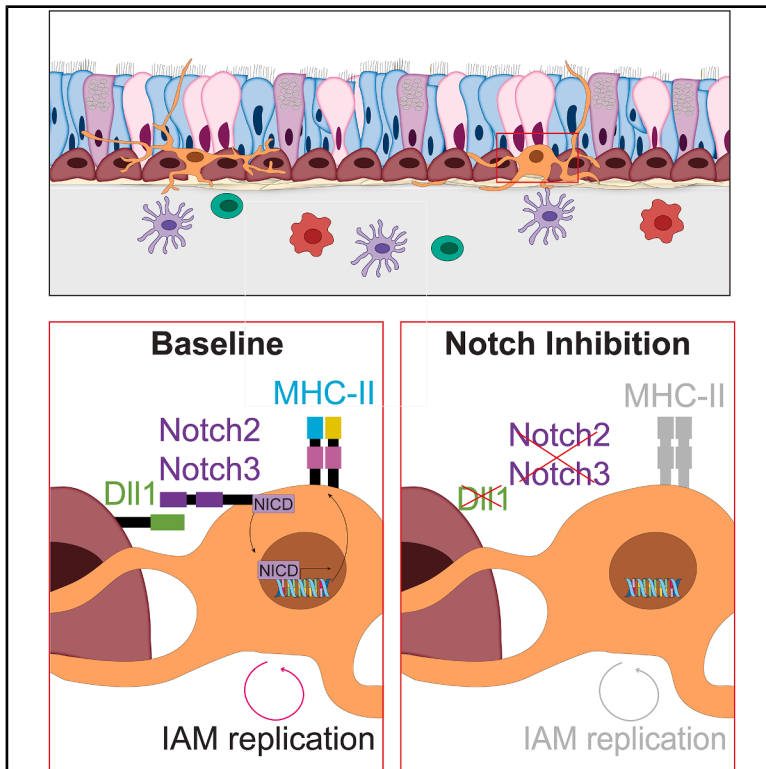


Airway basal stem cells are necessary for the maintenance of functional intraepithelial airway macrophages

Graphical abstract



Authors

Tristan Kooistra, Borja Saez, Marly Roche, ..., Jayaraj Rajagopal, Benjamin D. Medoff, Ana Pardo-Saganta

Correspondence

bmedoff@mgh.harvard.edu (B.D.M.), ana.pardo-saganta@innere.med.uni-giessen.de (A.P.-S.)

In brief

Kooistra et al. demonstrate that airway stem cells are necessary to maintain functional IAMs. Specifically, basal cell-derived Notch signaling is required for MHC II expression by IAMs. Indeed, impaired crosstalk results in loss of antigen-induced inflammation. As such, human IAM-like cells may play a role in pathologic airway inflammation.

Highlights

- Stem cell signaling regulates intraepithelial airway macrophage (IAM) function
- Basal cell-derived DII1 is required for IAM maturation
- IAMs are necessary for antigen-induced airway inflammation
- IAM-like cells are present in human conducting airways



Article

Airway basal stem cells are necessary for the maintenance of functional intraepithelial airway macrophages

Tristan Kooistra,^{1,2,15} Borja Saez,^{3,4,5,6,15} Marly Roche,^{1,2,15} Alejandro Egea-Zorrilla,^{5,6} Dongzhu Li,^{5,6} Dilanjan Anketell,^{1,2} Nhan Nguyen,^{1,2} Jorge Villoria,^{3,4} Jacob Gillis,^{1,2} Eva Petri,⁷ Laura Vera,⁷ Zuriñe Blasco-Iturri,^{5,6,7} Neal P. Smith,² Jehan Alladina,^{1,2} Yanting Zhang,⁸ Vladimir Vinarsky,^{3,4} Manjunatha Shivaraju,^{3,4} Susan L. Sheng,^{1,2} Michelle Chen,^{1,2} Meryem Gonzalez-Celeiro,^{3,4} Hongmei Mou,^{3,4,9} Avinash Waghray,^{3,4} Brian Lin,^{3,4,10} Azadeh Paksa,^{3,4} Kilangsungla Yanger,^{3,4} Purushotama Rao Tata,^{3,4,11} Rui Zhao,^{3,4} Benjamin Causton,^{1,2} Javier J. Zulueta,¹² Felipe Prosper,^{7,13} Josalyn L. Cho,¹⁴ Alexandra-Chloe Villani,² Adam Haber,⁸ Jayaraj Rajagopal,^{1,3,4,16} Benjamin D. Medoff,^{1,2,16,*} and Ana Pardo-Saganta^{1,3,4,5,6,16,17,*}

¹Division of Pulmonary and Critical Care Medicine, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA

²Center for Immunology and Inflammatory Diseases, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA 02129, USA

³Center for Regenerative Medicine, Massachusetts General Hospital, Boston, MA 02114, USA

⁴Harvard Stem Cell Institute, Harvard University, Cambridge, MA 02138, USA

⁵Institute for Lung Health (ILH), Department of Internal Medicine, Justus-Liebig University, Universities of Giessen and Marburg Lung Center (UGMLC), German Center for Lung Research (DZL), 35392 Giessen, Germany

⁶Cardio-Pulmonary Institute (CPI), Department of Internal Medicine, Justus Liebig University, 35392 Giessen, Germany

⁷Department of Regenerative Medicine, CIMA Universidad de Navarra, 31008 Pamplona, Spain

⁸Department of Environmental Health, Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA

⁹The Mucosal Immunology & Biology Research Center, Massachusetts General Hospital, Boston, MA 02114, USA

¹⁰Department of Developmental, Molecular and Chemical Biology, School of Medicine, Tufts University, Boston, MA, USA

¹¹Departments of Cell Biology and Medicine, Duke Regeneration Center, Duke University School of Medicine, Durham, NC 27710, USA

¹²Department of Pulmonology, Clínica Universidad de Navarra, 31008 Pamplona, Spain

¹³Department of Hematology and Cell Therapy, Clínica Universidad de Navarra, 31008 Pamplona, Spain

¹⁴Division of Pulmonary, Critical Care and Occupational Medicine, University of Iowa Carver College of Medicine, Iowa City, IA 52242, USA

¹⁵These authors contributed equally

¹⁶These authors contributed equally

¹⁷Lead contact

*Correspondence: bmedoff@mgh.harvard.edu (B.D.M.), ana.pardo-saganta@innere.med.uni-giessen.de (A.P.-S.)

<https://doi.org/10.1016/j.celrep.2025.115860>

SUMMARY

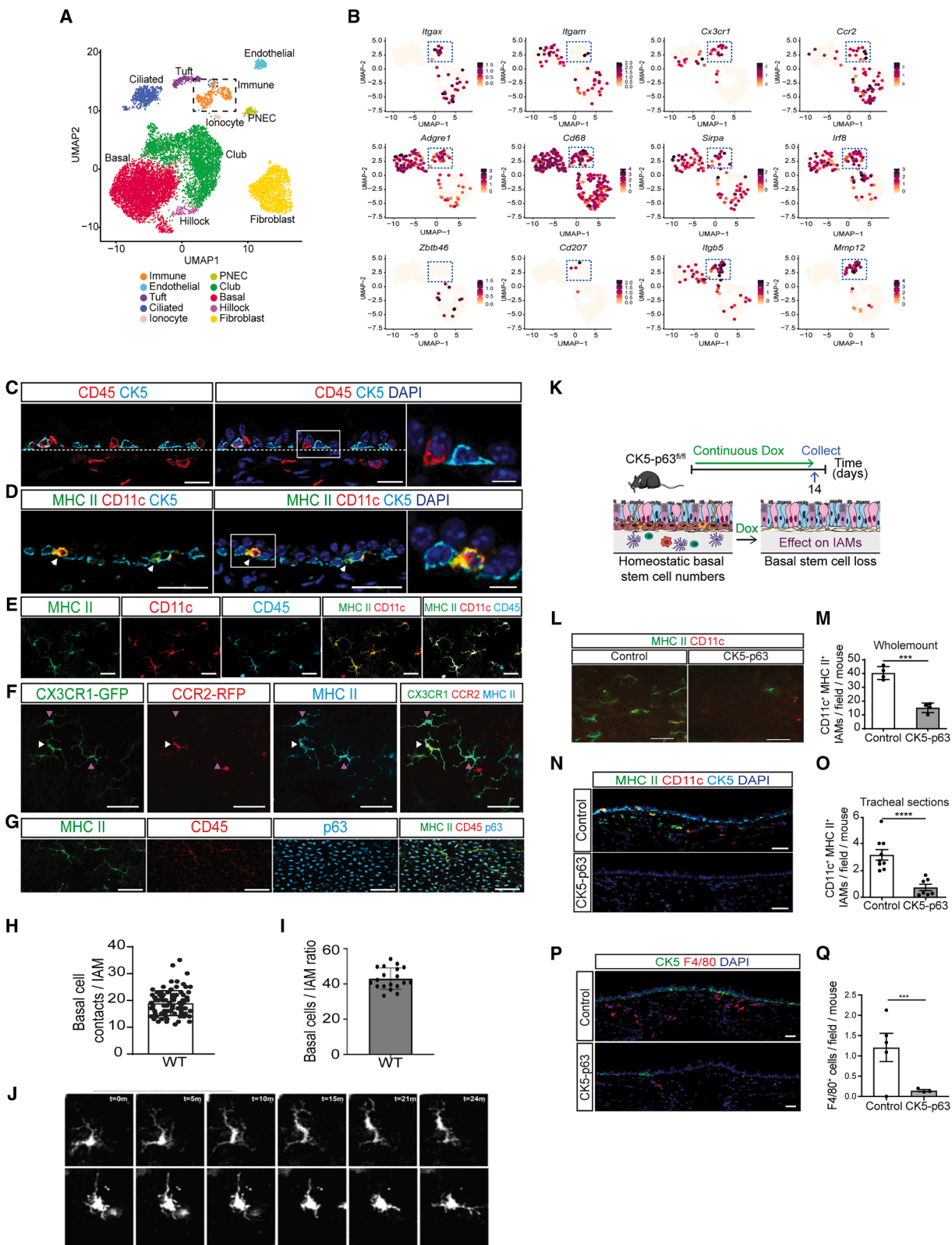
Stem cells are known to provide signals that contribute to the maintenance and function of neighboring cells. We demonstrate that Notch signaling arising from airway basal stem cells is necessary for the function of a unique population of intraepithelial airway macrophages (IAMs) in the murine trachea. Without this stem cell signaling, IAMs lose MHC II expression, which in turn prevents antigen-induced allergic inflammation. Distal murine airways do not harbor basal stem cells, and, in this region of the lung, allergic inflammation proceeds unperturbed. We speculate that the functional coupling of specific anatomically restricted stem cell populations and adjacent immune cells is one mechanism for ensuring that inflammatory responses are compartmentalized to regions of injury. Basal stem cells are found throughout the human airway tree and we demonstrate the existence of human IAM-like cells, suggesting that their interaction may influence airways disease.

INTRODUCTION

Airway epithelial basal stem cells are multipotent stem cells that are essential for the long-term maintenance of the airway epithelium.^{1–3} It has been demonstrated that basal cells signal to their own daughter cells by direct cell-to-cell contact through Notch signaling, effectively serving as niches for their own progeny.^{1,2} Basal cells are also known to mediate inflammation via the secretion of chemokines and cytokines.^{4–14}

The murine tracheal epithelium mirrors the corresponding epithelial cell conformation of human conducting airways.^{15,16} In particular, the murine trachea and proximal bronchi, unlike more distal mouse airways, contain the basal stem cells, which are abundant throughout the human conducting airways where airway pathology is often centered in diseases such as asthma.¹⁷ Thus, the murine trachea is well suited for the study of disease-relevant stem cell to immune cell communication in airway disorders such as asthma.





(legend on next page)

Communication between airway epithelial cells and airway myeloid cells, including dendritic cells (DCs) and macrophages, is critical for pulmonary immunity.^{4–10} Elucidating the biological basis of this communication has led to specific therapies that target epithelial cell-derived cytokines, such as thymic stromal lymphopoietin (TSLP) and interleukin (IL)-33 in asthma.^{18,19} However, we are only beginning to understand the heterogeneity of airway immune cells and the mechanisms that govern airway epithelial cell and immune cell interactions.^{20–25}

Myeloid cells within the airway are organized into a network composed of both intraepithelial and subepithelial compartments. Intraepithelial myeloid cells physically contact airway epithelial cells, and it is thought that they participate in immunity by directly surveilling the airway lumen.^{26,27} Recently, a unique population of intraepithelial airway macrophages (IAMs) has been identified in the trachea and proximal bronchi of mice.^{13,25} These cells are the predominant major histocompatibility complex (MHC) II⁺ myeloid cell population within the murine tracheal epithelium and promote airway epithelial repair following injury.^{13,14} In addition, there is evidence that airway macrophages can present inhaled antigens to airway-resident T cells or transfer antigens to DCs located in the subepithelial space.²⁵

Herein, we show that depletion of airway basal stem cells functionally disrupts the local network of IAMs and thereby alters antigen-driven inflammatory responses. Interestingly, the responses in the distal airways of the mouse, which lack basal stem cells, remain unaffected, suggesting that the stem cell effect is local. We show this compartmentalized response is explained by the necessity for contact-mediated signaling in which basal cell-derived Dll1 activates Notch receptors on IAMs. Furthermore, IAMs require tonic Notch signaling from basal cells to maintain their differentiated state. Notch blockade in IAMs not only decreases MHC II expression but is also associated with reduced allergic inflammation in the trachea.

RESULTS

A unique IAM population

A body of work has described a network of dendriform airway myeloid cells that previously were thought to be DCs.^{4–7,27} However, a recent study of the murine trachea identified a population of IAMs with dendriform morphology within the epithelium.¹³ We re-analyzed published single-cell datasets^{2,13} and employed immunohistochemistry to verify that intraepithelial MHC II⁺ dendriform cells are IAMs (Figures 1 and S1). IAMs are adjacent to CK5⁺ basal stem cells and express markers of conventional DC type 2 cells (cDC2), including CD11c (*Itgax*), MHC II, and signal regulatory protein α (*SIRP α*) (Figures 1A–1E and S1A–S1D). IAMs lack expression of the cDC transcription factor *Zbtb46* but do express *Irf8* (Figures 1B, S1A, and S1B). Additionally, IAMs express the macrophage-associated markers *Mafb*, *CX3CR1*, *F4/80* (*Adgre1*), *CD68*, *C1q*, and *C5ar1* (Figures 1B and S1A–S1F). Importantly, we confirm the previously reported expression of *Scimp* and *Tgfb β 1* in IAMs¹³ (Figure S1B) and validated the integrin *Itgb5* as a discriminatory IAM gene (Figures 1B, S1A, and S1G). Furthermore, lineage tracing of cDCs²⁸ using *Zbtb46-Cre*; *mTmG* reporter mice does not result in the labeling of IAMs. We only found rare cDCs within the epithelium at homeostasis, and the vast majority of intraepithelial MHC II⁺ cells were IAMs (Figure S1H). Using a *Cx3cr1-GFP*; *Ccr2-RFP* dual-reporter mouse line, we found that IAMs express *CX3CR1* and include a subfraction that co-expresses *CCR2* (Figure 1F), suggesting that IAMs undergo homeostatic turnover and are replaced by circulating *CCR2*⁺ monocytic precursors, in agreement with prior data in the setting of injury.^{9,13}

We also noted that IAMs are located adjacent to basal stem cells (Figures 1C and 1D). Using fixed whole-mount preparations of the murine trachea, we determined that each IAM is in direct, simultaneous contact with 19 ± 5 p63⁺ basal cells on average (Figures 1G and 1H). In addition, quantification of the ratio of basal cells and IAMs in the murine trachea revealed the presence of 43 ± 6 p63⁺ basal cells per IAM (Figures 1G and 1I), suggesting

Figure 1. Basal stem cells are necessary for the maintenance of IAMs

- (A) Uniform manifold approximation and projection (UMAP) clustering of mouse tracheal cells isolated from wild-type (WT) mice with immune cells highlighted by black dashed box.
- (B) Featureplots of tracheal immune cell gene expression. IAMs are highlighted using the blue dashed box.
- (C) Immunostaining for basal stem cells (CK5, cyan) and immune cells (CD45, red) on WT tracheal sections.
- (D) Immunostaining for CK5 (cyan) basal cells and IAM markers MHC II (green) and CD11c (red). White arrowheads indicate CD11c⁺/MHC II⁺ IAMs.
- (E) Whole-mount confocal imaging for MHC II (green), CD11c (red) and CD45 (cyan) on WT tracheal explants at the epithelial plane.
- (F) Whole-mount immunostaining for GFP (*CX3CR1*, green), RFP (*CCR2*, red), and MHC II (cyan) on tracheal explants from *CX3CR1-GFP*; *CCR2-RFP* mice. White arrows indicate *CCR2*⁺ IAMs. Purple arrows indicate *CX3CR1*⁺/MHC II⁺ cells within the epithelium.
- (G) Whole-mount immunostaining for MHC II (green), CD45 (red), and basal cell marker p63 (cyan).
- (H) Quantification of the number of basal stem cells (p63⁺) in contact with one IAM.
- (I) Quantification of the ratio of basal stem cells (p63⁺) per IAM.
- (J) Time-lapse images of an IAM (white) in a *CD11c-Cre*; *mTmG* tracheal explant every 5 min.
- (K) Schematic representation of basal cell depletion in *CK5rtTA*; *TetO-Cre*; *p63^{fl/fl}* mice (CK5-p63). Dox, doxycycline.
- (L) Whole-mount immunostaining for MHC II (green) and CD11c (red) in control (left) or CK5-p63 mice (right).
- (M) Quantification of CD11c⁺/MHC II⁺ IAMs per 20 $\times$ field in control or CK5-p63 tracheal whole mounts ($n = 4$ /group).
- (N) Immunostaining for MHC II (green), CD11c (red), and CK5 (cyan) in control or CK5-p63 mice.
- (O) Quantification of CD11c⁺/MHC II⁺ IAMs per 20 $\times$ field in control ($n = 9$) or CK5-p63 ($n = 7$) mice.
- (P) Immunostaining for CK5 (green) and *F4/80* (red) in control or CK5-p63 mouse tracheas.
- (Q) Quantification of intraepithelial *F4/80*⁺ cells per 20 $\times$ field in control ($n = 9$) or CK5-p63 ($n = 7$) mouse tracheas.
- Experiments were repeated three times (three independent experiments). *** $p < 0.001$, **** $p < 0.0001$. Data shown in the graphs are means $\pm$ SEM. Nuclei, DAPI (blue). Scale bar, 20 μ m. See also Figures S1 and S2.

that, on average, roughly 50% of basal cells are in contact with an IAM. Additionally, live imaging of *ex vivo* trachea from *CD11c-Cre; Rosa26-mTmG* mice demonstrates that the dendriform projections of IAMs are dynamic, extending and retracting through the interstices of the epithelium (Figure 1J).

Thus, our findings confirm the presence of a distinct population of myeloid cells with dendriform morphology characterized by an intraepithelial airway location adjacent to basal stem cells, a gene signature that aligns with a macrophage identity, and high expression of MHC II.

Airway basal stem cells are necessary for the maintenance of MHC II⁺ IAMs

Due to the proximity of IAMs to basal stem cells, we sought to establish whether airway stem cells regulate adjacent IAMs. We employed *CK5rtTA; TetO-Cre; p63^{fl/fl}* mice (CK5-p63 mice hereafter) to eliminate CK5-expressing airway basal cells from the epithelium through inducible deletion of the basal cell transcription factor p63, leading to basal cell loss over 2–3 weeks as previously reported (Figure 1K).²⁹ Tracheas from doxycycline (Dox)-treated CK5-p63 mice display a loss of the basal cell layer resulting in decreased epithelial thickness (Figure S2A). However, a continuous layer of differentiated epithelial cells remains with intact expression of tight junction proteins zonula occludens-1 (ZO1), claudin-1, and occludin (Figures S2B and S2C). We confirmed basal cell loss in Dox-treated CK5-p63 mice compared to Dox-treated *p63^{fl/fl}* control mice with p63 and CK5 staining (67 ± 18 vs. 501 ± 94 p63⁺ cells per tracheal section, and 160 ± 20 vs. 663 ± 81 CK5⁺ cells per tracheal section respectively), while club cell numbers were unchanged (Figure S2D). We observed a slight increase in acetylated tubulin⁺ ciliated cells, suggesting precocious differentiation following p63 deletion in basal cells as previously reported,²⁹ although the number of ciliated cells identified by FoxJ1⁺ staining was unchanged (Figure S2F). We also verified that the numbers of other epithelial cell types with immunomodulatory roles including tuft cells and ionocytes were unchanged following depletion of basal cells by analyzing tracheal sections (Figures S2G–S2J) and whole-mount tracheal preparations (Figures S2K–S2P).^{2,30–33} Neuroendocrine (NE) cell numbers were slightly increased in CK5-p63 tracheas (Figure S2J), although not significantly changed when counted on whole-mount tracheal preparations (Figure S2K). Thus, in these experiments using CK5-p63 mice, basal cells were the only epithelial population that was substantially changed in frequency.

IAMs, identified as intraepithelial CD11c⁺/MHC II⁺ cells, were decreased in tracheas of basal stem cell-depleted mice using analysis of whole-mount (15.1 ± 1.3 vs. 40 ± 6.6 cells per field, respectively) (Figures 1L and 1M) and sectioned tracheas (0.72 ± 0.2 vs. 3.2 ± 0.6 cells per field, respectively) (Figures 1N and 1O). Notably, quantification of F4/80⁺ cells and SIRPα⁺/MHC II⁺ cells also revealed a reduction of IAM numbers in CK5-p63 tracheas (Figures 1P, 1Q, and S2Q). To confirm that the loss of IAMs was not from systemic effects of Dox delivered by drinking water, we administered nebulized Dox to CK5-p63 mice (Figure S2R) to restrict Cre activation to the mouse airways.³⁴ Consistent with the prior data, we again

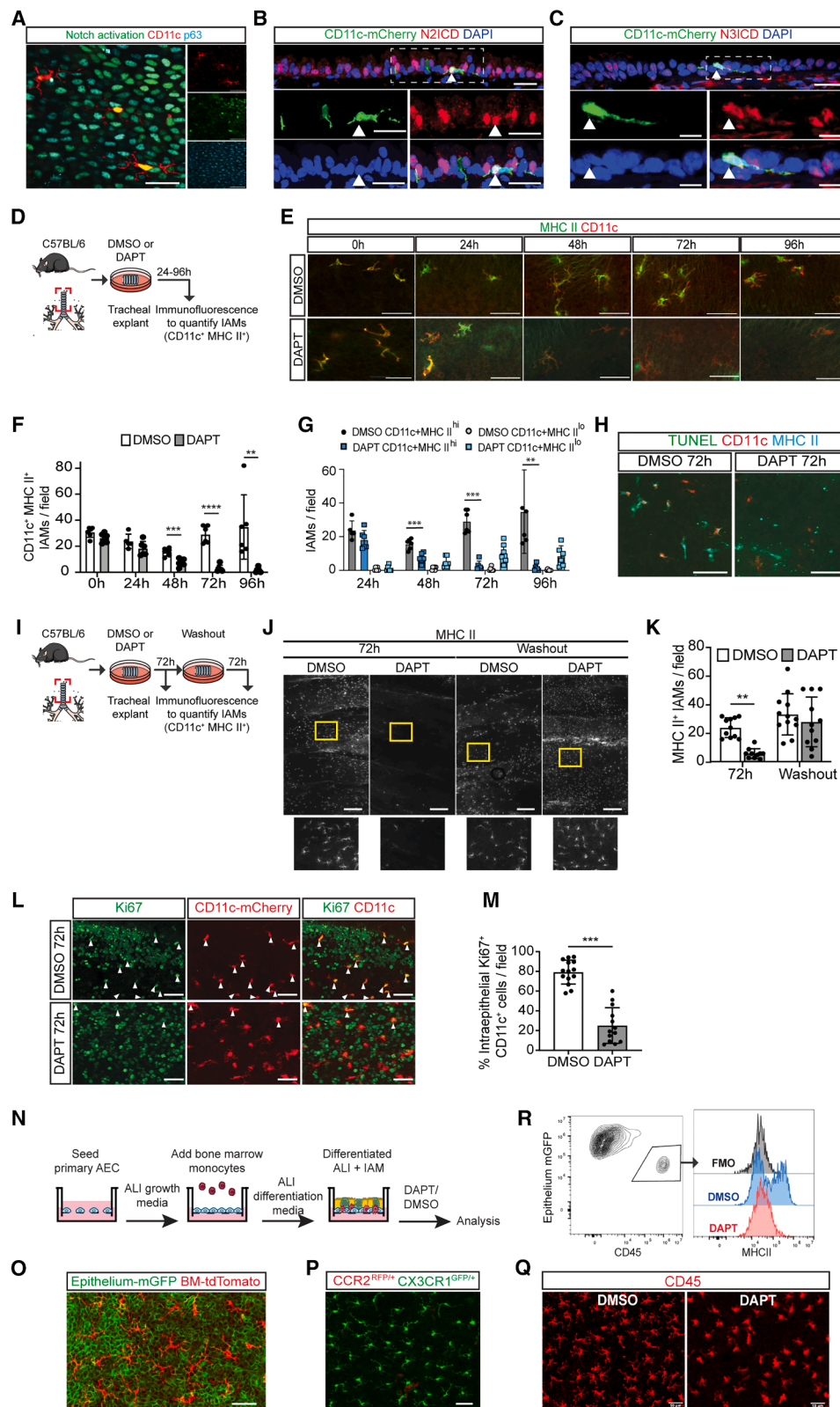
observed fewer CD11c⁺/MHC II⁺ IAMs in basal cell-depleted tracheas (Figures S2S and S2T).

Tonic Notch activation is required to maintain a network of MHC II⁺ IAMs

To probe the mechanisms by which basal stem cells interact with IAMs, we considered that Notch signaling may play an important role, particularly given the cell-cell contact between IAMs and airway basal cells (Figures 1C–1E and 1G–1I). Prior studies have implicated the Notch pathway in the development of several myeloid cell subsets,^{35–38} and we have previously shown that basal cell-derived Notch signals directly regulate neighboring epithelial cells.¹ To investigate this hypothesis, we crossed a *CD11c-mCherry* reporter mice with Notch signaling *CBF:H2B-Venus* mice to assess canonical Notch signaling activity. CD11c⁺ IAMs displayed Notch signaling activity as identified by nuclear Venus reporter signal (Figure 2A) and expression of Notch target gene *Hes1* (Figures S3A and S3B). More specifically, we detected nuclear Notch2 intracellular domain (N2ICD) (Figures 2B and S3C) and Notch3 intracellular domain (N3ICD) (Figures 2C and S3D) by immunofluorescent staining in IAMs, while Notch1 intracellular domain staining was rare (data not shown). This suggests tonic Notch2 and Notch3 activation in IAMs at homeostasis.

To further assess the importance of Notch signaling for IAMs, we excised tracheas from wild-type (WT) mice and treated them *ex vivo* with two different Notch inhibitors, DAPT and DBZ.³⁹ Tracheal explants were then fixed, stained for CD11c and MHC II, and analyzed using whole-mount confocal microscopy (Figure 2D). Notch inhibition with DAPT or DBZ caused a dramatic decrease in IAMs identified by CD11c⁺/MHC II⁺ staining (Figures 2E, 2F, S3E, and S3G). This decrease was rapid and progressive, starting at 48 h after treatment and reaching peak effect 72 h after treatment (Figures 2F and 2G). Of note, we did not detect apoptotic IAMs using TUNEL staining (Figure 2H). A CD11c⁺/MHC II^{lo} cell population with similar morphology transiently appeared between 48 and 72 h in low numbers (Figure 2G), suggesting the continual presence of IAMs with decreasing expression of a key functional marker. Interestingly, washout of DAPT treatment led to a recovery of MHC II⁺ IAMs within 72 h (Figures 2I–2K). Since there are no circulating monocytes to replenish IAMs in our explant model, this finding suggests that IAMs that had initially lost MHC II expression regained it following DAPT washout. In addition, Notch inhibition also resulted in a significant decrease in the fraction of Ki67⁺ proliferating CD11c⁺ IAMs in the explants (Figures 2L and 2M).

To assess whether the effects of DAPT on IAM MHC II expression were mediated through epithelial-immune interactions, we developed a co-culture model combining primary mouse tracheal epithelial cells and bone-marrow-derived monocytes. Trachea-derived epithelial cells were seeded onto Transwells to generate air-liquid interface (ALI) cultures. Purified bone-marrow monocytes were added to the apical chamber the day prior to establishing an ALI (Figure 2N). A two-color fluorescent reporter system using epithelium from mGFP mice and mTdT-mato-labeled monocytes revealed that monocytes integrate between epithelial cells and morphologically resemble IAMs (Figure 2O). Differentiation into CX3CR1⁺ macrophages was



(legend on next page)

verified using bone-marrow monocytes derived from the dual CCR2-RFP/CX3CR1-GFP mice seeded onto tracheal epithelial cells derived from B6 mice (Figure 2P). Addition of DAPT to mature cultures for 72 h eliminated detectable MHC II expression in monocyte-derived macrophages and promoted a rounded morphology (Figures 2Q and 2R).

To further investigate the role of IAM Notch activation *in vivo*, we used an inducible mouse line to target Notch signaling in established IAMs. We assessed the efficiency of the *Cx3cr1-CreER*; *Rosa26-tdTomato* reporter mouse line for labeling IAMs (Figure 3A). Four weeks following tamoxifen (Tam) administration, flow cytometry revealed the vast majority of tdTomato⁺ cells in CX3CR1-tdTomato tracheas are CD11b⁺ CD64⁺ F4/80⁺ macrophages (Figures S3H and S3I) with a small population (~5% of labeled cells) representing CD26⁺ DCs (Figure S3J). We confirmed that intraepithelial tdTomato⁺ cells displayed a dendriform morphology and co-expressed IAM markers including MHC II, CD11c, CD11b, and F4/80 (Figures 3B, 3C, 3K, and 3L). The CX3CR1-tdTomato reporter system resulted in the initial labeling of 80% of IAMs. After 24 weeks, only 20% of IAM remained labeled (Figure 3D), suggesting IAMs are continually replaced by circulating precursors and consistent with our observation of a CCR2⁺ subset of IAMs (Figure 1F). Interestingly, we observed that approximately 20% of IAMs remain labeled at 52 weeks after Tam administration (Figures 3D and 3E), indicating that there may be an IAM population that is either long lived or self-renewing.

Having demonstrated the utility of the *Cx3cr1-CreER* driver line to target IAMs, we next generated *Cx3cr1-CreER*; *mTmG*; *RBPJK^{fl/fl}* (hereafter CX3CR1-RBPJk) mice in which canonical Notch signaling can be conditionally disrupted in concomitantly lineage-labeled CX3CR1⁺ IAMs (Figures 3F and S3M). When compared with *Cx3cr1-CreER*; *mTmG*; *RBPJK^{+/+}* controls, we noted that the number of lineage-labeled IAMs per field was unchanged (21.2 ± 2 vs. 24.2 ± 1.4 Lineage⁺ IAMs per field in con-

trol tracheas) (Figures 3), suggesting that Notch activation was dispensable for IAM tissue localization. However, there was a substantial reduction in lineage-labeled IAMs that expressed MHC II (6.8 ± 1.3 vs. 22.8 ± 1.3 Lineage⁺ IAMs per field in control tracheas) (Figures 3G and 3I), suggesting that, while IAMs remain present in the tissue in the absence of Notch activation, MHC II expression is reduced. We did not observe any apoptotic IAMs based on Caspase3 detection in CX3CR1-RBPJk tracheas (Figure 3J). Although the numbers of lineage-labeled IAM cells were unchanged in CX3CR1-RBPJk airways, the percentage of proliferating Ki67⁺ IAMs was reduced compared to controls ($6.3\% \pm 3.2\%$ vs. $21.8\% \pm 2.1\%$ per field, respectively, Figures 3K and 3L), consistent with the effects of pharmacologic Notch inhibition *ex vivo* (Figures 2L and 2M). To assess whether IAM MHC II expression was specifically dependent on epithelial-derived Notch activation signals, we utilized our *in vitro* intraepithelial macrophage co-culture system. Bone-marrow monocytes from CX3CR1-RBPJk mice or Cre⁻ littermates were added to B6-derived tracheal epithelial cell cultures. At maturity, cultures were treated with 4-hydroxytamoxifen (4-OHT) to induce Cre-recombination, which resulted in complete loss of MHC II expression within the lineage-labeled macrophage population (Figures S3N and S3O). Altogether, these results suggest that Notch signaling is required to maintain IAM functional differentiation (MHC II-based antigen presentation) and promotes IAM proliferation.

Airway basal stem cell-derived Dll1 is necessary to maintain MHC II⁺ IAMs

Murine airway basal stem cells express high levels of the Notch ligands Jag2 and Dll1^{1,39} and have extensive contact with neighboring IAMs (Figures 1C, 1D, and 1G–1I). Thus, we hypothesized that IAM MHC II expression and proliferation are regulated through Notch ligands supplied by airway basal cells. Using a *Dll1-mCherry* reporter mouse, we found that Dll1 is uniformly

Figure 2. Notch activity is required for IAM functional markers and proliferation

(A) Whole-mount staining of *CD11c-mCherry*; *NotchCBF-H2B Venus* mouse tracheas for p63 (cyan) imaged in the epithelial plane. mCherry (CD11c, red), Venus (Notch activity, green).
(B and C) Immunostaining for Notch2 intracellular domain (N2ICD) (B) or N3ICD (C) (red) and mCherry (CD11c, green) on *CD11c-mCherry* mouse tracheas. Arrowheads indicate N2ICD⁺ or N3ICD⁺ IAMs.
(D) Schematic representation of Notch inhibition on WT tracheal explants after treatment with DAPT (2.5 μ M).
(E) Immunostaining for MHC II (green) and CD11c (red) on whole mounts of tracheal explants treated with DMSO or DAPT at different time points.
(F) Quantification of the absolute numbers of CD11c⁺/MHC II^{hi} IAMs per 20 $\times$ field at different treatment time points ($n = 5$ –6 images/group).
(G) Quantification of the absolute numbers of CD11c⁺/MHC II^{hi} and CD11c⁺/MHC II^{lo} cells per 20 $\times$ field at different treatment time points ($n = 5$ –6 images/group).
(H) Assessment of apoptosis by TUNEL assay (green) in IAMs (CD11c [red], MHC II [cyan]), on tracheal explants treated for 72 h.
(I) Schematic representation of Notch inhibition on tracheal explants for 72 h, followed by washout.
(J) Immunostaining for MHC II on whole mounts of tracheal explants treated with DMSO or DAPT for 72 h (left), and 72 h after washout (right).
(K) Quantification of CD11c⁺/MHC II⁺ IAMs (per 20 $\times$ field; $n = 10$ –12 images/group).
(L) Whole-mount staining for proliferating cells (Ki67⁺, green) in *CD11c-mCherry* (red) tracheal explants treated with DMSO (upper panels) or DAPT (bottom panels) for 72 h, with proliferating CD11c⁺ cells marked by white arrows.
(M) Percentage of proliferating IAMs (Ki67⁺ CD11c⁺) per field ($n = 13$ –14 images/group).
(N) Schematic representation of Notch inhibition on a co-culture system generated from airway epithelial cells and bone-marrow-derived monocytes followed by treatment with DAPT (2.5 μ M) for 72 h.
(O) Whole-mount staining of a co-culture for mGFP (epithelium, green) and tdTomato (intraepithelial macrophages, red).
(P) Whole-mount staining for GFP (CX3CR1⁺) and RFP (CCR2⁺) of a co-culture generated from *CX3CR1-GFP*; *CCR2-RFP* mouse bone marrow.
(Q) Whole-mount staining for CD45 (red) after treatment with DMSO or DAPT.
(R) Flow cytometry analysis of MHC II in CD45⁺ cells from the co-culture treated with DMSO or DAPT. FMO, fluorescence minus one control. Experiments *in vivo* were repeated three times; experiments *ex vivo* were repeated twice. ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Data shown in the graphs are means $\pm$ SEM. Nuclei, DAPI (blue). Scale bar, 5 μ m (B and C insets), 20 μ m (A–C, E, and H), 50 μ m (L and O–Q), 200 μ m (J). See also Figure S3.

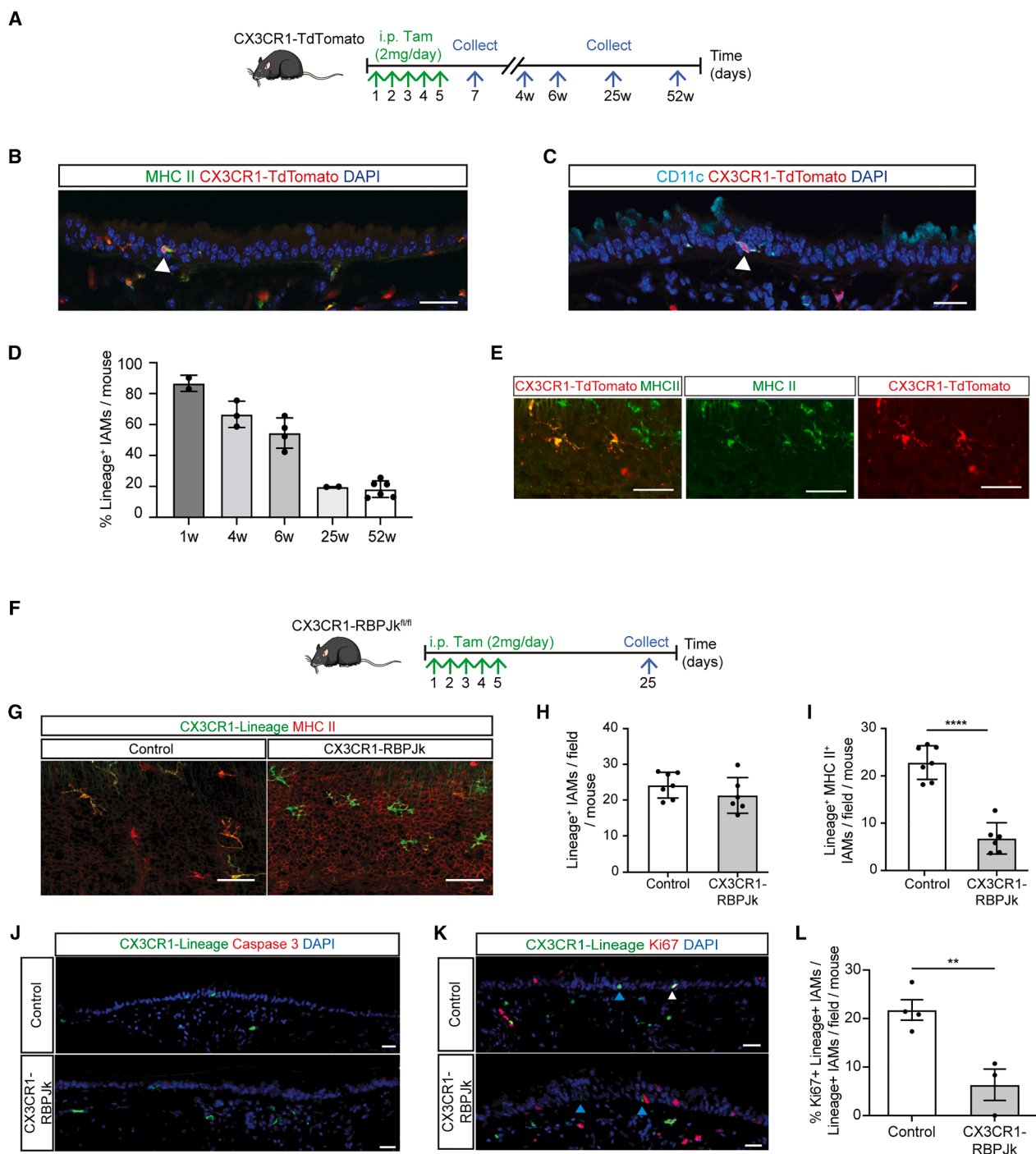


Figure 3. In vivo Notch inhibition in IAMS causes a loss of functional maturation status

(A) Schematic representation of lineage labeling of IAMS in CX3CR1-TdTomato mice. i.p., intraperitoneal; Tam, tamoxifen. (B and C) Immunostaining for CX3CR1-lineage-labeled IAMS (TdTomato⁺, red) and MHC II (B; green) or CD11c (C; cyan). Arrowheads indicate IAMS. (D) Percentage of CX3CR1-lineage-labeled IAMS per trachea at time points after Tam administration ($n = 2-4$ /group). (E) Whole-mount staining for CX3CR1-lineage-labeled IAMS (TdTomato⁺, red) and MHC II (green) 52 weeks after Tam administration. (F) Schematic representation of RBPJk (canonical Notch) inhibition in CX3CR1⁺ cells using CX3CR1-RBPJk mice. (G) Immunostaining for MHC II (red) and GFP (CX3CR1⁺, green) on CX3CR1-RBPJk tracheal whole mounts. (H and I) Quantification of intraepithelial CX3CR1-lineage-labeled cells (GFP⁺) (H) and CX3CR1⁺/MHC II⁺ cells (I) per 20 $\times$ field in control ($n = 7$) and CX3CR1-RBPJk ($n = 6$) tracheas.

(legend continued on next page)

expressed across all basal cells (Figure 4A). We also noted Dll1 expression in NE cells and tuft cells (Figures S3P and S3Q), but not in ionocytes (Figure S3R). We next assessed the effects of deleting Dll1 from basal cells by employing *CK5-CreER; Rosa26-LSL-eYFP; Dll1^{fl/fl}* mice (CK5-Dll1 hereafter) to remove Dll1 from CK5⁺ basal cells and concomitantly mark these cells with a yellow fluorescent protein (YFP) reporter following Tam administration (Figures 4B and S3S). We verified that common epithelial cell populations were unchanged in frequency 10 days after intraperitoneal (i.p.) Tam (Figure S3T). CK5-Dll1 mice displayed fewer CD11c⁺/MHC II⁺ IAMs compared to Tam-treated control mice when quantified on sectioned tracheas (1.4 ± 0.4 vs. 4.6 ± 0.9 IAMs per field, respectively) (Figures 4C and 4D), and whole-mount tracheas (25.1 ± 1.8 vs. 39.5 ± 2.6 IAMs per field, respectively) (Figures 4E and 4F). We observed residual intraepithelial dendriform CD45⁺ leukocytes that lacked MHC II expression in CK5-Dll1 tracheas (Figure 4G), consistent with a model in which stem cells deploy a contact-mediated Notch signal that is required for the ability of adjacent IAMs to present antigen. Finally, we targeted Jag2 expression in airway basal stem cells using *CK5-CreER; Rosa26-LSL-eYFP; Jag2^{fl/fl}* mice (CK5-Jag2 hereafter) (Figure 4H). There was no difference in CD11c⁺/MHC II⁺ cells in the trachea of Tam-treated mice compared to control mice (Figures 4I and 4J), suggesting that Dll1 is the dominant basal cell-derived Notch ligand engaging IAMs.

Airway basal stem cell loss prevents antigen-induced airway inflammation

Since airway myeloid immune cells continuously sample and process antigens,^{4–7} we assessed the functional consequences of basal stem cell deficiency on IAMs using a model of airway inflammation dependent on antigen presentation. Ovalbumin (OVA) is a classic model antigen to which mice can be sensitized through i.p. injection of alum-conjugated OVA. Subsequently, antigen challenge by nebulization of OVA alone results in airway antigen presentation, leading to allergic inflammation⁴⁰ (Figure 5A). Compared to other models of allergic airway inflammation, such as the house dust mite model, the OVA model has the advantage of decreased stimulation of airway epithelial cells, innate immune responses, and pathogen recognition receptors.⁴¹ After Dox-induced deletion of p63 and OVA sensitization and challenge, we confirmed that basal cells were effectively depleted and that other epithelial cell populations were not substantially changed in frequency (Figures S4A and S4B), while CD11c⁺/MHC II⁺ cells were decreased (Figures 5B and 5C). Additionally, there was a decrease in mucous metaplasia, the hallmark epithelial finding in antigen-induced inflammation, as assessed by Alcian blue staining (Figure 5D). This was confirmed by immunofluorescent stains of mucous cell differentiation markers (Figure 5E), with 73.3 ± 16.3 Muc5AC⁺ cells per

CK5-p63 tracheal section vs. 222 ± 44.5 cells in controls (Figure 5F).

We hypothesized that the lack of mucous metaplasia seen following basal stem cell loss reflected a diminished local type-2 inflammatory response.¹⁷ Since eosinophilic infiltration is a hallmark of a type-2 immune response, we examined tracheal sections for eosinophils using a staining panel of SiglecF⁺/CD11b⁺/CD11c[−]. We found that airway basal cell-depleted tracheas contained fewer eosinophils compared to controls after OVA sensitization and challenge (7.1 ± 1.1 vs. 14.9 ± 1.4 eosinophils per field, respectively; Figures 5G–5I). Of note, the number of eosinophils and activated CD4⁺ T cells in the lung compartment and bronchoalveolar lavage (BAL) of OVA-sensitized and challenged CK5-p63 mice was comparable to that in control mice (Figures S4C–S4F). There was also no change in distal airway goblet cell formation (Figures S4G and S4H). Thus, the loss of airway basal cells, which are found exclusively in the large airway of the mouse, correspondingly alters the local immune response, consistent with the Notch-based contact-mediated stem cell to immune cell signaling that is required for the ability of IAMs to present antigen.

Deletion of Dll1 on airway basal stem cells prevents antigen-induced airway inflammation

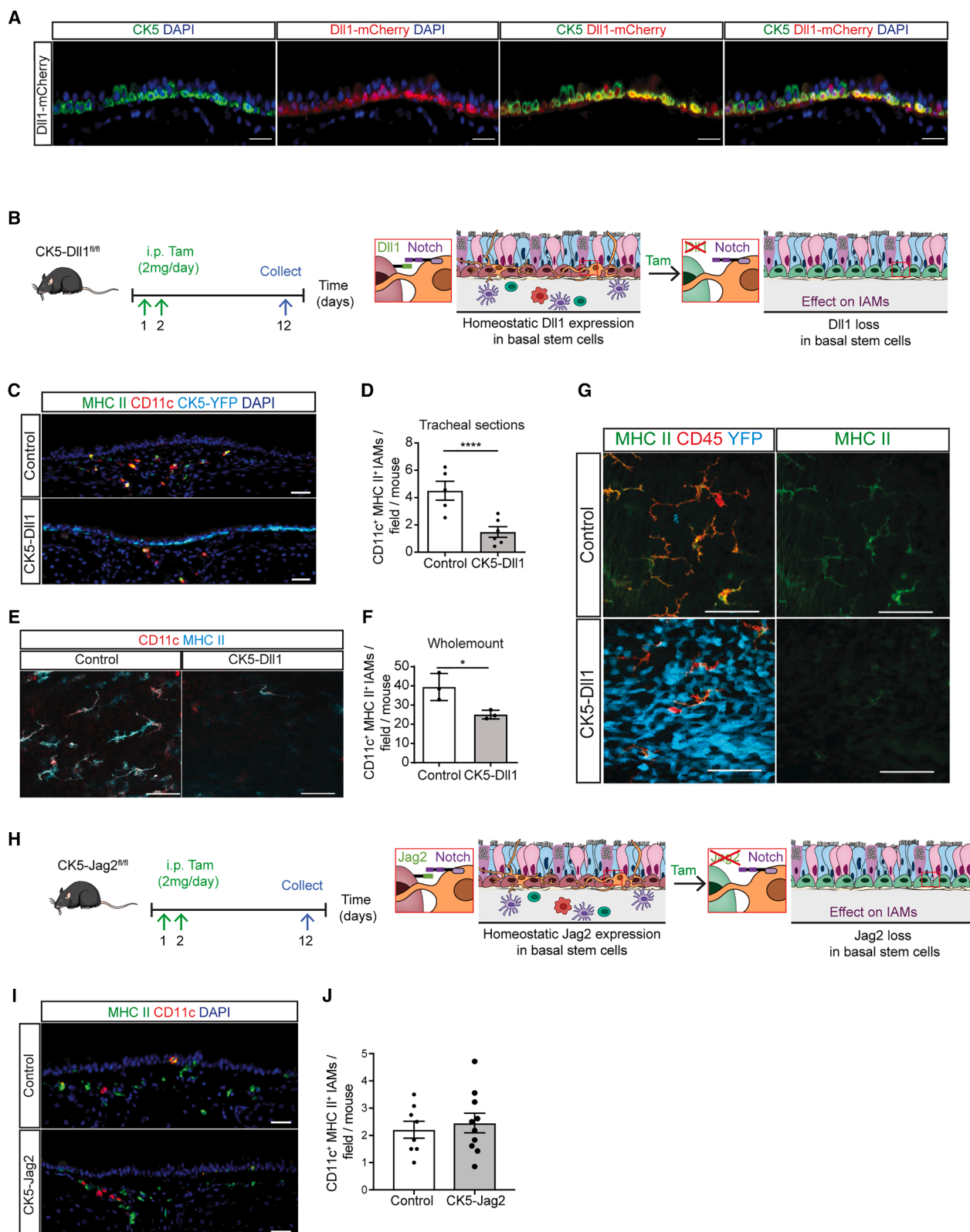
We next evaluated whether basal stem cell Dll1 loss would similarly blunt the response to OVA challenge (Figure 5J). Tracheas from Tam-treated CK5-Dll1 OVA-sensitized and challenged mice displayed reduced CD11c⁺/MHC II⁺ cell numbers (1.6 ± 0.5 vs. 4.4 ± 0.8 cells per field, respectively) (Figures 5K and 5L), eosinophils (3.1 ± 0.7 vs. 16.11 ± 2 cells per field, respectively) (Figures 5M and 5N), and mucous metaplasia ($13.2 \pm 1.6\%$ vs. $34.2 \pm 2.3\%$ mucous cells per total DAPI⁺ cells in tracheas, respectively) (Figures 5O and 5P). In contrast, the BAL and lungs of CK5-Dll1 mice still developed mucous metaplasia (Figure S4I), and eosinophilic inflammation similar to controls (Figures S4J–S4L), mirroring the responses seen in CK5-p63 mice. This suggests the effect of altering basal stem cell signaling specifically affects the local immune signaling in the trachea but not distal lung tissues. In contrast, deletion of Jag2 on airway basal stem cells did not prevent mucous metaplasia following OVA sensitization and challenge (Figures S4M and S4N), consistent with the lack of effect on IAMs (Figures 4I and 4J).

To directly assess whether IAMs are necessary for OVA-induced tracheal inflammation, we employed *Cx3cr1-CreER; R26-DTA^{fl/+}* mice (CX3CR1-DTA hereafter) to deplete airway macrophages in the setting of OVA sensitization and challenge (Figure S5A). Intranasal 4-hydroxytamoxifen (4-OHT) administration leads to ablation of approximately half of IAMs in the trachea as measured by CD11c⁺/MHC II⁺ staining (Figure S5B). When combined with OVA sensitization and challenge, IAM depletion

(J) Immunostaining for CX3CR1-lineage marker GFP (green) and apoptotic marker Caspase 3 (red) in control or CX3CR1-RBPJk mice.

(K) Immunostaining for GFP (green) and proliferation marker Ki67 (red) on control or CX3CR1-RBPJk mice. White arrowheads indicate proliferating (Ki67⁺) IAMs, blue arrowheads indicate non-proliferating (Ki67[−]) IAMs.

(L) Quantification of the percentage of Ki67⁺ CX3CR1-lineage⁺ IAMs per Lineage⁺ IAMs per 20× field of control ($n = 4$) or CX3CR1-RBPJk ($n = 3$) tracheas. Experiments were repeated twice. ** $p < 0.01$ and **** $p < 0.0001$. Data shown in the graphs are means $\pm$ SEM. Nuclei, DAPI (blue). Scale bar, 20 μ m (B, C, J, and K), 50 μ m (E and G). See also Figure S3.



(legend on next page)

led to a decrease in tracheal eosinophils (386 ± 27 vs. 561 ± 76 eosinophils per mm^2 , respectively) (Figures S5C and S5D) and mucous cells ($17.27 \pm 2.6\%$ vs. $33.8 \pm 2.8\%$ Muc5ac⁺ cells per total DAPI⁺ cells, respectively) (Figures S5E and S5F), while distal lung inflammation was preserved (Figures S5G and S5H).

To more specifically assess the relevance of Notch activation in IAMs in this context, we subjected CX3CR1-RBPJk mice to the OVA model (Figure 6A). OVA-treated CX3CR1-RBPJk tracheas showed a decrease in intraepithelial CD11c⁺/MHC⁺ cells (1.5 ± 0.7 vs. 6.2 ± 0.5 cells per field in control tracheas) (Figures 6B and 6C), whereas the overall number of lineage-labeled IAMs was unchanged (2.9 ± 0.5 vs. 2.5 ± 0.2 Lineage⁺ IAMs per field in control tracheas) (Figures 6D and 6E). Consistently, there was a reduction in MHC II-expressing lineage-labeled IAMs (1.1 ± 0.4 vs. 2.3 ± 0.1 Lineage⁺ MHC II⁺ IAMs per field in control tracheas; $38.8\% \pm 6.2\%$ vs. $90.8\% \pm 3.6\%$ Lineage⁺ MHC II⁺ per Lineage⁺ IAMs in control tracheas) (Figures 6D, 6F, and 6G). Similar to OVA-treated CK5-Dll1 and CX3CR1-DTA mice, we observed reduced eosinophilic inflammation in CX3CR1-RBPJk tracheas (1.8 ± 0.5 vs. 7.5 ± 0.7 eosinophils per field in control tracheas) (Figures 6H and 6I) and attenuated mucous metaplasia ($3\% \pm 1.6\%$ vs. $16.8\% \pm 3.2\%$ Muc5ac⁺ cells per total DAPI⁺ cells in control tracheas) (Figures 6J and 6K). Thus, Notch signaling in IAMs is necessary for eliciting antigen-induced inflammatory responses.

Finally, we evaluated whether differences in goblet cell formation in CK5-p63 and CK5-Dll1 mice are related to an intrinsic altered capacity of epithelial secretory cells to undergo mucous cell differentiation independent from regulating the degree of antigen-induced tracheal inflammation. Notably, overexpression of active Notch1 intracellular domain in basal stem cells or secretory cells can directly induce mucous cell differentiation,^{39,42} while blocking Notch2 and Notch3 has been shown to reduce mucous metaplasia.^{43–45} Furthermore, we previously demonstrated that basal stem cells can regulate secretory cell fate using Jag2 to send a feedforward signal.¹ To assess whether basal cell-derived Dll1 alters epithelial intrinsic mechanisms of goblet cell formation, we used lentiviral-delivered short hairpin RNA (shRNA) and overexpression constructs in primary mouse airway basal stem cells in the absence of immune cells. After expansion and maturation at ALI, cultures were treated with recombinant IL-13 to induce mucous cell formation (Figure S6A).^{42,44,46,47} Mucous metaplasia was not impacted by shRNA inhibition (Figures S6B and S6C) or overexpression of Dll1 (Figures S6D–S6F), indicating that basal stem cell-derived Dll1 is neither suffi-

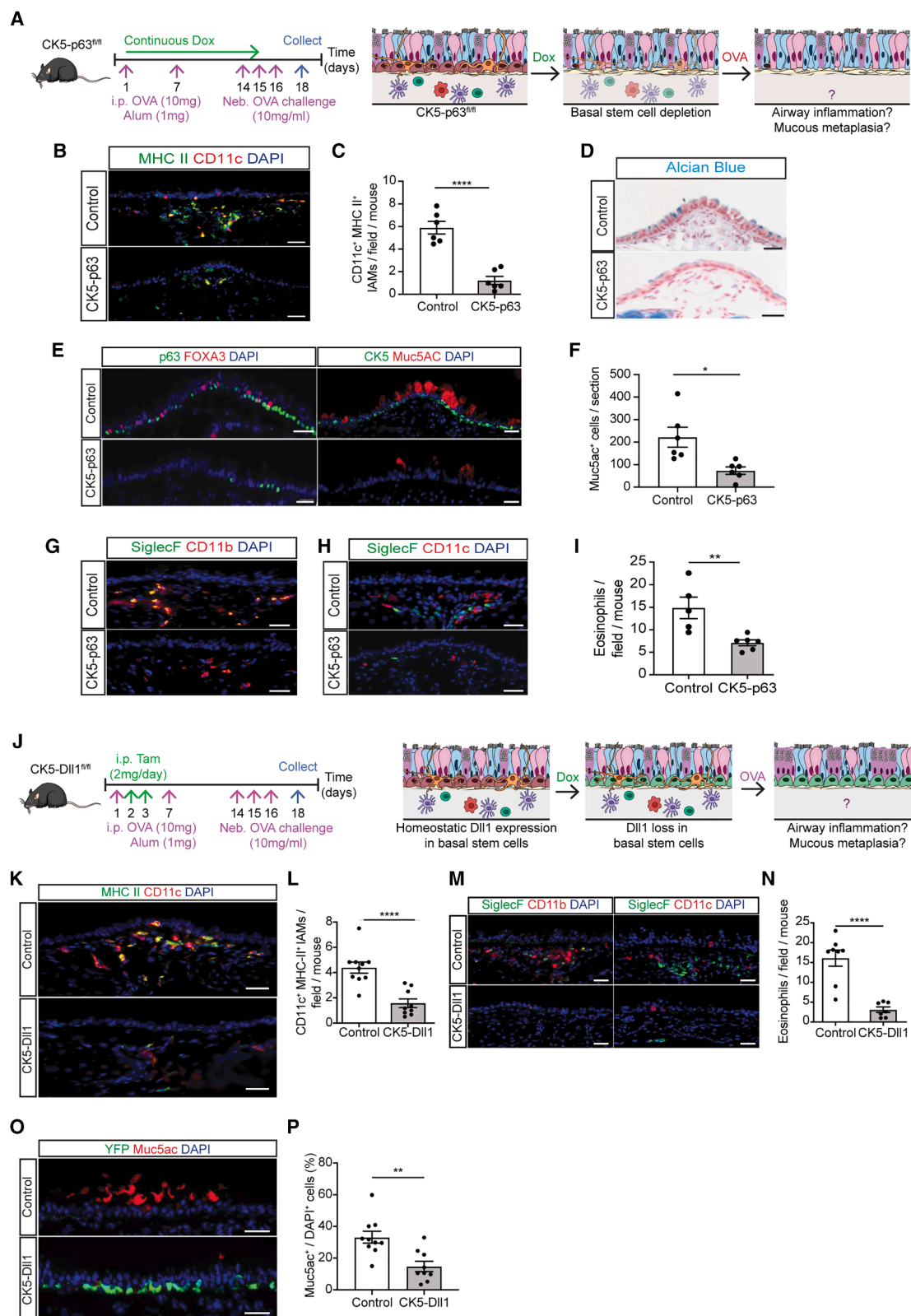
cient nor required for mucous metaplasia. Finally, we tested whether basal stem cells are necessary *in vivo* for IL-13-induced mucous metaplasia in the trachea. Intranasal administration of recombinant IL-13 resulted in extensive mucous metaplasia in Dox-treated CK5-p63 mouse tracheas (Figures S6G–S6I). Therefore, the absence of basal stem cells does not cause an underlying defect in the intrinsic capacity for secretory cells to differentiate into mucous cells *in vivo*. In aggregate, our combined observations lead us to conclude that allergen-induced mucous metaplasia does not require a feedforward signal from basal stem cells. Thus, our findings suggest an essential role for airway basal stem cell-mediated Notch signaling in the maintenance of a functional pool of IAMs that are required for local antigen-induced airway inflammation and mucous metaplasia.

Notch signaling components are highly expressed in human airway basal stem cells and airway mononuclear phagocytes

To assess whether human basal stem cells possess the capacity to regulate airway mononuclear phagocytes (aMNP) via Notch signaling, we re-analyzed single-cell transcriptomes from baseline and allergen-challenged airway specimens from allergic human subjects with and without asthma. Specifically, previously deposited single-cell RNA-seq (scRNA-seq) profiles, obtained from 21 lower airway brushes collected from eight human subjects with systemic sensitization to aeroallergens,⁴⁸ were found to contain 8,510 LYZ⁺ aMNP cells split into 14 clusters that included DCs, macrophages, and monocyte-derived cells (MCs). The DC clusters corresponded to well-defined DC1, DC2, AXL⁺/Siglec6⁺ DC, and plasmacytoid DC populations.⁴⁹ The monocyte-derived clusters (MC1–4) showed expression of classical monocyte markers, including CD14, but otherwise displayed a combination of DC and macrophage markers, consistent with known monocyte plasticity. Tissue macrophage subclusters expressed *MAFB*, high levels of complement family genes (*C1QA*, *C5AR1*),^{50–53} and low levels of *ZBTB46* compared to DC populations (Figure 7A). We next evaluated whether any of the aMNP clusters had a transcriptomic profile akin to that of murine IAMs. Discriminatory genes for murine IAMs¹³ were mapped onto human orthologs to define an IAM signature. Gene set enrichment analysis was performed to evaluate for an enrichment of the IAM signature across all aMNP clusters. Notably, only the macrophage cluster Mac2 showed positive enrichment for IAM marker genes (Figures 7B, S7A, and S7B). Mac2 and IAM signatures showed considerable overlap with genes that serve

Figure 4. Basal stem cell-derived Notch ligand Dll1 but not Jag2 is necessary for the maintenance of IAM function

- Immunostaining for Dll1 (mCherry, red) and CK5 (green) in *Dll1-mCherry* mouse tracheas.
 - Schematic representation of the inhibition of the Notch ligand Dll1 in basal cells using CK5-Dll1 mice. i.p., intraperitoneal; Tam, tamoxifen.
 - Immunostaining for CK5-lineage-labeled YFP (cyan) cells and IAMs (MHC II [green] and CD11c [red]) in control or CK5-Dll1 mice.
 - Quantification of CD11c⁺/MHC II⁺ IAMs per 20 $\times$ field in control ($n = 5$) or CK5-Dll1 ($n = 6$) mice.
 - Whole-mount staining for CD11c (red) and MHC II (cyan) on control and CK5-Dll1 tracheas.
 - Quantification of CD11c⁺/MHC II⁺ IAMs per 20 $\times$ field in control or CK5-Dll1 ($n = 3$ /group) whole-mount tracheas.
 - Whole-mount staining for YFP (CK5-lineage, cyan), CD45 (red), and MHC II (green) using control or CK5-Dll1 tracheas.
 - Schematic representation of the use of CK5-Jag2 mice to inhibit the Notch ligand Jag2 in basal cells.
 - Immunostaining for MHC II (green) and CD11c (red) in control or CK5-Jag2 mice.
 - Quantification of CD11c⁺/MHC II⁺ IAMs per 20 $\times$ field in control or CK5-Jag2 mice ($n = 8$ –10/group).
- Experiments were repeated three times (three independent experiments). * $p < 0.05$ and **** $p < 0.0001$. Data shown in the graphs are means $\pm$ SEM. Nuclei, DAPI (blue). Scale bar, 20 μm (A, C, and I), 50 μm (E and G). See also Figure S3.



(legend on next page)

as canonical macrophage markers, including complement genes (*C1q*) and *MAFB* (Figures 7A and S7B). Like murine IAMs, Mac2s express CD11c (*ITGAX*), high MHC II (*HLA-DQB1*), and other genes associated with antigen presentation (*CIITA*, *CD74*) (Figures 7A and S7B). Mac2 also express the genes for Langerin (CD207) and MerTK (Figure S7B). Additionally, Mac2 and IAMs express *MMP12* and *AXL*, further suggesting similar functional roles involving tissue remodeling and efferocytosis, both implicated in asthma pathobiology (Figure 7A).^{54,55}

We next analyzed the expression of Notch signaling components within the human aMNP and airway basal stem cell subclusters. Human basal stem cells showed robust expression of Notch ligands *DLL1*, *JAG1*, and *JAG2* (Figure 7C). Correspondingly, we identified strong expression of Notch receptors Notch1 and Notch2 across most aMNP populations (Figure 7D), including Mac2. We could identify Mac2 cells in human airway tissue sections as Langerin⁺ intraepithelial cells that co-express MerTK and C1q (Figures S7C and S7D). Like IAMs, Langerin⁺ Mac2 are found adjacent to CK5⁺ and CK17⁺ basal cells (Figures 7E–7G). Subsets of intraepithelial Langerin⁺ Mac2 showed evidence of Notch activation *in vivo* based upon nuclear staining for N1ICD, N2ICD, and N3ICD in both healthy and asthmatic human distal airways (Figures 7E–7J and S7E–S7H). Detection of the Notch target genes *Hes1* (Figures 7K, 7M) and *HeyL* (Figures 7L and 7N) in Langerin⁺ intraepithelial cells confirms Notch activation in Mac2 cells in healthy (Figures 7K and 7L) and asthmatic airways (Figures 7M and 7N). These results suggest that Mac2 are the human counterparts of murine IAMs with a similar pattern of gene expression, location, and evidence of tonic Notch activation.

DISCUSSION

Here, we show that airway basal stem cells help establish a functional niche for MHC II⁺ IAMs. Although previous work characterized these cells as DCs, IAMs are distinct from the Zbtb46⁺ DC lineage (Figures 1B, S1A, S1B, and S1H) and possess a signature characteristic of macrophages. Interestingly, a small fraction of IAMs persist over a year, suggesting that subsets of

IAMs may be long lived or capable of self-renewal at homeostasis, consistent with the robust proliferation of IAMs in tracheal explants (Figure 2M). The signals driving this heterogeneity and whether there are functional differences in IAM subsets have not yet been elucidated.

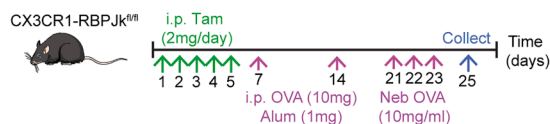
Within the airway, we show that basal stem cell Notch signaling through Dll1 is necessary for IAM MHC II expression and function. Importantly, our findings reveal an essential role for tonic Notch signaling between airway basal stem cells and IAMs, suggesting that airway basal stem cells serve as an immune cell niche. Previous work has underscored the interaction of epithelial stem cells and local macrophage populations. Within the alveolus, alveolar type 2 (AT2) epithelial stem cells stimulate and maintain alveolar macrophage maturation through secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF),⁵⁶ and disruption of this interaction results in dysfunctional immature macrophages causing the lung disease pulmonary alveolar proteinosis.⁵⁷ In addition, CD200 signaling to alveolar macrophages via CD200R restrains macrophage inflammatory activity.⁵⁸ Thus, in both the proximal airways and alveoli, stem cells are essential for the maintenance of local functional immune cell populations. Interestingly, mammary gland stem cells have also been shown to maintain macrophages using Notch signaling, potentially pointing to a conserved arrangement in which direct communication between stem cells and adjacent immune cells allows for coupled regeneration and inflammation.³⁷

We demonstrate that airway basal stem cells signal to dendritic form MHC II⁺ IAMs via Notch and that this cell-to-cell communication is required for locally restricted antigen-induced inflammation. Prior work has shown that broad inhibition of Notch receptors with blocking peptides or antibodies impairs allergic airway inflammation in a murine model of asthma,^{43–45} although these studies do not identify the specific mechanism involved since the strategies utilized are not cell specific. In addition, previous studies have highlighted roles for Notch signaling in type 2 inflammation; however, these studies focused on the direct effects of Notch signaling on T cells, affecting their polarization and egress from lymph nodes.^{59–62} In the OVA model, we observe that loss of Notch signaling in IAMs leads to a reduction

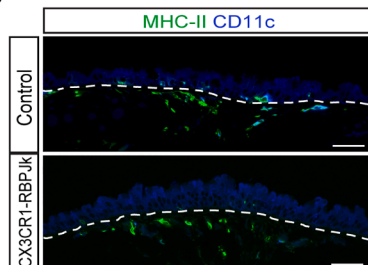
Figure 5. Basal stem cells are necessary for allergen-induced mucous metaplasia

(A) Schematic representation of ovalbumin (OVA) sensitization and challenge to induce airway inflammation and mucous metaplasia in CK5-p63 mice. Neb, nebulized.
(B) Immunostaining for IAMs (MHC II [green] and CD11c [red]) in OVA-treated control or CK5-p63 mice ($n = 4/\text{group}$).
(C) Quantification of CD11c⁺/MHC II⁺ IAMs per 20 $\times$ field in OVA-treated mice ($n = 6/\text{group}$).
(D) Alcian blue staining of tracheas from OVA-treated mice.
(E) Immunostaining for basal stem cells (p63, CK5; green) and mucous cells (FOXA3, Muc5AC; red) in OVA-treated control or CK5-p63 mice.
(F) Quantification of Muc5AC⁺ cells per tracheal section in OVA-treated mice ($n = 6/\text{group}$).
(G and H) Immunostaining for SiglecF (green) and CD11b (G) or CD11c (H) (red) in OVA-treated control and CK5-p63 mouse tracheas ($n = 6$).
(I) Quantification of eosinophils (SiglecF⁺ CD11b⁺ CD11c[−]) per 40 $\times$ field in OVA-treated control or CK5-p63 mice ($n = 5–6/\text{group}$).
(J) Schematic representation of the inhibition of basal cell Dll1 with OVA sensitization and challenge.
(K) Immunostaining for IAMs (MHC II [green] and CD11c [red]) in OVA-treated control or CK5-Dll1 mice.
(L) Quantification of IAMs per 20 $\times$ field of the trachea of OVA-treated control ($n = 10$) or CK5-Dll1 ($n = 9$) mice.
(M) Immunostaining for eosinophils (SiglecF, green) and CD11b (red) (left panels) or CD11c (red) (right panels) in OVA-treated control or CK5-Dll1 mice.
(N) Quantification of eosinophils (SiglecF⁺ CD11b⁺ CD11c[−]) per 40 $\times$ field in OVA-treated tracheas.
(O) Immunostaining for CK5-lineage YFP (green) and Muc5AC (red) in OVA-treated mice.
(P) Quantification of the percentage of Muc5AC⁺ mucous cells per DAPI⁺ cells in OVA-treated control or CK5-Dll1 mice ($n = 9–10/\text{group}$).
Experiments were repeated three times. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$. Data shown in the graphs are means $\pm$ SEM. Nuclei, DAPI (blue). Scale bar, 20 μm . See also Figures S4–S6.

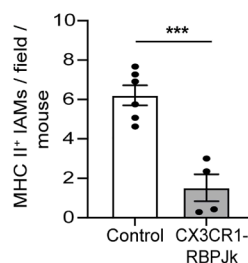
A



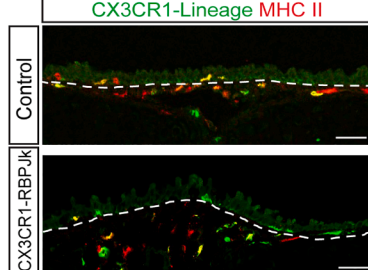
B



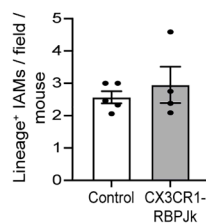
C



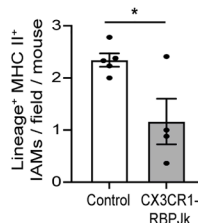
D



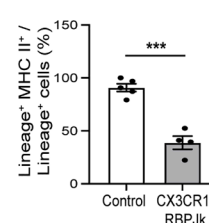
E



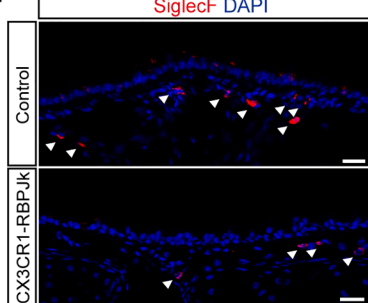
F



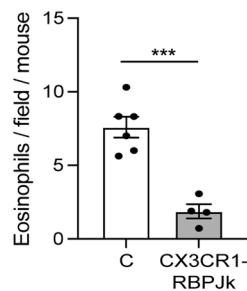
G



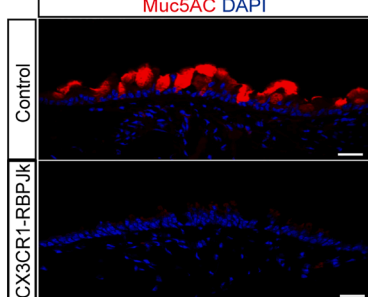
H



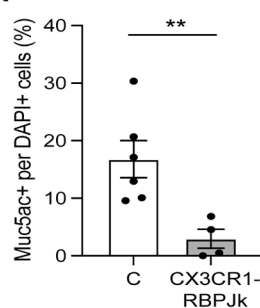
I



J



K



(legend on next page)

in both tracheal allergic inflammation and goblet cell metaplasia. We suspect that this is related to reduced airway IL-4/-13 production as a consequence of impaired antigen presentation to T cells. It has been previously shown that MHC II-expressing airway macrophages may present antigens to airway-resident T cells and can transfer antigens to subepithelial DCs that then migrate to draining lymph nodes.²⁵ This suggests a model wherein IAMs perform local antigen presentation in the airway, guided by direct signals from their surrounding tissue, while DCs propagate responses in draining lymphoid tissues. The tissue specificity of our findings suggest that the conducting airways may have distinct regulatory pathways governing antigen presentation compared to more distal airways and the alveoli, highlighting the importance of utilizing the murine trachea as a model for airway disease.

Finally, we identify a class of human intraepithelial airway HLA-positive macrophages (Mac2) that resemble murine IAM morphologically, and are also adjacent to human basal stem cells. Interestingly, human counterparts to IAMs similarly exhibit tonic Notch activation, suggesting that they may receive Notch signals from basal stem cells. Furthermore, these data suggest that there may be a similar direct stem-immune cell interaction that is required for the maintenance and function of these macrophages in human airways. Altogether, our data suggest that manipulation of airway basal stem cell-IAM crosstalk could be used therapeutically to regulate immune responses in the airway.

Limitations of the study

Myeloid cells express overlapping markers and we currently lack a specific Cre driver line that exclusively targets IAMs without impacting other myeloid sub-populations. Thus, we cannot exclude contributions of these cell types to our experimental paradigms. In particular, the CX3CR1-CreERT system can target both dendritic cells as well as other macrophage populations. The kinetics of our Tam administration largely eliminates the effects on short-lived dendritic cells, but the effects mediated by other macrophages, including airway subepithelial macrophages, cannot be fully excluded. Notably, we observe changes in subepithelial immune cells in our CK5-p63 and CK5-Dll1 mice suggesting that some effects of basal stem cell Notch signaling may proceed through intermediate migratory cell populations, cytokine/chemokine signals, or that subepithelial immune cells may themselves transit through the epithelium. Although all our results using a combi-

nation of *in vivo*, *ex vivo*, and *in vitro* models support a direct Notch-mediated effect of basal stem cells on IAMs, indirect effects cannot be excluded. Whether basal cell depletion may indirectly influence IAMs via changes in other airway epithelial or mesenchymal cells, including compensatory signals, is an extremely difficult question to address *in vivo* that would require extensive and sophisticated methods to provide rigorous conclusions.

RESOURCE AVAILABILITY

Lead contact

The lead contact is Ana Pardo-Saganta (ana.pardo-saganta@innere.med.uni-giessen.de).

Material availability

This study did not generate unique reagents.

Data and code availability

The following publicly available datasets in Gene Expression Omnibus under accession numbers GSE103354² and GSE193816⁴⁸ were re-analyzed. Codes for analysis of scRNA-seq data are available at GitHub (<https://github.com/tristankooistra/NotchAirwayMacs>).

ACKNOWLEDGMENTS

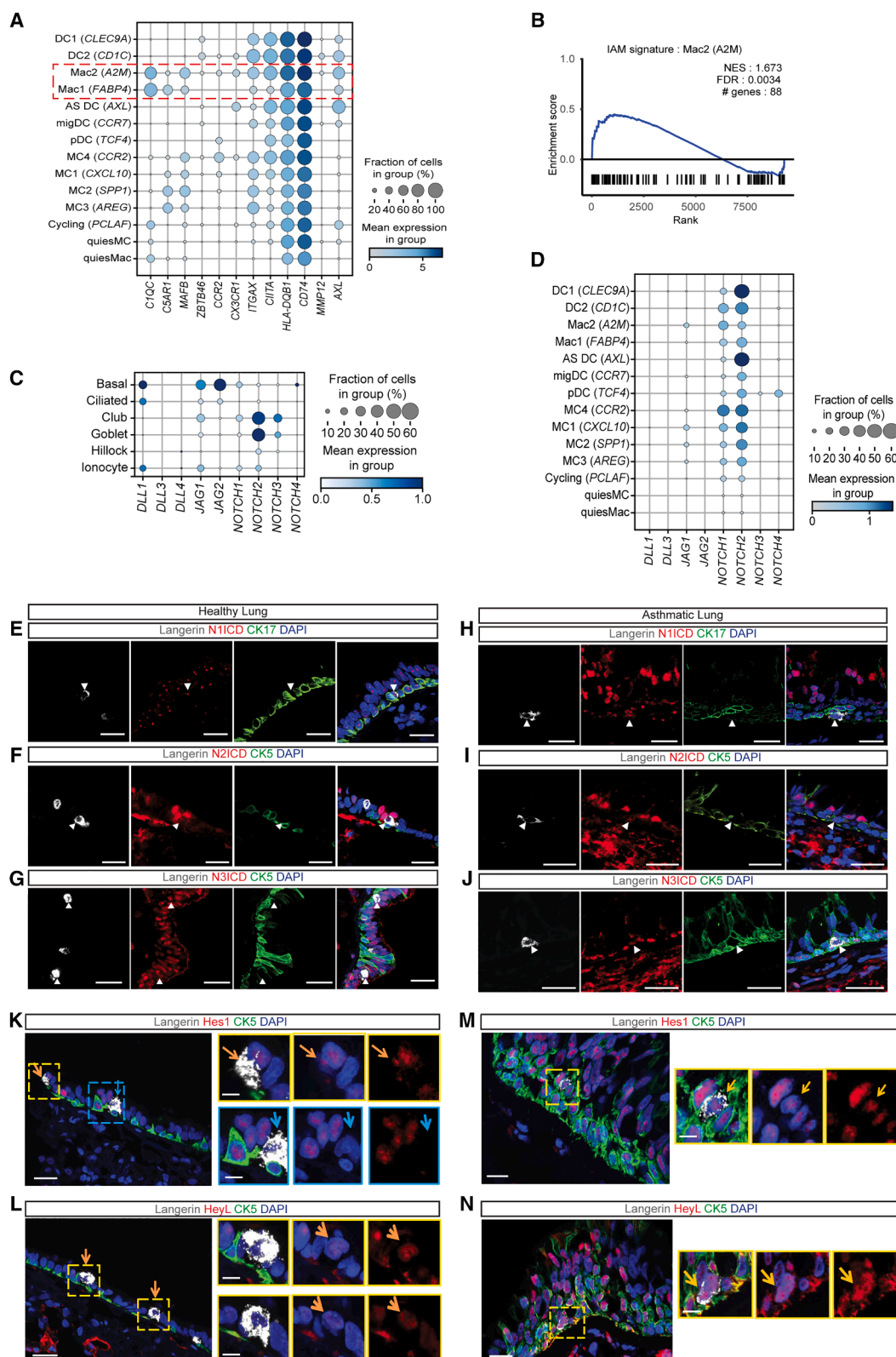
We thank Adam Glick for providing the *CK5rtTA* mice, Brigid Hogan for providing *CK5-creER* mice, Alea A. Mills for kindly sharing the *p63 floxed* mice, Rafi Kopan for the *Dll1 floxed* mice, and Yibin Kang and Hans Clevers for the *Dll1-mCherry* mice. We also thank Barry Stripp for providing the goat anti-SCGB1A1 antibody. We wish to extend our thanks to all of the current and former members of the Rajagopal Laboratory, the Medoff Laboratory, the Pardo-Saganta Lab, people involved in the maintenance of mouse colonies, the CIMA and HSCI flow cytometry core facility, the histology core at MGH and CIMA, the UGMLC Giessen Biobank, and all the colleagues that provided their feedback, especially Carrie Sokol and Mikael Pittet for their comments, guidance, and help in the presentation of the results.

This research was supported by the New York Stem Cell Foundation (J.R. is a New York Stem Cell Foundation-Robertson Investigator); by a National Institutes of Health-National Heart, Lung, and Blood Institute Early Career Research New Faculty (P30) award (5P30HL101287-02); RO1s (RO1HL118185 and RO1HL164563) from NIH-NHLBI (to J.R.); a Harvard Stem Cell Institute (HSCI) Junior Investigator Grant (to J.R.); a grant from the Department of Defense (W81XWH) and a Sanofi iAward to B.D.M.; an NIH-NHLBI grant T32HL116275 and American Thoracic Society Award to T.K.; a grant from Instituto de Salud Carlos III ISCIII (PI17/01346), co-funded by ERDF, "A way to make Europe" (to B.S.); an AEFAT award (to B.S.); an AECC Junior Investigator Grant exp. AIO16163636SAEZ (to B.S.); a Ramon y Cajal Award RYC-2015-18580 co-funded by FSE (to A.P.-S.); a grant from Gobierno de España SAF2017-89908-R (MINECO/AEI/FEDER, UE.) (to

Figure 6. Notch inhibition in IAMs attenuates OVA-induced airway inflammation and mucous metaplasia

- (A) Schematic representation of Notch inhibition in IAMs, and induction of allergic inflammation and mucous metaplasia after OVA challenges.
(B) Immunostaining for IAMs (MHC II [green] and CD11c [blue]) in OVA-treated control or CX3CR1-RBPJk mice.
(C) Quantification of IAMs (CD11c⁺/MHC II⁺) per 20× field in OVA-treated control (n = 6) or CX3CR1-RBPJk (n = 4) mice.
(D) Immunostaining for MHC II (red) and GFP (CX3CR1⁺, green) in OVA-treated mice.
(E and F) Quantification of intraepithelial CX3CR1-lineage-labeled cells (GFP⁺) (E) and double-positive CX3CR1⁺ (GFP⁺) MHC II⁺ cells (F) per 20× field in OVA-treated control (n = 5) and CX3CR1-RBPJk (n = 4) tracheas.
(G) Quantification of the percentage of intraepithelial CX3CR1-lineage-labeled cells (GFP⁺) per total Lineage⁺ cells.
(H) Immunostaining for eosinophils (SiglecF⁺, red) in OVA-treated mice.
(I) Quantification of eosinophils (SiglecF⁺) per 20× field in OVA-treated control (n = 6) or CX3CR1-RBPJk (n = 4) mice.
(J) Immunostaining for Muc5AC (red) in OVA-treated mice.
(K) Quantification of the percentage of Muc5AC⁺ mucous cells per DAPI⁺ cells in OVA-treated control (n = 6) or CX3CR1-RBPJk (n = 4) mice.

*p < 0.05, **p < 0.01, and ***p < 0.001. Data shown in the graphs are means ± SEM. Nuclei, DAPI (blue). Scale bars, 20 μm. See also Figure S5.



(legend on next page)

A.P.-S.); and a grant from Gobierno de Navarra GNS80/2016 co-funded by FEDER (to A.P.-S.).

AUTHOR CONTRIBUTIONS

T.K. designed and performed the experiments, analyzed and interpreted the data, optimized protocols for whole-mount staining and imaging of tracheal explants, and edited the manuscript. B.S. and M.R. performed *in vivo* experiments and analysis, contributed to the *in vitro* experiments, helped with the interpretation of the data, and edited the manuscript. A.E.-Z. and D.L. processed samples, performed immunostaining analysis, and generated final images. D.A., N.N., J.V., J.G., and M.C. helped with *in vivo* experiments. E.P., L.V., and Z. B.-I. processed samples from *in vivo* experiments, optimized immunostaining protocols, generated tools, and helped with *in vitro* experiments. N.P.S., A.-C.V., J.A., Y.Z., and A.H. re-analyzed scRNA-seq datasets. V.V. optimized the detection of IAMs on tracheal explants. M.S. analyzed NE cells. S.L.S. generated cartoons, reorganized figures, and helped edit the manuscript. M.G.-C. contributed to the analysis of the phenotype of CK5-p63 mice. H.M. and A.W. provided protocols and reagents and helped perform the *in vitro* experiments. B.L., A.P., and K.Y. organized, reestablished, and provided different mouse lines. P.R.T. and R.Z. contributed to the *in vivo* experiments with the basal cell depletion model. B.C. helped with the analysis of the inflammatory response. J.J.Z., F.P., and J.L.C. contributed to data interpretation and provided input on the manuscript. J.R. and B.D.M. co-designed the study, supervised the project, and co-wrote the manuscript. A.P.-S. conceived the study, designed and performed the experiments, analyzed and interpreted the data, supervised the project, coordinated the entire team, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
 - Animals
 - Human samples
- METHOD DETAILS
 - Mouse model of allergic airway inflammation
 - *In vivo* induction of mucous metaplasia
 - Cell dissociation, FACS, and flow cytometry analysis
 - Sectioned tissue preparation, immunohistochemistry, and immunofluorescence
 - Wholemount tissue preparation and staining
 - Microscopy and imaging
 - Time-lapse microscopy
 - RNA extraction and quantitative RT-PCR

- Cell culture and viral transduction
- Notch inhibition on an explant system
- Murine co-culture model
- Notch inhibition in an *in vitro* co-culture system
- Confocal/flow analysis of co-cultures
- Mouse single cell RNA sequencing (scRNAseq) analysis
- Human single cell RNA sequencing and gene set enrichment analysis (GSEA)

● QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2025.115860>.

Received: June 25, 2024

Revised: April 16, 2025

Accepted: May 27, 2025

Published: June 12, 2025

REFERENCES

1. Pardo-Saganta, A., Tata, P.R., Law, B.M., Saez, B., Chow, R.D.W., Prabhu, M., Gridley, T., and Rajagopal, J. (2015). Parent stem cells can serve as niches for their daughter cells. *Nature* 523, 597–601. <https://doi.org/10.1038/nature14553>.
2. Montoro, D.T., Haber, A.L., Biton, M., Vinarsky, V., Lin, B., Birket, S.E., Yuan, F., Chen, S., Leung, H.M., Villoria, J., et al. (2018). A revised airway epithelial hierarchy includes CFTR-expressing ionocytes. *Nature* 560, 319–324. <https://doi.org/10.1038/s41586-018-0393-7>.
3. Rock, J.R., Onaitis, M.W., Rawlins, E.L., Lu, Y., Clark, C.P., Xue, Y., Randell, S.H., and Hogan, B.L.M. (2009). Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc. Natl. Acad. Sci. USA* 106, 12771–12775. <https://doi.org/10.1073/pnas.0906850106>.
4. Hammad, H., and Lambrecht, B.N. (2008). Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat. Rev. Immunol.* 8, 193–204. <https://doi.org/10.1038/nri2275>.
5. Lambrecht, B.N., and Hammad, H. (2012). The airway epithelium in asthma. *Nat. Med.* 18, 684–692. <https://doi.org/10.1038/nm.2737>.
6. Lambrecht, B.N., and Hammad, H. (2014). Dendritic Cell and Epithelial Cell Interactions at the Origin of Murine Asthma. *Ann. Am. Thorac. Soc.* 11, S236–S243. <https://doi.org/10.1513/AnnalsATS.201405-218AW>.
7. Deckers, J., De Bosscher, K., Lambrecht, B.N., and Hammad, H. (2017). Interplay between barrier epithelial cells and dendritic cells in allergic sensitization through the lung and the skin. *Immunol. Rev.* 278, 131–144. <https://doi.org/10.1111/imr.12542>.
8. Aurora, A.B., and Olson, E.N. (2014). Immune Modulation of Stem Cells and Regeneration. *Cell Stem Cell* 15, 14–25. <https://doi.org/10.1016/j.stem.2014.06.009>.

Figure 7. Stem-immune Notch signaling in the human airway epithelium

(A) Dot plot depicting gene expression levels and percentage of cells expressing genes across MNP clusters, with red box highlighting macrophage populations Mac1 and Mac2.

(B) Gene set enrichment analysis of Mac2 based on an IAM gene set from Engler et al.¹³ FDR, false discovery rate; NES, normalized enrichment score.

(C and D) Dot plot depicting Notch ligand and receptor expression levels and percentage of cells expressing genes across airway epithelial cell clusters (C) and MNP clusters (D).

(E–J) Immunostaining for Notch1 intracellular domain (N1ICD) (E and H), N2ICD (F and I), or N3ICD (G and J) (red), Langerin (white), and CK5 or CK17 (green) in the human small airway of two healthy donors (E–G) or two asthma patients (H and J). Arrows indicate Langerin⁺ cells with Notch activity.

(K and L) Immunostaining for the Notch target genes Hes1 (K) and HeyL (L) (red) with Langerin (white) and CK5 (green) in healthy human airways. Orange arrows point to Hes1⁺ (K) or HeyL⁺ (L) Langerin⁺ cells. Blue arrow points to a Hes1-negative Langerin⁺ cells (K).

(M and N) Immunostaining for Hes1 (M) and HeyL (N) (red) with Langerin (white) and CK5 (green) in asthmatic human airways. Orange arrows point to Hes1⁺ (M) or HeyL⁺ (N) Langerin⁺ cells.

Nuclei, DAPI (blue). Scale bars, 20 μm. Scale bars in insets in (K)–(N), 5 μm. See also Figure S7.

9. Lechner, A.J., Driver, I.H., Lee, J., Conroy, C.M., Nagle, A., Locksley, R.M., and Rock, J.R. (2017). Recruited Monocytes and Type 2 Immunity Promote Lung Regeneration following Pneumectomy. *Cell Stem Cell* 21, 120–134.e7. <https://doi.org/10.1016/j.stem.2017.03.024>.
10. Whitsett, J.A., and Alenghat, T. (2015). Respiratory epithelial cells orchestrate pulmonary innate immunity. *Nat. Immunol.* 16, 27–35. <https://doi.org/10.1038/ni.3045>.
11. Byers, D.E. (2014). Defining the roles of IL-33, thymic stromal lymphopoietin, and IL-25 in human asthma. *Am. J. Respir. Crit. Care Med.* 190, 715–716. <https://doi.org/10.1164/rccm.201408-1539ED>.
12. Gauvreau, G.M., O'Byrne, P.M., Boulet, L.P., Wang, Y., Cockcroft, D., Bigler, J., FitzGerald, J.M., Boedigheimer, M., Davis, B.E., Dias, C., et al. (2014). Effects of an anti-TSLP antibody on allergen-induced asthmatic responses. *N. Engl. J. Med.* 370, 2102–2110. <https://doi.org/10.1056/NEJMoa1402895>.
13. Engler, A.E., Ysasi, A.B., Pihl, R.M.F., Villacorta-Martin, C., Heston, H.M., Richardson, H.M.K., Thapa, B.R., Moniz, N.R., Belkina, A.C., Mazzilli, S.A., and Rock, J.R. (2020). Airway-Associated Macrophages in Homeostasis and Repair. *Cell Rep.* 33, 108553. <https://doi.org/10.1016/j.celrep.2020.108553>.
14. Ysasi, A.B., Engler, A.E., Bawa, P.S., Wang, F., Conrad, R.D., Yeung, A.K., Rock, J.R., Beane-Ebel, J., Mazzilli, S.A., Franklin, R.A., et al. (2024). A specialized population of monocyte-derived tracheal macrophages promote airway epithelial regeneration through a CCR2-dependent mechanism. *iScience* 27, 110169. <https://doi.org/10.1016/j.isci.2024.110169>.
15. Hogan, B.L.M., Barkauskas, C.E., Chapman, H.A., Epstein, J.A., Jain, R., Hsia, C.C.W., Niklason, L., Calle, E., Le, A., Randell, S.H., et al. (2014). Repair and Regeneration of the Respiratory System: Complexity, Plasticity, and Mechanisms of Lung Stem Cell Function. *Cell Stem Cell* 15, 123–138. <https://doi.org/10.1016/j.stem.2014.07.012>.
16. Konkimala, A., Tata, A., and Tata, P.R. (2022). Lung Regeneration: Cells, Models, and Mechanisms. *Cold Spring Harb. Perspect. Biol.* 14, a040873. <https://doi.org/10.1101/cshperspect.a040873>.
17. Hammad, H., and Lambrecht, B.N. (2021). The basic immunology of asthma. *Cell* 184, 1469–1485. <https://doi.org/10.1016/j.cell.2021.02.016>.
18. Menzies-Gow, A., Corren, J., Bourdin, A., Chupp, G., Israel, E., Wechsler, M.E., Brightling, C.E., Griffiths, J.M., Hellqvist, Å., Bowen, K., et al. (2021). Tezepelumab in Adults and Adolescents with Severe, Uncontrolled Asthma. *N. Engl. J. Med.* 384, 1800–1809. <https://doi.org/10.1056/nejmoa2034975>.
19. Wechsler, M.E., Ruddy, M.K., Pavord, I.D., Israel, E., Rabe, K.F., Ford, L. B., Maspero, J.F., Abdulai, R.M., Hu, C.C., Martincova, R., et al. (2021). Efficacy and Safety of Itepekimab in Patients with Moderate-to-Severe Asthma. *N. Engl. J. Med.* 385, 1656–1668. <https://doi.org/10.1056/nejmoa2024257>.
20. Aran, D., Looney, A.P., Liu, L., Wu, E., Fong, V., Hsu, A., Chak, S., Naikawadi, R.P., Wolters, P.J., Abate, A.R., et al. (2019). Reference-based analysis of lung single-cell sequencing reveals a transitional profibrotic macrophage. *Nat. Immunol.* 20, 163–172. <https://doi.org/10.1038/s41590-018-0276-y>.
21. Mould, K.J., Jackson, N.D., Henson, P.M., Seibold, M., and Janssen, W.J. (2019). Single cell RNA sequencing identifies unique inflammatory airspace macrophage subsets. *JCI Insight* 4, e126556. <https://doi.org/10.1172/jci.insight.126556>.
22. Mould, K.J., Moore, C.M., McManus, S.A., McCubbrey, A.L., McClendon, J.D., Griesmer, C.L., Henson, P.M., and Janssen, W.J. (2021). Airspace Macrophages and Monocytes Exist in Transcriptionally Distinct Subsets in Healthy Adults. *Am. J. Respir. Crit. Care Med.* 203, 946–956. <https://doi.org/10.1164/rccm.202005-1989OC>.
23. Deprez, M., Zaragosi, L.E., Truchi, M., Becavin, C., Ruiz García, S., Arguel, M.J., Plaisant, M., Magnone, V., Lebrigand, K., Abelanet, S., et al. (2020). A Single-cell Atlas of the Human Healthy Airways. *Am. J. Respir. Crit. Care Med.* 202, 1636–1645. <https://doi.org/10.1164/rccm.201911-2199OC>.
24. Ural, B.B., Yeung, S.T., Damani-Yokota, P., Devlin, J.C., de Vries, M., Vera-Licona, P., Samji, T., Sawai, C.M., Jang, G., Perez, O.A., et al. (2020). Identification of a nerve-associated, lung-resident interstitial macrophage subset with distinct localization and immunoregulatory properties. *Sci. Immunol.* 5, eaax8756. <https://doi.org/10.1126/sciimmunol.aax8756>.
25. Tang, X.Z., Kreuk, L.S.M., Cho, C., Metzger, R.J., and Allen, C.D.C. (2022). Bronchus-associated macrophages efficiently capture and present soluble inhaled antigens and are capable of local Th2 cell activation. *eLife* 11, e63296. <https://doi.org/10.7554/eLife.63296>.
26. Huh, J.C., Strickland, D.H., Jahnsen, F.L., Turner, D.J., Thomas, J.A., Napoli, S., Tobagus, I., Stumbles, P.A., Sly, P.D., and Holt, P.G. (2003). Bidirectional interactions antigen-bearing respiratory tract dendritic cells (DCs) and T cells precede the late phase reaction in experimental asthma: DC activation occurs in the airway mucosa but not in the lung parenchyma. *J. Exp. Med.* 198, 19–30. <https://doi.org/10.1084/jem.20021328>.
27. Holt, P.G., Schon-Hegrad, M.A., Oliver, J., Holt, B.J., and McMenamin, P. G. (1990). A contiguous network of dendritic antigen-presenting cells within the respiratory epithelium. *Int. Arch. Allergy Appl. Immunol.* 91, 155–159. <https://doi.org/10.1159/000235107>.
28. Loschko, J., Schreiber, H.A., Rieke, G.J., Esterházy, D., Meredith, M.M., Pedicord, V.A., Yao, K.H., Caballero, S., Pamer, E.G., Mucida, D., and Nussenzweig, M.C. (2016). Absence of MHC class II on cDCs results in microbial-dependent intestinal inflammation. *J. Exp. Med.* 213, 517–534. <https://doi.org/10.1084/jem.2016062>.
29. Zhao, R., Fallon, T.R., Saladi, S.V., Pardo-Saganta, A., Villoria, J., Mou, H., Vinarsky, V., Gonzalez-Celeiro, M., Nunna, N., Hariri, L.P., et al. (2014). Yap tunes airway epithelial size and architecture by regulating the identity, maintenance, and self-renewal of stem cells. *Dev. Cell* 30, 151–165. <https://doi.org/10.1016/j.devcel.2014.06.004>.
30. Plasschaert, L.W., Žilionis, R., Choo-Wing, R., Savova, V., Knehr, J., Roma, G., Klein, A.M., and Jaffe, A.B. (2018). A single-cell atlas of the airway epithelium reveals the CFTR-rich pulmonary ionocyte. *Nature* 560, 377–381. <https://doi.org/10.1038/s41586-018-0394-6>.
31. Sui, P., Wiesner, D.L., Xu, J., Zhang, Y., Lee, J., Van Dyken, S., Lashua, A., Yu, C., Klein, B.S., Locksley, R.M., et al. (2018). Pulmonary neuroendocrine cells amplify allergic asthma responses. *Sci. Technol. Humanit.* 360, eaan8546. <https://doi.org/10.1126/science.aan8546>.
32. Branchfield, K., Nantie, L., Verheyden, J.M., Sui, P., Wienhold, M.D., and Sun, X. (2016). Pulmonary neuroendocrine cells function as airway sensors to control lung immune response. *Sci. Technol. Humanit.* 351, 707–710. <https://doi.org/10.1126/science.aad7969>.
33. Schneider, C., O'Leary, C.E., and Locksley, R.M. (2019). Regulation of immune responses by tuft cells. *Nat. Rev. Immunol.* 19, 584–593. <https://doi.org/10.1038/s41577-019-0176-x>.
34. Tata, P.R., Pardo-Saganta, A., Prabhu, M., Vinarsky, V., Law, B.M., Fontaine, B.A., Tager, A.M., and Rajagopal, J. (2013). Airway-specific inducible transgene expression using aerosolized doxycycline. *Am. J. Respir. Cell Mol. Biol.* 49, 1048–1056. <https://doi.org/10.1165/rcmb.2012-0412OC>.
35. Caton, M.L., Smith-Raska, M.R., and Reizis, B. (2007). Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen. *J. Exp. Med.* 204, 1653–1664. <https://doi.org/10.1084/jem.20062648>.
36. Lewis, K.L., Caton, M.L., Bogunovic, M., Greter, M., Grajkowska, L.T., Ng, D., Klinakis, A., Charo, I.F., Jung, S., Gommerman, J.L., et al. (2011). Notch2 receptor signaling controls functional differentiation of dendritic cells in the spleen and intestine. *Immunity (Camb., Mass.)* 35, 780–791. <https://doi.org/10.1016/j.immuni.2011.08.013>.

37. Chakrabarti, R., Celià-Terrassa, T., Kumar, S., Hang, X., Wei, Y., Choudhury, A., Hwang, J., Peng, J., Nixon, B., Grady, J.J., et al. (2018). Notch ligand Dll1 mediates cross-talk between mammary stem cells and the macrophageal niche. *Sci. Technol. Humanit.* 360, eaan4153. <https://doi.org/10.1126/science.aan4153>.
38. Hoshino, N., Katayama, N., Shibasaki, T., Ohishi, K., Nishioka, J., Masuya, M., Miyahara, Y., Hayashida, M., Shimomura, D., Kato, T., et al. (2005). A novel role for Notch ligand Delta-1 as a regulator of human Langerhans cell development from blood monocytes. *J. Leukoc. Biol.* 78, 921–929. <https://doi.org/10.1189/jlb.1204746>.
39. Rock, J.R., Gao, X., Xue, Y., Randell, S.H., Kong, Y.Y., and Hogan, B.L.M. (2011). Notch-dependent differentiation of adult airway basal stem cells. *Cell Stem Cell* 8, 639–648. <https://doi.org/10.1016/j.stem.2011.04.003>.
40. Pardo-Saganta, A., Law, B.M., Gonzalez-Celeiro, M., Vinarsky, V., and Rajagopal, J. (2013). Ciliated cells of pseudostratified airway epithelium do not become mucous cells after ovalbumin challenge. *Am. J. Respir. Cell Mol. Biol.* 48, 364–373. <https://doi.org/10.1165/rcmb.2012-0146oc>.
41. Jacquet, A. (2021). Characterization of Innate Immune Responses to House Dust Mite Allergens: Pitfalls and Limitations. *Front. Allergy* 2, 662378. <https://doi.org/10.3389/falgy.2021.662378>.
42. Guseh, J.S., Bores, S.A., Stanger, B.Z., Zhou, Q., Anderson, W.J., Melton, D.A., and Rajagopal, J. (2009). Notch signaling promotes airway mucous metaplasia and inhibits alveolar development. *Development* 136, 1751–1759. <https://doi.org/10.1242/dev.029249>.
43. Kang, J.H., Kim, B.S., Uhm, T.G., Lee, S.H., Lee, G.R., Park, C.S., and Chung, I.Y. (2009). Gamma-secretase inhibitor reduces allergic pulmonary inflammation by modulating Th1 and Th2 responses. *Am. J. Respir. Crit. Care Med.* 179, 875–882. <https://doi.org/10.1164/rccm.200806-893oc>.
44. Danahay, H., Pessotti, A.D., Coote, J., Montgomery, B.E., Xia, D., Wilson, A., Yang, H., Wang, Z., Bevan, L., Thomas, C., et al. (2015). Notch2 is required for inflammatory cytokine-driven goblet cell metaplasia in the lung. *Cell Rep.* 10, 239–252. <https://doi.org/10.1016/j.celrep.2014.12.017>.
45. Reid, A.T., Nichol, K.S., Chander Veerati, P., Moheimani, F., Kicic, A., Stick, S.M., Bartlett, N.W., Grainge, C.L., Wark, P.A.B., Hansbro, P. M., and Knight, D.A. (2020). Blocking Notch3 Signaling Abolishes MUC5AC Production in Airway Epithelial Cells from Asthmatics. *Am. J. Respir. Cell Mol. Biol.* 62, 513–523. <https://doi.org/10.1165/rcmb.2019-0069oc>.
46. Mou, H., Vinarsky, V., Tata, P.R., Brazauskas, K., Choi, S.H., Crooke, A.K., Zhang, B., Solomon, G.M., Turner, B., Bihler, H., et al. (2016). Dual SMAD Signaling Inhibition Enables Long-Term Expansion of Diverse Epithelial Basal Cells. *Cell Stem Cell* 19, 217–231. <https://doi.org/10.1016/j.stem.2016.05.012>.
47. Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., and Donaldson, D.D. (1998). Interleukin-13: central mediator of allergic asthma. *Sci. Technol. Humanit.* 282, 2258–2261. <https://doi.org/10.1126/science.282.5397.2258>.
48. Alladina, J., Smith, N.P., Kooistra, T., Slowikowski, K., Kernin, I.J., De-guine, J., Keen, H.L., Manakongtreecheep, K., Tantivit, J., Rahimi, R.A., et al. (2023). A human model of asthma exacerbation reveals transcriptional programs and cell circuits specific to allergic asthma. *Sci. Immunol.* 8, eabq6352. <https://doi.org/10.1126/sciimmunol.abq6352>.
49. Chloé-Villani, A., Satija, R., Reynolds, G., Sarkizova, S., Shekhar, K., Fletcher, J., Griesbeck, M., Butler, A., Zheng, S., Lazo, S., et al. (2017). Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Sci. Technol. Humanit.* 356, eaah4573. <https://doi.org/10.1126/science.aah4573>.
50. Geissmann, F., Manz, M.G., Jung, S., Sieweke, M.H., Merad, M., and Ley, K. (2010). Development of monocytes, macrophages, and dendritic cells. *Sci. Technol. Humanit.* 327, 656–661. <https://doi.org/10.1126/science.1178331>.
51. Jakubzick, C.V., Randolph, G.J., and Henson, P.M. (2017). Monocyte differentiation and antigen-presenting functions. *Nat. Rev. Immunol.* 17, 349–362. <https://doi.org/10.1038/nri.2017.28>.
52. Guillems, M., Ginhoux, F., Jakubzick, C., Naik, S.H., Onai, N., Schraml, B. U., Segura, E., Tussiwand, R., and Yona, S. (2014). Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat. Rev. Immunol.* 14, 571–578. <https://doi.org/10.1038/nri3712>.
53. Leach, S.M., Gibbings, S.L., Tewari, A.D., Atif, S.M., Vestal, B., Danhorn, T., Janssen, W.J., Wager, T.D., and Jakubzick, C.V. (2020). Human and Mouse Transcriptome Profiling Identifies Cross-Species Homology in Pulmonary and Lymph Node Mononuclear Phagocytes. *Cell Rep.* 33, 108337. <https://doi.org/10.1016/j.celrep.2020.108337>.
54. Lanone, S., Zheng, T., Zhu, Z., Liu, W., Lee, C.G., Ma, B., Chen, Q., Homer, R.J., Wang, J., Rabach, L.A., et al. (2002). Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling. *J. Clin. Investig.* 110, 463–474. <https://doi.org/10.1172/jci14136>.
55. Grabiec, A.M., Denny, N., Doherty, J.A., Happonen, K.E., Hankinson, J., Connolly, E., Fife, M.E., Fujimori, T., Fujino, N., Goenka, A., et al. (2017). Diminished airway macrophage expression of the Axl receptor tyrosine kinase is associated with defective efferocytosis in asthma. *J. Allergy Clin. Immunol.* 140, 1144–1146.e4. <https://doi.org/10.1016/j.jaci.2017.03.024>.
56. Gschwend, J., Sherman, S.P.M., Ridder, F., Feng, X., Liang, H.E., Locksley, R.M., Becher, B., and Schneider, C. (2021). Alveolar macrophages rely on GM-CSF from alveolar epithelial type 2 cells before and after birth. *J. Exp. Med.* 218, e20210745. <https://doi.org/10.1084/jem.20210745>.
57. Mc, C.C., Carey, B.C., and Trapnell, B.C. (2022). Autoimmune pulmonary alveolar proteinosis. *Am. J. Respir. Crit. Care Med.* 05, 1016–1035. <https://doi.org/10.1164/rccm.202112-2742so>.
58. Snelgrove, R.J., Goulding, J., Didierlaurent, A.M., Lyonga, D., Vekaria, S., Edwards, L., Gwyer, E., Sedgwick, J.D., Barclay, A.N., and Hussell, T. (2008). A critical function for CD200 in lung immune homeostasis and the severity of influenza infection. *Nat. Immunol.* 9, 1074–1083. <https://doi.org/10.1038/ni.1637>.
59. Fang, T.C., Yashiro-Ohtani, Y., Del Bianco, C., Knoblock, D.M., Blacklow, S.C., and Pear, W.S. (2007). Notch directly regulates Gata3 expression during T helper 2 cell differentiation. *Immunity (Camb., Mass.)* 27, 100–110. <https://doi.org/10.1016/j.immuni.2007.04.018>.
60. Amsen, D., Antov, A., Jankovic, D., Sher, A., Radtke, F., Souabni, A., Buslinger, M., McCright, B., Gridley, T., and Flavell, R.A. (2007). Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch. *Immunity (Camb., Mass.)* 27, 89–99. <https://doi.org/10.1016/j.immuni.2007.05.021>.
61. Tindemans, I., van Schoonhoven, A., KleinJan, A., de Bruijn, M.J., Lukkes, M., van Nimwegen, M., van den Branden, A., Bergen, I.M., Corneth, O.B., van IJcken, W.F., et al. (2020). Notch signaling licenses allergic airway inflammation by promoting Th2 cell lymph node egress. *J. Clin. Investig.* 130, 3576–3591. <https://doi.org/10.1172/jci128310>.
62. Aegerter, H., Lambrecht, B.N., and Jakubzick, C.V. (2022). Biology of lung macrophages in health and disease. *Immunity (Camb., Mass.)* 55, 1564–1580. <https://doi.org/10.1016/j.immuni.2022.08.010>.
63. Brooker, R., Hozumi, K., and Lewis, J. (2006). Notch ligands with contrasting functions: Jagged1 and Delta1 in the mouse inner ear. *Development* 133, 1277–1286. <https://doi.org/10.1242/dev.02284>.
64. Khanna, K.M., Blair, D.A., Vella, A.T., McSorley, S.J., Datta, S.K., and Le-françois, L. (2010). T cell and APC dynamics in situ control the outcome of vaccination. *J. Immunol.* 185, 239–252. <https://doi.org/10.4049/jimmunol.0901047>.

65. Vera, L., Garcia-Olloqui, P., Petri, E., Viñado, A.C., Valera, P.S., Blasco-Iturri, Z., Calvo, I.A., Cenzano, I., Ruppert, C., Zulueta, J.J., et al. (2021). Notch3 deficiency attenuates pulmonary fibrosis and impedes lung function decline. *Am. J. Respir. Cell Mol. Biol.* 64, 465–476. <https://doi.org/10.1165/rcmb.2020-0516oc>.
66. Pardo-Saganta, A., Law, B.M., Tata, P.R., Villoria, J., Saez, B., Mou, H., Zhao, R., and Rajagopal, J. (2015). Injury induces direct lineage segregation of functionally distinct airway basal stem/progenitor cell subpopulations. *Cell Stem Cell* 16, 184–197. <https://doi.org/10.1016/j.stem.2015.01.002>.
67. Kwok, S.J.J., Montoro, D.T., Haber, A.L., Yun, S.-H., and Vinarsky, V. (2023). Single-cell transcriptomics of a dynamic cell behavior in murine airways. *eLife* 12, e76645. <https://doi.org/10.7554/elife.76645>.
68. Hegab, A.E., Ha, V.L., Attiga, Y.S., Nickerson, D.W., and Gomperts, B.N. (2012). Isolation of basal cells and submucosal gland duct cells from mouse trachea. *J. Vis. Exp.* 67, e3731. <https://doi.org/10.3791/3731>.
69. Haber, A.L., Biton, M., Rogel, N., Herbst, R.H., Shekhar, K., Smillie, C., Burgin, G., Delorey, T.M., Howitt, M.R., Katz, Y., et al. (2017). A single-cell survey of the small intestinal epithelium. *Nature* 551, 333–339. <https://doi.org/10.1038/nature24489>.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD45.2-AF700	Biologend	Cat# 109822
CD16/CD32	BD Biosciences	Cat# 553141
CD45-FITC	Biologend	Cat# 103108
CD45-PE	Biologend	Cat# 103105
SiglecF-PE	BD Biosciences	Cat# E50-2440
CD11c-BV605	Biologend	Cat# 117333
CD3-BV421	Biologend	Cat# 100335
CD19-BV510	Biologend	Cat# 115545
CD4-BV785	Biologend	Cat# 100551
CD8a-PECy7	Biologend	Cat# 100721
CD69-APC	Biologend	Cat# 104513
CD11b-BV785	Biologend	Cat# 101243
F4/80-APC	Biologend	Cat# 123115
CD64-BV421	Biologend	Cat# 139309
CD26-PE-Cy7	Biologend	Cat# 137809
MHC II I-A/I-E AF647	Biologend	Cat# 107618
EpCAM-Bv421	Biologend	Cat# 118225
GSI β 4 (Griffonia Simplicifolia Isolectin beta4)-Biotin	Sigma	Cat# L2140
rabbit anti-cytokeratin 5	Abcam	Cat# ab53121
mouse anti-FOXJ1	eBioscience	Cat# 14-9965
chicken anti-green fluorescent protein	Aves Labs	Cat# GFP-1020
goat anti-GFP	Novus Biologicals	Cat# NB-100-1770
goat anti-SCGB1A1	Barry Stripp	N/A
goat anti-CC10	Santa Cruz Biotechnology	Cat# sc-9772
mouse IgG1 anti-Muc5ac	Thermo Fisher Scientific	Cat# MS-145
goat anti-Foxa3	Santa Cruz Biotechnology	Cat# sc-5361
rabbit anti-p63	Biologend	Cat# 619002
mouse anti-tubulin, acetylated	Sigma	Cat# T6793
rat anti-CD45	eBioscience	Cat# 14-0451
Syrian hamster anti-podoplanin	Developmental Studies Hybridoma Bank	Cat# 8.1.1
goat anti-mCherry	SicGEN	Cat# AB0040-200
rabbit anti-BSND	Abcam	Cat# ab242408
rabbit anti-BSND	Abcam	Cat# ab196017
rabbit anti-Trmp5	Alomone	Cat# ACC-045
rabbit anti-Gng13	Abcam	Cat# ab126562
rabbit anti-UCHL1/PGP9.5	Proteintech	Cat# 14730-1-AP
anti-mouse CD11c-PE	Biologend	Cat# 117308
anti-mouse CD11c-APC	eBioscience	Cat# 17-0114-81
anti-mouse MHC-II I-A/I-E 488	Biologend	Cat# 107615
anti-mouse MHC-II I-A/I-E AF647	Biologend	Cat# 107618
anti-mouse F4/80 PE	Biologend	Cat# 123109
anti-mouse CD11b-APC	eBioscience	Cat# 17-0112-81

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
anti-mouse CD11b-PE	Thermo Fisher Scientific	Cat# 12-0112-82
rat anti-SiglecF	BD Pharmingen	Cat# 565527
anti-mouse SiglecF-AF647	BD Biosciences	Cat# 562680
rat anti-Ki67	eBioscience	Cat# 145698
rabbit anti-caspase 3	Cell Signaling	Cat# 9661S
rat anti-F4/80	Biolegend	Cat# 123101
anti-CD172 (SIRPα) Alexa 594	Biolegend	Cat# 144020
anti-mouse SIRPα PE	Biolegend	Cat# 144011
rat anti-RBPJk	CosmoBio	Cat# 2ZRBP2
mouse anti-ZO1	Thermo Fisher Scientific	Cat# 33-9100
mouse anti-occludin	Thermo Fisher Scientific	Cat# 33-1500
mouse anti-claudin1	Thermo Fisher Scientific	Cat# 37-4900
rabbit anti-RFP	Abcam	Cat# ab240297
rat anti-Langerin/CD207	Dendritics	Cat# DDX0362P-100
rat anti-C1q	Abcam	Cat# ab11861
anti-mouse Mertk FITC	Biolegend	Cat# 151503
rabbit anti-Notch2	Cell Signaling	Cat# D67C8
mouse anti-Notch3	Santa Cruz Biotechnology	Cat# sc-515825
donkey anti-Rabbit-HRP conjugated	Thermo Fisher Scientific	Cat# 31458
donkey anti-mouse-HRP conjugated	Life Technologies	Cat# A16011
Rabbit anti-Notch1ICD	Abcam	Cat# ab8925
Mouse anti-Hes1	Abcam	Cat# ab119776
Rabbit anti-HeyL	Invitrogen	Cat# PA5-85014
Rabbit IgG Isotype	Thermo Fisher Scientific	Cat# 02-6102
Mouse IgG Isotype	Thermo Fisher Scientific	Cat# 10400C
chicken anti-CK5	Biolegend	Cat# 905901
rabbit anti-p63	Abcam	Cat# ab124762
sheep anti-integrin beta 5 (ITGB5)	R&D	Cat# AF8035
Bacterial and virus strains		
One Shot™ Stbl3™ Chemically Competent <i>E. coli</i>	Thermo Fisher Scientific	Cat# C737303
Lentiviral vectors KO.1 sh mDII1	This study	N/A
Lentiviral vectors mDII1	This study	N/A
Biological samples		
Human healthy lung tissue	DZL Biobank Giessen	N/A
Human healthy and asthmatic lung tissue	Mass General Brigham	N/A
Chemicals, peptides, and recombinant proteins		
Doxycycline	Sigma-Aldrich	Cat# D9891
4-hydroxytamoxifen	Sigma-Aldrich	Cat# H6278
Tamoxifen	Sigma-Aldrich	Cat# T5648
D-PBS	Gibco	Cat# 14190-094
Albumin from chicken egg white (Ovalbumin (grade V))	Sigma-Aldrich	Cat# A5503
Aluminium hydroxide hydrate	Sigma-Aldrich	Cat# A1577
Recombinant murine IL-13	PeptoTech	Cat# 210-13
RPMI 1640 medium, GlutaMAX™ supplement	Gibco	Cat# 61870036
Liberase TM Research Grade	Roche Applied Science	Cat# 5401119001
DNase I	Sigma-Aldrich	Cat# 10104159001
ACK RBC lysis buffer	Sigma	Cat# R7757
Viability staining	eBioscience	Cat# 65-0865-14

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Paraformaldehyde (PFA) Solution, 4% in PBS	Thermo Fisher Scientific	Cat# J19943-K2
FBS Fetal Bovine Serum, Qualified	Gibco	Cat# 10437-028
Streptavidin APC	Biolegend	Cat# 405207
TRI Reagent	Sigma	Cat# T9424
Sucrose	Sigma-Aldrich	Cat# S1888
O.C.T Compound	Thermo Fisher Scientific	Cat# 23-730-571
Harris Hematoxylin solution	PanReacAppliChem	Cat# 2539491211
Eosin Yellowish hydroalcoholic solution 1%	PanReacAppliChem	Cat# 2513011211
Alcian Blue solution	Sigma-Aldrich	Cat# B8438
Citric acid ACS reagent >99.5%	Sigma-Aldrich	Cat# 251275
Sodium citrate tribasic dihydrate	Sigma-Aldrich	Cat# S4641
Hydrogen peroxide solution	Sigma-Aldrich	Cat# H1009
Triton X-100	Sigma-Aldrich	Cat# T8787
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat# A9647
streptavidin-488	Life Technologies	Cat# S-32354
DAPI	Sigma-Aldrich	Cat# D9542
SYBR Green	Invitrogen	Cat# S33102
SAGM	Lonza	Cat# CC-3118
Rock inhibitor Y-27632	Selleckbio	Cat# S1049
Rock inhibitor Y-27632	Tocris	Cat# 1254
6.5 mm ² Transwell® with 0.4 µm Pore Polyester Membrane Insert, Sterile	Corning	Cat# 3470
PneumaCult-ALI Medium	StemCell	Cat# 05001
KAPA2G Fast HotStart ReadyMix	Kapa Biosystems	Cat# KK5608
Spel	New England Biolabs	Cat# R3133
EcoRI	New England Biolabs	Cat# R3101
Gelfoam Sterile Sponge	Pfizer	Cat# GTIN 00300090342104 (0009-0342-10)
DMEM/F12 (1X)	Gibco	Cat# 11320-033
0.25% Trypsin-EDTA (1X)	Gibco	Cat# 25200-056
PenStrep	Gibco	Cat# 15140-122
HEPES (1M) Buffer solution	Gibco	Cat# 15630-080
Primocin	InVivoGen	Cat# ant-pm-1
DAPT	Tocris	Cat# TB2634-RMU
DBZ	Calbiochem	Cat# 565789
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich	Cat# D2650
A-8301	Tocris	Cat# 2939
DMH-1	Tocris	Cat# 4126
CHIR99021	Tocris	Cat# 4423
Dithiothietol (DTT)	Sigma	Cat# D0632
TrypLE select	Gibco	Cat# 12563-029
0.5M EDTA pH8.0	Thermo Fisher Scientific	Cat# AM9260G
UltraPure Distilled Water (Dnase/Rnase Free)	Gibco	Cat# 10977-035
Critical commercial assays		
DeadEnd™ Fluorometric TUNEL System	Promega	Cat# G3250
Tyramide signal amplification fluorescein Kit	PerkinElmer	Cat# NEL701A001KT
Tyramide signal amplification biotin Kit	PerkinElmer	Cat# NEL700A001KT
RNeasy Micro Kit	QIAGEN	Cat# 74004

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
QuantiTect Reverse Transcription Kit	QIAGEN	Cat# 205311
Papain Dissociation System	Worthington Biochemical Corporation	Cat# LK003150
Monocyte Isolation Kit (BM), mouse	Miltenyi Biotec	Cat# 130-100-629
Deposited data		
Single-cell RNA-seq data mouse trachea	Montoro et al. ²	GSE103354
Single-cell RNA-seq data mouse trachea	Engler et al. ¹³	GSE152501
Single-cell RNA-seq data human lung	Alladina et al. ⁴⁸	GSE193816
Experimental models: Cell lines		
HEK293T cells	N/A	N/A
804G rat bladder cell line	N/A	N/A
Primary airway basal cells	N/A	N/A
Purified murine bone-marrow monocytes	N/A	N/A
Experimental models: Organisms/strains		
CK5-rtTA	Pardo-Saganta et al. ¹	N/A
tet(O)cre	Jackson Laboratory	Cat# 006224
CK5-creER	Pardo-Saganta et al. ¹	N/A
Rosa26R-EYFP	Jackson Laboratory	Cat# 006148
Rosa26R-CAG- <i>Isl</i> -tdTomato	Jackson Laboratory	Cat# 007909
Zbtb46-Cre	Jackson Laboratory	Cat# 028538
Rosa26R-mTmG	Jackson Laboratory	Cat# 007576
CD11c-Cre	Jackson Laboratory	Cat# 008068
p63 ^{fl/fl}	Zhao et al. ²⁹	N/A
Jag2 ^{fl/fl}	Pardo-Saganta et al. ¹	N/A
Dll1 ^{fl/fl}	Brooker et al. ⁶³	N/A
Dll1-mCherry	Chakrabarti et al. ³⁷ Yibin Kang (Princeton)	N/A
CD11c-mCherry	Khanna et al. ⁶⁴	N/A
Rosa26R- <i>Isl</i> -DTA	Jackson Laboratory	Cat# 009669
Cx3cr1-GFP; Ccr2-RFP	Jackson Laboratory	Cat# 032127
Cx3cr1-CreER	Jackson Laboratory	Cat# 021160
RBPJk ^{fl/fl}	Pardo-Saganta et al. ¹	N/A
CBF:H2B-Venus	Jackson Laboratory	Cat# 020942
FoxJ1-Cre	Mouse Genome Informatics (MGI)	Cat# MGI 3797103
C57Bl/6	Jackson Laboratory	Cat# 000664
Oligonucleotides		
Gapdh F	Invitrogen – Thermo Fisher Scientific	AACCTTTGGCATTGTGGAAGG
Gapdh R	Invitrogen – Thermo Fisher Scientific	ACACATTGGGGGTAGGAACA
β-2-Microglobulin F	Invitrogen – Thermo Fisher Scientific	CTGACCGGCCTGTATGCTAT
β-2-Microglobulin R	Invitrogen – Thermo Fisher Scientific	CAGTCTCAGTGGGGGTGAAT
Dll1 F	Invitrogen – Thermo Fisher Scientific	TTAGCATCATTGGGGCTACC
Dll1 R	Invitrogen – Thermo Fisher Scientific	TAAGTGTGGGGCGATCTTC
mDll1-SpeI	Invitrogen – Thermo Fisher Scientific	GTAAGTAGTatgggcgcgcggagc
mDll1-EcoRI	Invitrogen – Thermo Fisher Scientific	AGCGAATTcttacacctcagtcgctataac
Recombinant DNA		
MISSION® shRNA Dll1 NM_007865	SIGMA	NM_007865
pLKO.1	Addgene	Plasmid 10878
mDll1	OriGene	Plasmid MC221075
pSin-EF2-Nanog-Pur	Addgene	Plasmid 16578

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
FlowJo software (version 10.8)	NIH	https://www.flowjo.com
FIJI	NIH	https://fiji.sc
GraphPad Prism	Graph Pad	https://www.graphpad.com
igraph R package	R software	https://github.com/igraph/igraph
fgsea (v1.24.0, R)	Bioconductor	https://bioconductor.org/packages/release/bioc/html/fgsea.html

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

CK5-rtTA,¹ *tet(O)cre* (JAX 006224), *CK5-creER*,¹ *LSL-YFP* (JAX 006148), *TdTomato* (JAX7909), *Zbtb46-Cre* (JAX028538), *mTmG* (JAX007576), *CD11c-Cre* (JAX008068), *p63*^{fl/fl},²⁹ *Notch2*^{fl/fl} (JAX 010525), *Notch3* KO (JAX 010547), *Jag2*^{fl/fl},¹ *Dll1*^{fl/fl},⁶³ *Dll1-mCherry*,³⁷ *CD11c-mCherry*,⁶⁴ *Rosa DTA* (JAX 009669), *Cx3cr1-GFP;Ccr2-RFP* (JAX 032127), *Cx3cr1-CreER* (JAX 021160), *RBPJk*^{fl/fl},¹ *CBF:H2B-Venus* (JAX 020942) and *C57BL6/J* (JAX 000664) mice were previously described. *CK5-rtTA* and *tet(O)cre* mice were crossed to generate *CK5-rtTA;tet(O)cre* mice. *CK5-rtTA;tet(O)cre* mice were crossed with *p63*^{fl/fl} mice to generate basal stem cell specific *CK5-rtTA;tet(O)Cre;p63*^{fl/fl} conditional knockout mice upon doxycycline administration. Doxycycline administration was performed through drinking water (1 mg/mL) for 2 weeks as described previously.²⁹ Inhaled doxycycline (iDox) (50mg/ml in PBS) was administered by aerosol nebulization for three consecutive days as described.³⁴ *CX3CR1-CreER* mice were crossed with *Rosa-DTA* mice. Cre activation in *CX3CR1-CreER*; *Rosa-DTA* mice was achieved through seven consecutive days of administering 4-hydroxytamoxifen (4-i) (2μM) intranasally as two drops of 30 μL each. To allow for lineage tracing, *RBPJk*^{fl/fl} and *Notch2*^{fl/fl} mice were crossed with *mTmG* and *TdTomato* mice, respectively, then mated to *CX3CR1-CreER* mice. Tamoxifen was administered by intraperitoneal injection (i.p.) (2 mg per day) for five consecutive days to induce the cre-mediated recombination and the expression of the reporter. *CK5-creER*; *LSL-YFP* mice were crossed with *Dll1*^{fl/fl} and *Jag2*^{fl/fl} mice to generate basal cell specific *CK5-creER*; *LSL-YFP*; *Dll1*^{fl/fl} and *CK5-creER*; *LSL-YFP*; *Jag2*^{fl/fl} conditional knockout mice. *CK5-creER*; *LSL-YFP*; *Dll1*^{fl/fl} and *CK5-creER*; *LSL-YFP*; *Jag2*^{fl/fl} mice were then treated, with 2mg of i.p. tamoxifen (Tam) per day for 2 consecutive days. Mice were sacrificed 10 days after the last tamoxifen injection.

Mice aged 6–10 weeks old were used for experiments with similarly aged mice used for both control and treated animals. Litter-mate controls that lacked the Cre driver were used in all the experiments with the exception of Tam-treated *CK5-creER*; *LSL-YFP*; *Jag2*^{+/+} mice, intranasal 4-OHT-treated *C57BL6* for *Cx3cr1-CreER*; *R26-DTA*^{fl/+} mice, Tam-treated *Cx3cr1-CreER*; *mTmG*; *RBPJk*^{+/+} for *Cx3cr1-CreER*; *mTmG*; *RBPJk*^{fl/fl} mice, and *CX3CR1-CreER*; *Rosa26-TdTomato* as control for *Cx3cr1-CreER*; *Rosa26-TdTomato*; *Notch2*^{fl/fl}. *Dll1-mCherry* mice were kindly provided by Yibin Kang (Princeton) and tracheas were harvested with no previous treatment and processed as indicated. *CD11c-mCherry* mice were provided by Kamal Khanna (NYU) and were crossed with *CBF:H2B-Venus* mice and harvested with no treatment. We analyzed at least 3–7 mice per condition in each experiment and all the experiments were repeated twice or three times as indicated for each case in figure legends. All procedures and protocols were approved by the MGH Subcommittee on Research Animal Care in accordance with NIH guidelines and by the Animal Care Committee of the University of Navarra.

Human samples

Human lung tissue was obtained from the Division of Pulmonary and Critical Care Medicine (Massachusetts General Brigham) and from explanted lungs through the UGMLC Giessen Biobank in compliance with the current regulation for the use of human samples and guaranteeing ethical and legal documentation, data protection and all the procedures associated with quality control and data management. Informed consent was obtained in written form from each subject and all procedures were approved by the Ethical Committee at Justus Liebig University and by the Massachusetts General Brigham Institutional Review Board.

METHOD DETAILS

Mouse model of allergic airway inflammation

Acute allergic airway inflammation using OVA (grade V, Sigma-Aldrich, St. Louis, MO) was induced in mice as previously described.⁴⁰ Briefly, mice were immunized with two i.p. injections of 10 mg OVA bound to 1 mg aluminum hydroxide (alum; Sigma-Aldrich) in 0.5 mL PBS on days 1 and 7. Starting on day 14, mice were challenged by aerosol nebulization with 10 mg/mL OVA in PBS or PBS alone (control mice) for 20 min daily for 3 consecutive days. Mice were sacrificed 24h after the last aerosol challenge for the analysis of immune cell populations or 48h after the last aerosol challenge for the analysis of mucous metaplasia.

***In vivo* induction of mucous metaplasia**

CK5-p63 mice received 2 μ g of recombinant murine IL-13 (PeproTech #210-13), for three consecutive days intranasally following Doxycycline administration in the drinking water for 14 days. They were harvested 48h after the last IL-13 challenge.

Cell dissociation, FACS, and flow cytometry analysis

To exclude intravascular leukocytes from analysis of lung homogenates, mice were administered of 3 μ g of CD45-AF700 antibody (Biolegend #109822) diluted in 100 μ L sterile saline by retro-orbital injection 3 min prior to euthanasia. Lungs were cut into pieces and then digested for 45 min in RPMI 1640 with 0.28 U/ml Wunsh U/ml Liberase TM (Roche Applied Science) and DNase (30 U/ml; Sigma-Aldrich) at 37°C to extract leukocytes from lung tissue. The digested tissues were then strained through a 70- μ m filter before ACK RBC lysis (Sigma #R7757). Samples were blocked with purified CD16/CD32 mAb (1:100, 553141, BD Biosciences) and then stained with the following fluorescently labeled antibodies: CD45-FITC (103108, Biolegend), SiglecF-PE (562068, BD Biosciences), CD11c-BV605 (117333, Biolegend), CD3-BV421 (100335, Biolegend), CD19-BV510 (115545, Biolegend), CD4-BV785 (100551, Biolegend), CD8a-PECy7 (100721, Biolegend), and CD69-APC (104513, Biolegend). Afterward samples underwent viability staining (eBioscience #65-0865-14) followed by fixation in paraformaldehyde (PFA). Flow cytometry was performed on an BD CytoflexS analytical flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (version 10.8).

Tracheas from CX3CR1-TdTomato and CX3CR1-RBPJk mice were cut into pieces and then processed to a single-cell suspension using the same protocol as used for lungs. Samples were blocked with purified CD16/CD32 mAb (1:100, 553141, BD Biosciences) and then stained with fluorescently labeled antibodies including CD45-FITC (Biolegend #103108), CD11b-BV785 (Biolegend #101243), F4/80-APC (Biolegend #123115) or CD11c-APC (Biolegend #117310), CD64-BV421 (Biolegend #139309), CD26-PE-Cy7 (Biolegend #137809 Clone H194-112), and MHC II I-A/I-E AF647 (Biolegend 107618, M5/114.15.2).

Airway epithelial basal cells from trachea and major bronchi of CK5-DII1 mice were isolated using papain as described^{1,40} and stained with EpCAM-Bv421 (1:100; 118225, Biolegend), GSI β 4 (Griffonia Simplicifolia Isolectin beta4)-Biotin (1:250; L2140, Sigma) for 30 min in 2.5% FBS in PBS on ice followed by an incubation with streptavidin APC (1:500; 405207, Biolegend) for 10 min in 2.5% FBS in PBS on ice. After washing, EpCAM+ GSI β 4+ basal cells were gated and eYFP+ and eYFP- basal cells were sorted out using a FACS Aria flow cytometer (BD, San Jose, CA). Sorted cells were lysed immediately in TRI Reagent (Sigma) and RNA was extracted as previously described.^{1,40}

Sectioned tissue preparation, immunohistochemistry, and immunofluorescence

Mouse tracheas were removed using sterile technique and then fixed in 4% PFA for 1–2 h at 4°C, washed with PBS, and transferred to a 30% sucrose solution overnight. For immunofluorescence, airways were embedded in OCT and cryosectioned as longitudinal 7 μ m sections. Hematoxylin-eosin and Alcian blue stainings were performed on frozen sections following standard protocols. Cryosections were stained with the previously described protocol described.^{1,41} The following antibodies were used: rabbit anti-cytokeratin 5 (1:1000; ab53121, Abcam); mouse anti-FOXJ1 (1:500; 14–9965, eBioscience); chicken anti-green fluorescent protein (1:500; GFP-1020, Aves Labs); goat anti-GFP (1:100; NB-100-1770, Novus Biologicals); goat anti-SCGB1A1 (1:500; kindly provided by Barry Stripp); goat anti-CC10 (1:100; sc-9772, Santa Cruz Biotechnology); mouse IgG1 anti-Muc5ac (1:250; MS-145, Thermo Fisher Scientific); goat anti-Foxa3 (1:100; sc-5361, Santa Cruz Biotechnology); rabbit anti-p63 (1:100; 619002, Biolegend); mouse anti-tubulin, acetylated (1:100; T6793, Sigma), rat anti-CD45 (1:100; 14–0451, eBioscience); Syrian hamster anti-podoplanin (T1 α) (1:50. 8.1.1, Developmental Studies Hybridoma Bank); goat anti-mCherry (1:500, AB0040-200, SicGEN); rabbit anti-BSND (1:200; ab242408, abcam); rabbit anti-Trmp5 (1:200; ACC-045, Alomone); rabbit anti-Gng13 (1:100; ab126562, abcam); rabbit anti-Pgp9.5 (1:200; 14730-1-AP, Proteintech); anti-mouse CD11c-PE (1:100, 117308, Biolegend), anti-mouse CD11c-APC (1:100, 17-0114-81, eBioscience), anti-mouse MHC II I-A/I-E 488 (1:200, 107615, Biolegend), anti-mouse CD11b-APC (1:100, 17-0112-81, eBioscience), anti-mouse CD11b-PE (1:100, 12-0112-82, Thermo Fisher Scientific), rat anti-SiglecF (1:100, 565527, BD Pharmingen), rat anti-Ki67 (1:200, 145698, eBioscience), rabbit anti-caspase 3 (1:100, 9661S, Cell Signaling), rat anti-F4/80 (1:100, 123101, Biolegend), anti-CD172 (SIRP α) Alexa 594 (1:100, 144020, Biolegend), rat anti-RBPJk (1:200, 2ZRBP2, CosmoBio), mouse anti-ZO1 (1:100, 33–9100, Thermo Fisher Scientific), mouse anti-occludin (1:100, 33–1500, Thermo Fisher Scientific), mouse anti-claudin1 (1:100, 37–4900, Thermo Fisher Scientific), rabbit anti-RFP (1:100, ab240297, abcam), rat anti-Langerin/CD207 (1:100, DDX0362P-100, Dendritics), rat anti-C1q (1:100, ab11861, abcam), anti-Merk FITC (1:50, 151503, Biolegend). Appropriate secondary antibodies (Life Technologies' Alexa Fluor series 488, 594, or 647) were diluted 1:500. Apoptosis was also measured by TUNEL staining (DeadEnd Fluorometric TUNEL System, Promega) following manufacturer's instructions. Immunofluorescent detection of Notch2 intracellular domain (N2ICD)¹ and Notch3 intracellular domain (N3ICD)⁶⁵ required signal amplification for rabbit anti-Notch2 (1:1500; D67C8, Cell Signaling) and mouse anti-Notch3 (1:1500; sc-515825, Santa Cruz Biotechnology). Antigen retrieval was performed using a pressure cooker for 2 h and a citrate buffer with a pH adjusted to 6.2 (for Notch2) and 6.3 (for Notch3). Endogenous peroxidases were then blocked by incubation for 10 min in 3% H₂O₂ and primary antibodies incubated overnight in PBS 0.1% Triton X-100 and 3% BSA. Following primary antibody incubation, sections were washed and incubated with donkey anti-Rabbit-HRP conjugated (1:500; 31458, Thermo Fisher Scientific) or donkey anti-mouse-HRP conjugated (1:500; A16011, Life Technologies) in PBS 0.1% Triton X-100 and 3% BSA for 2 h at room temperature followed by tyramide signal amplification (PerkinElmer) biotin conjugated in the case of Notch2 (NEL700A001KT) or fluorescein conjugated in the case of Notch3 (NEL701A001KT). Sections were then washed and incubated for 30 min at room temperature with streptavidin-488 (1:500; S-32354, Life Technologies) in the case of Notch2 staining.

Immunodetection of rabbit anti-Notch1ICD (1:1500, ab8925, abcam), mouse anti-Hes1 (1:1500, ab119776, abcam) and rabbit anti-HeyL (1:1500, PA5-85014, Invitrogen) was performed following the same protocol used for N2ICD. N2ICD staining represents nuclear and non-nuclear localized portions of the ICD as previously described.⁶⁶ Rabbit IgG Isotype (#02-6102, Thermo Fisher Scientific) and Mouse IgG Isotype (10400C, Thermo Fisher Scientific) control antibodies were used as control. Further details concerning validation and optimized protocols for detection of Notch1, 2 and 3 ICD can be found in prior publications^{1,65,66} and <https://www.rajabopallab.com/lab-protocols>.

Wholemount tissue preparation and staining

Tracheas were isolated and a ventral incision was made followed by excision of 10 cartilaginous rings using a dissecting microscope. Tissues were fixed in 4% paraformaldehyde for 15 min at room temperature. For analysis of epithelial cell populations, antigen retrieval was performed by incubating in citrate buffer pH 6 in a 95°C heat block for 15 min. Tracheas were blocked for 60 min in PBS with 0.3% Triton X-100 and 1% BSA. Samples were then incubated with primary antibodies at 4°C overnight. The following antibodies were used: chicken anti-CK5 (1:400, 905901, Biolegend), rabbit anti-p63 (1:400, ab124762, Abcam), rabbit anti-UCHL1/PGP9.5 (1:400, 14730-1-AP, Proteintech), rabbit anti-BSND (1:400, ab196017, Abcam), rabbit anti-GNG13 (1:200, ab126562, Abcam), mouse-anti Muc5ac (1:250; MS-145, Thermo Fisher Scientific), sheep anti-integrin beta 5 (ITGB5) (15 µg/mL, AF8035, R&D). Following primary antibody incubations, tracheas were washed and incubated with compatible secondaries diluted to 1:400 (Life Technologies' Alexa Fluor series 488 or 594) in PBS 0.3% Triton X-100 and 1% BSA for 2 h at room temperature. Specimens were then washed for at least 4 h and flat mounted onto slides.

To stain for IAMs, tracheas underwent the same process of sample isolation and fixation, but no antigen retrieval was used. Specimens were blocked in PBS 0.3% Triton X-100 and 1% BSA for 60 min. Tracheas were then stained with a combination of directly conjugated CD11c PE (1:50, 117308, Biolegend) and MHC II I-A/I-E AF488 or AF647 (1:50, 107615 or 107618, Biolegend) F4/80 PE (1:50, 123109, Biolegend), SIRPα PE (1:50, 144011, Biolegend) overnight at 4°C. Afterward, samples were washed for several hours and then flat mounted onto slides. When staining for eosinophils by wholemount, SiglecF-AF647 (1:30, 562680, BD) was combined with CD11c and MHC II-AF488 staining as above.

Microscopy and imaging

Tissue was imaged using an Olympus FluoView FV10i confocal microscope (Olympus Corporation), an IX7181 (Olympus), an FV3000 confocal microscope (Olympus), an AxioImager.M1 (Zeiss), a LSM780 confocal microscope (Zeiss) and a LSM800 confocal microscope (Zeiss). We performed two methods to quantify immune cells. The first method to quantify cells was on sectioned tracheas. Cells were manually counted based on immunofluorescence staining of markers for each of the respective cell types.^{1,40} Briefly, cell counting was performed on the basis of nuclear staining with DAPI (nuclei) and specific cell markers. Cells were counted using 40x or 20x magnification fields (each field represented 250 µm or 500 µm of epithelium, respectively) covering the whole tracheal epithelium, from cartilage ring 1 to 12, of each mouse. For CK5-p63 baseline experiments, between 10 and 15 fields per trachea were counted; for OVA-treated CK5-p63, between 15 and 17 fields per trachea were counted; for CK5-DII1 baseline, between 7 and 12 fields per trachea were counted; for OVA-treated CK5-DII1, between 9 and 12 fields per trachea were counted; for CK5-Jag2 baseline, between 11 and 13 fields per trachea were counted; for CX3CR1-RBPJk baseline, 8 fields per trachea were counted; for OVA-treated CX3CR1-RBPJk, between 10 and 20 fields per trachea were counted. The second method to quantify cells was through using tracheal wholemount preparations. For analysis of epithelial cell populations, all antibody staining was observed to be isolated to the epithelial layer. The entire surface of wholemounted tracheas was imaged using a 10X objective to tilescan z-stacks containing the epithelium followed by image flattening through maximal intensity projection (MIP). Cells were counted on flattened MIP images, and the tracheal surface area was measured. Rare cells were counted manually with the exception of GNG13+ Tuft cells for which there were several thousand per trachea and a script was written to perform automated cell counting based on the "Find Maxima" function in ImageJ/Fiji (available upon request).

For analysis of intraepithelial myeloid populations, there was a substantial amount of mesenchymal cell staining such that different counting methods were required. Tracheal z-stacks were obtained through the epithelium using 20X objectives covering the majority of central, non-membranous portions of the wholemount preparations. The boundary between the epithelium and subepithelium was determined based on collagen autofluorescence of basement membrane fibers. Cells located within the epithelial layer were manually counted within the z stack over each 20X field, representing an approximate surface area of 425 µm by 425 µm. Dual CD11c⁺/MHC II⁺ staining was used to initially quantify IAMs. Subsequently, targeting efficiency of *CX3CR1-CreER* was calculated by counting the fraction of MHC II⁺ intraepithelial cells that were tdTomato labeled. In following *CX3CR1-CreER* based experiments, Lineage⁺ IAM were defined as mGFP⁺ cells within the epithelium and scored according to MHC II⁺ staining. Images were processed and analyzed using ImageJ/Fiji (NIH).

Time-lapse microscopy

Tracheas from *CD11c-mCherry* reporter mice were explanted and sutured into a custom imaging platform as previously described.⁶⁷ In brief, the trachea was affixed to a silicone o-ring which was placed on top of an inverted transwell insert (Corning #3470) attached to a 60mm tissue culture dish with PDMS. This dish was placed in a CO₂ and temperature controlled live imaging chamber (TokaiHit) on the microscope stage. DMEM/F12 media supplemented with Primocin (InvivoGen) and 15mM HEPES contacted the explant from

below through the ALI membrane. Time lapse imaging over 25 min was performed using an Olympus FVMPE-RS multiphoton laser scanning microscope equipped with a MaiTai HPDS-O IR pulsed laser and INSIGHT X3-OL IR pulsed laser using a 25 X water immersion lens (NA 1.05). Images were exported into ImageJ for processing to generate maximum intensity projections from z-stacks spanning the epithelial layer to best capture all IAM processes.

RNA extraction and quantitative RT-PCR

Total RNA was extracted from sorted cell populations as previously described.^{1,40} *Dll1* deletion was confirmed extracting total RNA from lineage labeled (YFP+) and YFP negative basal cells isolated from Tam-treated *CK5-creER;LSL-YFP;Dll1^{fl/fl}* mice. The comparative Ct method for relative quantification of gene expression was used ($2^{\Delta\Delta Ct}$, where ΔCt represents the difference in threshold cycle between the target and control genes). For control genes, the geometric mean of cycle thresholds for GAPDH and β -2-microglobulin was used. Otherwise, data was normalized to GAPDH mRNA levels. The following primers were used: *Gapdh*: Forward 5' AACTTTGGCATTGTGGAAGG 3' and reverse 5' ACACATTGGGGGTAGGAACA 3'; *β -2-Microglobulin*: Forward 5' CTGACCGGCCTGTATGCTAT 3' and reverse 5' CAGTCTCAGTGGGGGTGAAT 3'; *Dll1*: Forward 5' TTAGCATCATTGGGGCTACC 3' and reverse 5' TAAGTGTGGGGCGATCTTC 3'.

Cell culture and viral transduction

Mouse tracheal epithelial cells were isolated using papain for tissue dissociation and stained for EpCAM and GSI β 4 to sort them as previously described.^{1,40} Cells were cultured and expanded in complete SAGM (small airway epithelial cell growth medium; Lonza, CC-3118) containing TGF- β /BMP4/WNT antagonist cocktails and 5mM Rock inhibitor Y-27632 (Selleckbio, S1049). To initiate air-liquid interface (ALI) cultures, airway basal cells were dissociated and seeded onto transwell membranes. After confluence, media was removed from the upper chamber. Mucociliary differentiation was performed with PneumaCult-ALI Medium (StemCell, 05001). Differentiation of airway basal cells on an air-liquid interface was followed by directly visualizing beating cilia in real time after 8 days. Mouse basal cells were infected with lentiviral vectors carrying shRNAs targeting mouse *Dll1*. Four different clones were obtained from Sigma (MISSION® shRNA *Dll1* NM_007865, clones TRCN0000028890, TRCN0000028865, TRCN0000028845, TRCN0000028864, TRCN0000028910), and cloned into pLKO.1 vector (Addgene Plasmid 10878). Murine *Dll1* was amplified from the plasmid MC221075 (OriGene) using a high fidelity Taq polymerase and the following primers: *Forward* 5'-GTAAC TAGTatggcgccgtcggagc-3'; *Reverse*: 5'-AGCGAATTCttacacctcagtcgtataac-3', to be next cloned into pSin-EF2-Nanog-Pur (Addgene Plasmid 16578) following Spel and EcoRI digestion. Insert into the plasmid was confirmed by PCR and sequencing. Lentiviral production was performed in HEK293 cells following standard protocols. Concentrated viruses were used at a MOI of 6 to infect murine basal cells for 9h at 37C in 5% CO₂. Basal cells were selected by puromycin administration in the case of *Dll1* overexpression, or by GFP after FACS sorting in the case of *Dll1* inhibition and then the cells were allowed to grow to confluence before being transferred onto transwell membranes. 24d (*Dll1* overexpression) or 26d (*Dll1* inhibition) after ALI initiation, cells were washed, fix with 4% PFA and immunostained to detect the development of mucous cells. For *Dll1* inhibition experiments, 20d after ALI initiation mucous cell formation was induced by administering recombinant murine IL-13 (20ng/ml; 210-13, PeproTech) for 6d. To test initially the efficiency of m*Dll1* overexpression and shRNA *Dll1*, non-purified infected cells were collected 72h after infection and lysed in TRI Reagent for analysis of *Dll1* expression.

Notch inhibition on an explant system

Tracheas were excised from mice using a dissecting microscope as described above for wholemount harvesting. However, in addition to a longitudinal ventral incision, each trachea was divided into two equal halves with another longitudinal dorsal incision along the posterior membrane. Tracheas were placed on gelfoam sponges (Pfizer) in Petri dishes and cultured at air-liquid interface in DMEM/F12 based media supplemented with 15mM HEPES (Thermo Fisher Scientific). For Notch inhibition, the media contained either 2.5 μ M DAPT (Tocris) or 10 μ M DBZ (Calbiochem) while control media contained an equivalent volume of DMSO (Sigma), <0.1% v/v. Daily media changes were performed and tracheas were cultured for 3 days unless otherwise specified followed by wholemount fixation and staining.

Murine co-culture model

Tracheal epithelial cells from Membrane-GFP (mGFP) mice were isolated to better visualize immune integration into the epithelium. mGFP mice were generated by breeding *Rosa26R-mTmG* mice (JAX 007576) to *FoxJ1-Cre* mice (MGI 3797103). *FoxJ1-Cre* activity in spermatocyte precursors resulted in reporter recombination to produce mGFP sperm, leading to mGFP offspring when bred to C57BL/6 mice. The *FoxJ1-Cre* allele was subsequently backcrossed out of mice expressing mGFP through additional matings to C57BL/6 mice. In subsequent experiments, C57BL6/J mice (JAX 000664) were used to generate epithelial cells lines. Primary tracheal epithelial cells were isolated by digesting tracheas as previously described.⁶⁸ Freshly isolated epithelium was seeded onto plates pre-coated with laminin-rich matrix derived from filtered supernatant of the 804G rat bladder cell line. The cells were then expanded in Small Airway Growth Media (SAGM, Lonza #CC-3118) supplemented with ROCK inhibitor Y-27632 (Tocris), dual SMAD inhibitors A-8301/DMH-1 (Tocris), and WNT activator CHIR99021 (Tocris).⁴⁶ Passage 2–5 cells were used to seed transwell inserts (Corning #3470) pre-coated with 804G-derived matrix as above. When confluence was achieved, media was aspirated from apical and basolateral chambers and basolateral media was replaced with PneumaCult ALI media (StemCell Technologies

#05001). Purified murine bone-marrow monocytes were then added to the apical surface in PneumaCult ALI media at a concentration of $1.5 \times 10^5/\text{mm}^2$. Bone marrow monocytes were isolated for use according to the manufacturers protocol (Miltenyi Biotec # 130-100-629). Rosa26R-mTmG (JAX 007576), CCR2-RFP/CX3CR1-GFP (Jax 032127), and CX3CR1-RBPJk mice were used as sources of bone marrow for experiments. Cultures were maintained in Pneumacult ALI media until maturity was reached. No supplemental exogenous cytokines/chemokines were added to the media.

Notch inhibition in an *in vitro* co-culture system

Mature cultures were treated with $2.5 \mu\text{M}$ DAPT (Tocris) while control cultures were treated with an equivalent volume of DMSO for 72h before analysis by immunofluorescent staining or flow cytometry. To induce deletion *in vitro* from CX3CR1-RBPJk mice, $2 \mu\text{M}$ 4-OHT (Sigma) was added to basolateral media of mature cultures for 5 consecutive days. 4-OHT treated wells with monocytes from *RBPJk^{fl/fl};mTmG* littermates lacking *CX3CR1-CreER* were used as controls. Two weeks later, cultures were analyzed by flow cytometry.

Confocal/flow analysis of co-cultures

Live transwell cultures were maintained on a temperature and climate controlled stage and imaged with a Zeiss LSM780 confocal laser scanning microscope using a $10\times$ objective to capture fluorescent reporter signal. For flow cytometry, mucus was removed from the apical surface by a 5 min incubation in dithiothietol (DTT, Sigma). The apical surface was then washed two times with PBS and TrypLE select (Gibco) was added to apical and basolateral chambers for 20 min at 37°C to generate a single cell suspension. The digested cultures were passed through a $100 \mu\text{m}$ filter and resuspended in MACS buffer (PBS supplemented with 2mM EDTA and 1% bovine serum albumin). Samples were blocked with purified CD16/CD32 mAb (1:100, 553141, BD Biosciences) and then stained with fluorescently labeled antibodies CD45-PE (Biolegend 103105, 30-F11), MHC II I-A/I-E AF647 (Biolegend 107618, M5/114.15.2) followed by fixation in PFA. Flow cytometry was performed on an BD CytoflexS analytical flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (version 10.8).

Mouse single cell RNA sequencing (scRNAseq) analysis

Single-cell profiles for murine tracheal myeloid cells were obtained by re-analyzing data collected as part of our prior study of the tracheal epithelium.² Previously, non-epithelial cells (fibroblasts, endothelial cells, and immune cells) were considered contaminating populations and were computationally filtered out and excluded from the prior analysis.² To examine the immune populations, the 10X digital gene expression matrices were downloaded (GSE103354, GSE152501) and analyzed using standard processing pipelines while still including non-epithelial populations. The top 3,000 most variable genes were identified based on a previously described outlier detection approach.⁶⁹ PCA was performed and the top 50 principal components were used to construct a Shared Nearest Neighbor (SNN) graph, using approximate nearest neighbors ($k = 20$, $\text{eps} = 0.5$). 10,744 total cells were allocated into communities (cell type clusters) using the Leiden algorithm (implemented using the *igraph* R package, resolution = 0.25). Two *Krt5^{high}* and two *Scgb1a1^{high}* clusters were merged to define basal and club cells respectively. All other clusters detected represented a distinct cell type (or lineage, in the case of immune cells). To examine heterogeneity of marker gene expression in immune cells, we recomputed variable genes, PCA, and UMAP on only the cluster of 304 immune cells and visualized the indicated genes by projected them onto the resulting UMAP embedding.

Human single cell RNA sequencing and gene set enrichment analysis (GSEA)

For full details regarding subject recruitment, sample processing, and cell clustering was previously described.⁴⁸ An IAM gene set was derived using the list of IAM discriminatory markers from Engler et al.¹³ and mapping them to their human orthologs. GSEA was then performed with this IAM gene set, to look for an enrichment of an IAM signature in human airway mononuclear phagocyte clusters, using *fgsea* (v1.24.0, R). Cluster gene lists were defined and ordered as previously described with a pseudobulk one-vs-all approach. Only genes that were expressed in $>5\%$ of cells were included in the ranking lists with 10,000 permutations to test for independence. Based on multiple hypothesis testing a false discovery rate was calculated using the Benjamini-Hochberg method. Dot plots and violin plots to show functional specialization of airway mononuclear phagocytes and expression of Notch ligands and receptors was performed using the *dotplot* function in *scanpy* and plotted using *matplotlib* as performed.⁴⁸

QUANTIFICATION AND STATISTICAL ANALYSIS

The standard error of the mean was calculated from the average of the indicated number of samples in each case (n = biological replicates/condition/experiment). All the experiments were repeated two or three times. The number of samples per condition is indicated in each case in the figure legend. Multiple $20\times$ or $40\times$ magnification fields per sample were counted and is indicated in detail in the [Microscopy and imaging](#) section. No animals were excluded from the analysis. Data was analyzed and compared among groups by Student's *t*-test (unpaired, two-tailed test) or two-way ANOVA. In the experiments where multiple fields per trachea were counted, the data was analyzed by nested *t* test. A *p*-value of less than 0.05 was considered significant. The analysis was performed with GraphPad Prism 10.0 software.