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A Critical Role for Dendritic Cells in the Evolution of IL-1β–Mediated Murine Airway Disease

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Chronic airway inflammation and fibrosis, known as airway remodeling, are defining features of chronic obstructive pulmonary disease and are refractory to current treatments. How and whether chronic inflammation contributes to airway fibrosis remain controversial. In this study, we use a model of chronic obstructive pulmonary disease airway disease utilizing adenoviral delivery of IL-1β to determine that adaptive T cell immunity is required for airway remodeling because mice deficient in α/β T cells (*tcra*$^{-/-}$) are protected. Dendritic cells (DCs) accumulate around chronic obstructive pulmonary disease airways and are critical to prime adaptive immunity, but they have not been shown to directly influence airway remodeling. We show that DC depletion or deficiency in the crucial DC chemokine receptor *ccr6* both protect from adenoviral IL-1β–induced airway adaptive T cell immune responses and fibrosis in mice. These results provide evidence that chronic airway inflammation, mediated by accumulation of α/β T cells and driven by DCs, is critical to airway fibrosis. ***The Journal of Immunology*****, 2015, 194: 000–000.**

Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the United States (1). Peribronchial chronic inflammation and fibrotic scarring of small airways, known as airway remodeling, is refractory to steroid-based therapies and causes airflow obstruction in COPD (2, 3). The mechanisms leading to steroid-refractory chronic inflammation and whether that inflammation causes fibrotic scarring in COPD remain areas of active investigation (4).

Cigarette smoke (CS) is the major cause of airway remodeling by inducing cellular injury and increasing the susceptibility to respiratory pathogens, in particular viruses (5). Rodents when exposed to CS, viruses, or both together demonstrate exaggerated airway remodeling (6, 7). CS and viruses engage similar host danger pathways leading to inflammasome activation and enhanced IL-1β secretion (6, 8, 9). IL-1β protein levels are increased in human COPD biospecimens (10–13), delivery of IL-1β to the airways causes experimental airway remodeling (14, 15), and perturbation of IL-1 signaling protects against experimental CS-induced airway remodeling (16, 17).

Primary human or mouse lung fibroblasts upon IL-1β stimulation become prosynthetic, increase TGF-β activation, and highly express the potent dendritic cell (DC) chemokine CCL20 (15). Proteomic cytokine analysis of lungs from intratracheal (i.t.) adenovirus (Ad)–IL-1β-treated mice reveal elevated levels of CCL20. Increased CCL20 levels are associated with airway remodeling induced by CS in combination with the viral mimetic poly(I:C) (15).

CCL20 is increased in COPD samples and is the only known ligand for chemokine receptor CCR6 (18). Thus, *ccr6*-deficient mice can be used to interrogate the functions of CCL20 in vivo. CCR6, which is expressed by DCs, has been shown to be required for DC recruitment and CS-induced emphysema in mice (19). DCs are critical APCs that have been implicated in the pathogenesis of COPD through priming pathologic adaptive T cell immune responses (20). DC accumulation surrounding airways correlate with COPD disease severity (18). In mice, IL-1β or CS exposure induces CCL20 expression, which correlates with lung (DC) numbers, adaptive Th1 and Th17 immune responses, and expression of the profibrotic cytokines IL-4, IL-13, and IL-17 (15, 21).

The causal role of the immune response in airway remodeling remains to be fully elucidated. Recent evidence suggests that the adaptive immune response may be important for CS-induced lung pathology, as mice deficient in IL-17RA are protected from CS-induced emphysema (22). Clodronate depletion of both macrophages and DCs has recently been found to protect against CS-induced airway remodeling (23). As critical players in innate and adaptive inflammation, the roles and mechanisms of DC in the pathogenesis of airway remodeling are important to define.

In this study, we elucidate a causal linkage between immune and fibrotic pathology by demonstrating: 1) DCs are required for airway inflammation and fibrosis, 2) the DC chemokine receptor CCR6 is required for pathology adaptive T cell immunity and airway remodeling, and 3) α/β T cells are required for airway remodeling. Taken together, these data suggest that therapeutically targeting lung DCs may represent a strategy to prevent or treat airway remodeling in COPD.

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Abbreviations used in this article: Ad, adenovirus; AM, alveolar macrophage; BAL, bronchoalveolar lavage; COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; DC, dendritic cell; DT, diphtheria toxin; DTR, diphtheria toxin receptor; i.t., intratracheal(ly); WT, wild-type; YFP, yellow fluorescent protein.

Materials and Methods

Mice

All mice were bred and housed in specific pathogen-free housing under an Institutional Review Board–approved protocol and in accordance with the guidelines of the Laboratory Animal Resource Center of the University of California, San Francisco (San Francisco, CA). Cd11c-dtr [B6.FVB-Tg(Itgax-DTR/GFP)57Drl], *ccr6*$^{-/-}$ (B6.129P2-*ccr6*^{tm1Dgen}/J), *tcra*$^{-/-}$ (B6.129S2-tcra^{tm1Mom}/J), and wild-type (WT) mice, all in the C57BL/6 background, were obtained from The Jackson Laboratory (Bar Harbor, ME). Mouse CD11c–enhanced yellow fluorescent protein (YFP) (24) transgenic reporter mice were provided by M. Nussenzweig (The Rockefeller University, New York, NY). Approval for the use of mice in this study was obtained from the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Recombinant adenovirus

The recombinant E1–E3-deleted type 5 adenovirus, either empty (Ad-C) or expressing human active IL-1β (Ad-IL-1β), has been described in detail elsewhere (25). The replication-deficient virus was commercially amplified and purified by cesium chloride gradient centrifugation and PD-10 Sephadex chromatography, plaque titered on 293 cells, and checked for WT contamination (ViraQuest, North Liberty, IA). Recombinant type 5 adenoviral vectors expressing Cre–enhanced GFP fusion protein, enhanced GFP, or LacZ were obtained from the Gene Transfer Vector Core (University of Iowa, Iowa City, IA)

Intratracheal injections

Mice were anesthetized with i.p. injection of Avertin (250 mg/kg, i.p.). Then, Ad-hIL-1β or Ad-LacZ (2.5×10^8 PFU in 75μl sterile PBS) was instilled i.t. with a needle (Popper 24G-1′ Straight 1.25-mm ball) using the direct visualized instillation technique (26). The control was Ad-LacZ.

Mouse organ harvests and bronchoalveolar lavage

For bronchoalveolar lavage (BAL) and organ harvest the trachea was cannulated and the lungs were lavaged five times using 0.8 ml sterile PBS with 5 mM EDTA, and the BAL or organs were harvested as described (27).

Airway morphometry

Measurements of airway inflammation were estimated using H&E-stained slides, and wall fibrosis was assessed by the presence of thick collagen bundles stained by the trichrome method essentially as described by Hogg et al. (28), which estimates wall fibrosis as a measure of the trichrome-stained area from the airway lumen to the outer edge of adventitial connective tissue and expressed as wall area/basement membrane length. The wall area and basement membrane lengths are determined using image analysis software (ImageJ v1.36b). Microtome sections from H&E- or trichrome-stained sections of paraffin-embedded mouse lungs were digitally imaged at ×200 magnification (QCapture v2.68.2; QImaging, Surrey, BC, Canada). The slides were coded and an investigator blinded to the experimental groups acquired five digital images representing each lung lobe (and two images from the largest lobe) and the images were coded and cataloged. Airway inflammation was defined as the inflammatory infiltrate extending from the airway basement membrane toward the lung parenchyma. Airway fibrosis was defined as thick collagen bundles (stained blue in trichrome stains). A minimum of 12 airways was examined per mouse.

Preparation of lung sections for live cell imaging in the lung

Mice were given a lethal overdose of Avertin and exsanguinated by cutting the renal artery. The lungs and trachea were exposed by cutting through the diaphragm and chest wall. The mice were intubated by tracheotomy with the sheath from an 18-gauge i.v. catheter. Lungs were inflated with 1 ml 2% low melting temperature agarose in sterile PBS maintained at 37°C, and the solution was solidified by briefly rinsing the inflated lungs with PBS at 4°C. Inflated lungs were then excised from the mouse and placed in a sterile 50-ml conical containing room temperature RPMI 1640 without phenol red (Invitrogen/Life Technologies). The left lobe was isolated, cut into 360-μm sections using a vibratome filled with cool PBS, mounted on plastic slides with Vectabond (3M), and placed in a dish containing RPMI 1640 without phenol red before imaging.

Real-time two-photon imaging

A custom resonant-scanning two-photon instrument (29) contains a four-photomultiplier tube detector and collects data at video rate. Where indicated, lung sections were stained with Hoechst for 10 min at a concentration of 10 μg/ml and then maintained at 36°C in RPMI 1640 medium bubbled with 95% O_2 and 5% CO_2 for up to 8 h. The health of lung sections was assessed by ciliary movement in large airways. Samples were excited with a 10-W Mai Tai Ti:Sapphire laser (Spectra-Physics) tuned to a wavelength of 910 nm, and emission wavelengths of 440/40 nm (for Hoechst) and 542/27 nm (for YFP) were collected. Micro-Manager (Vale Laboratory, University of California, San Francisco) was used for image acquisition. Each lung section was first surveyed in a raster scan spanning $1567 \times 1300 \times 175$ μm in *x*,*y*,*z*.

Imaris-based analysis of morphology

Images were analyzed with Imaris software (Bitplane) using isosurface with masking and spot tracker applications. Three-dimensional images were rendered by Imaris or MetaMorph software (Molecular Devices), and sphericity was calculated by Imaris using the ratio of the surface area of a sphere (with the same volume as the given particle) to the surface area of the particle (29).

Flow cytometry

Lung cell collection, staining, and gating were performed essentially as described, with the following modifications (15). Cells were stained without stimulation, and cytokine capture assays for IL-17A and IFN-γ were performed using cytokine secretion assay kits, as per the manufacturer's instructions (Miltenyi Biotec, Auburn, CA).

ELISA assays

Lung specimens were uniformly homogenized with stainless steel ball bearings and the TissueLyser II (Qiagen, Valencia, CA) homogenizer. Homogenates were obtained in PBS with 1% Triton X-100 and protease inhibitors (protease inhibitor mixture set I; Calbiochem, Billerica, MA), with 1 mM Na_3VO_4 and 2 mM PMSF final concentrations. Homogenates were thoroughly standardized to a working concentration of 1 mg/ml total protein using the BCA assay (Thermo Scientific, Waltham, MA). IL-17A sandwich ELISAs were performed using the human ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical analysis

All data are reported as means ± SE. Comparisons between two different groups were determined using a Student *t* test for parametric data or a Mann–Whitney test for nonparametric data. A one-way ANOVA was used for multiple comparisons and Tukey, Dunn, or Bonferroni post hoc tests were used to test for statistical significance. Significance was defined as $p < 0.05$. Logistic regression analysis was performed using Stata (v12.1). All other statistical analyses were performed using the software package Prism 4.0b (GraphPad Software, San Diego, CA).

Results

Depletion of DCs protects mice from IL-1β–induced airway inflammation and fibrosis

Because DCs are crucial for adaptive T cell immune responses, we sought to test the hypothesis that DCs were important for Ad-IL-1β–mediated airway inflammation and fibrosis. To deplete DCs we used transgenic mice expressing the simian diphtheria toxin receptor (DTR) under the control of the murine CD11c promoter (30). In this system, the DTR is mostly confined to the DC compartment, and most murine lung DCs express the transgene and in the presence of DT rapidly become apoptotic within 1 d owing to inhibition of protein synthesis (30). The complete depletion lasts 2 d before DCs gradually repopulate (30). Murine cells do not possess a high-affinity receptor for DT and are thus insensitive (31).

We treated CD11c-DTR mice with i.t. injection of Ad-IL-1β, an airway remodeling system that recapitulates key features of human airway remodeling in COPD, including increased numbers of neutrophils, macrophages, DCs, and $CD4^+$ Th1 and Th17 cells, accompanied by increased localization of inflammatory cells surrounding the airways with accompanying fibrosis (15). In this system, 1 wk after i.t. Ad-IL-1β instillation, IL-1β levels and lung inflammation peak (15). We therefore treated mice with DT on day 5 after Ad-IL-1β so that depletion would coincide with peak IL-1β-levels.

We confirmed the depletion of CD11c$^+$ DCs cells by crossing the CD11c-DTR mice to transgenic CD11c-YFP mice, which express YFP in DCs and alveolar macrophages (AMs). We used a size and scatter gating strategy to discriminate DCs (CD11c^{high}, CD11b^{high}, MHC class II^{high}, Ly6C$^+$, F480$^-$) from AMs (CD11b^{high}, Gr1$^-$, Ly6c^{high}, MHC class II^{high}, CD11c^{low}) (Supplemental Figs. 1, 2). Two days after DT, lung DCs were depleted (Fig. 1A–C), as were AMs, as previously reported (Fig. 1D, 1F) (32). To confirm the airway localization of DCs and their depletion by DT, we used two-photon microscopy of living lung sections of compound transgenic mice (CD11c-DTR;CD11c-YFP). We found that Ad-IL-1β treatment caused a dramatic increase in the localization of DCs within 100 μm of the airways; DT significantly reduced DCs surrounding the airways (Fig. 2).

We next measured the effect of depletion of CD11c-DTR$^+$ cells on Ad-IL-1β–induced airway inflammation and fibrosis. CD11c-DTR mice were treated with DT 5 d after i.t. Ad-IL-1β and euthanized 4 d later. Intratracheal Ad-IL-1β caused a significant increase in total BAL cell count, macrophages, neutrophils, and lymphocytes (Fig. 3), as well as airway inflammation and airway wall fibrosis (Fig. 4); these increases were all significantly reduced by DT treatment (Figs. 3, 4). The fibrotic response was confined to airways and was not significantly increased around vessels ($p = 0.99$). These data demonstrate that CD11c$^+$ cells, which consist of both DCs and AMs, are required for pathologic airway inflammation and fibrosis.

Deficiency of ccr6, *the chemokine receptor for CCL20, protects mice from IL-1β–induced airway inflammation and fibrosis*

Mice deficient in *ccr6* are expected to have defective DC influx, because *ccr6* is expressed by DCs and is the only receptor for CCL20 (33). Intratracheal Ad-IL-1β caused significantly less airway wall inflammation (Fig. 5) and fibrosis (Fig. 5) compared with WT, suggesting that a CCR6/CCL20-dependent inflammatory response was coupled to the subsequent fibrotic response.

α/β T cells are required for airway fibrosis

We next sought to determine whether T cells, which have been shown to be involved in the remodeling response (15), were required for airway fibrosis in the Ad-IL-1β model. T cells, of which >90% in mice and humans express the α/β heterodimeric TCR, have been implicated in fibrotic responses through the elaboration of cytokines such as IL-17 (34). Thus, we used *tcra*$^{-/-}$ mice to test the role of T cells in IL-1β–mediated airway fibrosis. Compared to WT mice (Fig. 5), *tcra*$^{-/-}$ mice failed to develop either airway inflammation (Fig. 5) or fibrosis (Fig. 5) 14 d after i.t. Ad-IL-1β. These data support a role for adaptive immunity, specifically α/β T cells in fibrotic airway disease.

CCR6-expressing cell types in the lungs of Ad-IL-1β–treated mice

The cell types reported to express CCR6 include DCs, AMs, CD4$^+$ Th17 cells, B cells, and neutrophils (35–38). We detected obvious surface staining of CCR6 on CD11b$^+$ DCs and B cells, only trace expression on AMs and CD4$^+$ Th17 cells, and no expression on neutrophils from WT compared with *ccr6*$^{-/-}$ mice (Fig. 6). There were significant increases in numbers of *ccr6*$^+$ DCs in WT mice treated with Ad-IL-1β compared with Ad-LacZ (Fig. 6B). There was a slight nonsignificant increase in *ccr6*$^+$ B cells (18%, $p = 0.11$) and no increase in *ccr6*$^+$ AMs or CD4$^+$ Th17 cells in WT mice treated with Ad-IL-1β compared with Ad-LacZ. These data suggest that DCs are the most likely *ccr6*-expressing cell type that is involved in Ad-IL-1β–mediated airway fibroinflammatory responses.

DC expression of ccr6 *is required for i.t. Ad-IL-1β–induced increases in innate and adaptive immune effectors*

We sought to understand how CCR6-expressing DCs and adaptive α/β T cell immunity are coupled to a fibrotic airway response. DCs, as well as the profibrotic CD4–T cell subsets, Th1 and Th17 cells, are characteristically increased in COPD samples as well as in the Ad-IL-1β model (15). Resident DCs in the murine lung are comprised of two major subsets, with the most numerous characterized by high expression of CD11c, CD11b, and absence of CD103 (CD11b$^+$), whereas the minor subset expresses CD11c and CD103, but not CD11b (CD103$^+$) (39). It is the former DC subset, CD11b$^+$, that predominates in the lung in the Ad-IL-1β model (15). This subset expresses intermediate levels of Ly6c, suggesting a monocytic lineage (20). We found that CD11b$^+$ DC numbers were significantly increased 3 d after Ad-IL-1β treatment and this increase was sustained (Fig. 7A). Significant increases in numbers of Th1 and Th17 cells actively secreting IFN-γ or IL-17, respectively, were not significantly increased until day 9 (Fig. 7B). These day 9 increases in CD11b$^+$ DCs and Th1 and Th17 cells

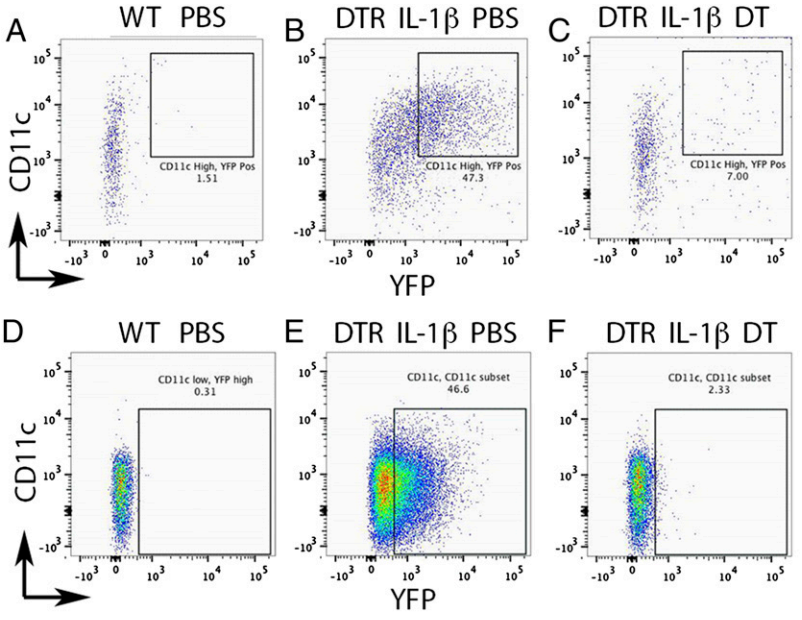

FIGURE 1. DCs and AMs are depleted in i.t. Ad-IL-1β–treated CD11c-DTR mice. Percoll-enriched lung immune cell populations were analyzed by multicolor flow cytometry multicolor staining of lung immune cell populations from WT, untreated (**A** and **D**), or compound transgenic (CD11c-DTR;CD11c-YFP) mice (**B**, **C**, **E**, and **F**) treated with i.t. Ad-IL-1β. Five days postinfection, CD11c-DTR;CD11c-YFP mice were treated with PBS or DT. At day 7, at the peak of IL-1β expression, mice were euthanized and lung cell suspensions were stained for CD11c, CD11b, MHC class II, F480, Ly6c, and Gr1. Staining for CD11c on the *y*-axis and YFP fluorescence on the *x*-axis are indicated for lung DC populations (A–C) and AMs (D–F). The gates used to determine depletion of DCs or AMs are shown and the gating strategy is shown in Supplemental Figs. 1 and 2.

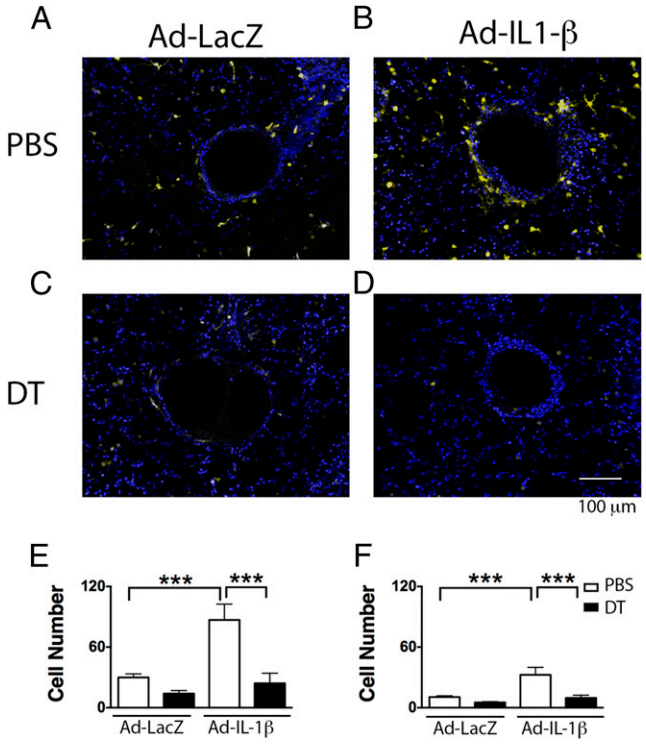

FIGURE 2. DC airway localization is increased by i.t. Ad-IL-1β treatment of CD11c-DTR;CD11c-YFP mice and completely blocked by DT. CD11c-DTR mice were treated with Ad-LacZ (**A** and **C**) or i.t. Ad-IL-1β (**B** and **D**). Five days postinfection, CD11c-DTR;CD11c-YFP mice were treated with PBS (A and B) or DT (C and D). At day 7, at the peak of IL-1β expression, mice were euthanized and vibratome living lung sections were analyzed using two-photon microscopy. The YFP$^+$ cells are shown as yellow cells against a Hoechst (blue) counterstained background. The number of DCs within 100 μm (**E**) or outside of 100 μm (**F**) from the airway basement membrane was assessed using automated cell analysis (Imaris). Scale bar, 100 μm.

were significantly attenuated in *ccr6*$^{-/-}$ mice (Fig. 8A, 8C, 8D). Interestingly, AM numbers were also significantly increased in i.t. Ad-IL-1β–treated *ccr6*$^{-/-}$ mice to comparable levels as seen in i.t. Ad-IL-1β–treated WT mice (Fig. 8B). However, steady-state AM numbers were significantly decreased in *ccr6*$^{-/-}$ compared with WT Ad-LacZ control mice (Fig. 8B). This decrease was related to the Ad-LacZ virus, as the decrease was not seen in untreated *ccr6*$^{-/-}$ mice (in BAL, AM numbers were: WT, $7.1 \times 10^5 \pm 5.8$; *ccr6*$^{-/-}$, $7.1 \times 10^5 \pm 6.8$; p = NS). Evaluation of BAL revealed a significant increase in neutrophils in i.t. Ad-IL-1β–treated compared with Ad-LacZ–treated WT mice, and this increase was significantly attenuated in *ccr6*$^{-/-}$ mice (Fig. 8E). Analysis of whole-lung lysates revealed a significant increase in IL-17A protein in i.t. Ad-IL-1β–treated WT but not *ccr6*$^{-/-}$ mice (Fig. 8F). These data suggest that CCL20/CCR6 is essential for increasing DC but not AM numbers in response to IL-1β. Furthermore, in the Ad-IL-1β model, DCs are critical for Th1 and Th17 adaptive immunity.

Discussion

This study has addressed the mechanistic connection between innate and adaptive immunity with airway wall fibrosis in COPD. In the present study, we have determined that CD11c$^+$ cells that accumulate around airways in response to IL-1β are required for airway inflammation and fibrosis. The IL-1β–induced DC accumulation is *ccr6*-dependent and required for enhanced adaptive immune responses, of which α/β T cells are critical for airway remodeling. The temporal relationship between DC accumulation, adaptive immunity, and airway fibrosis suggest that DC accumulation is critical for establishing profibrotic adaptive immune responses (18, 28).

How or whether immune responses contribute to the development of fibrosis remains hotly debated. Clodronate depletion of both macrophages and DCs has recently been found to protect against CS-induced airway remodeling (23). Because DCs and macrophages express CD11c and are depleted in the CD11c-DTR model, we cannot definitively rule out a role for macrophages in airway fibrosis. However, in Ad-IL-1β–treated mice, of the CCR6-expressing cells types (35–38) AMs were not decreased by *ccr6* deficiency and DCs were the only CCR6$^+$-expressing cells that were increased by Ad-IL-1β. These findings suggest that DCs and not macrophages are required for the fibrotic airway responses.

In Ad-IL-1β–treated *ccr6*$^{-/-}$ mice, AMs were increased nearly to WT levels in contrast to DCs, which were markedly reduced in

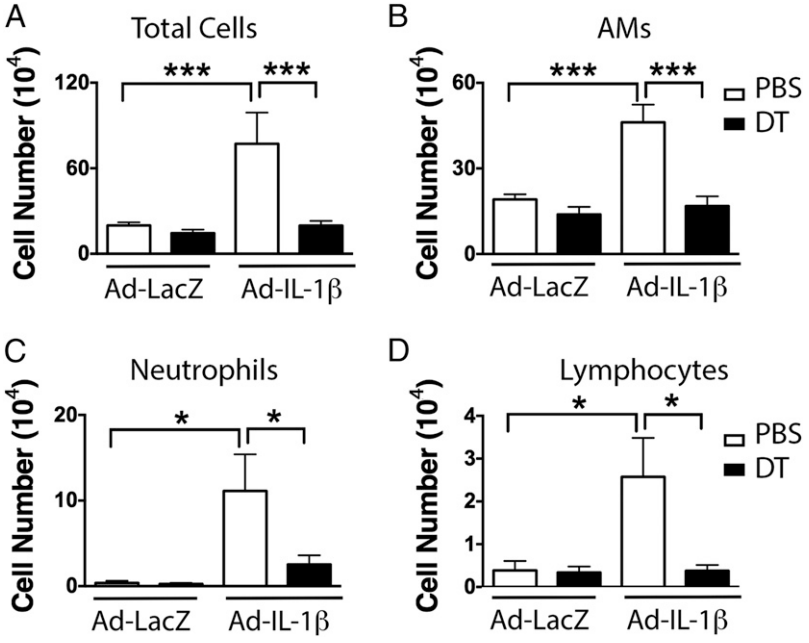

FIGURE 3. Lung inflammation is blocked by depletion of CD11c-expressing cells. BAL of CD11c-DTR mice that were treated with Ad-LacZ or i.t. Ad-IL-1β is shown. Five days postinfection, CD11c-DTR; CD11c-YFP mice were treated with PBS (open bars) or DT (filled bars). Cytospin preparations were assessed for total cells count (**A**), AMs (**B**), neutrophils (**C**), or lymphocytes (**D**). (n = 4 in PBS and 8 in Ad-IL-1β groups). *$p < 0.05$, ***$p < 0.001$ by ANOVA and a Bonferroni posttest.

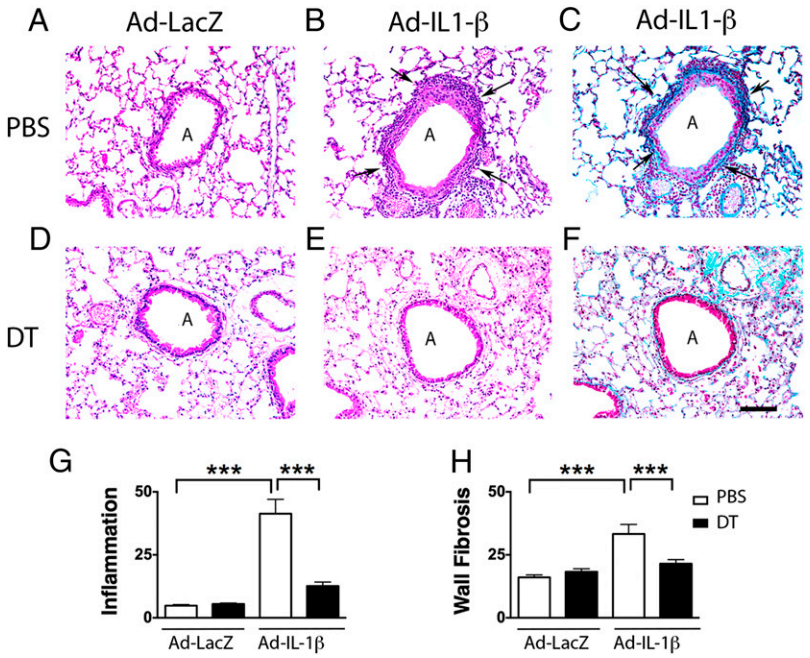

FIGURE 4. Depletion of CD11c-expressing cells protects against Ad-IL-1β–mediated airway remodeling. Representative histologic images from CD11c-DTR mice treated with either Ad-LacZ (**A** and **D**) or Ad-IL-1β (**B**, **C**, **E**, and **F**). Five days after adenoviral administration, mice were treated with PBS (A–C) or DT i.p. (4 ng/g body weight) (D–F) and mice were euthanized on day 9. Scale bar, 100 μm. Airway morphometry of H&E-stained sections was performed to determine the area of inflammation around the airways (A, B, D, E, and **G**) or the extent of fibrosis around the airway wall (C, F, and **H**), as determined by trichrome staining and airway morphometry as described by Hogg et al. (28) and adapted to mice in Kitamura et al. (15). Error bars are SEM. $***p < 0.001$ as determined by ANOVA and a Bonferroni posttest. A, airway lumen.

numbers. These data confirm that Ad-IL-1β–dependent DC accumulation in mice is mainly dependent on *ccr6* whereas AM accumulation is *ccr6*-independent. We have not yet determined the compensatory mechanism explaining increased macrophage influx in Ad-IL-1β–treated $ccr6^{-/-}$ mice, but we speculate that the CCL2/*ccr2* axis is involved because Ad-IL-1β–treated mice express high levels of CCL2 in the lung (15). Additionally, AMs in *ccr6*-deficient mice have other notable differences from WT mice, as $ccr6^{-/-}$ AMs in Ad-LacZ–treated mice were decreased compared with untreated WT mice, suggesting that *ccr6*-deficient AM survival was influenced by the Ad-LacZ virus. Whether this difference in survival is linked to compensatory increases in other chemokine receptors such as *ccr2* remains to be determined.

DCs have the capacity to directly produce profibrogenic cytokines and thus could directly contribute to airway fibrosis. DCs express the integrin $\alpha_v\beta_8$, which is a potent and critical activator of latent TGF-β,

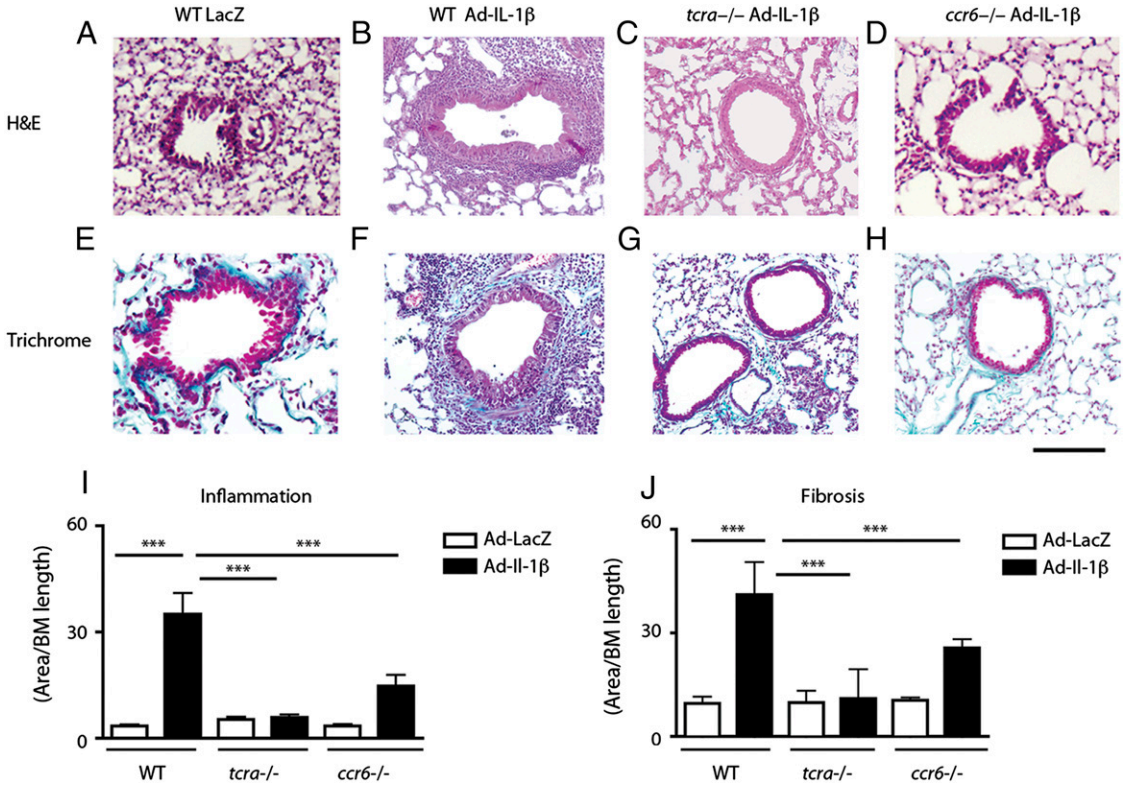

FIGURE 5. α/β T cells and *ccr6* are required for IL-1β–induced airway fibrosis. WT (**A**, **B**, **E**, and **F**), $tcra^{-/-}$ (**C** and **G**), or $ccr6^{-/-}$ (**D** and **H**) mice were treated with i.t. Ad-IL-1β (B–D and F–H) or Ad-LacZ (A and E) as a control. Scale bar, 100 μm. After 14 d, inflammation (A–D, H&E) and fibrosis (E–H, trichrome) were assessed by airway morphometry, as above (**I** and **J**). $***p < 0.001$ by one-way ANOVA and a Bonferroni posttest; $n \geq 8$ for every group.

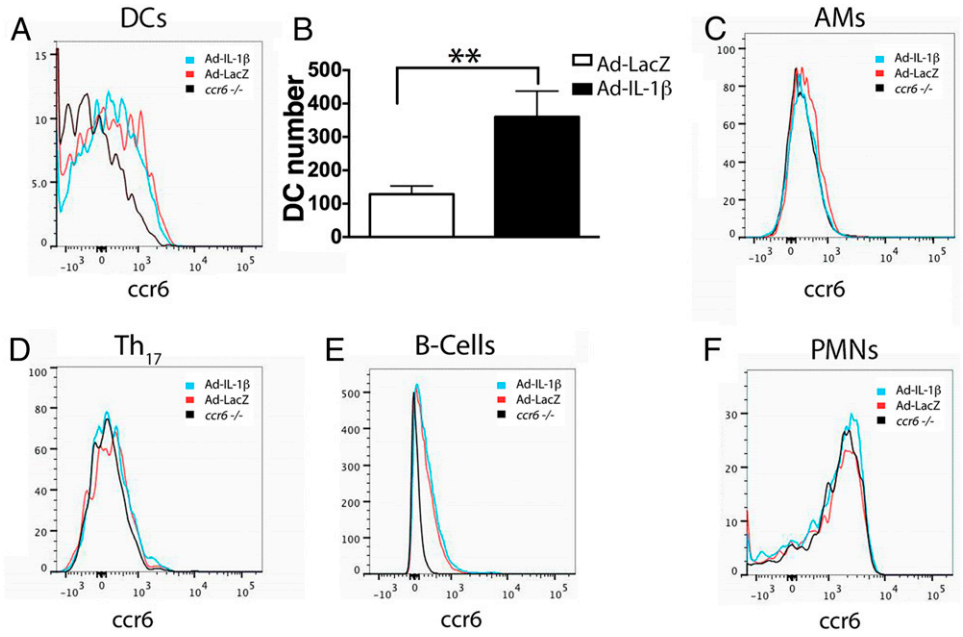

FIGURE 6. DCs are the major *ccr6*-expressing lung immune cell type in Ad-IL-1β–treated mice. WT or $ccr6^{-/-}$ (**A**–**F**) mice ($n \geq 7$) were treated with i.t. Ad-IL-1β or Ad-LacZ as a control. After 9 d, Percoll-enriched lung immune cell populations were analyzed by multicolor flow cytometry for (A and B) $CD11b^+$ DCs ($CD11c^+$, $CD11b^+$, Ia^{high}, $Ly6c^{int}$, nonautofluorescent), (C) AMs ($CD11c^+$, Ia^{low}, autofluorescent), and (D) $CD4^+$ Th17 cells ($TCR\beta^+$, Ia^{low}, IL-17A surface positive). Surface cytokine expression was determined using unstimulated cells stained using a cytokine secretion assay (Miltenyi Biotec). (E) B cells (CD19, Ia^+, $Nk1.1^-$, $TCR\beta^-$). (F) PMNs ($Gr1^{high}$, $Ly6c^{high}$, $CD11b^{high}$, Ia^-). Represenative histogram overlays (A and C–F) of CCR6 expression of lung immune populations from WT mice treated with Ad-IL-1β (blue) or Ad-LacZ (red), compared with $ccr6^{-/-}$ mice (black) as a negative control, are shown. In (B), the number of $CCR6^+$ lung DCs in Ad-IL-1β–treated (filled) or Ad-LacZ–treated (open) mice are shown. $n = 7$. $^{**}p < 0.01$ by Student *t* test.

a profibrotic cytokine that is expressed in a latent form that must be activated to function. Genetic deletion of $\alpha_v\beta_8$ on DCs has been shown to block airway smooth muscle contraction, but not airway remodeling (21). In contrast, conditional deletion of $\alpha_v\beta_8$ on fibroblasts or global inhibition of $\alpha_v\beta_8$ using neutralizing Abs efficiently blocks airway remodeling, suggesting that TGF-β activated by cell types other than DCs contributes to airway fibrosis (7, 15).

Our present and past studies support an indirect role for *ccr6*-expressing DCs in airway fibrosis. In the present study, we have begun to address the components downstream of a DC-mediated profibrotic adaptive immune response in airway remodeling. We find a temporal increase in DCs precedes an adaptive $CD4^+$ Th1 and Th17 response. These temporal relationships are likely to not only apply to the IL-1β airway remodeling system but also to other airway remodeling induced by CS, because IL-17A is also increased during CS-induced airway remodeling (7). IL-17A is mainly secreted by α/β $CD4^+$ T cells (i.e., Th17 cells) (34), and deficiency of all α/β T cells protects against IL-1β–induced airway fibrosis. IL-17A is increased in expression in COPD and mediates bleomycin-induced pulmonary fibrosis in mice (40). Therefore, we speculate that IL-17A is critical in the role that DCs play in airway fibrosis. However, we cannot exclude that other α/β T cells, such as $CD4^+$ Th1, or neutrophils also contribute independently to airway fibrosis.

The mechanisms driving DC accumulation around airways are important to define to understand the genesis of the subsequent profibrotic inflammatory responses. We have recently determined that airway fibroblasts are an important source of the chemokine ligand for CCR6, CCL20 (O.J. Brand, S. Somanath, H. Yanagisawa, C. Moermans, M. Hashimoto, S.M. Cambier, J. Markovics, A. Hill,

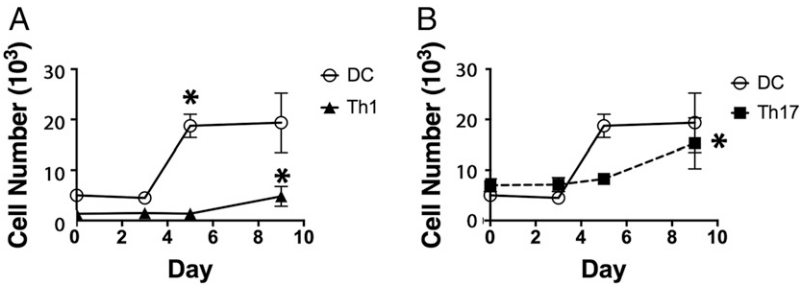

FIGURE 7. $CD11b^+$ DC numbers increase prior to $CD4^+$ Th1 and Th17 cells after Ad-IL-1β treatment. WT mice ($n \geq 3$ at each time point) were treated with i.t. Ad-IL-1β and lung immune cell populations were assessed by multicolor flow cytometry 3, 5, and 9 d after Ad-IL-1β treatment. Day 0 mice were WT untreated mice. Percoll-enriched lung immune cell populations were analyzed for (**A** and **B**) $CD11b^+$ DCs ($CD11c^+$, $CD11b^+$, Ia^{low}, $Ly6c^{int}$, nonautofluorescent) and compared with (A) $CD4^+$ Th1 ($TCR\beta^+$, Ia^-, IFN-γ surface positive) or (B) $CD4^+$ Th17 cells ($TCR\beta^+$, Ia^-, IL-17A surface positive). Surface cytokine expression was determined using unstimulated cells stained using a cytokine secretion assay (Miltenyi Biotec). $^*p < 0.05$ by ANOVA and a Dunn multiple comparisons test.

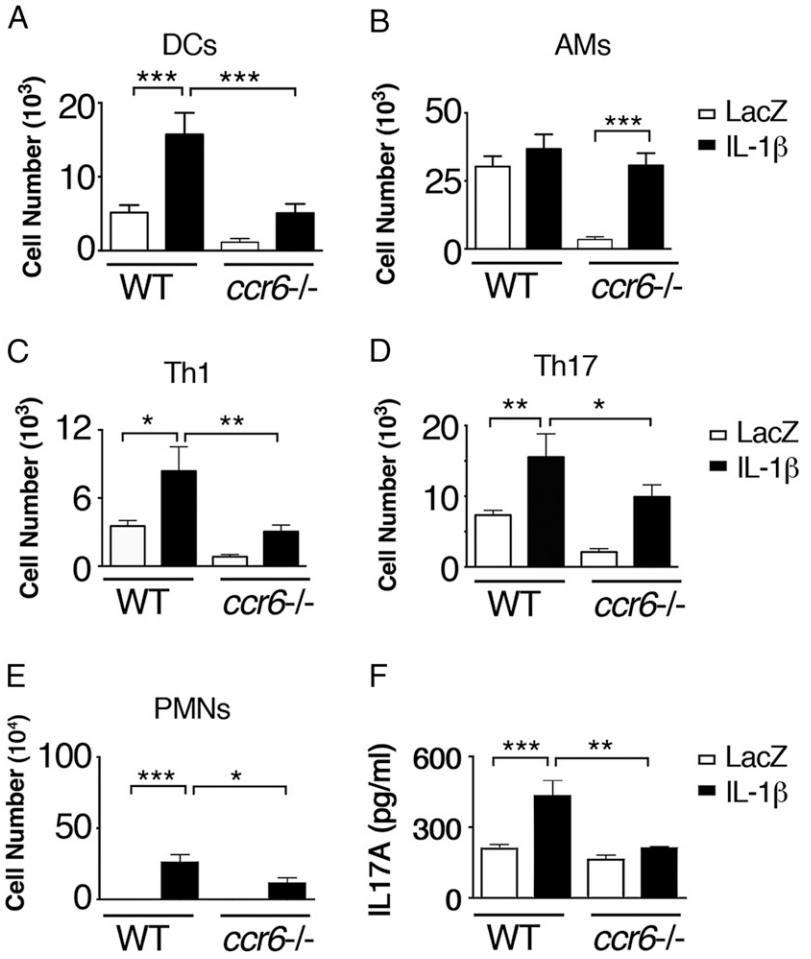

FIGURE 8. *ccr6* is required for innate and Th1 and Th17 adaptive immunity in Ad-IL-1β–treated mice. WT or $ccr6^{-/-}$ (**A**–**F**) mice ($n \geq 7$) were treated with i.t. Ad-IL-1β or Ad-LacZ as a control. After 9 d, Percoll-enriched lung immune cell populations were analyzed by multicolor flow cytometry for (A) DCs ($CD11c^+$, $CD11b^+$, Ia^{high}, $Ly6c^{int}$, nonautofluorescent), (B) AMs ($CD11c^+$, Ia^{low}, autofluorescent), (C) $CD4^+$ Th1 ($TCR\beta^+$, Ia^{low}, IFN-γ surface positive), or (D) $CD4^+$ Th17 cells ($TCR\beta^+$, Ia^{low}, IL-17A surface positive). Surface cytokine expression was determined using unstimulated cells stained using a cytokine secretion assay (Miltenyi Biotec). BAL (E) was performed to assess neutrophil numbers (n = 5/group). IL-17A ELISA (F) was performed using whole-lung lysates to assess total IL-17A levels (n = 6/group repeated three times with similar results). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by ANOVA with a Bonferroni posttest.

D. Jablons, P. Wolters, J. Lou, J.D. Marks, J.L. Baron, and S.L. Nishimura, submitted for publication). Furthermore, we have determined that an important mechanism driving CCL20 expression from fibroblasts involving integrin $\alpha_v\beta_8$ mediated activation of TGF-β. TGF-β activated by an $\alpha_v\beta_8$-dependent mechanism initiates signaling through the canonical TGF-β signaling pathway (i.e., SMADs); SMAD4 binds to an upstream enhancer element on the CCL20 promoter that forms a complex with a proximal NF-κB subunit that is required for CCL20 expression (Brand et al., submitted for publication). We have recently used two-photon imaging of living lung tissue to determine that $\alpha_v\beta_8$- or *ccr6*-dependent DC chemokinesis in response to Ad-IL-1β or CS drives the microanatomic localization of DCs around large and small airways (M. Hashimoto, H. Yanagisawa, S. Minagawa, D. Sen, R. Ma, L.M. Murray, P. Tsui, J. Lou, J.D. Marks, J.L. Baron, M.F. Krummel, and S.L. Nishimura, submitted for publication).

Two-photon microscopy has provided a resolution not afforded by static immunohistochemical studies and has provided additional novel insights into DC airway localization, in addition to confirming that DCs are depleted in the CD11c-DTR model. In the Ad-IL-1β model, DCs preferentially localize within 100 μm of the airway in close proximity to airway fibroblasts, and airway epithelial cells, CCL20-expressing cell types, and very few, if any, DCs were observed interdigitating with the airway epithelium. Of the two major resident lung DC subsets ($CD103^+$ or $CD11b^+$ DCs), intraepithelial DCs have been suggested to represent the $CD103^+$ subset (20). Although we cannot exclude a role for $CD103^+$ DCs in the fibroinflamamtory responses in the Ad-IL-1β model, the paucity of intraepithelial DCs and the larger relative increase in numbers of the $CD11b^+$ DC subset suggest that $CD11b^+$ DCs are more likely to play a role in pathologic airway remodeling. In Ad-IL-1β–treated mice the $CD11b^+$ subset expresses Ly6c, which provides evidence of a hematogenous origin and monocytic lineage and suggests that these cells are newly recruited and replace the steady-state resident $CD11b^+$ DCs. Such newly recruited $CD11b^+$ DCs are also the dominant lung DC population following influenza infection and at the peak of infection they are the major DC subset involved in Ag presentation and T cell cross-priming in the draining lymph node (41). In the Ad-IL-1β model, the $CD11b^+$ DC population is the major *ccr6*-expressing lung DC subset, is efficiently deleted in DT-treated DTR mice, appears in the lung before $CD4^+$ Th1 and Th17 cells, and is the most numerous lung DC subset that migrates to the draining lymph node (15, 41). For these reasons, we hypothesize that the $CD11b^+$ DC subset is the airway-adjacent DC subset seen on two-photon microscopy and is also the subset most likely to play a major role in driving *ccr6*-dependent pathologic adaptive $CD4^+$ Th1 and Th17 cell responses.

In summary, our data provide compelling evidence that $CD11b^+$ DCs play a role in the evolution of murine airway disease.

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Disclosures

The authors have no financial conflicts of interest.

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