL-12 and IL-17 by acting on Gα_i in CD11b⁺ DCs. *Mucosal Immunol.* 8: 815–827.
- Klein Wolterink, R. G., A. Kleinjan, M. van Nimwegen, I. Bergen, M. de Bruijn, Y. Levani, and R. W. Hendriks. 2012. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *Eur. J. Immunol.* 42: 1106–1116.
- Akbari, O., P. Stock, E. Meyer, M. Kronenberg, S. Sidobre, T. Nakayama, M. Taniguchi, M. J. Grusby, R. H. DeKruyff, and D. T. Umetsu. 2003. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat. Med.* 9: 582–588.
- Murdoch, J. R., and C. M. Lloyd. 2010. Resolution of allergic airway inflammation and airway hyperreactivity is mediated by IL-17-producing gamma delta T cells. *Am. J. Respir. Crit. Care Med.* 182: 464–476.
- Motomura, Y., H. Kitamura, A. Hijikata, Y. Matsunaga, K. Matsumoto, H. Inoue, K. Atarashi, S. Hori, H. Watarai, J. Zhu, et al. 2011. The transcription factor E4BP4 regulates the production of IL-10 and IL-13 in CD4+ T cells. *Nat. Immunol.* 12: 450–459.
- Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley, and B. Stockinger. 2006. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24: 179–189.
- Torabi-Parizi, P., N. Vrisekoop, W. Kastanmüller, M. Y. Gerner, J. G. Egen, and R. N. Germain. 2014. Pathogen-related differences in the abundance of presented antigen are reflected in CD4+ T cell dynamic behavior and effector function in the lung. *J. Immunol.* 192: 1651–1660.
- Ginhoux, F., K. Liu, J. Helft, M. Bogunovic, M. Greter, D. Hashimoto, J. Price, N. Yin, J. Bromberg, S. A. Lira, et al. 2009. The origin and development of nonlymphoid tissue CD103+ DCs. *J. Exp. Med.* 206: 3115–3130.
- Plantinga, M., M. Guillems, M. Vanheerswynghe, K. Deswarte, F. Branco-Madeira, W. Toussaint, L. Vanhoutte, K. Neyt, N. Killeen, B. Malissen, et al. 2013. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity* 38: 322–335.
- Takeda, K., M. Kamanaka, T. Tanaka, T. Kishimoto, and S. Akira. 1996. Impaired IL-13-mediated functions of macrophages in STAT6-deficient mice. *J. Immunol.* 157: 3220–3222.
- Thornton, E. E., M. R. Looney, O. Bose, D. Sen, D. Sheppard, R. Locksley, X. Huang, and M. F. Krummel. 2012. Spatiotemporally separated antigen uptake by alveolar dendritic cells and airway presentation to T cells in the lung. *J. Exp. Med.* 209: 1183–1199.
- Hasegawa, A., K. Hayashi, H. Kishimoto, M. Yang, S. Tofukuji, K. Suzuki, H. Nakajima, R. M. Hoffman, M. Shirai, and T. Nakayama. 2010. Color-coded real-time cellular imaging of lung T-lymphocyte accumulation and focus formation in a mouse asthma model. *J. Allergy Clin. Immunol.* 125: 461–468.e6.
- Hondowicz, B. D., D. An, J. M. Schenkel, K. S. Kim, H. R. Steach, A. T. Krishnamurthy, G. J. Keitany, E. N. Garza, K. A. Fraser, J. R. Moon, et al. 2016. Interleukin-2-dependent allergen-specific tissue-resident memory cells drive asthma. *Immunity* 44: 155–166.
- Halim, T. Y. F., R. H. Krauss, A. C. Sun, and F. Takei. 2012. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* 36: 451–463.
- Halim, T. Y., C. A. Steer, L. Mathä, M. J. Gold, I. Martinez-Gonzalez, K. M. McNagny, A. N. McKenzie, and F. Takei. 2014. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 40: 425–435.
- Gold, M. J., F. Antignano, T. Y. Halim, J. A. Hirota, M.-R. Blanchet, C. Zaph, F. Takei, and K. M. McNagny. 2014. Group 2 innate lymphoid cells facilitate sensitization to local, but not systemic, TH2-inducing allergen exposures. *J. Allergy Clin. Immunol.* 133: 1142–1148.

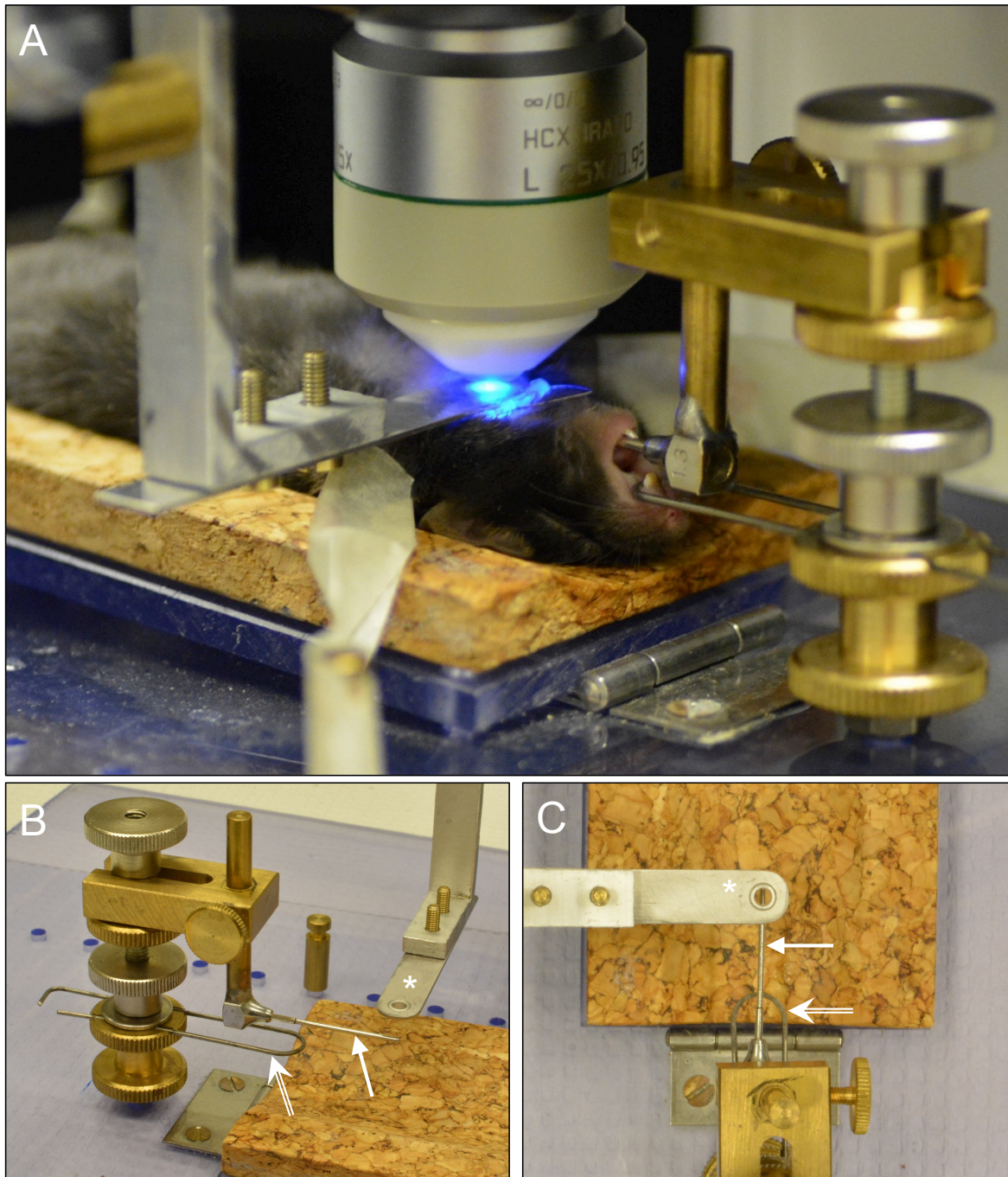
52. Perona-Wright, G., K. Mohrs, and M. Mohrs. 2010. Sustained signaling by canonical helper T cell cytokines throughout the reactive lymph node. *Nat. Immunol.* 11: 520–526.
53. Liu, Z., M. Y. Gerner, N. Van Panhuys, A. G. Levine, A. Y. Rudensky, and R. N. Germain. 2015. Immune homeostasis enforced by co-localized effector and regulatory T cells. *Nature* 528: 225–230.
54. Ingram, J. L., and M. Kraft. 2012. IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. *J. Allergy Clin. Immunol.* 130: 829–842 quiz 843–844.
55. Li, L., Y. Xia, A. Nguyen, Y. H. Lai, L. Feng, T. R. Mosmann, and D. Lo. 1999. Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *J. Immunol.* 162: 2477–2487.
56. Olvera-Gomez, I., S. E. Hamilton, Z. Xiao, C. P. Guimaraes, H. L. Ploegh, K. A. Hogquist, L. Wang, and S. C. Jameson. 2012. Cholera toxin activates nonconventional adjuvant pathways that induce protective CD8 T-cell responses after epicutaneous vaccination. *Proc. Natl. Acad. Sci. USA* 109: 2072–2077.
57. Datta, S. K., M. Sabet, K. P. Nguyen, P. A. Valdez, J. M. Gonzalez-Navajas, S. Islam, I. Mihajlov, J. Fierer, P. A. Insel, N. J. Webster, et al. 2010. Mucosal adjuvant activity of cholera toxin requires Th17 cells and protects against inhalation anthrax. *Proc. Natl. Acad. Sci. USA* 107: 10638–10643.

Supplemental Figure 1



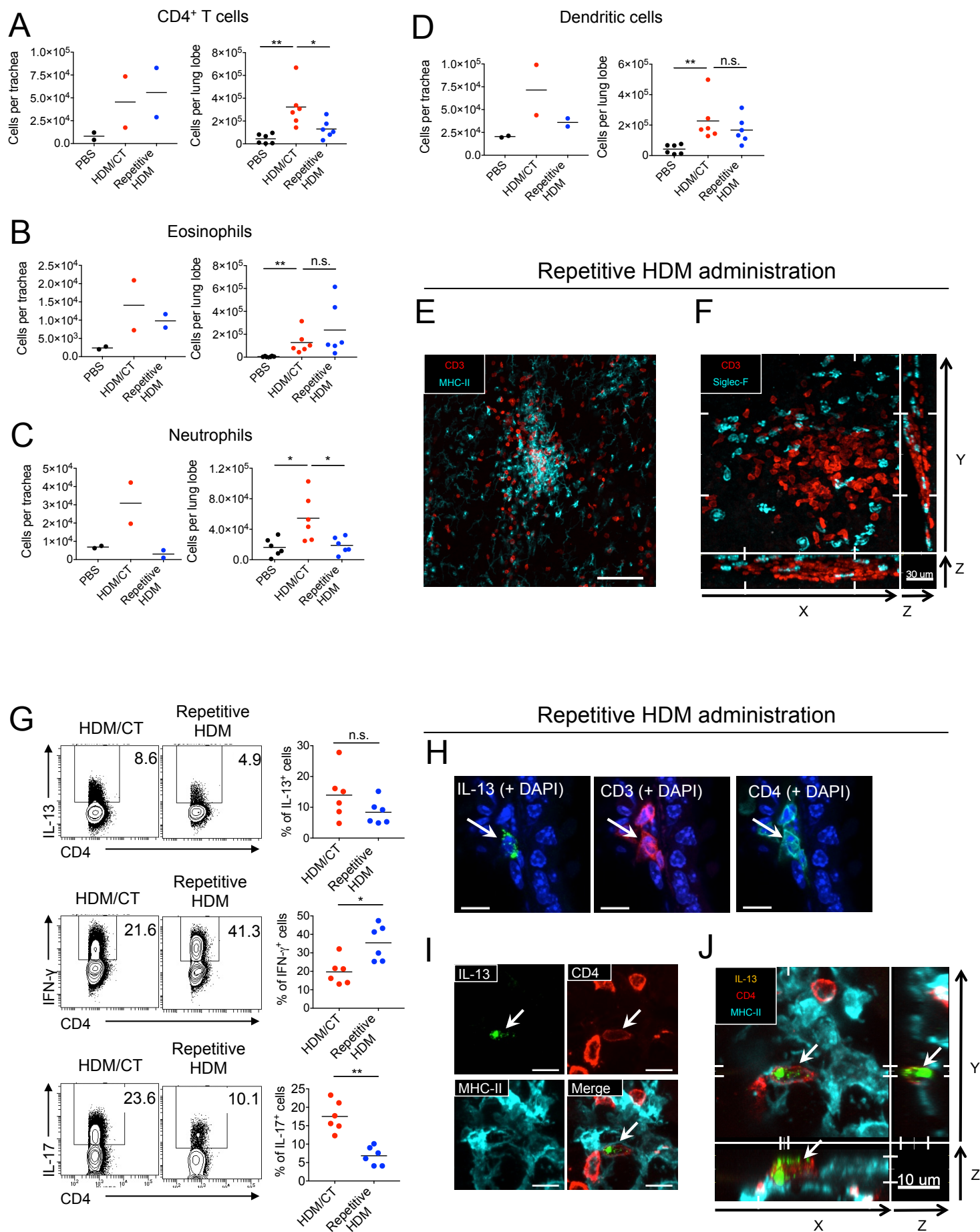
Supplemental Figure 1: Image processing steps applied to confocal microscopic data involving p-STAT6 stainings. Raw confocal Z-stacks with p-STAT6⁺ single-positive (arrows) and p-STAT6⁺ DsRed⁺ double-positive cells (double arrows) were processed via “3D surface rendering” of the DsRed (T-cell) channel, using Imaris. Within these surfaces, intensity values of the cyan (p-STAT6) channel were set to zero using the “Mask Channel” function of Imaris. As a result, only p-STAT6⁺ DsRed⁻ cells appeared in the processed image (arrows). The same image before and after these processing steps is presented below (bar=20μm).

Supplemental Figure 2



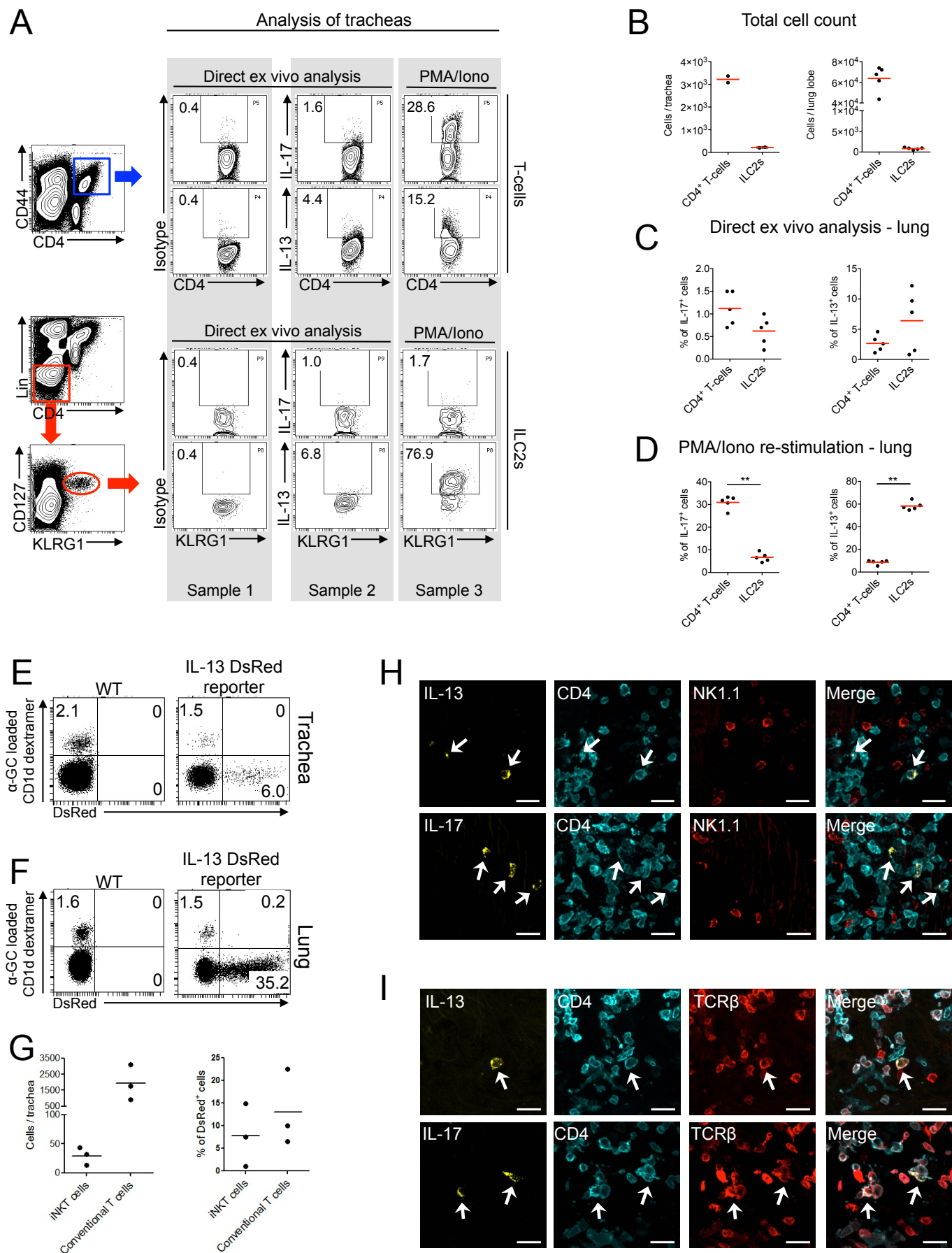
Supplemental Figure 2: Intravital microscopic setup for imaging the mouse trachea. (A) Snapshot of the complete experimental setup ready for MP-IVM analysis. (B, C) Key components of the custom-made stage as viewed from the side (B) or from above (C): imaging “window” (asterisk), intubation cannula (arrow) and wire loop for holding the teeth (double arrow).

Supplemental Figure 3



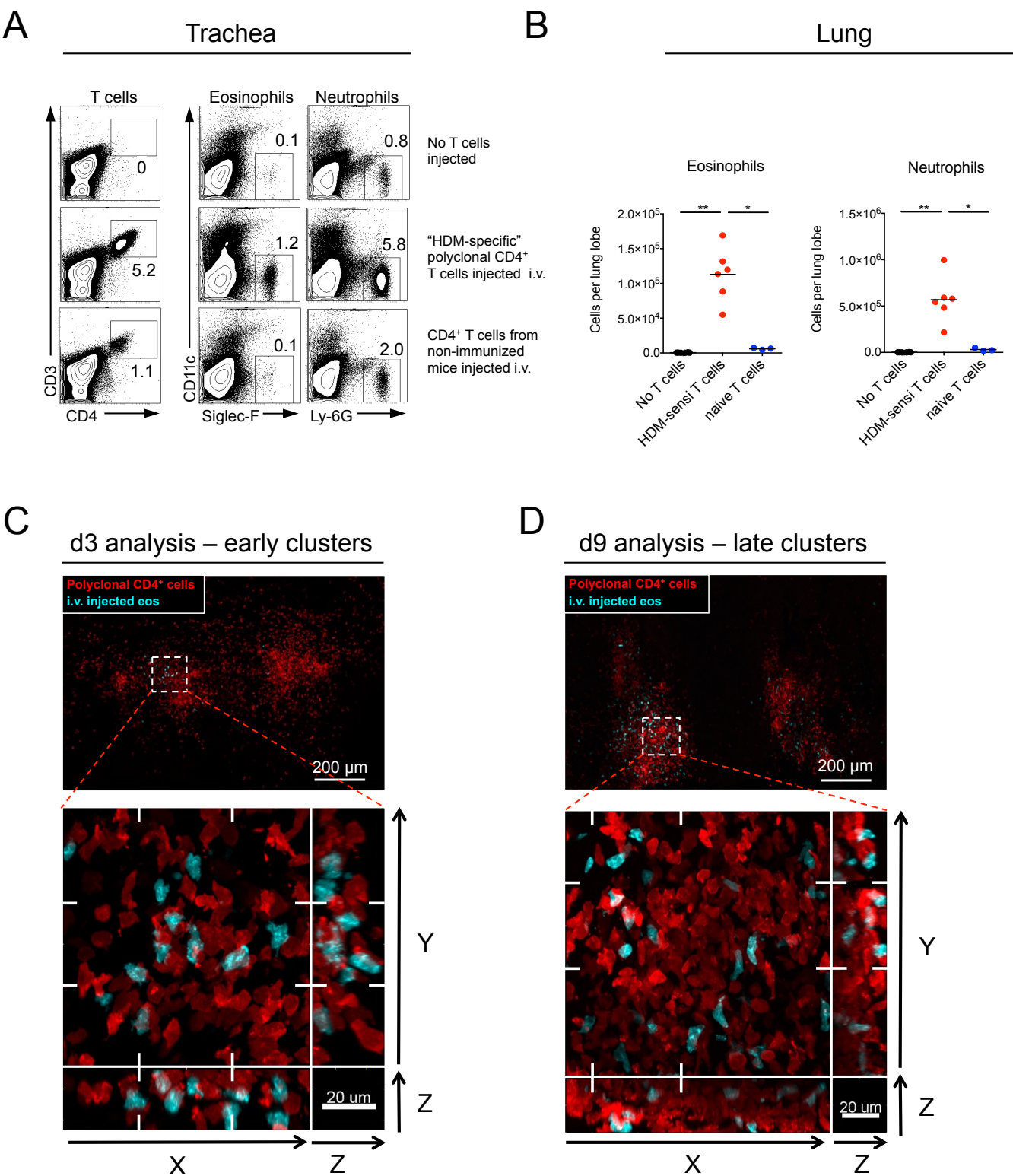
Supplemental Figure 3: Comparison of the new model involving HDM/CT-sensitization with a commonly used model based on adjuvant-free HDM sensitization and repetitive challenge. C57Bl/6 mice were sensitized with HDM/CT on d0 and analyzed on d8 (“HDM-CT-model”) or sensitized with HDM on d0 and challenged daily with HDM on days 7-11, followed by analysis on d14 (“repetitive HDM-model”). Controls were repetitively treated with PBS, respectively. **(A-D)** comparison of cellular infiltration in the tracheas (3 tracheas pooled per measurement, n=2 measurements) and lungs (n=6 mice; *:p < 0.05; **:p < 0.01 as determined using Mann-Whitney test). **(E, F)** Co-clustering of T cells and DCs (left panel, bar=100 μ m) or T cells and eosinophils (right panel; XY, XZ and YZ representations, bar=30 μ m) in the tracheal mucosa of mice treated according to the “repetitive HDM” protocol. **(G)** Comparison of cytokine responses of CD4⁺ T cells from lung digests after PMA/Iono restimulation (n=6 mice, *:p < 0.05; **:p < 0.01 as determined using Mann-Whitney test). **(H)** Visualizing IL-13⁺ CD3⁺CD4⁺ T cells in the airway mucosa after repetitive HDM administration (arrow shows a cytokine-producing cell, bar=10 μ m). **(I, J)** IL-13⁺ T cells are engaged in contact with MHC-II⁺ DCs in the tracheal mucosa after repetitive HDM administration (**I**: Z-projections of individual channels, **J**: XY, XZ and YZ representations. Arrow shows an IL-13⁺ T cell in direct contact with a DC. Bar=10 μ m). Results were pooled from two independent experiments.

Supplemental Figure 4



Supplemental Figure 4: Characterization of cytokine-producing cell populations. (A, B, C, D) Comparison of CD4⁺ T-cell and ILC2 cytokine production in tracheal and lung tissue digests. C57Bl/6 mice were sensitized with HDM/CT on d0 and received HDM challenge on d7. Tracheas and lungs were analyzed on d9. (A) Identification of tracheal CD4⁺CD44^{hi} effector T cells and Lin⁻CD127⁺KLRG1⁺ ILC2s in the same sample and measurement of cytokine-producing cells directly ex vivo or after PMA/Ionomycin re-stimulation. Five tracheas were pooled for each measurement (isotype control vs. specific anti-cytokine staining was done using the same digest). (B) Total number of CD4⁺ T cells and ILCs per trachea (left plot) and in the right upper lung lobe (right plot). (C, D) Quantitation of cytokine production by CD4⁺ T cells vs. ILC2s in the lungs as measured directly ex vivo (C) or after PMA/Ionomycin re-stimulation (D). Results were pooled from two independent experiments (n=2 measurements for pooled tracheas, n=5 measurements for single lung lobes; **:p < 0.01 as determined using Mann-Whitney test). (E, F, G) Analysis of IL-13 production by CD1d-restricted iNKT cells vs. conventional T cells. Plots indicate comparison of tracheal (E) and lung (F) digests from WT vs. IL-13 DsRed reporter mice (gated on CD3⁺ cells, numbers indicate percentages). (G) Quantification of total cell numbers and percentages of reporter-positive cells. Each dot represents one measurement (3 pooled tracheas). (H, I): Characterization of prominent IL-13⁺ and IL-17⁺ CD4⁺ T cells in tracheal sections. Arrows indicate cytokine-positive cells (bar=20μm). Results of a single experiment are shown.

Supplemental Figure 5



Supplemental Figure 5: The T-cell dependent recruitment of eosinophils occurs within effector T-cell clusters. (A, B) CAG-DsRed mice were sensitized with HDM/CT o.p.h. and on d4, CD4⁺ T cells from the lung-draining LN were isolated and i.v. transferred into Rag^{-/-} mice. Control mice were injected with LN T cells from non-immunized mice or received no T cells. Recipient mice were sensitized one week later with HDM/CT. At d7 after HDM/CT sensitization, mice received another HDM challenge and digested lung tissue was analyzed on d9. **(A)** analysis of tracheal digests (3 tracheas pooled per analysis). Numbers indicate percentages. **(B)** analysis of lung digests (individual lung lobes were analyzed, n=3-6 mice per group; *:p < 0.05; **:p < 0.01 as determined using Mann-Whitney test). Results of a single experiment are shown.

(C, D) “HDM-specific” polyclonal CD4⁺ T cells were isolated from the BrLN of CAG-DsRed mice and 0.5 x 10⁶ cells (for d3 analysis) or 1 x 10⁵ cells (for d9 analysis) were adoptively transferred into Rag-1^{-/-} mice on d-7. At d0, recipients received HDM/CT. At d2 or d8, 5 x 10⁷ fluorescently labeled eosinophils were injected i.v. and one day later, perfusion-fixed tracheas were collected for whole-mount confocal analysis (on d3 or d9). Images show the localization of eosinophils into effector CD4⁺ cell clusters, formed at d3 **(C)** or at d9 **(D)**. Boxed regions in the low-magnification images (upper panels, bar=200 μm) have been enlarged (lower panels) and shown as XY, XZ and YZ images (a range of slices is shown, as indicated by tick marks). Two mice were analyzed at each time point. Results of a single experiment are shown.

Supplemental Video Legends

Supplemental Video 1: 3D-exploration of the two-photon microscopic dataset shown in Figure 1, D-F. An XY “ortho-slicer” shows sequential optical sections starting from the tracheal lumen, pausing in the epithelial layer (red) to show intraepithelial DCs (yellow) and progressing towards the outer surface of the trachea. Following a rotation of the dataset by 90°, an XZ “ortho-slicer” shows a sequential series of cross-sections. Grid spacing=50µm.

Supplemental Video 2: A novel two-photon intravital microscopic setup for the visualization of cellular dynamics in the tracheal mucosa. Probing behavior of DCs (yellow) in the trachea of a CD11c-EYFP mouse was visualized together with i.v. injected blood tracer (red) and SHG (cyan) indicating collagen-rich outer surface of the trachea. XY, XZ and YZ representations are shown. Time is shown as hh:mm:ss.

Supplemental Video 3: Visualization of blood flow in deep tracheal vessels. DCs (yellow) in the trachea of a CD11c-EYFP mouse were visualized together with i.v. injected blood tracer (red) and SHG (cyan) indicating collagen-rich outer surface of the trachea. XY, XZ and YZ representations are shown. Time is shown as hh:mm:ss.

Supplemental Video 4: Visualization of DC-T-cell interaction dynamics in the tracheal mucosa in vivo. Ubiquitously fluorescent CAG-ECFP mice were sensitized with HDM/CT on d-13. On d-7, lung-draining lymph nodes were collected, CD4⁺ T cells were isolated and injected into CD11c-EYFP Rag-1^{-/-} recipients. Recipients were sensitized on d0 with HDM/CT o.p.h. and challenged with HDM on d7. Two-photon imaging was performed on d9. Movie shows the dynamics of a cluster formed by DCs (yellow) and “HDM-specific” polyclonal CD4⁺ T cells (cyan). XY, XZ and YZ representations are shown. Time is shown as hh:mm:ss.

Supplemental Video 5: Visualization of DC-T cell interaction dynamics in the tracheal mucosa in vivo. The same experimental setup is shown as in **Supplemental Video 4**. Circles indicate two interacting DC-T cell conjugates. Time is shown as hh:mm:ss.

Supplemental Video 6: Comparison of polyclonal vs. control, non-antigen-specific T-cells. CAG-ECFP mice were sensitized with HDM/CT on d-13. On d-7, lung-draining lymph nodes were collected, CD4⁺ T cells were isolated and injected into CD11c-EYFP Rag-1^{-/-} recipients. Recipients were sensitized on d0 with HDM/CT o.ph. and challenged with HDM on d7. On day 8, animals were i.v. injected with fluorescently labeled OT-II Rag-1^{-/-} effector T cells. Two-photon imaging was performed on d9. Movie shows simultaneous visualization of “HDM-specific” polyclonal CD4⁺ T cells (cyan), OT-II Rag-1^{-/-} effector T cells (red) and DCs (green) in the tracheal mucosa. Time is shown as hh:mm:ss.

Supplemental Video 7: Visualization of eosinophil migration to CD4⁺ T-cell clusters. CAG-ECFP mice were sensitized with HDM/CT on d-13. On d-7, lung-draining lymph nodes were collected; CD4⁺ T cells were isolated and injected into TCR α ^{-/-} recipients. Recipients were sensitized on d0 with HDM/CT o.ph. and challenged with HDM on d7. Two-photon imaging was performed on d9. Just before imaging, fluorescently labeled eosinophils (isolated from NJ.1638 mice) were i.v. injected. Movie shows several eosinophils (red) rolling in blood vessels (visualized by FITC-dextran, green) and “HDM-specific” polyclonal CD4⁺ T cells (cyan) in the mucosal tissue. An extravasated eosinophil (arrow, then shown by circle) migrates towards a group of T cells. Time is shown as hh:mm:ss.