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Aeroallergen Challenge Promotes Dendritic Cell Proliferation in the Airways

Tibor Z. Veres,^{*,†,1} Sabrina Voedisch,^{*,†} Emma Spies,^{*,†} Joona Valtonen,[‡] Frauke Prenzler,^{*,†} and Armin Braun^{*,†}

Aeroallergen provocation induces the rapid accumulation of CD11c⁺MHC class II (MHC II)⁺ dendritic cells (DCs) in the lungs, which is driven by an increased recruitment of blood-derived DC precursors. Recent data show, however, that well-differentiated DCs proliferate in situ in various tissues. This may also contribute to their allergen-induced expansion; therefore, we studied DC proliferation in the airways of mice in the steady state and after local aeroallergen provocation. Confocal whole-mount microscopy was used to visualize proliferating DCs in different microanatomical compartments of the lung. We demonstrate that in the steady state, CD11c⁺MHC II⁺ DCs proliferate in both the epithelial and subepithelial layers of the airway mucosa as well as in the lung parenchyma. A 1-h pulse of the nucleotide 5-ethynyl-2'-deoxyuridine was sufficient to label 5% of DCs in both layers of the airway mucosa. On the level of whole-lung tissue, 3–5% of both CD11b⁺ and CD11b[−] DC populations and 0.3% of CD11c⁺MHC II^{low} lung macrophages incorporated 5-ethynyl-2'-deoxyuridine. Aeroallergen provocation caused a 3-fold increase in the frequency of locally proliferating DCs in the airway mucosa. This increase in mucosal DC proliferation was later followed by an elevation in the number of DCs. The recruitment of monocyte-derived inflammatory DCs contributed to the increasing number of DCs in the lung parenchyma, but not in the airway mucosa. We conclude that local proliferation significantly contributes to airway DC homeostasis in the steady state and that it is the major mechanism underlying the expansion of the mucosal epithelial/subepithelial DC network in allergic inflammation. *The Journal of Immunology*, 2012, 190: 000–000.

The critical role of dendritic cells (DCs) in pulmonary immune responses has been well established during the last decade (1). Airway DCs form a network beneath the epithelium (2), ideally localized to capture inhaled Ag and subsequently migrate to the draining lymph nodes to present their antigenic cargo to naive T cells (3). Because of their effective Ag transporting and presenting function, airway DCs have been implicated to mediate sensitization to inhaled allergens under specific circumstances, which then results in a Th2-biased immune response as observed, for example, in allergic asthma. Pathogenic allergen-specific Th2 cells migrate to the airways and give rise to long-lived memory T cells (4). Upon allergen inhalation, reactivation of memory T cells contributes to the development of allergic inflammation (5).

Repeated exposure to allergen induces a rapid accumulation of DCs in the airway mucosa in both animal models (6) and human asthmatics (7), suggesting that local Ag presentation via DCs is

an important means of memory T cell reactivation (8). However, accumulation of airway mucosal DCs not only occurs after allergen exposure, but also following inhalation of bacterial and viral Ags, thus presenting a universal feature of the acute cellular response to inhalational challenge (9, 10). This expansion of the mucosal DC network presumes an increased recruitment of DCs or DC precursors from the blood. Indeed, an increase in CD31^{hi} Ly-6C[−] bone marrow precursors has been observed to support the increased demand for DCs in the inflamed airways (11). In addition to this mechanism, however, the de novo appearance of DCs in the steady state can also occur via in situ proliferation of tissue-resident DCs in both lymphoid (12, 13) and nonlymphoid organs (14–16).

In most nonlymphoid organs, DC homeostasis relies on continuous replenishment by CD11c⁺MHC class II (MHC II)[−] DC progenitors (14) and monocytes (17–19), with two exceptions; that is, Langerhans cells (LCs) of the epidermis (15, 20) and microglia cells of the brain (21) are capable of self-renewal via in situ proliferation independently of precursors arriving from the blood. Apart from these two sites, cells locally arising from blood-borne precursors constantly replace dying or emigrating DCs in nonlymphoid tissues, including the lungs. However, before being replaced by newly recruited cells, well-differentiated DCs undergo a limited number of divisions in situ (14, 16), similar to lymphoid organ DCs (12, 13). In the lungs, divisions of both major CD11c⁺MHC II⁺ DC populations, namely CD103[−] and CD103⁺ DCs, have been recently suggested (14); however, DC proliferation in different tissue compartments of the respiratory tract and its contribution to the expansion of the DC network following local allergen challenge has not been addressed.

In this study we demonstrate that airway DCs constantly proliferate in the steady state in all anatomical compartments. This basal proliferation was massively increased in the airway mucosa after allergen inhalation, suggesting that local proliferation contributes

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Abbreviations used in this article: Alum, aluminum hydroxide; DC, dendritic cell; EdU, 5-ethynyl-2'-deoxyuridine; EYFP, enhanced yellow fluorescent protein; LC, Langerhans cell; MHC II, MHC class II.

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to supporting the increased demand for DCs under inflammatory conditions.

Materials and Methods

Animals

CD11c-enhanced yellow fluorescent protein (EYFP) transgenic mice (22) and CAG-DsRed mice on a C57BL/6 background, CX₃CR1^{gfp/+} mice (23) on a BALB/c background, and C57BL/6 wild-type mice were used at 8–12 wk age. Animal experiments were performed in concordance with the German animal protection law under a protocol approved by the appropriate governmental authority (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit).

Immunization and aeroallergen challenge

Experimental allergic airway inflammation was induced as described earlier (24). Briefly, mice were sensitized with 10 µg OVA (grade VI; Sigma-Aldrich, St. Louis, MO) adsorbed to 1.5 mg aluminum hydroxide (Alum) diluted in PBS on days 0, 14, and 21 via i.p. injection. Controls received Alum in PBS. Animals were exposed to 1% OVA aerosol in PBS for 20 min on day 27.

Labeling DNA of proliferating cells with 5-ethynyl-2'-deoxyuridine in vivo

The thymidine nucleotide analog 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen) dissolved in DMSO at 25.5 mg/ml and further diluted in PBS to 5 mg/ml was used for the in vivo labeling of cells within the S phase. Animals received a bolus i.p. injection of 1 mg EdU in a volume of 200 µl 1 h before sacrifice. Controls received DMSO/PBS, respectively.

Tissue processing and whole-mount immunostaining

At the indicated time points after allergen provocation, animals were sacrificed using an overdose of i.p. administered pentobarbital. The chest cavity was opened, the trachea was cannulated, and the lungs were inflated in situ with 2% paraformaldehyde at a pressure of 20 mm H₂O. After ligation of the trachea, the lungs were removed and fixed overnight in the same fixative at 4°C. Next day, the left and the right superior lobe were separated, and the left lobe was pinned to the bottom of a petri dish coated with Sylgard (Dow Corning, Midland, MI). The main axial pathway of the left lobe was carefully dissected under a stereomicroscope (Wild Heerbrugg, Heerbrugg, Switzerland). The right superior lobe was cut to 300-µm-thick slices using a vibratome (OTS-5000; Electron Microscopy Sciences). Specimens were washed and permeabilized with 0.3% Triton X-100. Following EdU detection using the Click-IT EdU Alexa Fluor 647 kit (Invitrogen), specimens were immunostained as whole mounts. Primary Abs included chicken anti-GFP (Novus Biologicals), rabbit anti-cytokeratin (Abcam), rat anti-MHC II (clone M5/114.15.2; BD Biosciences), and rabbit anti-Ki67 (clone SP6; Innovative Diagnostik-Systeme). Secondary detection was performed with donkey anti-chicken Cy2 and anti-rabbit or anti-rat Cy3 Abs (Jackson ImmunoResearch).

Confocal microscopy and quantitative image analysis

Images were acquired using an LSM 510 META (Carl Zeiss) confocal microscope using $\times 20$ and $\times 40$ (water immersion) objectives. Image stacks for the quantitative analysis were scanned with an XYZ resolution of $1024 \times 1024 \times 100$ with dimensions of $325.8 \times 325.8 \times 50$ µm, respectively (see Supplemental Video 1). Two image stacks were taken per mouse at the proximal part of the main bronchus of the left lung. Each stack was divided into an epithelial and subepithelial part (see Supplemental Fig. 1), which were separately analyzed using Imaris 6.2.1 (Bitplane). DCs were enumerated using the automated object detection feature of Imaris software as described earlier (25). First, surface objects were generated using optimal threshold settings in the CD11c-EYFP (DC) channel via "region growing." This resulted in individual surface objects for every cell, with an accurate separation of touching cells. "Quality" filter was used for the detection of seed points. Filter settings were optimized by visually comparing the result with the maximum-intensity projection of the same dataset. We identified EdU⁺ cells by filtering DC-representing surface objects according to fluorescence intensity in the far-red (EdU) channel.

Flow cytometry

Murine lungs were excised, minced to small pieces manually, and digested with 2 mg/ml collagenase type III (Worthington Biochemical) and 0.1% DNase I (Roche). Erythrocytes and cell debris were removed using Lympholyte-M gradient (Cedarlane Laboratories), followed by enrichment of CD11c⁺ cells

via magnetic separation using MACS technology (Miltenyi Biotec). Bronchoalveolar lavage fluid was retrieved by inserting an 18-gauge cannula into the trachea and flushing lungs with 2×0.8 ml PBS. EdU-incorporating cells were detected using the Click-IT EdU Alexa Fluor 647 flow cytometry assay kit (Invitrogen) according to the manufacturer's description. Subsequently, samples were blocked with anti-CD32/16 (BD Biosciences) and stained with FITC-labeled anti-MHC II (M5/114.15.2), PE-labeled anti-CD11c (N418), and PE-Cy7-labeled CD11b (M1/70) (all from eBioscience) and analyzed with an FC-500 flow cytometer (Beckman Coulter).

Adoptive transfer of monocytes

CD11c-EYFP mice were crossed with CAG-DsRed mice (both as homozygotes) and the resulting offspring were used to purify CD115⁺ monocytes from bone marrow as described earlier (18). Briefly, the femur and tibia were flushed with RPMI 1640/10% FCS and mononuclear cells were enriched using Lympholyte-M density gradient. CD115⁺ cells were enriched from the cell suspension using a CD115⁺ MicroBead kit (Miltenyi Biotec). Cells ($1-2 \times 10^6$) were i.v. transferred into OVA/Alum-sensitized and sham-sensitized control CD11c-EYFP mice 3 d before OVA aerosol challenge.

Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical differences in the number of DCs as well as in the percentage of proliferating DCs between OVA-sensitized animals and the control group or between different analysis time points were analyzed with an unpaired *t* test using GraphPad Prism 4.03. Differences with *p* < 0.05 were considered as statistically significant.

Results

Steady-state proliferation of airway DCs

To identify proliferating DCs in the airways, we combined in vivo EdU labeling with confocal microscopy of whole-mount preparations using CD11c-EYFP reporter mice (Fig. 1). Systemic administration of the thymidin analog EdU 1 h prior to analysis assured the detection of locally proliferating cells. The CD11c-EYFP transgene revealed a network of DCs within the epithelial layer of the airway mucosa, visualized by cytokeratin staining (Fig. 1A), and in the alveolar septa of parenchymal lung tissue (Fig. 1C). In both anatomical compartments, single CD11c-EYFP⁺ cells with an EdU⁺ nucleus were found (Fig. 1A, 1C, enlarged regions), suggesting the presence of DCs within the S phase of the cell cycle.

CD103⁺CD11c⁺MHC II⁺ DCs of the liver and kidney have been shown to mainly arise from CX₃CR1⁺CD11c⁺MHC II⁺ pre-conventional DCs, which migrate from the bone marrow through the blood to peripheral tissues. In the bronchial epithelium, proliferating CD11c-EYFP⁺ cells uniformly expressed MHC II and they were indistinguishable from nonproliferating CD11c-EYFP⁺ cells on the basis of their morphology and MHC II expression (Fig. 1B, upper panel). To study the expression of CX₃CR1 on epithelial DCs, we examined GFP⁺ intraepithelial cells of CX₃CR1^{gfp/+} reporter mice. This reporter system revealed a morphologically similar network of GFP⁺MHC II⁺ DCs, in which EdU⁺ cells were indistinguishable from their EdU[−] counterparts on the basis of MHC II expression and morphology (Fig. 1B, lower panel). In the subepithelial layers of the airway mucosa, EdU⁺CD11c-EYFP⁺ cells mainly had a rounded shape and also expressed MHC II. In the lung parenchyma, however, both MHC II⁺ and MHC II[−] EdU⁺ CD11c-EYFP⁺ cells were identified (see Supplemental Fig. 2).

Steady-state mouse CD11c⁺MHC II⁺ respiratory tract DCs can be divided into two major subpopulations on the basis of CD11b and/or CD103 expression: CD103⁺CD11b[−] and CD103[−]CD11b⁺ DCs (26, 27). We attempted to use these markers to visualize the two DC subsets using whole-mount microscopy, but the Ab labeling was not successful in our whole-mount immune-labeling setup (as was the case with most anti-integrin Abs). CD103⁺ DCs are also known to express Langerin and this marker has been previously used to visualize LCs in epidermal whole-mount prep-

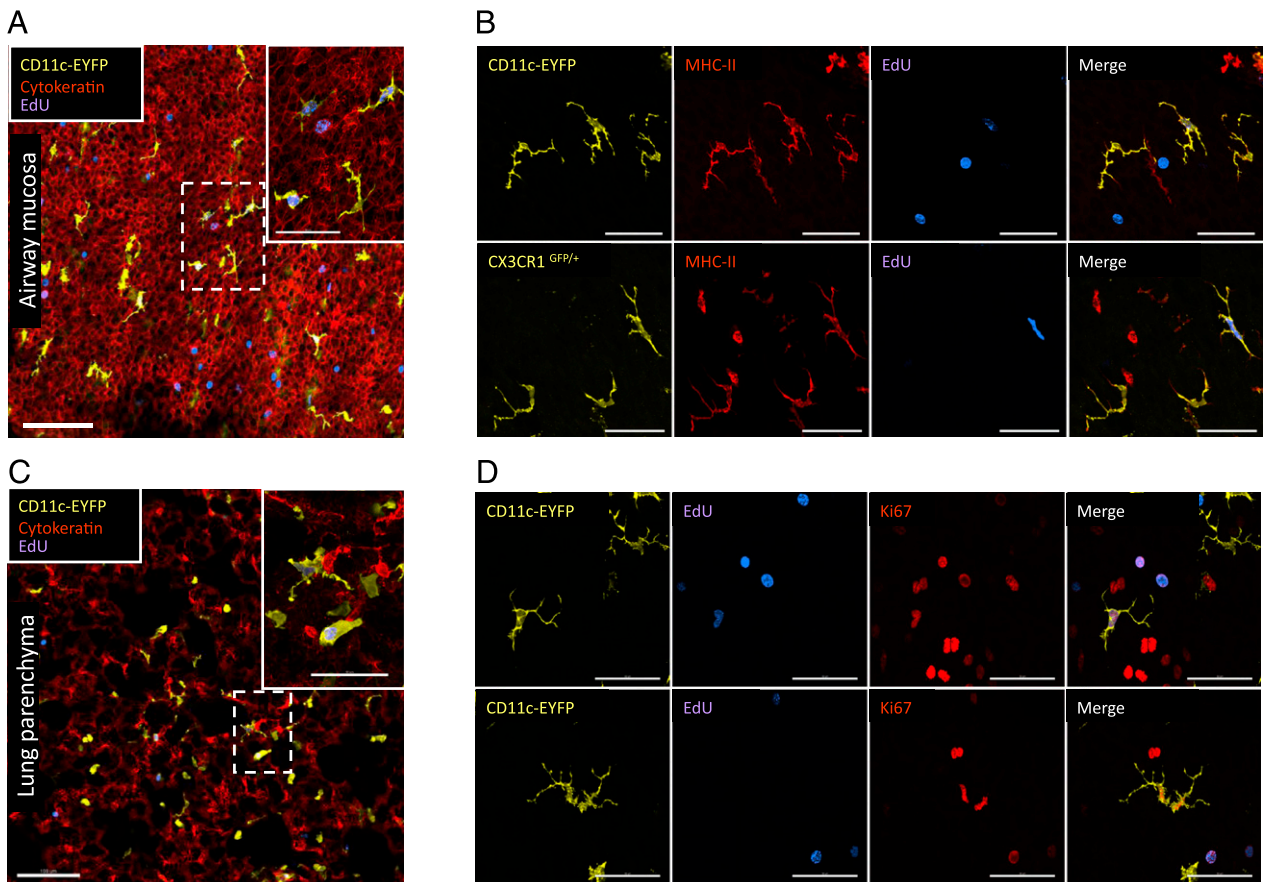


FIGURE 1. Steady-state proliferation of DCs in the airway mucosa and lung parenchyma. Lungs of CD11c-EYFP and CX₃CR1^{GFP/+} mice were analyzed after a 1-h pulse of i.p. administered EdU. Whole-mount preparations of the airway mucosa or lung parenchyma were immunostained for cytokeratin, MHC II, or Ki67, combined with fluorescent EdU detection. Maximum intensity projections of confocal z-stacks are shown. (**A** and **C**) EdU⁺ DCs in the airway mucosa and lung parenchyma, low-magnification overviews (scale bars, 100 μ m). Regions indicated by dashed boxes were scanned at higher resolution (*enlarged insets*; scale bars, 50 μ m). (**B**) Airway mucosa of CD11c-EYFP (*upper panel*) or CX₃CR1^{GFP/+} mice (*lower panel*) showing EdU⁺ and EdU⁻ DCs positive for MHC II. Scale bars, 50 μ m. (**D**) Proliferating DCs are EdU⁺Ki67⁺ double-positive (*upper panel*) or Ki67⁺ single-positive (*lower panel*). Scale bars, 50 μ m).

arations (15). Although Langerin staining revealed the LC network in the ear epidermis in our setup as well, we were unable to identify the Langerin⁺ DC network in the airway mucosa (see Supplemental Fig. 3). However, staining of frozen sections revealed that most CD11c⁺ DCs associated with the bronchial epithelium coexpress Langerin as observed in earlier studies, suggesting again a technical reason for not finding these cells in bronchial whole mounts (see Supplemental Fig. 3). According to frozen-section immunostainings of ours and others, we assume that most epithelial CD11c⁺ DCs belong to the CD103⁺ subtype, whereas most DCs in the deeper layers of the airway mucosa are CD103⁻ DCs. Therefore, our method of separately analyzing the epithelial and subepithelial compartments (as used also in the following experiments) is a valid approach to study CD103⁺CD11b⁻ versus CD103⁻CD11b⁺ DCs in the airway mucosa.

Because EdU incorporation only reveals cells within the S phase, we performed immunostaining for the general cell proliferation marker Ki67 to identify proliferating DCs within other phases of the cell cycle. EdU⁺CD11c-EYFP⁺ DCs were uniformly Ki67⁺ (Fig. 1D, *upper panel*); however, Ki67 also labeled EdU⁻ DCs, some of which were found in pairs (Fig. 1D, *lower panel*).

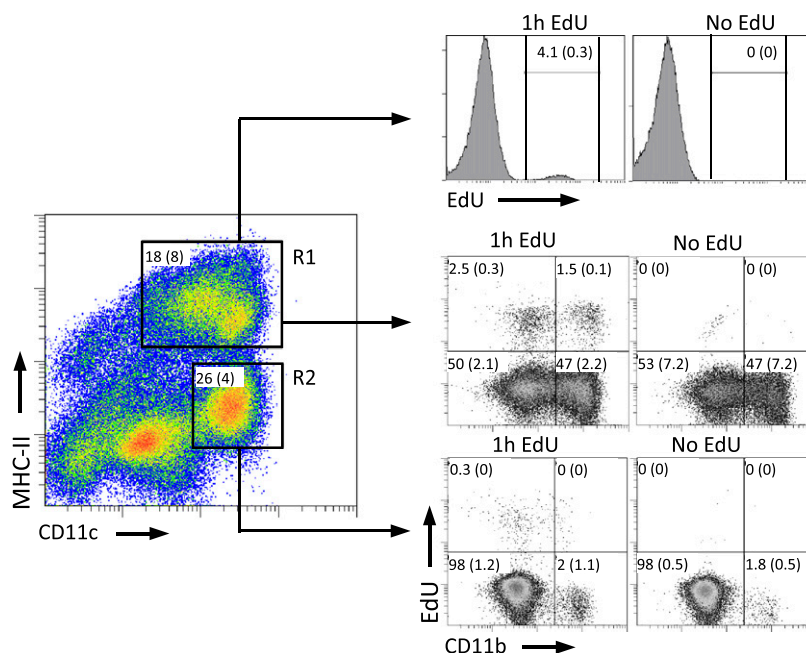
Next we used flow cytometry to measure the proportion of EdU⁺ cells within CD103⁺CD11b⁻ and CD103⁻CD11b⁺ subpopulations in enzymatically digested lung tissue. In these experiments we primarily relied on CD11b as a marker to separate the two populations. As shown in Fig. 2, ~4% of all CD11c⁺MHC II⁺ DCs

incorporated EdU 1 h after a single i.p. bolus EdU administration. Proliferation was detected in both populations, whereas the proportion of EdU⁺ cells was somewhat higher in the CD11b⁻ compared with the CD11b⁺ population. Interestingly, ~0.3% of CD11c⁺MHC II^{low} lung macrophages also incorporated EdU. We conclude that both DCs (located in different anatomical compartments of the respiratory tract) and lung macrophages continuously proliferate in the steady state.

DC proliferation following allergen inhalation

It has been well documented that the numbers of respiratory tract DCs as well as their migration to the draining lymph nodes increase following allergen inhalation (6, 8). This increase in DC number has been attributed to the recruitment of DCs or DC precursors from the blood to the airway mucosa. Accordingly, mice with airway inflammation displayed an expansion of myeloid precursors in the bone marrow (11). However, the number of lung DCs is also a function of local proliferation and cell death, in addition to cell influx and efflux (28). Thus, we examined the hypothesis that an allergen-induced increase in local DC proliferation contributes to the expansion of the DC network. First, the kinetics of airway mucosal DC accumulation and local proliferation following OVA aerosol challenge in OVA-sensitized animals were assessed using quantitative whole-mount microscopy. The total number of CD11c-EYFP⁺ DCs as well as the percentage of EdU⁺ CD11c-EYFP⁺ DCs both in the epithelial and subepithelial com-

FIGURE 2. Steady-state proliferation of the major myeloid DC populations and lung macrophages. Flow cytometric analysis of cells from lung tissue of wild-type mice after a 1-h pulse of i.p. administered EdU. CD11c⁺ cells were enriched via magnetic separation. *Left panel*, Identification of CD11c⁺MHC II⁺ DCs (region R1) and CD11c⁺MHC II^{low} macrophages (region R2). *Right panel*, Percentage of proliferating cells within region R1 (histograms); proportion of EdU⁺ proliferating CD11b⁺ and CD11b⁻ cells within the respective regions R1 and R2 (dot plots). Numbers indicate mean percentage (SD) of cells within the respective gates. Lung cells from two mice were pooled for each measurement in three independent experiments.



partments of the airway mucosa was analyzed 12, 24, 36, and 48 h after a single OVA aerosol challenge. The number of epithelial DCs gradually increased and was significantly higher at 36 h after aerosol challenge compared with 12 h, subsequently decreasing at 48 h (Fig. 3A), following kinetics similar to earlier observations (8). Subepithelial DC numbers significantly increased at 36 h (compared with 12 h) and remained elevated at all later time points (Fig. 3B). The changes in the proportion of EdU⁺ proliferating DCs of the epithelial compartment showed similar kinetics as the total DC number with a significant increase at 36 h after aerosol challenge compared with the 12 h value and decreasing afterward (Fig. 3C). However, the proliferation rate of DCs in the subepithelial

compartment, although slightly elevated at 24 h, did not change significantly at the time points examined. These initial data suggested that the expansion of the airway mucosal DC network is accompanied by an increased local proliferation. To confirm this increase in airway mucosal DC proliferation upon aeroallergen challenge, we performed a second, independent experiment comparing sham-sensitized versus OVA-sensitized animals 24 h after a single OVA aerosol challenge, because the previous experiment suggested an accelerating proliferation rate at this specific time point. As shown in Fig. 4A, the number of DCs did not yet increase in OVA-sensitized animals compared with controls in either anatomical compartment, but the proportion of proliferating DCs

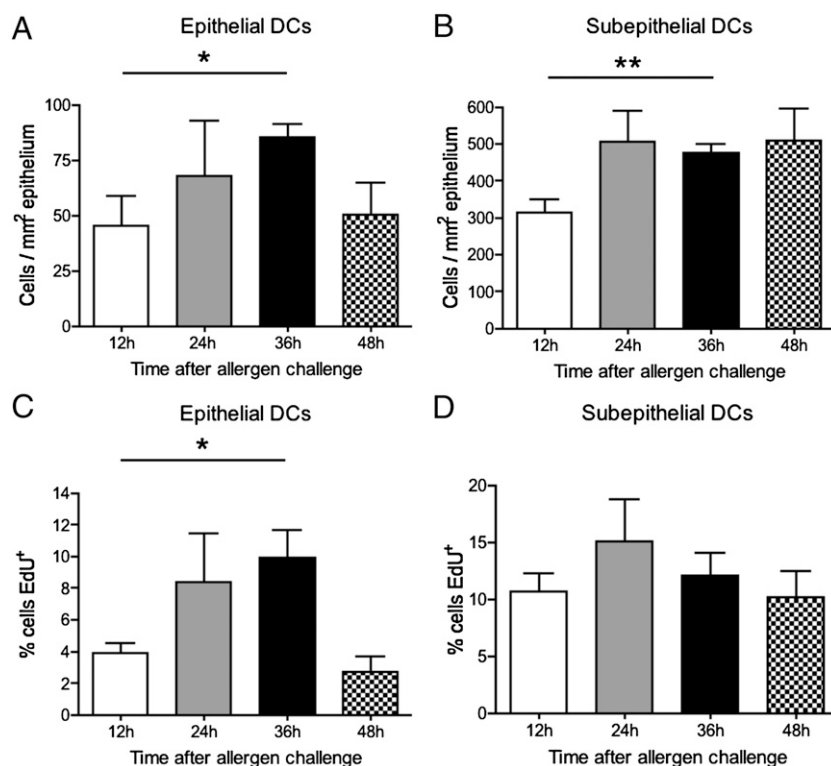
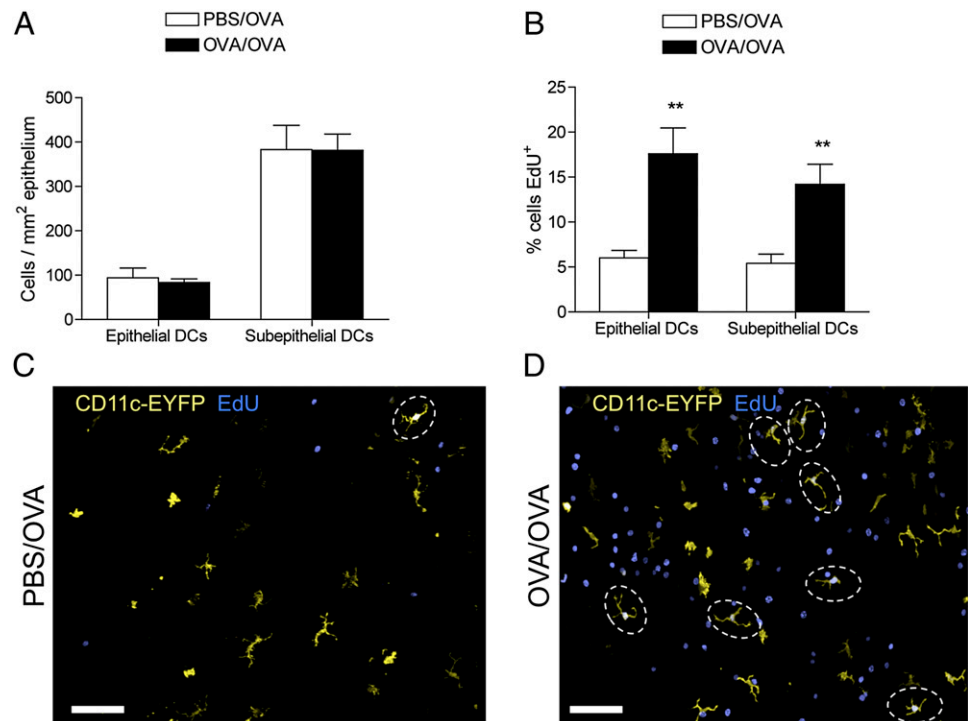


FIGURE 3. Kinetics of airway mucosal DC proliferation after aeroallergen challenge. CD11c-EYFP mice were sensitized with OVA/Alum i.p. on days 0, 14, and 21 and received OVA aerosol exposure on day 27 and sacrificed after 12, 24, 36, and 48 h. All animals received 1 mg EdU i.p. 1 h prior to sacrifice. Lungs were fixed and whole-mount preparations of the airway mucosa were made via microdissection. After performing fluorescent EdU detection, samples were stained against GFP. High-resolution confocal images (two z-stacks of each mouse) were acquired, followed by quantitative analysis. Four mice were analyzed at each time point. (A and B) The number of CD11c-EYFP⁺ DCs (calculated as cells beneath 1 mm² airway epithelium) within the epithelial (A) and subepithelial (B) compartments at the respective time points are displayed. (C and D) Graphs show the percentage of EdU⁺CD11c-EYFP⁺ cells (calculated from the total number of CD11c-EYFP⁺ DCs) within the epithelial (C) and subepithelial (D) compartments at the respective time points. Data are displayed as means $\pm$ SEM. * p < 0.05, ** p < 0.01.

FIGURE 4. Airway mucosal DC proliferation at 24 h following aero-allergen challenge. Comparison of OVA-sensitized (OVA/OVA) and sham-sensitized (PBS/OVA) CD11c-EYFP mice 24 h after OVA aerosol exposure (five mice were used in both groups). EdU administration and confocal microscopic analysis were performed as described above. The number of CD11c-EYFP⁺ DCs (**A**) and the percentage of EdU⁺CD11c-EYFP⁺ cells (**B**) are shown. Data are displayed as means $\pm$ SEM. $^{**}p < 0.01$ versus PBS/OVA). (**C** and **D**) Confocal z-projections of the airway epithelial layer showing EdU⁺CD11c-EYFP⁺ cells (dotted circles); images are representative of the airway mucosa from animals in the PBS/OVA (**C**) and OVA/OVA (**D**) groups. Scale bars, 100 μ m.



was ~3-fold increased in this group compared with nonsensitized controls (Fig. 4B; see also representative confocal images in Fig. 4C and 4D). We next intended to find out whether an increase in local proliferation following allergen challenge is limited to the airway mucosal compartment or whether it can be observed also in parenchymal lung DC populations. Therefore, we analyzed by flow cytometry the proliferation rate of DCs from total lung tissue, which mainly represent DCs located within the lung parenchyma. In contrast to the initially unaltered DC numbers observed in the airway mucosa, we observed an ~1.5-fold increase in the frequency of DCs in lung tissue 24 h after OVA aerosol challenge (Fig. 5A). This increase mainly affected the CD11b⁺ DC population (Fig. 5B), which is a mixed population of DCs containing lung-resident CD11b⁺ conventional DCs as well as CD11b⁺ monocyte-derived inflammatory DCs (29). The proliferation rate of total lung tissue DCs did not show an increase in the OVA/OVA group compared with controls (Fig. 5C), further suggesting that the increase in DC numbers did not result from local proliferation but rather by the recruitment of nonproliferating CD11b⁺ monocyte-derived DCs from the bone marrow. Because we were not able to distinguish the newly recruited monocyte-derived DC population from resident CD11b⁺ DCs that were already present before the allergen challenge, we were unable to measure whether allergen challenge affected the proliferation of resident DCs within the lung parenchyma.

We next intended to confirm our assumption that monocyte-derived inflammatory DCs do not proliferate *in situ* and they are mainly located in the lung parenchyma and not in the airway mucosa. Therefore, we performed adoptive transfer of monocytes into CD11c-EYFP mice 3 d before allergen challenge and studied their distribution in the lung 24 h after the challenge using whole-mount microscopy. CD11c-EYFP mice were crossed with CAG-DsRed mice (which ubiquitously express DsRed) and the resulting offspring served as donors of CD115⁺ monocytes that we purified from the bone marrow. As shown in Fig. 6A, the transferred cells could be identified in the lungs by their DsRed expression where approximately half of them differentiated into CD11c⁺MHC II⁺

DCs. DsRed⁺ monocyte-derived DCs mainly populated the parenchymal lung tissue (Fig. 6B) and they did not appear in the airway mucosa (not shown). Additionally, we were not able to identify proliferating DsRed⁺ cells although the surrounding tissue contained several EdU⁺ endogenous CD11c-EYFP⁺ cells. These findings confirm that monocyte-derived inflammatory DCs do not contribute to the expansion of the DC network in the airway mucosa but it is rather the result of the local proliferation of resident epithelial and subepithelial DCs.

Discussion

Our results demonstrate that in the steady state, CD11c⁺MHC II⁺ DCs proliferate in both the epithelial and subepithelial compartments of the airway mucosa as well as in the lung parenchyma. This basal proliferation was observed in both major subpopulations that were previously distinguished on the basis of CD11b (or CD103) expression, and it accounted for ~4% of the respective DC populations, consistent with a recent report by Ginhoux et al. (14). However, CD11c⁺MHC II^{low} lung macrophages were also found to proliferate, although to a lesser extent, suggesting that proliferation of tissue-resident cells is a general feature of the DC/macrophage lineage in the lungs and is not limited to DCs.

It is noteworthy that a 1-h pulse of the nucleotide EdU was sufficient to label 4% of all DCs. Because nucleotide incorporation only occurs in cells within the S phase of the cell cycle, it is likely that this method underestimates the true extent of steady-state proliferation. Indeed, Ki67 staining revealed an additional 30% of cycling cells in the airway mucosa (results not shown). Although DC proliferation in different lymphoid and nonlymphoid organs has meanwhile become a well-documented phenomenon (28), the division of well-differentiated APCs in the airway mucosa still appears unexpected. In the subepithelial layers, small, rounded MHC II⁺ cells were previously regarded as recently recruited small immature “monocyte-like” cells with low endocytic ability, compared with larger, more dendritiform DCs in the epithelium with high endocytic activity (10). Thus, in the present study, proliferating subepithelial CD11c⁺ cells would ideally represent a local pre-DC

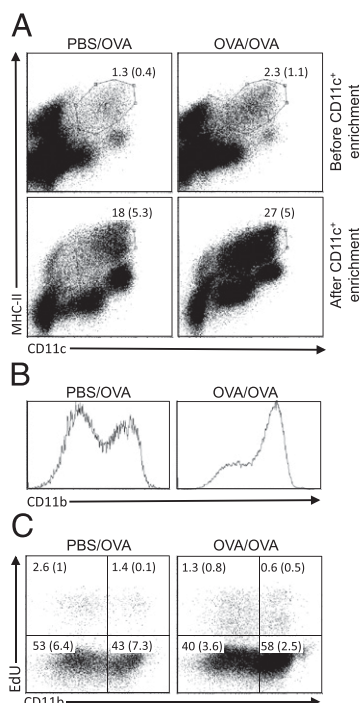


FIGURE 5. Proliferation of DCs obtained from total lung tissue after aeroallergen challenge. Comparison of sham-sensitized (PBS/OVA) and OVA-sensitized (OVA/OVA) wild-type mice 24 h after OVA aerosol exposure. Animals received 1 mg EdU i.p. 1 h prior to sacrifice. Cells from total lung digests were analyzed via flow cytometry. **(A)** Plots show CD11c⁺MHC II⁺ DCs in PBS/OVA and OVA/OVA mice before and after enrichment of CD11c⁺ cells with paramagnetic beads. **(B)** Histograms show the distribution of CD11b⁺ and CD11b⁻ DCs and **(C)** plots display the proportion of EdU⁺ proliferating CD11b⁺ and CD11b⁻ DCs in the two treatment groups after enrichment of CD11c⁺ cells (all gated on CD11c⁺MHC II⁺ cells). Numbers indicate mean percentage (SD) of cells within the respective gates. Lung cells from two mice were pooled for each measurement in three independent experiments.

population, which gives rise to functional, nonproliferating epithelial DCs. However, our results did not confirm this presumption, as both epithelial and subepithelial CD11c⁺ cells 1) divided at the same rate and 2) expressed high amounts of MHC II, in contrast to pre-DCs, which were previously identified as CD11c⁺MHC II⁻ cells (14). This suggests that the EdU⁺CD11c⁺MHC II⁺ cells identified in the present study are indeed well-differentiated DCs.

The unexpectedly high rate of local DC proliferation poses the question whether this mechanism is sufficient for the maintenance of the DC network without the recruitment of bone marrow-derived precursors. Recent studies utilizing parabiosis experiments helped to understand at which rate replacement by blood-derived precursors contributes to DC turnover (12, 14). The study by Ginhoux et al. (14) shows that in parabiotic mice with separate organs and shared circulation, a chimerism for both CD103⁺ and CD11b⁺ DCs occurs in the lungs. After separation of parabionts, lung DCs had a much longer half-life (30 d for CD103⁺ DCs and 15 d for CD11b⁺ DCs) than did DCs in other tissues (7 d). Because this analysis was done with DCs from total lung tissue, it does not exclude the possibility that in distinct anatomical compartments, local proliferation substantially (if not exclusively) contributes to the steady-state renewal of DCs. The mucosal epithelial DC network could be a candidate for such a population, because they seem comparable to the epidermal LC population. Bronchial epithelial DCs presumably belong to the CD103⁺ airway DC population,

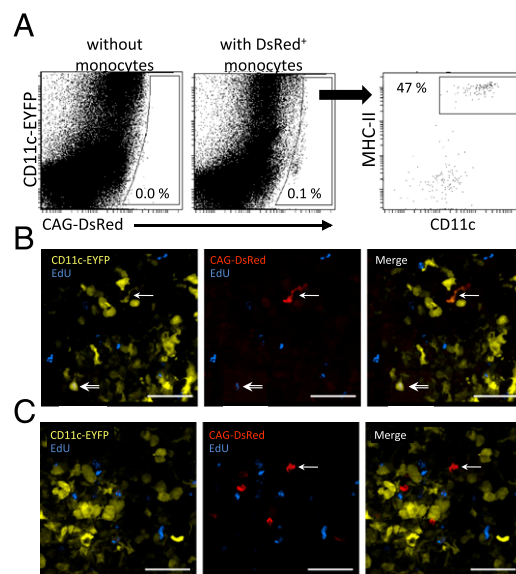


FIGURE 6. Visualization of monocyte-derived inflammatory DCs in the lungs after aeroallergen challenge. CD115⁺ monocytes (isolated from bone marrow) of CD11c-EYFP × CAG-DsRed mice were adoptively transferred into OVA/Alum-sensitized and sham-sensitized CD11c-EYFP mice 3 d before OVA aerosol challenge. **(A)** Identification of the DsRed⁺ transferred monocyte population in the lungs of OVA/OVA mice using flow cytometry. Plots show cells previously gated on 7-aminoactinomycin D⁻ (live) events. Numbers indicate percentages of cells within the respective gates. **(B and C)** Confocal z-projections of peripheral lung tissue from OVA/OVA mice showing the distribution of the adoptively transferred DsRed⁺ cell population. In **(B)**, arrow shows a monocyte-derived DC expressing both EYFP and DsRed, but which has no EdU⁺ nucleus. Double arrow shows an endogenous DsRed⁻EYFP⁺ DC with an EdU⁺ nucleus. In **(C)**, arrow points to one of three DsRed⁺EYFP⁻ monocytes that have not differentiated into DCs. Scale bars, 50 μm. Images are representative of an experiment involving three mice per group.

which have an unusually long half-life (14). In an earlier study by Holt et al. (30), whole body x-ray irradiation caused a rapid decline of tracheal epithelial DCs. The rate of decline could be reduced by locally shielding the head and neck area. In the shielded group of animals, a subsequent repopulation of the epithelial DC network was observed even in the absence of bone marrow transplantation, whereas no repopulation was seen in the unshielded group. The self-renewing capacity of the epithelial DC network might explain this observation; however, the relative contribution of blood-derived precursors and local proliferation will remain unclear until future studies combine parabiosis experiments with histology to address this question in detail.

Allergen provocation caused a 3-fold increase in the percentage of EdU-incorporating cells 1 d after allergen challenge both in the epithelial and the subepithelial layers of the airway mucosa, which was later followed by an increase in the number of DCs. This finding suggests that local proliferation contributes to the expansion of the mucosal DC network upon an inflammatory stimulus. However, under inflammatory conditions, also freshly recruited monocyte-derived inflammatory DCs contribute to increasing DC numbers in the lung. Our monocyte transfer experiments suggest that monocyte-derived DCs mainly accumulate in the lung parenchyma and not in the airway mucosa, and they appeared as nonproliferating cells in 1-h EdU labeling studies.

Comparable studies with LCs of the epidermis showed that the nature of the inflammatory stimulus determines whether the expansion of the LC network is driven by local proliferation or by blood-derived precursor recruitment. In skin inflammation models

involving UV treatment, dying LCs were replaced by monocytes, which locally differentiated into LCs (31). In cutaneous graft-versus-host disease lesions of patients after allogeneic hematopoietic cell transplantation, dermal DCs were fully replaced by donor-derived cells (16). However, in other inflammation models that preserved the integrity of the LC network, such as topical treatment with the vitamin D₃ analog MC903, LCs could be induced to massively proliferate (15), and this induction depended on a keratinocyte signal. In this sense, the induction of airway mucosal DC proliferation upon allergen inhalation could be driven by a similar, epithelial cell–derived mechanism. Indeed, airway epithelial cells were shown to activate mucosal DCs, causing allergic disease. In a house dust mite–induced asthma model, TLR4 triggering on structural cells caused DC activation and determined Th2 immunity to house dust mites (32). It is possible that the allergen-induced DC proliferation in our model was driven by a similar mechanism; however, the key epithelial signal has yet to be identified.

In summary, our data show that the major DC populations of the respiratory tract, together with lung macrophages, continuously proliferate in the steady state. In the airway mucosa, allergen challenge enhanced DC proliferation in a mouse model of allergic airways disease, resulting in more DCs. Interfering with DC proliferation to inhibit their activity as APCs coordinating the inflammatory response could be considered when developing antiasthmatic therapies in the future.

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Disclosures

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Aeroallergen challenge promotes dendritic cell proliferation in the airways

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Online data supplement

Video 1. Typical confocal Z-series of the airway mucosa. The video shows serial optical sections of the airway mucosa in a mouse with allergic inflammation (OVA/OVA-group). Proliferating EdU^+ CD11c-EYFP^+ DCs are visible within the epithelium and in the subepithelial layers. Tissue preparation and immunostaining was performed as described in "Methods". CD11c-EYFP^+ DCs are green, the epithelium (stained against cytokeratin) is red, EdU -incorporating cell nuclei are blue.

Figure S1. Distinction between epithelial and subepithelial DCs. CD11c-EYFP mice were treated and samples prepared as described for Figure 1A. **A:** confocal Z-stack is shown as Y-projection "side-view" to demonstrate the distinction of epithelial and subepithelial compartments of the airway mucosa. **B:** Z-projection "top-view" of the optical sections generated within the epithelial layer. **C:** Z-projection of the optical sections generated within the subepithelial layer. These two parts of the confocal Z-stack were separately analyzed during the quantitative studies.

Figure S2. MHCII co-expression of CD11c-EYFP^+ cells in different anatomical compartments. CD11c-EYFP mice were treated and samples prepared as described for Figure 1. Confocal Z-stacks were taken at the indicated anatomical compartments. EdU^+ CD11c-EYFP^+ cells co-expressing MHCII are indicated with continuous white circles,

MHCII⁺ cells are indicated with dashed circles. Maximum-intensity projections are shown (bar = 100 μ m).

Figure S3. Immunostaining approach for the visualization of Langerin expressing DCs in the airway mucosa. A and B: Untreated CD11c-EYFP mice were sacrificed with an overdose of i.p. pentobarbital. The ears were collected, the chest cavity was opened and the animals were fixed by vascular perfusion via the left heart ventricle with 3% PFA for 5 min. Subsequently, the trachea was collected and post-fixed in 3% PFA overnight at 4°C. Ears of the same animals were split with a forceps to dorsal and ventral halves and incubated in 2mM EDTA in PBS for 2h at 37°C. Then, the epidermis was carefully separated from the dermis using fine forceps. Epidermal sheets were washed with PBS and fixed in 3% PFA overnight at 4°C. Finally, epidermal sheets and tracheal whole-mount samples were stained with antibodies against GFP, MHCII and Langerin according to the whole-mount immunostaining protocol described under “Materials and Methods”. Images show projections of confocal Z-stacks of the ear epidermis (A) or the tracheal mucosa (B). C: Lungs of WT mice were filled with a 1:3 mixture of OCT/PBS and snap-frozen in liquid nitrogen. 5 μ m-thick cryosections were stained for CD11c and Langerin and finally labelled with the nuclear stain To-Pro-3. Images show the projection of a confocal Z-stack with the cross-section of a bronchus. Scale bars = 50 μ m.

Figure S1

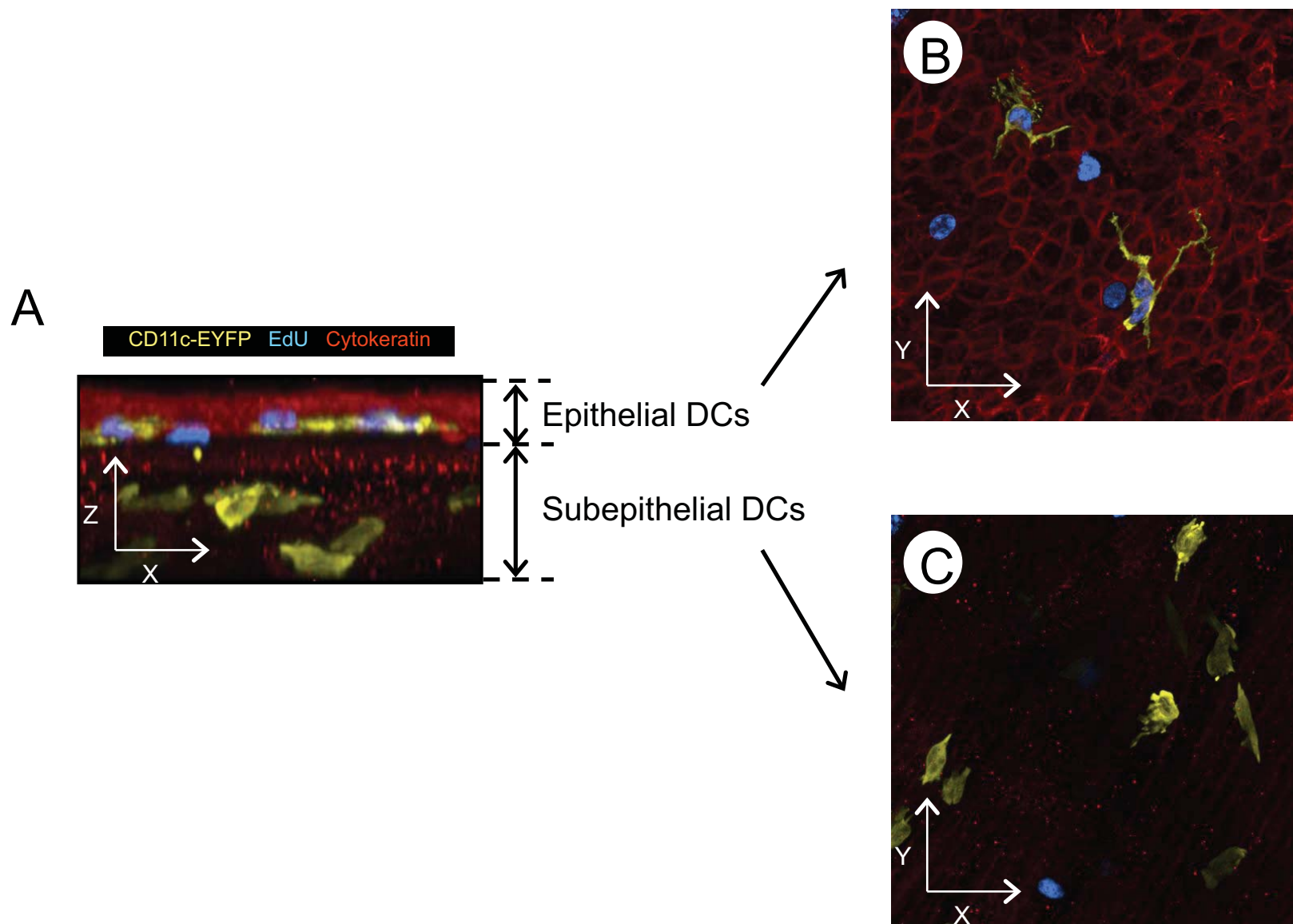


Figure S2

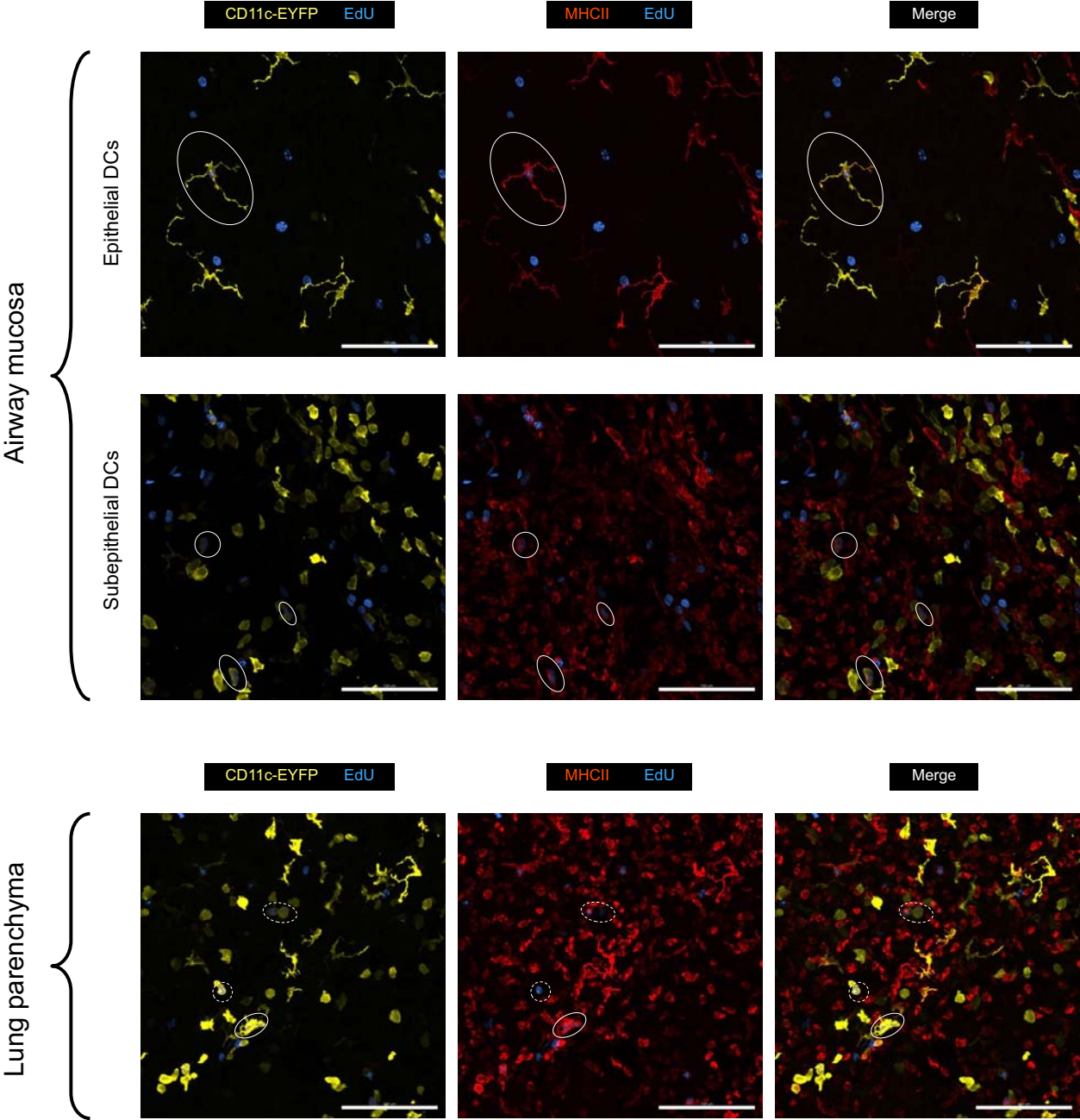
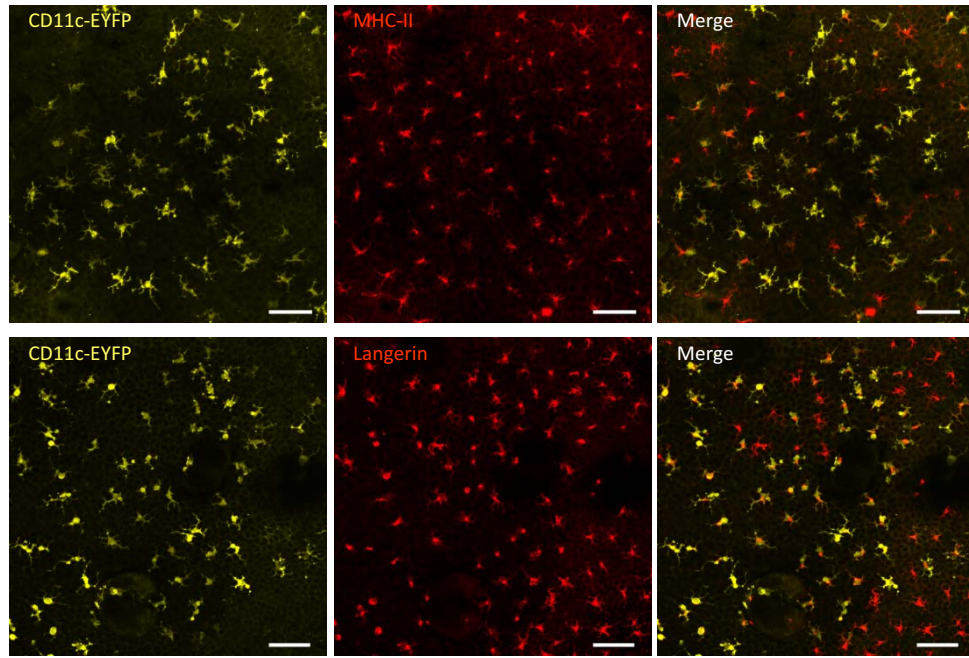


Figure S3

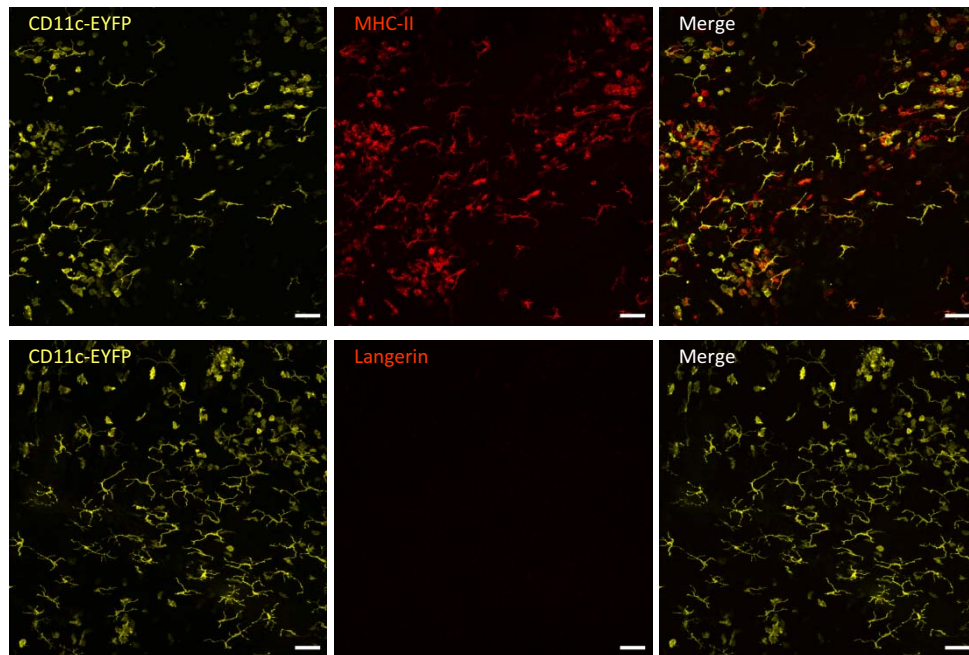
A

Ear epidermal sheets



B

Tracheal whole-mounts



C

Lung
frozen sections

