CD11b+ lung dendritic cells at different stages of maturation induce Th17 or Th2 differentiation

Gentaro Izumi1,3,4, Hideki Nakano1,4✉, Keiko Nakano1, Gregory S. Whitehead1, Sara A. Grimm2, Michael B. Fessler1, Peer W. Karmaus1 & Donald N. Cook1✉

Dendritic cells (DC) in the lung that induce Th17 differentiation remain incompletely understood, in part because conventional CD11b+ DCs (cDC2) are heterogeneous. Here, we report a population of cDCs that rapidly accumulates in lungs of mice following house dust extract inhalation. These cells are Ly-6C+, are developmentally and phenotypically similar to cDC2, and strongly promote Th17 differentiation ex vivo. Single cell RNA-sequencing (scRNA-Seq) of lung cDC2 indicates 5 distinct clusters. Pseudotime analysis of scRNA-Seq data and adoptive transfer experiments with purified cDC2 subpopulations suggest stepwise developmental progression of immature Ly-6C+Ly-6A/E+ cDC2 to mature Ly-6C-CD301b+ lung resident cDC2 lacking Ccr7 expression, which then further mature into CD200+ migratory cDC2 expressing Ccr7. Partially mature Ly-6C+Ly-6A/E-CD301b- cDC2, which express Il1b, promote Th17 differentiation. By contrast, CD200+ mature cDC2 strongly induce Th2, but not Th17, differentiation. Thus, Th17 and Th2 differentiation are promoted by lung cDC2 at distinct stages of maturation.
**Results**

**HDE induces Ly-6C+ cDC2-like cell accumulation in the lung.**

Previous studies have shown that when inhaled together with ovalbumin (OVA), adjuvants such as lipopolysaccharide (LPS)\textsuperscript{30} and HDEs\textsuperscript{31,32}, can promote the in vivo development of OVA-specific Th17 cells that in turn drive airway neutrophilia upon subsequent exposure to that same protein. In agreement with this, levels of IL-17 and IL-13, as well as numbers of neutrophils and eosinophils, were significantly increased in the airways of mice after sensitization with HDE/OVA and challenge with OVA aerosol (Fig. 1a, b and Supplementary Fig. 1a, b). Helper T cell differentiation is known to be induced in the tissue-draining LNs\textsuperscript{28}, but it remains unclear whether this can also occur in the lung. To investigate whether Th17 differentiation can occur in the lung, we measured IL-17 in the lung and mediastinal LNs (mLNs) at various times post-HDE/OVA sensitization. In parallel, we measured IL-13 to assess Th2 differentiation. By 2 days post-sensitization, IL-13 was elevated in mLNs, but did not appear in the lung until 4 days sensitization (Supplementary Fig. 1c). This suggests Th2 cells arise in mLNs and subsequently migrate to the lung. By contrast, IL-17 was elevated in both the lung and mLNs by 2 days post-sensitization (Supplementary Fig. 1c), suggesting that Th17 cells can simultaneously develop in both locations. Taken together, these results suggest that lung-resident cDCs can promote the development of allergen-specific Th17 cells. In support of this, total lung DCs isolated from HDE/OVA-treated mice primed the development of IL-17-producing Th17 cells, as well as IL-13-producing Th2 cells (Fig. 1c), in agreement with a previous report\textsuperscript{33}.

To investigate the profiles of lung cDC populations, we used mass cytometry to compare lung cDCs at steady state and following HDE/OVA allergic sensitization. Unsupervised analysis revealed two cell populations within the CD45+ leukocyte gate that were present only in HDE/OVA-treated mice (Fig. 1d). tSNE analysis identified one of those two populations as interstitial macrophages (IMs), and the other as cDC2. A more restrictive analysis of mononuclear cells (CD45+CD3ε–CD19–NK1.1–Ly-6G+) (Supplementary Fig. 2a) showed that the second population of HDE-induced cells was similar to, but distinct from, traditional cDC2 (Fig. 1d and Supplementary Fig. 2b). These cells displayed low amounts of the macrophage markers, F4/80, CD88, and Siglec-F, but displayed high levels of Ly-6C (Fig. 1e), a marker usually associated with inflammatory monocytes\textsuperscript{20}. This display of Ly-6C allowed us to use flow cytometry to distinguish the HDE-induced cDC2-like cells from conventional cDC2 (Fig. 1f). To evaluate the cDC2-like Ly-6C+ cells by flow cytometry, we gated for cDCs (CD45+CD11c+MHC-II+C/88/F4/80Siglec-F−), excluding Siglec-F+ alveolar macrophages (AMs), C/88/F4/80+ IMs, F4/80+ monocytes, and C/88+MHC-II–neutrophils (Supplementary Figs. 1d and 3a, b). At steady state, there were very few Ly-6C+ antigen-presenting cells (APCs), but their numbers increased dramatically by 12 h post-treatment, peaked between 18 and 24 h, then declined rapidly and stabilized by 48 h (Fig. 1f, g). cDCs having low display of Ly-6C (Ly-6C–cDC2) were abundant at steady state, but their numbers also increased by 24 h post-HDE/OVA inhalation, and returned to baseline by 48 h (Fig. 1g). Number of monocytes and IMs also increased after HDE/OVA inhalation, whereas those of cDC1 and AMs were much less affected by this treatment (Fig. 1g and Supplementary Fig. 3c).

**Ly-6C+ HDE-induced APCs are cDC2.** Like cDC1 and cDC2, HDE-induced Ly-6C+ APCs also formed dendrites typical of DCs (Fig. 2a), but their high display levels of Ly-6C prompted us to...
test whether these HDE-induced APCs were indeed bona fide cDCs, or were instead derived from monocytes. We purified multiple APC subsets and from the lung, prepared RNA from them, and performed bulk RNA-sequencing (RNA-Seq). Principal component analysis (PCA) and differentially expressed gene (DEG) analysis revealed that the transcriptional profile of Ly-6C\(^+\) HDE-induced APCs was very similar to that of cDC2 (relatively few DEGs), and more distantly related to the transcriptomes of other APCs (more DEGs), including cDC1s, preDCs and monocytes (Fig. 2b, c and Supplementary Fig. 4a). For example, the cDC signature genes, Dpp4, H2eb1, Itgax, Kmo, and Zbtb46\(^{34,35,13,36}\) were expressed in Ly-6C\(^+\) HDE-induced APCs and in Ly-6C\(^+\) cDC2 and cDC1, but not in monocytes (Fig. 2d and Supplementary Fig. 4b). Conversely, expression of macrophage-signature genes\(^{36,37}\) was lower in Ly-6C\(^+\) HDE-induced APCs than in monocytes (Supplementary Fig. 4b).
However, the cDC-associated genes, Dpp4 and Zbtb46, were not as highly expressed in Ly-6C+ HDE-induced APCs as in the other cDC subsets. Given that Ly-6C is also displayed on preDCs in the lung and bone marrow (BM) (Supplementary Fig. 5c)38, we reasoned that Ly-6C+ APCs might be immature cDCs. In agreement with this, accumulation of Ly-6C+ APCs in the lungs of HDE-treated animals was severely impaired in Cer2−/−Cx3cr1−/− double knockout (DKO) mice (Supplementary Fig. 5d), reminiscent of a similar finding for preDCs38.

Zbtb46 is exclusively expressed by cDCs34,35, and the promoter of this gene has been used to express diphtheria toxin receptor (DTR) transgenes, thereby conferring selective sensitivity to diphtheria toxin (DTX) in cDCs. As expected, administration of DTX to these Zbtb46-DTR transgenic mice significantly decreased the number of cDC1 and cDC2, and also decreased Ly-6C+ HDE-induced APCs (Fig. 2e). By contrast, monocytes, AMs, and IMs were not decreased. Similarly, Flt3−/− mice lacking FLT3L had significantly fewer cDC1, cDC2, and Ly-6C+ HDE-induced APCs compared with wild-type (WT) mice (Fig. 2f). Together, these observations suggest that the Ly-6C+CD11b+F4/80−CD86−Siglec-F− APCs induced by HDE inhalation are bona fide cDCs, and we henceforth refer to them as “Ly-6C+ cDC2”.

Ly-6C+ cDC2 promote Th17 cell differentiation. All cDC subsets, including Ly-6C+ cDC2, took up fluorescently labeled OVA upon its instillation into the airways of mice (Fig. 3a, b). The antigen processing ability of cDCs was also analyzed using DQ-OVA, which fluoresces upon its digestion by intracellular proteases. The frequency of OVA+Ly-6C+ cDC2 and their mean fluorescent intensity (MFI) were either similar to, or greater than, the corresponding values for the other cDC subsets (Supplementary Fig. 7a, b), suggesting that Ly-6C+ cDC2 have the potential to capture antigens, degrade them, and present antigen-derived peptides to T cells.

We next examined the expression of Il1b, Il6, and Tgfb1, which encode cytokines that promote Th17 differentiation26,27,39. The RNA-Seq data revealed that each of these three genes was more highly expressed in Ly-6C+ cDC2 than in either Ly-6C+ cDC2 or cDC1 (Fig. 3c). Ly-6C+ cDC2 also expressed high levels of Casp1 and Casp4/11, whose encoded proteins activate IL-1β (Supplementary Fig. 7c)40. Il23a, which encodes the IL-23A subunit that supports the survival of Th17 cells41, was highly expressed by Ly-6C− cDC2. By contrast, Il12b, which encodes the p40 subunit of the Th1-promoting cytokine IL-12, was lower in Ly-6C+ cDC2 than in other cDC subsets (Fig. 3c).

To directly test the Th17 cell-inducing ability of Ly-6C+ cDC2, we purified these cells and co-cultured them with naive OVA-specific CD4+ T cells from OT-II TCR transgenic mice. cDC1 and Ly-6C+ cDC2 were also tested for comparison. Compared with other cDC subsets, Ly-6C+ cDC2 induced only moderate proliferation of T cells (Fig. 3d). Consistent with this moderate proliferation, IL-2 production in the co-culture of T cells with Ly-6C+ cDC2 was lower than in co-cultures containing other cDC subsets (Supplementary Fig. 7d). The lower IL-2 production and moderate proliferation induced by Ly-6C+ cDC2 might be due to lower levels of MHC class II and co-stimulatory molecule CD86 on their surface compared with other cDC subsets (Supplementary Fig. 7e, f), in agreement with our results from mass cytometry analysis (Fig. 1e). Strikingly, however, Ly-6C+ cDC2 strongly promoted Th17 differentiation. By comparison, Ly-6C− cDC2 and cDC1 had only weak activity in this regard (Fig. 3d and Supplementary Fig. 7g). Neutralizing antibody (Ab)-mediated blockade of either IL-1β (Fig. 3e and Supplementary Fig. 7h) or IL-6 (Fig. 3f and Supplementary Fig. 7i) markedly suppressed Ly-6C+ cDC2-directed Th17 differentiation, while having either no effect or only modest effects on T cell proliferation.

Ly-6C+ cDC2 promote Th17 response in vivo. The induction of Th17 differentiation by Ly-6C+ cDC2 ex vivo prompted us to test whether Ly-6C+ cDC2 were sufficient to promote Th17 responses in vivo. To this end, we generated a mouse strain in which Ly-6C+ cDC2 represent the vast majority of cDCs in the lung. We first bred mice carrying a Cre recombinase-inducible gene encoding the diphtheria toxin alpha subunit (DTAΔ) with mice carrying a Cre gene under control of the Ilgax (CD11c) promoter (IlgaxCreX DTAΔ). In these mice, previously termed ΔDC12, many CD11c-expressing cells, including cDCs, are reported to undergo spontaneous cell death. Our experiments confirmed this, although some cDC1 and Ly-6C+ cDC2 remained (Supplementary Fig. 8a–d). To selectively reduce the number of BATF3-dependent cDC17, we crossed ΔDC mice with BATF3-deficient mice and found that the offspring of this cross (Batf3−/−ΔDC mice) essentially lacked all lung DC subsets except Ly-6C+ cDC2 (Fig. 4a–d). We then studied responses of Batf3−/−ΔDC mice in an HDE-mediated model of asthma32 (Fig. 4e). Compared with WT mice, Batf3−/−ΔDC animals displayed reduced accumulation of IL-13 (Fig. 4f) and eosiophils (Fig. 4g) in the airways, indicating diminished Th2 responses. By contrast, airway neutrophilia in Batf3−/−ΔDC mice was as high, or higher, than that seen in WT mice (Fig. 4g). Furthermore, IL-17 production from lungs of Batf3−/−ΔDC mice was comparable, or even higher, than in WT mice (Fig. 4f). IFN-γ production was elevated in Batf3−/−ΔDC mouse lungs, suggesting that these mutant mice can develop Th1 responses. Together, these results show that the Ly-6C+ cDC2 selectively retained in Batf3−/−ΔDC mice are sufficient to promote Th17 development, which in turn drives allergen-dependent airway neutrophilia.
Fig. 2 Lineage analysis of HDE-induced Ly-6C<sup>+</sup> APCs. a Morphology of HDE-induced Ly-6C<sup>+</sup> APCs, Ly-6C<sup>-</sup> cDC2, cDC1, and monocyes purified by flow cytometry. Dendrites are indicated by arrowheads. Bars denote 10 μm. A representative result from two independent experiments is shown. b Principal component analyses of RNA-Seq data for these same cell types isolated from mouse lungs at steady state and 16 h after HDE/OVA inhalation (HDE). c Number of DEGs between the indicated pairs of cell populations. Mo: monocyes. d Expression of cDC-signature genes is shown as transcripts per million (TPM) from RNA-Seq analysis. Data were analyzed by two-way ANOVA with Fisher’s LSD multiple comparison test (n = 3). Data are presented as mean ± SEM. e, f Cell numbers for indicated cell populations in HDE/OVA-treated Zbtb46-DTR mice (C57BL/6 background) with or without DTX treatment (e), or HDE/OVA-treated WT or FLT3L KO mice (C57BL/6 background) (f), as determined by flow cytometry. The gating strategy is shown in Supplementary Fig. 3b. Data were analyzed by unpaired two-tailed t-test (n = 4). Data are presented as mean ± SEM. A representative result from two independent experiments is shown. Source data are provided as a Source Data file.
Ly-6C+ cDC2 give rise to a subpopulation of Ly-6C- cDC2. Their short life span (Fig. 1g), gene expression profile (Fig. 2d), and display of Ly-6C (Fig. 1e, f) all suggested that Ly-6C+ cDC2 might represent an immature stage of cDC2. To study this, we purified cDC2 and monocytes from the lung and cultured them in vitro. During culture, monocytes maintained Ly-6C on their surface, whereas Ly-6C+ cDC2 lost Ly-6C, and, at least based on their display of surface markers, became even more similar to Ly-6C- cDC2 (Fig. 5a, b and Supplementary Fig. 9b). The transcriptome of Ly-6C+ cDC2 was more similar to that of Ly-6C- cDC2 than to either cDC1 or monocytes, before and after in vitro culture (Fig. 5c). Nonetheless, cultured Ly-6C+ cDC2 remained transcriptionally distinct from Ly-6C- cDC2 (Fig. 5c and Supplementary Fig. 10a), raising the possibility that Ly-6C+ cDC2 are not simply direct precursors of Ly-6C- cDC2.

Although valuable in many experimental settings, gene profiling of bulk cells has a limited capacity to detect heterogeneity in cell populations, including cDC2. We therefore sorted total cDC2 from the lung following HDE/OVA treatment, and studied them at the single-cell level using scRNA-Seq. Analysis of the data by Seurat software revealed seven distinct clusters within total cDC2 population (Fig. 5d). Examination of genes uniquely expressed by each cluster (Fig. 5e and Supplementary Fig. 10b), as well as comparisons to clusters previously identified in the lungs of naive mice by Han et al. (Fig. 5f) and lungs of virus infected mice by Bosteels et al. (Supplementary Fig. 10c), indicated that cluster 7 is mixture of Xcr1-expressing cDC1s and dividing DCs, whereas cluster 4 is mixture of AMs and IMs. As the latter cells had transcriptional properties of both macrophages and cDC2, they might be equivalent to monocyte-derived DCs recently reported by Menezes et al. By contrast, the remaining five clusters (1, 2, 3, 5, and 6) expressed cDC signature genes but not macrophage genes, suggesting they are all bona fide cDC2 (Fig. 5e, f and Supplementary Fig. 10–d).

Since Ly-6C is a surface marker on cDC2 that induce Th17 differentiation (Fig. 3d), we evaluated cell surface Ly-6C by labeling the cells prior to lysis with an oligonucleotide-labeled Ab directed at that protein (indexed scRNA-Seq). Integration of sequence data corresponding to this bar-coded oligonucleotide together with sequencing data for mRNA allowed us to measure cell surface Ly-6C on each cDC2 cluster. We found that although Ly6c2 RNA is only highly expressed in cluster 6, Ly-6C cell surface protein was present on clusters 1, 3, and 6 (Fig. 6a). This discrepancy between protein and RNA might result from the retention of Ly-6C protein on the cell surface after transcription of the Ly6c2 gene ceased. If so, clusters 1 and 3 may represent more mature forms of cDC2 than cluster 6. The majority of cells in cluster 2 and 5 were negative for both Ly6c2 mRNA and Ly-6C protein (Fig. 6a), suggesting that they might represent relatively immature cDC2.

To analyze the maturation pathway of the above-described cDC2 subpopulations, we needed to first develop a strategy for their purification. We therefore queried the scRNA-Seq data for DEGs encoding cell surface proteins that could be leveraged in flow cytometry experiments to purify cDCs corresponding to the different clusters (Fig. 6b). We found that clusters could be identified using antibodies (Abs) against the following markers: clusters 1 and 3 [Ly-6C-Ly-6A/E]; cluster 2 [CD200+]; cluster 4 [CD14+]; cluster 5 [CD301b+ (encoded by Mgl2)]; and cluster 6 [Ly-6C-Ly-6A/E+] (Fig. 6c). Using these Abs, we found that number of cDCs within clusters 1, 3, and 6 increased dramatically after HDE/OVA-mediated allergic sensitization, peaked around 18–24 h later, and then declined (Fig. 6d). The accumulation of
cDCs within each of these clusters was dependent on the chemokine receptors, CCR2 and CX3CR1 (Supplementary Fig. 12a), suggesting descendance of these cDCs from newly migrated preDCs. Cluster 5 was the major cDC2 population present at steady state, and numbers of these cells did not change dramatically post-sensitization, suggesting they are lung-resident cDC2.

Developmental trajectory (pseudotime) analysis of the clusters using Monocle suggested that cDC2 likely mature in the following order; clusters 6, 1, 3, and 5 (Fig. 7a, b). While cluster 2 might also descend from cluster 3, there is a large gap in pseudotime between those two clusters (Fig. 7a, b), and the transcriptome of cluster 2 is very different from those of the other clusters (Fig. 5e and Supplementary Fig. 10e). Cluster 2 might therefore represent a cDC2 population that is independent of Ly-6C\(^+\) cDC2. To study the maturation of cDC2 subpopulations in vivo, we isolated Ly-6C\(^+\)CD301b\(^-\)CD200\(^-\) cDC2 (corresponding to clusters 6, 1, and 3) from lungs of C57BL/6J (CD45.2) mice and adoptively transferred these cells to CD45.1 recipients (Fig. 7c). Analysis of donor-cDC2-derived CD45.2\(^+\) cells recovered from recipient mice revealed the transferred cells had lost Ly-6C, and had slightly increased CD200, but not CD301b, by 1 day post-transfer (Fig. 7d and Supplementary Fig. 12b). However, by 3 days post-transfer, some donor cDC2 had gained CD301b, whereas CD200 levels were unchanged compared with cells harvested at day 1. These data suggest that CD301b\(^+\) cells in cluster 5 descend from Ly-6C\(^+\) cells in clusters 6, 1, and 3.

Cluster 2 is a minor population in the lung at steady state (equivalent to cluster #29 in the study of Han et al.) (Fig. 5f), but number of these cells increased dramatically after HDE/OVA-mediated mouse model of asthma.  

Fig. 4 Ly-6C\(^+\) cDC2 are sufficient for induction of Th17-dependent neutrophilic airway inflammation. a – d Analysis of cDCs (CD45\(^+\)CD11c\(^+\)A\(^+\)CD88\(^+\)F4/80−Siglec-F−) in the lungs of WT C57BL/6, C57BL/6-Batl3\(^−/−\) and C57BL/6-Batl3\(^−/−\) ΔDC mice at steady state, including representative cytograms (a, c), and compiled data (b, d) showing percentages and cell numbers for total cDC, cDC2, and cDC1 (b), and for Ly-6C\(^+\) cDC2 (d). The gating strategy is shown in Supplementary Fig. 1d. Data were analyzed by ordinary one-way ANOVA with Dunnett’s multiple comparison test (n = 4). Data are presented as mean ± SEM. e Timeline for allergic sensitization and allergen challenge in HDE/OVA-mediated mouse model of asthma. f Cytokine production in lungs of challenged mice, as measured by ELISA. g Cell numbers for the indicated leukocyte subsets in BALF of allergen-challenged WT and Batf3\(^−/−\) ΔDC mice. Data were analyzed by ordinary one-way ANOVA with Tukey’s multiple comparison test (n = 14 WT and n = 13 Batf3\(^−/−\) ΔDC mice). Data are presented as mean ± SEM. Combined results of two independent experiments are shown. Source data are provided as a Source Data file.
mediated sensitization (Fig. 6d). Unlike clusters 1, 3, and 6, this increase was not dependent on CCR2 and CX3CR1 (Supplementary Fig. 12a), suggesting that cluster 2 cells are derived from lung-resident cDCs, which are in cluster 5. To test this, we adoptively transferred Ly-6C<sup>+</sup>cDC2 from C57BL/6J (CD45.2) mouse lung into CD45.1 recipients. The majority of donor-cDC2-derived CD45.2<sup>+</sup> cells recovered from the recipient lungs at 1 day post-transfer had lost CD301b, but had undergone dramatic increases in CD200 (Fig. 7e and Supplementary Fig. 12c). Very few donor-derived cDC2 were recovered from recipients at 3 days post-transfer, but these cells also displayed the CD301b<sup>+</sup>Ly-6C<sup>+</sup>CD200<sup>+</sup> phenotype (Fig. 7e).
and Supplementary Fig. 12c). These results demonstrate that CD301b+ cDC2 can give rise to fully mature CD200+ cDC2.

**Ly-6C+ cDC2 subpopulation stimulates Th17 differentiation.** Previous studies have demonstrated that specific transcription factors are required in cDCs for their promotion of different T helper cell lineages; Irf4 for Th2 and Th17; Klf4 and Relb for Th2; and Notch2 for Th17 responses20,22,23,47–49. Our scRNA-Seq analysis revealed selective expression of Relb in cluster 2, whereas Irf4, Klf4, and Notch2 were not expressed in a cluster-specific manner (Fig. 8a). However, we did observe selective expression of Il1b gene in clusters 3 and 5 (Fig. 8a), suggesting these cDCs might preferentially stimulate Th17 differentiation. To test this experimentally, we purified four cDC2 subpopulations; cluster 1 + 3 (Ly-6C+Ly-6A/E−), cluster 2 (CD200+), cluster 5 (CD301b+), and cluster 6 (Ly-6C+Ly-6A/E+) from mouse lungs after HDE/OVA instillation, and separately co-cultured them with naive CD4+ T cells from OT-II mice. Mature cDC2 in cluster 5 (CD301b+) and 2 (CD200+) potently induced T cell proliferation (Fig. 8b). Th17 differentiation was most strongly induced by cDC2 in cluster 1 + 3 (Ly-6C+Ly-6A/E−), followed by cluster 5 (CD301b+) and cluster 6 (Ly-6C+Ly-6A/E+) (Fig. 8c and Supplementary Fig. 14b). cDC2 in cluster 2 (CD200+) were the least effective in this regard. Cluster 2, while very poorly inducing Th17 differentiation, strongly induced Th2 differentiation. Cluster 5 had modest Th2-inducing activity, whereas the Ly-6C+ subpopulations performed poorly in this regard (Fig. 8c). Taken together, the data indicate that partially mature cDC2 (mainly cluster 3) expressing Il1b preferentially induce Th17 differentiation, while fully mature cDC2 in cluster 2 selectively induce Th2 cells.

**Human counterparts of mouse lung cDC2.** We next investigated the relationship between the above-described clusters of mouse lung cDC2 and scRNA-Seq data recently reported for cells in human lung50. Human CD1c+ cDC2 are heterogeneous and comprise at least two subpopulations (Supplementary Fig. 15a).
To identify potential human counterparts of individual mouse cDC2 clusters, we evaluated human cell expression of DEGs that defined the various cDC2 clusters we had identified in the mouse lung. A human cDC2 subpopulation expressed Ifitm1, Atf3, and Ccl17, which were also highly expressed in mouse cDC2 clusters 1, 3, and 5 (Supplementary Fig. 15b). Noteworthy, this human cDC2 subpopulation also highly expressed Il1b (Supplementary Fig. 15a). By contrast, a different human cDC2 subpopulation had relatively low levels of Il1b. This subpopulation was similar to mouse cDC2 cluster 2, which also expressed Marcksl. These results suggest that the former human cDC2 subpopulation might preferentially stimulate Th17 cell differentiation.

**Lung-resident cDCs induce Th17 cell differentiation.** Further analysis of our scRNA-Seq data revealed that expression of Ccr7 is restricted to the Th2-inducing cDC2 in cluster 2 (Supplementary Fig. 10e). Because CCR7 is the chemokine receptor primarily responsible for directing migration of cDC2s to lung-draining LNs,29 this finding suggests that most of the Th17-inducing cDC2 in clusters 1, 3, and 5 do not migrate to mLNs. To test this, we...
Data are presented as mean ± SEM. Source data are provided as a Source Data file.

**Discussion**

Identifying a consensus Th17-inducing cDCs in the lung has proven elusive, with different research groups reporting discrepant conclusions.\(^{18,22}\) Reasoning that the heterogeneity of cDC2 might be responsible for these discrepancies, we deeply analyzed lung cDCs using a combination of high-dimensional mass cytometry, conventional flow cytometry, and scRNA-Seq. Mass cytometry revealed that a cDC2 subset with cell surface display of Ly-6C rapidly accumulates in the lung post-allergic sensitization, and flow cytometry-based cell sorting showed that these cDC2 can potently stimulate Th17 differentiation ex vivo. It is possible that this cDC subset has gone unappreciated until now because Ly-6C\(^+\) cells are often assumed to be monocytes,\(^{20}\) and are therefore often excluded from cDC preparations. However, Ly-6C can be displayed by multiple cell types including preDC53.

Our use of indexed scRNA-Seq to simultaneously evaluate Ly6c2 gene expression and Ly-6C protein suggested that immature cDC2 (cluster 6) express this gene, and that during maturation Ly6c2 expression is lost first (clusters 1 and 3), followed by loss of Ly-6C protein (cluster 5). The immature status of cluster 6 is also suggested by its exclusive expression of Ly6a, which encodes the Ly-6A protein displayed on hematopoietic stem cells and DC progenitors.\(^{34,55}\) This suggests an order of maturation from cluster 6 (Ly-6C\(^+\)Ly-6A/E\(^+\)) → clusters 1/3 (Ly-6C\(^+\)Ly-6A/E\(^-\)) → cluster 5 (CD301b\(^+\)), a path that is also consistent with the developmental order obtained from the pseudotime analysis, and in agreement with results of our adoptive transfer experiments with Ly-6C\(^+\) cDC2. This developmental order might also explain the moderate Th17 promoting ability of Ly-6C\(^+\) cDC2, as they likely arise from Ly-6C\(^+\) cDC2 precursor cells. Although Ly-6C\(^+\) cDC2 are not as abundant at steady state as they are post-HDE,

fluorescently labeled lung cDCs in vivo by instilling PKH26 dye into the airways of mice together with HDE/OVA, and analyzed PKH26\(^+\) cDCs in lung-draining mLNs on the following day (Supplementary Fig. 16a). Among PKH26\(^+\) migratory cDC2, more than 90% were CD200\(^-\), including CD200\(^+\) single positive and CD200\(^+\)CD301b\(^+\) double positive cells (Supplementary Fig. 16a, b). By contrast, there were very few CD200\(^-\) cells among PKH26\(^+\) cDC2 in mLNs, consistent with their lack of CCR7. This raised the possibility that the latter cells, which promote Th17 differentiation ex vivo, might also fulfill this task in vivo as lung-resident cDCs. This would be consistent with our result that IL-17 production post-sensitization occurs in the lung with similar kinetics as production of that cytokine in lung-draining mLNs (Supplementary Fig. 1c). To confirm that Th17 cell differentiation can occur in the lung, we adoptively transferred IL-17 fate-mapping, OVA-specific (OT-II) CD4\(^+\) T cells to recipient mice and evaluated Th17 cell development in the lung and mLNs at various times post-HDE/OVA sensitization.\(^{31}\) The frequency of OVA-specific IL-17\(^+\) Th17 cells among donor-derived CD4\(^+\) T cells was higher in the lung than in mLNs at multiple time points post-HDE/OVA sensitization (Supplementary Fig. 16c, d).

Our findings do not exclude the possibility of Th17 induction by mLN cDCs such as blood-derived cDCs, but clearly indicate that lung-resident cDCs can promote the development of allergen-specific Th17 cells. To examine whether lung-resident cDCs are sufficient for the development of Th17-mediated neutrophilic allergic airway inflammation in vivo, we studied Lymphotixin alpha (Lta)–deficient mice, which lack peripheral LNs.\(^{32}\) Following splenectomy to avoid the confounding issue of sensitization in the spleen, animals were sensitized with LPS/OVA and subsequently challenged with aerosolized OVA (Supplementary Fig. 16e). WT mice developed eosinophilia, whereas Lta\(^/-\) mice did not (Supplementary Fig. 16f), suggesting that the spleen and/or LNs are necessary for Th2 cell development. By contrast, splenectomized Lta\(^/-\) mice developed robust neutrophilia after challenge, demonstrating that the spleen and LNs are dispensable for Th17-dependent neutrophilia.
they are nonetheless present in the lung and might constitutively differentiate into the more mature CD301b+ lung-resident cDC2. cDC2 in cluster 2 are almost absent at steady state, but their numbers increase dramatically following HDE/OVA inhalation. This accumulation was independent of CCR2 and CX3CR1, contrasting with Ly-6C+ cDC2 and preDCs, which are dependent on these chemokine receptors. This suggests that the cDC2 in cluster 2 arise from a pool of existing lung-resident preDCs or cDC2, but not from newly migrated preDCs, upon inhalation of agents such as HDE. Indeed, adoptive transfer experiment revealed that CD301b+ cDC2, which are the sole lung-resident cDC2 at steady state, mature to CD200+ cDC2, which are equivalent to the cells in cluster 2. These cDC2 were by far the most potent at promoting Th2 differentiation ex vivo, and they express many unique genes, including Relb, which encodes a transcription factor required in lung cDC for Th2 induction59. Thus, RELB might control the Th2-inducing function of these cDC2.

Another striking feature of cluster 2 in cDC2 is their exclusive expression of Ccr7. The limited expression of Ccr7 among total cDC2 might explain why they are generally less migratory than cDC129. It is not surprising that Ly-6C+ cDC2 (cluster 1, 3, and 6) do not express Ccr7, as they resemble immature or transitional cDCs, but the absence of appreciable Ccr7 in mature cDCs within cluster 5 was unexpected. cDC2 in cluster 5 and their immediate precursors in cluster 3 are Th17-inducing lung-resident cDCs, as they express Il17b and Tgfbi1 and induce Th17 cell differentiation ex vivo. The function of lung-resident cDC2 is supported by our findings that Th17 responses (but not Th2 responses) can be detected in the lung shortly after HDE/OVA inhalation and that secondary lymphoid tissue is dispensable for Th17-dependent neutrophilia in HDE/OVA-mediated mouse model of asthma. In that model, we found that in addition to robust neutrophilia, lymphocyte numbers were also increased in the LN-deficient Lta−/− mice. This observation is consistent with previous studies showing that migration of cDCs from the lung to mLN is required for the induction of tolerance to inhaled antigens28,56. mLN might therefore be critical to both promote Th2 responses and regulate inflammatory responses. By contrast, mLN are dispensable for Th17 differentiation, which can occur in the lung itself, and is likely driven by lung-resident cDCs.

Comparison of our scRNA-Seq data with previously published data allowed several parallels to be drawn. Han et al. identified multiple clusters of cDCs in the mouse lung at steady state24, with an abundance of Mgl2+ cDC2. That cluster is transcriptionally most similar to our cluster 5, which was the major cDC2 cluster at steady state, and is somewhat similar to our clusters 1 and 3, which are developmentally related to cluster 5. Gngt2+ cDCs in the study of Han et al. are similar to the Ly-6C+ clusters 6 and 1 in our analysis but different from cluster 5, suggesting these cells are at an immature stage. Han et al. also detected a very minor population, H2-M2+ cluster 29, in lungs of naive mice, and this population is likely equivalent to cluster 2 in our analysis. Very recently, Bosteels et al. reported an analysis of cDC2 populations in the mouse lung following infection with the RSV-related pneumonia virus of mice (PVM)25, and identified an inflammatory cDC2 population that has potent ability to induce proliferation and Th1 differentiation of CD4+ T cells. Comparing those data to our scRNA-Seq data revealed similarities between the inflammatory cDC2 reported by Bosteels et al. with cDC2 clusters 1 and 6 in our study. However, a counterpart of cluster 3, which preferentially stimulates Th17 cell differentiation, was not clearly seen in that comparison. Thus, depending on the stimulus to which they are exposed, cDCs might acquire different transcriptomic profiles and different functions.

Comparing our scRNA-Seq data with human lung cDC data reported by Vieira Braga et al.36 revealed likely human counterparts of mouse cDC2 clusters. Mouse cDC2 in clusters 1, 3, and 5 in our study are likely analogous to a subpopulation of human cDC2 expressing Il17b reported by Vieira Braga et al. Recently Dutertre et al. reported that human CD5−CD163+CD14+ cDCs isolated from human blood potently stimulate Th17 differentiation37. We could not identify an unambiguous counterpart of that cDC cluster in our mouse cDC2 populations, possibly due to both species- and tissue-specific differences between the cells analyzed. However, it is possible that the Il17b-expressing human cDC2 cluster29 overlaps with CD5−CD163+CD14+ cDC2. Finally, with regard to Th2 instruction, a subpopulation of human cDC2 that is distinct from Il17b+ cells expresses Markscl1. We found that this gene is uniquely expressed by mouse cluster 2, suggesting that the Il17b+ Markscl1+ human cDC2 subpopulation might be the human counterpart of the mouse cDC2 in cluster 2 (CD200+ cDC2) that we observed to drive Th2 differentiation. Further characterization of both human and mouse cDCs will be necessary to test this.

In conclusion, we have identified multiple subpopulations of mouse cDC2, including a non-migratory lung-resident population that preferentially stimulates allergen-specific Th17 cell differentiation. These findings should assist in the development of both preventive and therapeutic strategies to control the induction and severity of Th17-mediated neutrophilic asthma.

Methods

**Mice.** C57BL/6, C57BL/6-Baf53−/− (B6.129S-C(Baf53tm1Ifc)Ksny/J), C57BL/6-Ccr2−/− (B6.129S4-Ccr2tm1Ifc/J), C57BL/6-Cd11c−/− (B6.Cg-Tg(Ifgus-crf1-lacZ)1-Res/J), C57BL/6-CD45.1 (B6.SL-Tg(Cx3cr1-EGFP-BrdU)1Bdel/J), C57BL/6-DTA−/− (B6.129P2-Gr(ROSA)26Sorαi1(ΔTA/ΔTA)rd21118N(Sorαi1lacZ1015Ncre/+)Mak/J), C57BL/6-Tg(OT-2 TCR)425Cbn/J, and C57BL/6-D6C-DTR (B6.Cg(Zbhbgd461H6/2B6G12C10)M1v/J) mice were purchased from Jackson Laboratories. C57BL/6-FI3L−/− (C57BL/6-FI3Ltm1We珀/J) and C57BL/6-Cx3cr1−/− (B6.129-Cx3cr1tm1(dNeom)J) mice were purchased from Taconic Biosciences (Germantown, NY, USA)38. OVA-specific Il17a fate-mapping mice on C57BL6 background (B6.Cg-Ilt17a111111TfPcfy2892H2f Gr(ROSA)26Sorαi1(ΔTA/ΔTA)rd21118N(Sorαi1lacZ1015Ncre/+)Mak/J) and Tg(TcraTcrb)425Cbn/J mice were purchased from Taconic Biosciences (Germantown, NY, USA)58. OVA-specific IL17α-fate mapping mice on C57BL6 background (B6.Cg-Ilt17a111111TfPcfy2892H2f Gr(ROSA)26Sorαi1(ΔTA/ΔTA)rd21118N(Sorαi1lacZ1015Ncre/+)Mak/J) and Tg(TcraTcrb)425Cbn/J mice were generated as previously described41. Ccr2−/− Cx3cr1−/− DKO mice were generated by crossing Ccr2−/− and Cx3cr1−/− mice50. ΔDC mice were generated by crossing Batf3−/− and Batf3−/− ΔDC mice were generated by crossing Batf3−/− and Batf3−/− mice51. B6.Cg-Ilt17a111111TfPcfy2892H2f Gr(ROSA)26Sorαi1(ΔTA/ΔTA)rd21118N(Sorαi1lacZ1015Ncre/+)Mak/J mice were bred and housed in specific pathogen-free conditions at the NIHIEHS with the following housing condition: light cycle: 7:00 a.m. to 7:00 p.m., temperature: 72 ± 2 °F, humidity: 40–60%. Mice were used between 6 and 12 weeks of age. All animal procedures complied with institutional guidelines approved by the NIHIEHS Animal Care and Use Committee.

Allergic sensitization and mouse model of asthma. For allergic sensitization, mice were lightly anesthetized with isoflurane and given two oropharyngeal (o.p.) instillations, 1 week apart, of 100 µg LPS-free OVA (Worthington Biomedical) with 10 µl HDE or 100 ng LPS (Maplewood Sigma) in a total of sodium perborat (0.5 µl pm PBS (HDE/OVA or OVA)30). The HDE was prepared as previously described52,59. Briefly, vacuumed dust samples from homes in North Carolina were passed through a coarse sieve, then extracted at 100 mg/ml with PBS at 4°C with overnight mild agitation. The samples were centrifuged to remove insoluble debris, and supernatants were sterilized by passage through a 0.22 µm filter (Millipore Sigma). Endotoxin concentration was 50 ng LPS/10 µl HDE, as measured by a Limulus Amoebocyte Lysate assay (Lonza, catalog #50-648U). In some experiments, 100 µg Alexa Fluor (AF) 647-conjugated OVA or 10 µg DQ-OVA (ThermoFisher Scientific) was used to analyze antigen uptake50. In some experiments, DTX (20 mg/kg body weight) (List Biological Laboratories) was injected into peritoneal cavity (Vortech Pharmaceuticals) at 48 h after challenge, bronchoalveolar lavage fluid (BALF) and lung tissue were collected. BALF leukocytes were spun onto glass slides using Cytospin centrifuge (ThermoFisher Scientific), and stained with hematoxylin and eosin prior to microscopic analysis. Lung tissues were incubated in complete RPMI1640 (ThermoFisher Scientific) containing 10% fetal bovine serum (FBS; characterized, HyClone), 50 µM β-mercaptoethanol, penicillin, streptomycin, and 10 µg/ml OVA for 24 h. Cytokines in the supernatant were measured by ELISA.
Isolation of DCs, preDCs, and T cells. Lungs were harvested from untreated mice or 16 h after instillation of HDE/OVA unless specified. Lungs were perfused by PBS injection followed by air pressure. For DC purification to label dead cells, tissue was digested with Liberase TM (100 µg/mL) (Roche), Collagenase XI (250 µg/mL), Hyaluronic acid (1 mg/mL), and DNase I (200 µg/mL) (Sigma Aldrich) for 30 min (mass cytometry) or 60 min (flow cytometry) at 37 °C. The reaction was stopped by the addition of EDTA (20 mM final concentration). A single-cell suspension was prepared using Liberase TM (BD Biosciences). Cell suspensions were stained with anti-mouse CD16/CD32 Ab (2.4G2) (10% culture supernatant), 5% normal mouse, and 5% rat streptomycin in a 96-well U-bottom plate (BD Biosciences)62. In some experiments, puriﬁed small lymphocytes were stained with the following fluorochrome-conjugated Abs obtained from BD Biosciences (BD), BioLegend (BL), or eBioscience (ThermoFisher scientiﬁc) (eBio): anti-mouse CD3e (145-2C11, BD 553060; 0.5 µg/mL), CD11b (M170, BD 553309; 0.5 µg/mL), CD19 (6D5, BL 115504; 0.5 µg/mL), CD45R/B220 (RA3-6B2, eBio 12-4352-82; 0.5 µg/mL), CD49b (DX5, BD 553856; 0.5 µg/mL), Ly-6a/E (D7, BD 557404; 0.5 µg/mL), Ly-6G (1A8, BL 127604; 0.5 µg/mL), and TER119 (TER-119, BD 553672; 0.5 µg/mL)38. cDCs and preDCs were puriﬁed by ﬂow cytometric sorting.

Flow cytometric analysis and sorting. Cells were diluted to 1–2×10^6/100 µL and incubated with a non-speciﬁc binding blocking reagent cocktail of anti-mouse CD16/CD32 Ab (2.4G2) (10% culture supernatant), 3% normal mouse, and 5% rat serum (Jackson ImmunoResearch)38. Cell surface antigens were stained with the following fluorochrome-conjugated Abs obtained from BD Biosciences (BD), BioLegend (BL), or eBioscience/ThermoFisher Scientiﬁc (eBio): anti-mouse CD3e (145-2C11, BD 553060; 0.5 µg/mL), CD11b (M170, BD 553309; 0.5 µg/mL), CD19 (6D5, BL 115504; 0.5 µg/mL), CD45R/B220 (RA3-6B2, eBio 12-4352-82; 0.5 µg/mL), CD49b (DX5, BD 553856; 0.5 µg/mL), Ly-6a/E (D7, BD 557404; 0.5 µg/mL), Ly-6G (1A8, BL 127604; 0.5 µg/mL), and TER119 (TER-119, BD 553672; 0.5 µg/mL)38. cDCs and preDCs were puriﬁed by ﬂow cytometric sorting.

Mass cytometry. Low-density cells (3×10⁷) isolated from perfused lungs of C57BL/6 mice were incubated for 5 min with 1 µM of Cell-Id Cisplatin (Fluidigm) at room temperature to label dead cells, then washed with Maxpar Cell Staining Buffer (Fluidigm). To block non-speciﬁc Ab binding, cells were incubated with anti-mouse CD16/CD32 Abs, normal mouse serum, and rat serum, then incubated with 50 µL of metal-conjugated Abs (Supplementary Table 1) for 30 min. Staining of cells with ﬂuorescence-conjugated primary Abs was followed by metal-conjugated secondary Abs. Cells were then stained with 1 µM of Cell-Id Cisplatin for 60 min, washed with Maxpar Cell Staining Buffer, and then incubated in 125 nM Cell-ID Intercalator-Ir (Fluidigm) in Maxpar Fix and Perm Buffer (Fluidigm) overnight. On the following day, cells were washed and ﬁltered through BelArt SP Flowmix cell strainers (Fisher Scientiﬁc), and analyzed on a Helios mass cytometer (Fluidigm) with CyTOF 6.7 software (Fluidigm). Data were analyzed using Cytobank platform (Cytobank).

Detection of allergen-specific Th17 and Th2 cells. Lymphocytes were isolated from LNs and spleens of donor mice, including OT-II and IL-17a single-cell suspension using Cytomax (Miltenyi) by negative selection with the following biotinylated Abs obtained from BD Biosciences (BD), BioLegend (BL), or eBioscience (ThermoFisher Scientiﬁc) (eBio): PE-anti-mouse CD3e (145-2C11, BD 553060; 0.5 µg/mL), CD11b (M170, BD 553309; 0.5 µg/mL), CD19 (6D5, BL 115504; 0.5 µg/mL), CD45R/B220 (RA3-6B2, eBio 12-4352-82; 0.5 µg/mL), CD49b (DX5, BD 553856; 0.5 µg/mL), Ly-6a/E (D7, BD 557404; 0.5 µg/mL), Ly-6G (1A8, BL 127604; 0.5 µg/mL), and TER119 (TER-119, BD 553672; 0.5 µg/mL)38. cDCs and preDCs were puriﬁed by ﬂow cytometric sorting.

Co-culture of DCs and CD4⁺ T cells. Naive CD4⁺ T cells were puriﬁed from LNs and spleens with AutoMACS (Miltenyi) by streptavidin-conjugated MACS beads (Miltenyi) and a biotinylated Ab cocktail containing the following Abs obtained from BioLegend (BL) or eBioscience (ThermoFisher Scientiﬁc) (eBio): PE-anti-mouse CD3e (145-2C11, BD 553065; 1 µg/mL), APC-anti-mouse CD4 (RM4-5, BL 100516; 1 µg/mL), BV421-anti-mouse CD8α (253-22, BL 114013; 1 µg/mL), BV601-anti-mouse CD8β (327.15, BL 102406; 1 µg/mL), BV711-anti-mouse CD45R/B220 (RA3-6B2, eBio 12-4352-82; 0.5 µg/mL), CD86 (AF6.120.1, BL 116404; 0.5 µg/mL), Ly-6C/G (R6-808.5, BD 553125; 0.5 µg/mL), and CD44 (IM7, BL 100304; 0.05 µg/mL)60. Naive CD4⁺ T cells (5×10⁶ cells/well) and lung cDCs (5×10⁶ cells/well) were co-cultured in a 7.5% CO₂ incubator for 5 days in 200 µL complete Iscove’s modiﬁed Dulbecco’s medium (IMDM) containing 10% FBS (certiﬁed, Invitrogen), 50 µM β-mercaptoethanol, penicillin and streptomycin in a 96-well U-bottom plate (BD Biosciences)62. In some experiments, neutralizing Abs against mouse Il-1β (B122, BL 503514) or Il-6 (MP5-23, BL 503453) or hamster IgG (HTX886, BL 400990) or rat IgG (RTK2071, BL 400432) isotype control Abs (3 µg/mL) were added to co-cultures of cDCs and T cells. Culture supernatant was collected 3 days after culture, and Il-2 was measured by ELISA. Cells were harvested and washed 5 days after culture, and viable cells were counted using Luna-Fl cell counter (Lumos Biosystems). To elicit efector T cell responses, T cells were incubated (1×10⁶ cells/200 µL/well) for 24 h in a 96-well ﬂat-bottom plate coated with Abs to mouse CD3e (145-2C11, BD 100331; 1 µg/mL) and CD28 (37.51, BL 102116; 1 µg/mL). Cytokines in the supernatant of incubated T cells were measured by ELISA using Multispan Ascent plate reader with Ascent 2.6 software (Thermo Electron) or BioPlex Immunoassay (Bio-Rad, catalogue #171G5013M) according to manufacturer’s instruction.

Ex vivo DC maturation assay. Lung cDCs or monocytes were puriﬁed by ﬂow cytometry and cultured (2×10⁵ cells/200 µL/well) for up to 2 days in complete RPMI-10 in 96-well U-bottom plates. The phenotypes of cultured cells were analyzed by ﬂow cytometry. Total RNA was isolated from freshly isolated or cultured cells using Nucleoscript RNA XS kit according to the manufacturer’s instruction (Takara Bio, catalogue #740902.50). mRNA expression was examined using the NanoString platform utilizing the Mouse Myeloid Innate Immunity Panel v2 (NanoString Technologies) that measures 732 endogenous and 20 housekeeping RNAs. RNA expression data (as quantiﬁed on the nCounter Digital Analyzer) were adjusted using the manufacturer’s positive and negative experimental control probes as well as housekeeping genes with nSolver 4.0 software (NanoString Technologies). The data were further analyzed by Partek J.0 (Partek) and R 3.5.2 (R Foundation) software. The results (log2) are presented in Supplementary Data 1.

In vivo DC maturation assay. Ly-6C⁺ CD11c⁺ DC200⁺ or Ly-6C⁻CD11c⁻CD200⁻ DC2 from HDE/OVA-sensitized C57BL/6 mice (CD45.2) were puriﬁed by ﬂow cytometry. Cells were adoptively transferred into C57BL/6 mice (CD45.1) or Ly-6C⁻CD11c⁻CD200⁻ derived cells recovered from recipient lungs was analyzed by ﬂow cytometry.

**ARTICLE**

**NATURE COMMUNICATIONS** | doi:10.1038/s41467-021-25307-x | www.nature.com/naturecommunications
Transcriptome analysis of lung APCs. RNA from lung cDCs and monocytes and BM preDCs (1–10^6 cells/sample) harvested by tissue homogenization and counted according to the manufacturer’s instructions. Multiplexed cDNA libraries were sequenced by the NIEHS Epigenomics and DNA Sequencing Core Laboratory on an Illumina HiSeq 4000 (Illumina) according to the SELEX methods recommended in the BioLegend protocol (https://www.biolegend.com/en-us/totalseq)45. The cDNA for antibody-derived transcripts (ADT) and gene-derived transcripts was generated and amplified by the NIEHS Epigenomics and DNA Sequencing Core Laboratory on NextSeq 500 (Illumina) with paired-end sequencing (Read 1:30; Read 2:150). The data were processed using DESeq2 version 2.11.1. A total of 5.9 x 10^6 reads were obtained.

Indexed single-cell RNA sequencing. CD11c^+ cDC2 from HDE/OVA-treated C57BL/6 mice were purified by flow cytometry; BM preDCs (1 x 10^5 cells/mL concentration with 98% viability were loaded into the scRNA-Seq protocol. The raw data were filtered (Seurat) raw count matrices were subset on mito-score >20. Filtered reads were mapped to the mm10 reference genome via STAR alignment, barcode assignment, and unique mapping. The Gene Expression library and the ADT Library were sequenced by the NIEHS Epigenomics and DNA Sequencing Core Laboratory on a MiSeq5000 (Illumina) as single-end 76-mers. The data were processed using UMAP version 2.3.3. Reads were filtered to retain only those with mean base quality score >20. Filtered reads were mapped to the mm10 reference genome via STAR version 2.5 (parameters –outMultiMappers –outSortMattersStart -o –outFilterType ByStrand –outOrderNorm None) and subsequently normalized by size factors reported from DESeq265. PCA coordinates were determined using R function “heatmap2” with row-scaling of rlog-transformed scores as calculated by DESeq2. The RNA-Seq expression heatmap was generated by R function “heatmap.2” with row-scaling of rlog-transformed scores as calculated by DESeq2.

Analysis of scRNA-seq data. scRNA-seq raw data processing. Alignment, barcode assignment, and unique molecular identifier (UMI) counting was performed using Cell Ranger 3.0.1 and the “cellmark count” command. Alignment and UMI counting were performed with the minimum mm10-1.2.0 reference. The following feature libraries were included for antibody sequencing: Ly-6C (sequence: AAGTCGTGAGGCATG) and CD11b (sequence: TGGAGGCTCATTTGT). Outputs from filtered count matrices were used for subsequent analyses. From Cell Ranger an estimated 3891 cells, 144,076 mean reads per cell, and 2032 median genes per cell were recovered. 99.9% of reads mapped to genome and 97.7% barcodes were valid in antibody sequencing.

scRNA-Seq dimensionality reduction and clustering. Data from scRNA-Seq were processed using the Seurat v3.0 package in R version 3.6.2 (https://satijalab.org/seurat/). Data were filtered on characters for homogeneity, including number of features (high threshold: 4500; low threshold: 1000), total RNA counts (high threshold: 30,000; low threshold: 250), proportion cycling (high threshold: 0.06; low threshold: 0.01), and proportion of mitochondrial RNA (high threshold: 0.025; low threshold: 0.005). Data were normalized and scaled for number of RNA features, proportion cycling, and proportion of mitochondrial RNA. Normalized and scaled gene expression data were projected onto principal components (PCs). The first 30 PCs were used for non-linear dimensionality reduction using Uniform Manifold Approximation and Projection (UMAP)66. Gene expression and metadata were visualized using this UMAP projection. Clustering was performed using the “FindNeighbors” (k parameter = 50), followed by the “FindClusters” (resolution = 0.5) functions of the Seurat v3.0 package in R version 3.6.266. Cluster marker genes from res.0.5 in Seurat (described above) were generated by the “FindAllMarkers” function (Supplementary Table 2). Heatmaps corresponding to scRNA-Seq data reported by Han et al.24, Bostelo et al.25, and Dutertre et al.57 were generated with R version 3.6.2 package heatmap2 using normalized expression scores from Seurat analysis of lung cells with assigned cell type annotation.

scRNA-Seq pseudotime analysis. Pseudotime analysis was performed using Monocle246 according to instructions provided on GitHub (https://cole-trapnell-lab.github.io/monocle-release/docs/). In short, filtered (Seurat) raw count matrices were subset on Seurat res.0.5 clusters 1, 2, 3, 5, 6 and used to infer cellular developmental trajectories. The information was projected onto two-dimensional space using “DDIRTree.” In addition, inferred pseudotime from Monocle 2 was projected onto UMAP dimensions 1 and 2 from Seurat (described above).

Statistics. Data are presented as mean ± SEM. Statistics to analyze differences among groups using Prism software are indicated in figure legends. P < 0.05 was considered significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The NanoString data have been deposited in the Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo) under accession code GSE156763, and are also provided in Supplementary Data 1. The bulk RNA-Seq data of lung APCs in this study have been deposited in GEO under accession code GSE149778. DEGs in the bulk RNA-Seq analysis are provided in Supplementary Data 2. The scRNA-Seq data of CD11c^+ cDC2 have been deposited in GEO under accession code GSE156527. All other data supporting the findings of this study are available from the corresponding author upon request. Source data are provided with this paper.

Code availability. Custom codes were not created for data analyses in this study. Analysis followed publicly available instructions from Seurat (http://satijalab.org/seurat/) and Monocle (http://cole-trapnell-lab.github.io/monocle-release/docs/). Any additional information required for the analysis of data in this manuscript is available from the authors upon request.

Received: 21 September 2020; Accepted: 30 July 2021; Published online: 19 August 2021.

References.

1. Iwanaga, N. & Kolls, J. K. Updates on T helper type 17 immunity in respiratory disease. Immunology 156, 3–8 (2019).
2. McGrath, K. W. & et al. A large subgroup of mild-to-moderate asthma is persistently noneosinophilic. Am. J. Respir. Crit. Care Med. 185, 612–619 (2012).
3. Wenzel, S. E. & et al. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. Am. J. Respir. Crit. Care Med. 156, 737–743 (1997).
4. Al-Raml, W. et al. T(H17)-associated cytokines (IL-17A and IL-17F) in severe asthma. J. Allergy Clin. Immunol. 123, 1185–1187 (2009).
5. Bacher, P. & et al. Human anti-fungal Th17 immunity and pathology rely on cross-reactivity against Candida albicans. Cell 176, 1340–1355 e1315 (2019).
6. Chien, J. W. et al. Increased IL-17A secreting CD4^+ T cells, serum IL-17 levels and enhanced nitric oxide are correlated with childhood asthma severity. Clin. Exp. Allergy 43, 1018–1026 (2013).
7. McKinley, L. et al. TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice. J. Immunol. 181, 4089–4097 (2008).
8. Lambrecht, B. N. & Hammad, H. The role of dendritic and epithelial cells as master regulators of allergic airway inflammation. Lancet 376, 835–843 (2010).
9. Idoya, J. & Steinman, R. M. SnapShot: dendritic cells. Cell 146, 660–660 e662 (2011).
10. Sampathy, A. T., Wu, X., Albring, J. C. & Murphy, K. M. Re(de)fining the dendritic cell lineage. Nat. Immunol. 13, 1145–1154 (2012).
11. Liu, K. et al. In vivo analysis of dendritic cell development and homeostasis. Science 324, 392–397 (2009).
12. Plantinga, M. et al. Conventional and monocyte-derived CD11c^+ dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. J. Immunol. 184, 1335–1340 (2010).
13. Nakano, H. et al. Complement receptor C5AR1/CDS8 and dipeptidyl peptidase-4/CD26 define distinct hematopoietic lineages of dendritic cells. J. Immunol. 194, 3808–3819 (2015).
14. Bostelo, C., Lambrecht, B. N. & Hammad, H. Isolation of conventional murine lung dendritic cell subsets. Curr. Protoc. Immunol. 120, 3.7.1–3.7.16 (2018).
15. Sung, S. S. et al. A major lung CD103 (αE)-β7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. J. Immunol. 176, 2161–2172 (2006).
16. Crozet, K. et al. Cutting edge: expression of XCR1 defines mouse lymphoid-tissue resident and migratory dendritic cells of the CD8α+ type. J. Immunol. 187, 4411–4415 (2011).

17. Edelson, B. T. et al. Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8α+ conventional dendritic cells. J. Exp. Med. 207, 823–836 (2010).

18. Zelante, T. et al. CD103+ dendritic cells control Th17 cell function in the lung. Cell Rep. 12, 1789–1801 (2015).

19. del Rio, M. L., Bernhardt, G., Rodriguez-Barbosa, J. I. & Forster, R. Development and functional specialization of CD103+ dendritic cells. Immunol. Rev. 234, 268–281 (2010).

20. Nutt, S. L. & Chopin, M. Transcriptional networks driving dendritic cell differentiation and function. Immunity 52, 942–956 (2020).

21. Murphy, K. M. Transcriptional control of dendritic cell development. Adv. Immunol. 120, 239–263 (2017).

22. Schlitzer, A. et al. IRF4 transcription factor-dependent CD11b+ dendritic cells in human control of mouse colocal IL-17 cytokine responses. Immunity 38, 970–983 (2013).

23. Gao, Y. et al. IRF4-dependent dendritic cells. Immunology 39, 722–732 (2013).

24. Han, X. et al. Mapping the mouse cell atlas by Microwell-seq. Cell 172, 1091–1107 e1018 (2017).

25. Bosteels, C. et al. Inflammatory type 2 DCs acquire features of cDC1s and macrophages to orchestrate immune to respiratory virus infection. Immunology 165, 1039–1056 (2020).

26. Zhu, J. & Paul, W. E. Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. Immunochemistry 238, 247–262 (2012).

27. Littman, D. R. & Rudensky, A. Y. Th17 and regulatory T cells in mediating and restraining inflammation. Cell 140, 845–858 (2010).

28. Forster, R., Davalos-Missitte, A. C. & Rot, A. CCRT and its ligands: balancing immunity and tolerance. Nat. Rev. Immunol. 8, 362–371 (2008).

29. Nakano, H. et al. Migratory properties of pulmonary dendritic cells are determined by their developmental lineage. Microsc. Immunol. 6, 678–691 (2013).

30. Wilson, R. H. et al. Allergic sensitization through the airway primes Th17-dependent neutrophilia and airway hyperresponsiveness. Am. J. Respir. Crit. Care Med. 180, 720–730 (2009).

31. Whitehead, G. S., Thomas, S. Y. & Cook, D. N. Modulation of distinct asthmatic phenotypes in mice by dose-dependent inhalation of microbial products. Environ. Health Perspect. 122, 34–42 (2014).

32. Wilson, R. H. et al. The Toll-like receptor 5 ligand flagellin promotes asthma by priming allergic responses to indoor allergens. Nat. Med. 18, 1705–1710 (2012).

33. Hsa, B. J. et al. Trif-dependent induction of Th17 immunity by lung dendritic cells. Microsc. Immunol. 8, 186–197 (2015).

34. Meredith, M. M. et al. Expression of the zinc finger transcription factor zDC (Zbb46, Bdf4d) defines the classical dendritic cell lineage. J. Exp. Med. 209, 1183–1201 (2010).

35. Satpathy, A. T. et al. Zbb46 expression distinguishes classical dendritic cells and their committed progenitors from other immune lineages. J. Exp. Med. 209, 1135–1152 (2012).

36. Miller, J. C. et al. Deciphering the transcriptional network of the dendritic cell lineage. Nat. Immunol. 13, 888–899 (2012).

37. Gauthier, E. L. et al. Gene-expression profiles and transcriptional regulatory pathways that underlie the identity and diversity of mouse tissue macrophages. Nat. Immunol. 13, 1118–1128 (2012).

38. Nakano, H., Lyons-Cohen, M. R., Whitehead, G. S., Nakano, K. & Cook, D. N. Distinct functions of CXC4R, CCR2, and CXCR1 in dendritic cell precursors from the bone marrow to the lung. J. Leukoc. Biol. 101, 1143–1153 (2017).

39. Zhou, L., Chong, M. M. & Littman, D. R. Plasticity of CD4+ T cell lineage differentiation. Immunity 30, 646–655 (2009).

40. Martinon, F. & Tschopp, J. Inflammatory caspases and inflammasomes: master switches of inflammation. Cell Death Differ. 14, 10–22 (2007).

41. Maddur, M. S., Miossec, P., Kaveri, S. V. & Barry, J. Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies. Ann. Pathol. 181, 8–18 (2012).

42. Ohmacht, C. et al. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. J. Exp. Med. 206, 549–559 (2009).

43. Stuert, T. et al. Comprehensive integration of single-cell data. Cell 177, 1888–1902.e1821 (2019).

44. Menezes, S. et al. The heterogeneity of Ly6C(hi) monocytes controls their differentiation into iNOS+ macrophages or monocyte-derived dendritic cells. Immunity 45, 1205–1218 (2016).

45. Stoeckius, M. et al. Simultaneous epitope and transcriptome measurement in single cells. Nat. Methods 14, 865–868 (2017).
