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Implicating Exudate Macrophages and Ly-6C\textsuperscript{high} Monocytes in CCR2-Dependent Lung Fibrosis following Gene-Targeted Alveolar Injury

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The alveolar epithelium is characteristically abnormal in fibrotic lung disease, and we recently established a direct link between injury to the type II alveolar epithelial cell (AEC) and the accumulation of interstitial collagen. The mechanisms by which damage to the epithelium induces lung scarring remain poorly understood. It is particularly controversial whether an insult to the type II AEC initiates an inflammatory response that is required for the development of fibrosis. To explore whether local inflammation occurs following a targeted epithelial insult and contributes to lung fibrosis, we administered diphtheria toxin to transgenic mice with type II AEC–restricted expression of the diphtheria toxin receptor. We used immunophenotyping techniques and diphtheria toxin receptor–expressing, chemokine receptor-2–deficient (CCR2\textsuperscript{−/−}) mice to determine the participation of lung leukocyte subsets in pulmonary fibrogenesis. Our results demonstrate that targeted type II AEC injury induces an inflammatory response that is enriched for CD11b\textsuperscript{+} nonresident exudate macrophages (ExM) and their precursors, Ly-6C\textsuperscript{high} monocytes. CCR2 deficiency abrogates the accumulation of both cell populations and protects mice from fibrosis, weight loss, and death. Further analyses revealed that the ExM are alternatively activated and that ExM and Ly-6C\textsuperscript{high} monocytes express mRNA for IL-13, TGF-\(\beta\), and the collagen genes, COL1A1 and COL3A1. Furthermore, the accumulated ExM and Ly-6C\textsuperscript{high} monocytes contain intracellular collagen, as detected by immunostaining. Together, these results implicate CCR2 and the accumulation of ExM and Ly-6C\textsuperscript{high} monocytes as critical determinants of pulmonary fibrosis induced by selective type II AEC injury. The Journal of Immunology, 2013, 190: 000–000.

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive scarring disorder of the lung. Treatment options for IPF and other fibrotic lung diseases are limited, and the identification of new therapeutic targets would be aided by an improved understanding of disease pathogenesis. Numerous indirect observations suggest that abnormalities of the alveolar epithelium, and in particular type II alveolar epithelial cells (AEC), precipitate the fibrotic cascade. For example, epithelial cell hyperplasia and denudation are commonly observed overlying the fibroblast foci, the purported sites of active collagen deposition in the IPF lung (1–3). In addition, mutations in type II AEC–specific genes, including surfactant protein A2, surfactant protein C (SPC), and ATP-binding cassette protein A3, have been linked to familial fibrotic lung disease (4–8). Similar to the human disease, defects in the alveolar epithelium are prominent in the murine model of bleomycin-induced pulmonary fibrosis (9, 10). To substantiate the direct link between defects in the alveolar epithelium and fibrogenesis, we developed transgenic mice that express the human diphtheria toxin receptor (DTR) in a type II AEC–specific manner. We found that the administration of diphtheria toxin (DT) to these mice selectively injured their type II AEC and induced pulmonary fibrosis (11). The mechanism(s) by which AEC injury instigates pulmonary fibrosis remains uncertain. Although early studies implicated lung inflammation in the pathogenesis of IPF, a current prevailing hypothesis proposes that repetitive damage to the alveolar epithelium, with little contribution from inflammatory cells, results in collagen accumulation by preventing normal wound repair and by stimulating the secretion of profibrotic growth factors (12). However, recent reports have spurred a renewed scientific interest in the role of inflammation, and specifically macrophages, in the development of fibrotic lung disease. For example, macrophage accumulation can be a prominent feature in individuals with familial pulmonary fibrosis due to SPC and ATP-binding cassette protein A3 mutations (6, 8). In addition, macrophage accumulation was observed in several murine models of lung fibrosis (e.g., bleomycin adminis-

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Abbreviations used in this article: AEC, alveolar epithelial cell; AM, alveolar macrophage; Col1, Collagen-1; DT, diphtheria toxin; DTR, diphtheria toxin receptor; DTR\textsuperscript{−/−} mice, mice expressing the diphtheria toxin receptor under the control of the surfactant protein C promoter; ExM, exudate macrophage; iNOS, inducible NO synthase; IPF, idiopathic pulmonary fibrosis; PD-L1, programmed cell death ligand; qPCR, real time quantitative RT-PCR; SPC, surfactant protein C; WT, wild-type.
tration, γ-herpes virus infection, TGF-β overexpression, and IL-10 overexpression) (13–19). Despite the recognition that macrophages are present in areas of injury in these models, little is known about their phenotype or whether they are derived from local or circulating precursors. Our recent study (20) was the first to directly show that targeted alveolar injury was necessary and sufficient to induce nonresident exudate macrophage accumulation in mice that develop lung fibrosis. In this study, we implicated macrophage gene expression of PAI-1 and Collagen-1 (Col1) as potential mechanisms by which these cells promote lung scarring. Despite these advances, many questions still remain, including the mechanism by which the exudate macrophages (ExM) accrue within the lung microenvironment and how they promote fibrogenesis.

In the current study, we undertook a detailed kinetic analysis of macrophage and monocyte accumulation in mice in response to targeted type II AEC injury. Our results demonstrate that fibrosis following alveolar damage occurs in conjunction with the increased accrual of nonresident ExM and their precursors, Ly-6C<sup>high</sup> monocytes. A thorough phenotypic analysis reveals that the ExM and Ly-6C<sup>high</sup> monocyte subpopulations demonstrate a profibrotic phenotype characterized by alternative activation and the expression of both profibrotic cytokines and collagen. Furthermore, CCR2 deficiency limits the macrophage/monocyte accumulation and protects against collagen deposition. Collectively, our results provide important insights into how epithelial injury is translated into interstitial fibrosis, and we elucidate specific cell types and cell recruitment pathways that might serve as novel therapeutic targets for patients with IPF.

Materials and Methods

Animals

All animal experiments were performed in accordance with institutional guidelines set forth by the University of Michigan Committee on the Use and Care of Animals. Transgenic mice expressing the DTR under the control of the murine splicing promoter were generated in our laboratory at the University of Michigan on a C57BL/6 background (designated DTR+ mice) (11). Control C57BL/6 mice (wild-type [WT]) were purchased from The Jackson Laboratory (Bar Harbor, ME). We sequentially crossed DTR+ mice with CCR2−/− mice (C57BL/6 genetic background) to generate CCR2-deficient DTR+ (DTR+/CCR2−/−) mice.

Assessment of mouse genotypes

The presence of the DTR was detected using PCR, as previously described (11).

DT administration and experimental design

Six- to eight-week-old mice (from the strains identified above) were injected i.p. with DT (Sigma Chemical, St. Louis, MO) once daily for 14 d at a dose of 10.0 μg/kg in 100 μl PBS (or PBS alone). Note that DT was purchased from Rockland Immunochemicals (Gilbertsville, PA), and rabbit IgG (001-000-003) and donkey anti-rabbit Ig (711-166-152) were purchased from Jackson ImmunoResearch. The mAb were primarily conjugated with FITC, PE, PerCP-Cy5.5, allophycocyanin, allophycocyanin-Cy7, or Pacific Blue. Isotype-matched irrelevant control mAb (BioLegend) and rabbit IgG were purchased from Jackson ImmunoResearch. Transgenic mice expressing the DTR under the control of the murine splicing promoter were generated in our laboratory at the University of Michigan on a C57BL/6 background (designated DTR+ mice) (11). Control C57BL/6 mice (wild-type [WT]) were purchased from The Jackson Laboratory (Bar Harbor, ME). We sequentially crossed DTR+ mice with CCR2−/− mice (C57BL/6 genetic background) to generate CCR2-deficient DTR+ (DTR+/CCR2−/−) mice.

Multiplex quantitative PCR array

RNA was purified from lung leukocytes by the TRIZol method (Ambion), and cDNA was generated using the RT<sup>2</sup> First Strand Kit (QIAGEN). A total of 2.5 ng cDNA from each sample was loaded into the wells of a custom qPCR array (QIAGEN) and run on a LightCycler 480 system (Roche). Relative RNA expression levels were inferred from the C<sub>q</sub> values, using β-actin as the control. Significant genes were identified using R-package samr, which uses the false-discovery rate method employed in the software SAM (Significance Analysis of Microarrays) (22).

mAb

The following mAb were purchased from BioLegend (San Diego, CA): N418 (anti-murine CD11c, hamster IgG1); 2.4G2 (“Fc block”); (anti-murine CD16/CD32, rat IgG2b); 30-F11 (anti-murine CD45, rat IgG2b); 16-10A1 (anti-murine CD80, hamster IgG1); 5C7 (anti-murine CD86, rat IgG2b); M5/114.15.2 (anti-murine B7-H1/programmed cell death ligand (PD-L1), rat IgG2a); TY25 (anti-murine B7-DC/DP-L2, rat IgG2a); AMS-3.2.1 (anti-murine I-A<sup>d</sup>-mHC class II<sup>+</sup>, mouse IgG2b); 145-2C11 (anti-murine CD3e, hamster IgG1, κ); 6D5 (anti-murine CD19, rat IgG2a); M3/88 (anti-murine Mac-2, rat IgG2a); M3/84 (anti-murine CD107b (Mac-3), rat IgG1); I-A<sup>d</sup> (anti-Ly-6G, rat IgG2a); BM8 (anti-murine F4/80, rat IgG2a); and TG12 (anti-murine CXCR4, rat IgG2b). The following mAb were purchased from BD Biosciences PharMingen (San Diego, CA): AL-21 (anti-murine Ly-6C, rat IgM); M1/70 (anti-murine CD11b, rat IgG2b), and 2/3 (anti-murine CD40, rat IgG2a). The following mAb were purchased from Cedar Laboratories (Burlington, NC): 2F8 (anti-murine CD204, rat IgG2b) and NLDC-145 (anti-Murine CD205, rat IgG2a). Anti-collagen (#600-401-103) was purchased from Rockland Immunochemicals (Gilbertsville, PA), and rabbit IgG (001-000-003) and donkey anti-rabbit Ig (#711-166-152) were purchased from Jackson ImmunoResearch. The mAb were primarily conjugated with FITC, PE, PerCP-Cy5.5, allophycocyanin, allophycocyanin-Cy7, or Pacific Blue. Isotype-matched irrelevant control mAb (BioLegend) were tested simultaneously in all experiments.

Ab staining and flow cytometric analysis

Ab staining, including blockade of FcR, and analysis by flow cytometry were performed as described previously (20, 23, 24). Data were collected on a BD LSR II flow cytometer equipped with 488-nm blue, 405-nm violet, and 633-nm red lasers using FACSDiva software (both from Becton Dickinson Immunocytometry Systems, Mountain View, CA) and analyzed using FlowJo software (Tree Star, San Carlos, CA); 10,000–100,000 cells were analyzed per sample. In selected experiments, FACS of specific lung macrophage populations was performed using a BD ARIA flow cytometer and FACS Diva software.

Gating strategy used to identify lung leukocyte subsets including macrophages and monocytes

Single-cell suspensions enriched for lung leukocytes were obtained, stained with Ab, and assessed by flow cytometry (as above), using a previously published gating strategy (20, 24, 25) detailed in Supplemental Fig. 1. To identify lung macrophages and monocytes (Supplemental Fig. 1A), samples were stained with anti-Ly-6C (FITC), anti-F4/80 (PE), anti-CD3 and anti-CD19 (PerCP-Cy5.5), anti-Ly-6G (PE–allophycocyanin–Cy7), anti-CD45 (allophycocyanin), anti-CD11b (allophycocyanin–Cy7), and anti-CD11c (Pacific Blue). Thereafter, a series of gates was used to identify alveolar macrophages (AM; CD45+ CD3− CD19− Ly-6G− FSC<sub>med</sub/high autologous fluorescence<sup>+</sup> CD11c<sup>+</sup> CD11b<sup>+</sup>), ExM (CD45<sup>+</sup> CD3<sup>−</sup> CD19− Ly-6G− FSC<sub>med</sub/high autologous fluorescence<sup>+</sup> CD11c<sup>+</sup> CD11b<sup>+</sup>), Ly-6C<sub>high</sub> monocytes (CD45<sup>+</sup> CD3<sup>−</sup> CD19− SSC<sub>med</sub> CD11c<sup>−</sup> F4/80<sup>+</sup> CD11b<sup>+</sup> Ly-6C<sub>high</sub>), and Ly-6C<sub>low</sub> monocytes (CD45<sup>+</sup> CD3<sup>−</sup> CD19− SSC<sub>low</sub> CD11c<sup>−</sup> F4/80<sup>+</sup> CD11b<sup>+</sup> Ly-6C<sub>low</sub>). In some experiments, further Ab staining was performed to evaluate these leukocyte subsets for cell surface markers commonly expressed on macrophages and monocytes (Figs. 5A, 5B, 6E). To identify granulocytes (Supplemental Fig. 1B), a series of gates was set to identify neutrophils (CD45<sup>−</sup> SSC<sub>med</sub> Ly-6C<sub>high</sub> and eosinophils (CD45<sup>−</sup> SSC<sub>med</sub> Ly-6C<sub>low</sub>) and lymphocytes (Supplemental Fig. 1C), separate samples were stained with anti-CD8 (FITC), anti-CD4 (PE), anti-CD19 (PerCP-Cy5.5), and anti-CD45 (allophycocyanin) to identify CD8<sup>+</sup> T cells (CD45<sup>+</sup> CD4<sup>+</sup>), CD8<sup>−</sup> T cells (CD45<sup>+</sup> CD8<sup>+</sup>), and B cells (CD45<sup>+</sup> CD19<sup>+</sup>).

Monocyte/macrophage isolation

Myeloid cell subsets from DTR+ mice (n = 5) following 14 d of DT treatment were purified (∼95%) by FACS using the gating strategy described above (Supplemental Fig. 1A) (20, 24).

Quantitative RT-PCR on sorted macrophages and monocytes

Total macrophage or monocyte-specific RNA was prepared using RNeasy Plus Mini Kit (QIAGEN), and first-strand cDNA was synthesized using SuperScript III (Invitrogen, Carlsbad, CA). Specific qPCR were conducted...
using a SYBR Green–based detection and an MX 3000P system (Stratagene, La Jolla, CA). Forty cycles were performed (94°C for 15 s, followed by 60°C for 30 s and 72°C for 30 s) using each cDNA template. The mRNA levels were normalized to expression of the housekeeping gene GAPDH using the following formula: percentage GAPDH expression = 100/2^(-

Data are presented as the average ± SEM of three individual experiments (with each experiment representing gene expression on leukocyte subsets pooled from five DT-treated DTR+ mice). The following primer pairs were used for qPCR detection of mRNA expression: Arginase-1 sense, 5'-CAGAAGAATGGAGAAGACTCAG-3', Arginase-1 antisense, 5'-CAGATATTGCAGGGAGTCACC-3'; COL3a1 sense, 5'-TCTTCACTCATGCTTCGTT-3'; COL3a1 antisense, 5'-TTTCAGAGAGGATAATGGTT-3'; inducible NO synthase (iNOS) sense, 5'-TTTGCCTCTCCTGATACT-GCCAAAG-3'; iNOS antisense, 5'-GCTCTGGTGCTTCAAGGCC-3'; IL-13 sense, 5'-CTACAGTCCCTGGTTCCT-3'; IL-13 antisense, 5'-TTTGCCTCGCTCTAAATA-3'; TGF-β sense, 5'-TGACGGCACTCCCACTGTTA-3'; TGF-β antisense, 5'-CGCTGAAAG-3'; COL1a1 sense, 5'-ACCTCAAGATGGCCACCTCTGACTG-3'; COL1a1 antisense, 5'-GCAGCTTTGGTATGGTGAGCT-3'; COL3a1 sense, 5'-CAATCCCAATTCTCCTCAGTGCTTCC-3'; COL3a1 antisense, 5'-ACCCTGAGCTCTGAGACTACCA-3'; and GAPDH sense, 5'-TATGTGCTTCTGGAGATTCTGTC-3'; GAPDH antisense, 5'-GAGTGTTCAC-ATTTCCTGCT-3'.

Hydroxyproline assay

Hydroxyproline content of the lung was measured as previously described (26).

Lung histology

The left lung was inflation-fixed at 25 cm H2O pressure with 10% neutral-buffered formalin, removed en bloc, further fixed in 10% neutral-buffered formalin overnight, and then embedded in paraffin. Five-micron sections were stained using H&E or picrosirius red and visualized by bright-field and cross-polarized microscopy.

Statistical analysis

All data are expressed as mean ± SEM. Data were evaluated by the unpaired Student t test (for comparison between two samples) or by ANOVA with the Dunnett posttest (for multiple comparisons). Statistical difference was accepted at p < 0.05.

Results

Targeted type II AEC injury induces lung inflammation

In this murine model, repetitive administration (for 14 d) of DT to DTR+ mice selectively injures type II AEC and results in pulmonary fibrosis, as evidenced by increased hydroxyproline content and histologic evidence of collagen deposition in alveolar regions (11). Although we have found PAI-1 to be required, the mechanisms translating AEC injury into lung fibrosis in this model have not been determined. In the current study, we first investigated whether a targeted type II AEC injury would induce inflammation. Using flow cytometric analysis, we enumerated the total number of CD45+ leukocytes within the lungs of both WT and DTR+ mice (before day 0), during (days 7 and 14), and 1 wk after (day 21) DT treatment. Total lung leukocytes did not increase in WT mice treated with DT, whereas, in DTR+ mice, the number of CD45+ cells was increased significantly (~2-fold; Fig. 1A).

We next compared histologic features of injury on day 14 in DT-treated WT and DTR+ mice. As expected, no evidence of alveolar damage or inflammation was evident in the WT group (Fig. 1B, left panel). In contrast, patchy areas of interstitial thickening were prevalent in the lungs of DT-treated DTR+ mouse, and these regions contained numerous large cells with macrophage morphology (Fig. 1B, right panel and right inset). Lastly, we performed a limited gene array on the total lung leukocyte population obtained from DT-treated WT and DTR+ mice (at day 14) to further characterize this inflammatory response. Of the 20 genes assessed, we found an upregulation of Arginase-1 and a decreased expression of IFN-γ in DTR+ mice (relative to WT mice) (Fig. 1C).

ExM and Ly-6Chigh monocyte accumulation

The identification of large cells within the injured alveoli and the increase in Arginase-1 expression in DT-treated DTR+ mice suggested to us that macrophages might be accumulating in response to the targeted type II AEC injury. To evaluate this possibility, we used flow cytometric analysis with an established gating strategy [(20, 24) and as described in Materials and Methods and Supplemental Fig. 1; abbreviated gating shown in Fig. 2A–C] to distinguish and enumerate AM and ExM in the lungs of DT-treated WT and DTR+ mice. Prior studies indicated that AM are predominantly resident cells, whereas ExM accumulate in the lung in response to infection and inflammation (24, 27–29). Consistent with this, we found a modest increase in the AM population (~1–2-fold) in DTR+ mice at day 7 of DT treatment (relative to untreated DTR+ mice) that did not differ from DT-treated WT mice at any time point (Fig. 2D). In contrast, the number of ExM increased nearly 5-fold (relative to untreated DTR+ mice), peaked on day 14 of DT treatment, and differed significantly from DT-treated WT mice on days 14 and 21 of treatment (Fig. 2E). Note that DT treatment induced a small (~2-fold) and transient (day 7 only) increase in AM and ExM numbers in WT mice. The observed expansion of ExM in the DT-treated DTR+ mice was unique, because other lung leukocyte subsets either were not increased (CD4+ T cells and eosinophils) or were increased ~2-fold (CD8+ T cells, B cells, and neutrophils) relative to DT-treated WT mice (Supplemental Fig. 2).

We hypothesized that the observed increase in ExM population in response to alveolar injury was due to the recruitment of an ExM precursor. We (24) and other investigators (28, 30) previously demonstrated that ExM are derived from Ly-6Chigh monocytes. However, both Ly-6Chigh monocytes, which are CCR2+ CX3CR1- and often referred to as “inflammatory monocytes,” and Ly-6Clow monocytes, which are CCR2− CX3CR1+ and often referred to as “patrolling monocytes,” have been identified at sites of tissue fibrosis in murine models (15, 27). Using flow cytometric analysis (as described in Materials and Methods and Supplemental Fig. 1; abbreviated gating shown in Fig. 2F–H), we identified both Ly-6C+ and Ly-6Clow monocytes in the lungs of DT-treated DTR+ mice (Fig. 2F–H). The Ly-6Chigh monocytes increased >4-fold in DT-treated DTR+ mice after 7 d (relative to untreated DTR+ mice; Fig. 2I), immediately preceding the peak accumulation of ExM (Fig. 2E). In comparison, there was no increase in this leukocyte population in DT-treated WT mice. In contrast, Ly-6Clow monocytes were not increased at day 7 in DT-treated DTR+ mice, and they only increased 2-fold by day 14 of treatment (Fig. 2J). Collectively, these data demonstrate that a selective injury to the type II alveolar epithelium is sufficient to promote the accrual of Ly-6Chigh monocytes and imply that these cells locally differentiate into ExM following their recruitment.

The role of CCR2 in ExM and Ly-6Chigh accumulation

Prior studies indicate that the lung accumulation of ExM and Ly-6Chigh monocytes in response to infectious stimuli is CCR2 dependent (24, 25, 28, 29). To assess the role of CCR2 in our alveolar injury model, we evaluated macrophage and monocyte accumulation following DT treatment in CCR2-deficient DTR+ (DTR+: CCR2−/−) mice. Our results demonstrated the expected increase in ExM (Fig. 3A) and Ly-6Chigh monocytes (Fig. 3B) in the DTR+: CCR2-expressing mice relative to control animals (at day 14). In contrast, the numbers of ExM and Ly-6Chigh monocytes did not increase in CCR2-deficient DTR+ mice (Fig. 3A, 3B). Consistent with these cell counts, we did not observe any evidence of increased interstitial macrophage accumulation in the lungs of DT-treated CCR2-deficient DTR+ mice on day 14 using light microscopy.
FIGURE 1. Targeted injury to type II AEC promotes lung inflammation. DT was administered daily for 14 d to either WT (DTR+), or DTR+ mice. (A) Lung cells from individual mice were isolated from untreated mice (day 0) and at 7, 14, and 21 d after the onset of DT treatment. Cells were stained with Ab, and flow cytometric analysis was used to identify and enumerate total numbers of CD45+ lung leukocytes. Data are mean ± SEM of 8–12 DT-treated mice/time point (from three separate experiments) assayed individually. (B) Representative photomicrographs of lung sections obtained from DT-treated WT (left panel) and DTR+ mice (right panel) (H&E; original magnification ×800, left and right panels). Note the presence of increased cellularity in the alveolar regions of DTR+ mice with evidence of large cells both with alveolar airspaces (orange arrows in right panel) and within thickened interstitial infiltrates (green arrows in right panel). (C) A limited gene array was performed on RNA extracted from enriched lung leukocyte populations of WT mice or DTR+ mice following 14 d of DT treatment. Data are plotted as the cycle time difference (ΔCt) between the target gene and the β-actin gene. Solid arrows indicate significant differences in gene expression between WT and DTR+ mice. *p < 0.05, versus day 0 (uninfected) for mice of the same DTR-expression (DTR-:CCR2+/+), and CCR2-deficient (DTR-:CCR2-/-) groups. **p < 0.05, WT mice versus DTR+ mice at the designated time point.

The role of CCR2 in targeted type II AEC injury–induced lung fibrosis, weight loss, and mortality

We next assessed the influence of CCR2 on lung fibrosis following targeted type II AEC injury. We compared lung hydroxyproline content in the following four groups of mice treated with DT for 14 d: WT (DTR-:CCR2+/+), CCR2-deficient (DTR-:CCR2-/-), DTR+ (DTR+:CCR2+/+), and CCR2-deficient DTR+ (DTR+:CCR2-/-). A control group of PBS-treated WT mice was included to establish basal lung collagen content. As was the case in our original study, DT treatment of WT mice did not significantly increase lung hydroxyproline content (95% confidence interval: -64.09 to +8.724 μg/mg Fig. 4A) (11). CCR2-deficient mice (that were negative for DTR) also did not develop a significant increase in lung collagen content with DT exposure. In contrast, DT treatment of DTR+ mice with intact CCR2 expression induced a robust (≥2-fold) increase in lung hydroxyproline, consistent with our prior studies (11, 20). Importantly, this increase in the quantity of lung hydroxyproline in the DT-treated DTR+ mice was abrogated by the absence of CCR2 expression, and the lung hydroxyproline content of these DT-treated DTR-:CCR2-/- mice was not different from the DTR-treated control groups.

The quantitative changes in lung collagen content that we observed were associated with qualitative changes in alveolar histology. Specifically, the lungs of DT-treated DTR+ mice demonstrated mixed cellular and fibrotic regions characterized by thickened alveolar walls and evidence of increased alveolar wall collagen, as assessed by picrosirius staining using bright-field microscopy (Fig. 4B, middle panels). Histologic evidence of increased lung collagen was not observed in DT-treated WT (Fig. 4B, left panels) or CCR2-deficient DTR+ mice (Fig. 4B, right panels). Examination of lung sections obtained from DTR+ mice under cross-polarized light (in which cross-aligned collagen is birefringent) provided additional evidence that the observed picrosirius staining was attributable to collagen (Fig. 4C). The intensity of this staining was generally less than that observed surrounding larger airways and vascular structures, suggesting that the newly deposited collagen may be loosely organized. Collectively, these results show that fibrosis resulting from a targeted epithelial injury is CCR2 dependent.

We previously demonstrated that mice with targeted type II AEC injury experienced significant weight loss and decreased survival (11, 20). To determine the effect of CCR2 deficiency on these parameters, weight loss and survival were monitored in the following three groups of mice treated with DT for 14 d: WT mouse (DTR-:CCR2+/+), DTR+ mouse (DTR+:CCR2+/+), and CCR2-deficient DTR+ mouse (DTR+:CCR2-/-). As expected, DT treatment of DTR+ mice resulted in weight loss (Fig. 4D) and increased mortality (Fig. 4E) relative to WT mice. In contrast, DT-treated CCR2-deficient DTR+ mice lost an intermediate amount of weight (relative to DTR+ and WT mice) and were protected against the lethal effects of DT-mediated type II AEC injury.

Cell surface phenotype of the accumulated ExM and Ly-6C<sup>high</sup> monocytes

Our results revealed a strong association between the accumulation of ExM and Ly-6C<sup>high</sup> monocytes and the development of lung
FIGURE 2. ExM and Ly-6Chigh monocytes accumulate in mice with targeted injury to type II AEC. DT was administered per protocol for 14 d to either WT (DTR−) or DTR+ mice. (A–J) Lung cells isolated from untreated mice (day 0) and from mice at 7, 14, and 21 d after the onset of DT treatment were stained with specific Abs and analyzed by flow cytometric analysis, as described in Materials and Methods and Supplemental Fig. 1. (A) Gating strategy used to identify AM and ExM among lung leukocytes obtained from DTR+ mice after 14 d of DT treatment. Initial gating (data not shown) on CD45+ lung leukocytes excludes debris, lymphocytes, and CD11c+ cells (Supplemental Fig. 1). Within the CD11c+ populations, a plot of FL-3 versus FSC (left panel) distinguishes larger autofluorescent macrophages (AP+) from smaller nonautofluorescent cells. Among AP+ macrophages, plots of isotype controls (middle panel) and CD11c+ versus CD11b (right panel) distinguish AM (as AP+ CD11c+ CD11b−; gate AM) and ExM (as AP+ CD11c+ CD11b+; gate ExM). Representative photomicrographs of AM (B) and ExM (C) obtained by flow sorting of lung leukocytes at day 14 (H&E, original magnification ×1000). Total numbers of AM (D) and ExM (E) present in the lungs of WT or DTR+ mice at each time point. (F) Gating strategy used to identify Ly-6Clow and Ly-6Chigh monocytes among lung leukocytes obtained from DTR+ mice after 14 d of DT treatment. Initial gating (data not shown) on CD45+ lung leukocytes excludes debris, lymphocytes, granulocytes, and CD11c+ cells (Supplemental Fig. 1). Within the SSClow CD11c+ population (left panel), a gate of F4/80 versus FSC identifies F4/80+ monocytes. Next, plots of isotype controls (middle panel) and CD11b versus CD11c (right panel) identify Ly-6Chigh monocytes (gate Ly-6Chigh) and Ly-6Clow monocytes (gate Ly-6Clow). Representative photomicrographs of Ly-6Chigh monocytes (G) and Ly-6Clow monocytes (H) obtained by flow sorting of lung leukocytes at day 14 (H&E, original magnification ×1000). Total numbers of Ly-6Chigh monocytes (I) and Ly-6Clow monocytes (J) present in the lungs of WT or DTR+ mice at each time point. Total macrophage and monocyte numbers were calculated by multiplying the frequency of each population (using the gating strategy described above) by the total number of CD45+ lung leukocytes at each time point. Data represent mean ± SEM of 8–12 mice assayed individually/time point. *p < 0.05, versus day 0 (untreated) for mice of the same DTR-expression profile. ANOVA, **p < 0.05, WT versus DTR+ mice at the designated time point.

FIGURE 3. Accumulation of ExM and Ly-6Chigh monocytes in mice with targeted type II AEC injury is CCR2 dependent. DT was administered for 14 d protocol to WT mice (DTR−;CCR2+/+), DTR+ mice (DTR+;CCR2+/+), and DTR−;CCR2−/− mice. Lung leukocytes from individual mice were isolated and stained with Abs. Multiparameter flow cytometric analysis was used to identify and enumerate total numbers of ExM (A) and Ly-6Chigh monocytes (B). Data are mean ± SEM of four to five DT-treated mice/strain assayed individually. (C) Photomicrograph of a representative lung section from a DT-treated DTR+ mouse deficient in CCR2 (H&E, original magnification ×400). Note the relative absence of inflammation. *p < 0.05.

fibrosis following targeted alveolar injury. To gain additional insight into the phenotype and function of these cell populations, we next characterized their cell surface expression of a panel of macrophage-associated proteins (F4/80, Mac-2, Mac-3, CD204, CD205; Fig. 5A) and MHC class II and costimulatory molecules (including CD40, CD80, CD86, and PD-L1 and PD-L2; Fig. 5B). Relative to their Ly-6Chigh monocyte precursors, ExM expressed higher amounts of Mac-2, Mac-3, and CD204. Both populations expressed F4/80 but minimal CD205 (Fig. 5A). ExM also expressed higher amounts of MHC class II and costimulatory molecules than did Ly-6Chigh monocytes (Fig. 5B).

ExM are alternatively activated and express mRNA for profibrotic cytokines

In macrophages, increased arginase expression relative to iNOS is characteristic of alternative activation, and this phenotype has been associated with tissue repair and fibrosis (15–17, 19, 31). We identified that Arginase-1 gene expression was upregulated in the total lung leukocyte population obtained from DT-treated DTR mice (relative to DT-treated WT mice; Fig. 1C). To determine whether this reflected alternative macrophage activation, we isolated (by cell sorting) pooled populations of ExM from DT-treated DTR+ mice (at day 14) and used qPCR to assess mRNA expression of Arginase-1 and Galectin-3 (associated with alternative activation) and iNOS (associated with classical activation). Results show that expression of Arginase-1, Galectin-3 (Fig. 5C), and the
Arginase-1/iNOS ratio (Fig. 5D) were high in ExM (Fig. 5C) obtained from DTR+ mice, consistent with an alternatively activated phenotype. We also examined the expression of profibrotic mediators by the ExM and Ly-6Chigh monocytes and found that these cell populations expressed mRNA for IL-13 and TGF-β (Fig. 5E). Collectively, our finding that ExM in the lungs of mice with targeted alveolar injury are alternatively activated and express profibrotic cytokines suggests potential mechanisms by which these cells might contribute to pulmonary fibrosis.

ExM and Ly-6Chigh monocytes display evidence of collagen production

We next sought to determine whether lung leukocytes might directly influence the severity of fibrosis through collagen production. Detection of intracellular type I collagen by flow cytometric analysis has been used as evidence that CD45+ leukocytes express collagen (32–35). Therefore, we used Ab staining for intracellular Col1 and flow cytometric analysis to compare the percentage and total number of Col1+ cells among either CD45+ cells (leukocytes) or CD45− cells (nonleukocytes) obtained from DT-treated WT mice, DTR+ mice, or DTR+:CCR2−/− mice. The percentage of CD45+ cells staining positive for intracellular Col1 was similar among groups (WT mice = 4.4 ± 0.4%; DTR+ mice = 4.7 ± 0.2%; DTR+:CCR2−/− mice = 5.1 ± 0.4%). However, the total number of Col1+ cells (lung leukocytes) was significantly increased in DT-treated DTR+ mice but not DTR+:CCR2−/− mice relative to WT mice (Fig. 6A). The percentage of CD45− cells staining positive for intracellular Coll was similar among groups (WT mice = 4.4 ± 0.4%; DTR+ mice = 4.7 ± 0.2%; DTR+:CCR2−/− mice = 5.1 ± 0.4%). However, the total number of CD45− Coll+ cells (lung leukocytes) was significantly increased in DT-treated DTR+ mice but not DTR+:CCR2−/− mice relative to WT mice (Fig. 6A).

Next, we performed a similar analysis using our established gating strategy (Fig. 2, Supplemental Fig. 1) to assess for in-
tracellular Col1 staining on subsets of lung macrophages and monocytes. We observed that ExM consistently display the highest percentage of Col1+ staining (24.5 ± 2.5%) in DTR+ mice in comparison with AM (13.1 ± 1.4%), Ly-6Chigh monocytes (15.0 ± 1.4%), or Ly-6Clow monocytes (8.3 ± 1.9%) (Fig. 6B). The percentage of each subset staining positive for intracellular Col1 was similar among groups of mice (data not shown). However, the total numbers of Col1+ AM, ExM, and Ly-6Chigh monocytes, but not Ly-6Clow monocytes, increased in DT-treated DTR+ mice relative to WT mice (Fig. 6C). In contrast, the numbers of Col1+ ExM and Ly-6Chigh monocytes in the lungs of CCR2-deficient DTR+ mice, which are protected from fibrosis, were significantly lower and similar to that of the WT group.

To determine whether intracellular collagen staining might be explained solely by collagen ingestion, we measured type I and type III collagen gene expression (by qPCR) in these populations following their isolation (by FACS) from DTR+ mice following 14 d of DT treatment. Results demonstrate strong expression of COL1A1 and COL3A1 genes in all four macrophage and monocyte subsets (Fig. 6D), consistent with our findings using flow cytometric analysis.

Fibrocytes are bone marrow–derived cells implicated in tissue fibrosis. They are broadly defined as cells staining for both the leukocyte cell surface marker CD45 and intracellular collagen, and their accumulation in the lungs of mice subjected to bleomycin injury is CCR2 dependent (23, 36, 37). Thus, the Col1+ ExM and Ly-6Chigh monocytes that we observed in the lungs of DT-treated DTR+ mice qualify as fibrocytes by these criteria. To determine whether these cells expressed another fibrocyte marker, we assessed for CXCR4 mRNA (36). Our results demonstrate that ExM and Ly-6Chigh monocytes obtained from DT-treated DTR+ mice express low to modest amounts of CXCR4 (Fig. 6E).

**Discussion**

In the current study, we used transgenic mice expressing the DTR under the control of the SPC promoter to investigate whether DT-
mediated injury of the alveolar epithelium would result in lung inflammation and, if so, which specific inflammatory cell subsets participate in the development of lung fibrosis in this model system. We report the following novel findings: repetitive DT-mediated injury to type II AEC results in an inflammatory response enriched for nonresident ExM and Ly-6C<sup>high</sup> monocytes; ExM and Ly-6C<sup>high</sup> monocytes do not accumulate in the lungs of DTR<sup>+</sup> mice; these mice are protected from lung fibrosis, weight loss, and death in response to DT treatment; ExM and Ly-6C<sup>high</sup> monocytes demonstrate alternative activation and profibrotic cytokine production, potential mechanisms by which these cells might promote fibrogenesis; and ExM and Ly-6C<sup>high</sup> monocytes meet criteria for fibrocytes.

The pathogenic underpinnings of pulmonary fibrosis remain poorly understood. Consistently recognized abnormalities of the alveolar epithelium in areas of ongoing fibrosis have served as the

![Image of graphs and plots](http://www.jimmunol.org/)

**FIGURE 6.** CCR2 mediates the specific accumulation of Col1<sup>+</sup> ExM and Ly-6C<sup>high</sup> monocytes in mice with targeted type II AEC injury. (A-C) Flow cytometric analysis was performed on cells obtained from the lungs of DT-treated WT mice, DTR<sup>+</sup> mice, and DTR<sup>+</sup>:CCR2<sup>−/−</sup> mice (n = 4-5 mice/group at day 14 of DT treatment) for the detection of intracellular Col1 relative to isotype-control staining. (A) Total numbers of Col1<sup>+</sup> CD45<sup>+</sup> cells (lung leukocytes) and Col1<sup>+</sup> CD45<sup>−</sup> cells (nonleukocytes). Data are mean ± SEM of four or five DT-treated mice/strain assayed individually. (B) Representative plots depicting isotype-control staining (left panels) and Col1 staining (right panels) on each subset of lung macrophages and monocytes. Percentages indicate average Col1<sup>+</sup> staining for each subset ± SEM. (C) Total numbers of Col1<sup>+</sup> macrophages and monocytes. (D) Defined macrophage and monocyte subsets were purified (∼95% by cell sorting) from pooled populations of lung leukocytes obtained from DT-treated DTR<sup>+</sup> mice (n = 5 mice) at day 14 of DT treatment. mRNA obtained from each subset was obtained and assessed by qPCR for expression of Col1 and Collagen-3. Specific gene expression was expressed as a percentage of GAPDH (Materials and Methods). Values represent the average ± SEM from three separate experiments. (E) Representative graphs depicting the expression of CXCR4 on ExM (top panel) and Ly-6C<sup>high</sup> monocytes (bottom panel), as assessed by flow cytometric analysis performed on lung leukocytes obtained from DT-treated DTR<sup>+</sup> mice (n = 5) at day 14 of DT treatment. Shaded graphs, isotype staining; open graphs, CXCR4 staining. *p < 0.05.
basis for the popular hypothesis that pulmonary fibrosis results from aberrant wound repair (12). In this hypothesis, the contribution of inflammation to lung scarring is downplayed, in part, because anti-inflammatory treatments have not altered the disease course in IPF patients and because inflammatory cell accumulation is not a prominent feature of the histopathology (12). Instead, defective type II AEC are thought to drive fibrogenesis directly through their failure to reconstitute an intact epithelium and through their altered production of pro- and antifibrotic mediators.

The development of our model of DT-mediated injury to type II AEC confirmed the relationship between epithelial injury and lung fibrosis and afforded us the opportunity to investigate whether inflammation helps to translate the epithelial insult into a fibrotic response. To our knowledge, in the current study, we identify for the first time a direct relationship among targeted injury to the type II epithelium, the influx of inflammatory cells, and the deposition of lung collagen. Specifically, DT-mediated injury to type II AEC induced a sustained ∼2-fold increase in total lung leukocytes. Histologic evaluation of DT-treated DTR⁺mice revealed an increased cellularity within the alveolar walls, and this micro-anatomic location of inflammation coincides with the distribution of collagen deposition reported in the current study (Figs. 1B, 4B, 4C) and in our initial report of this model (11). This modest degree of inflammation in our model is reminiscent of the relatively sparse inflammation observed in histopathology sections obtained from IPF lungs and contrasts with the exuberant inflammation observed in the lungs of mice treated with bleomycin.

Within the context of this mild inflammatory response, we observed an early and sustained enrichment in the numbers of ExM and their precursors, Ly-6C⁺high monocytes. This finding confirms and extends the results of our recently published study and further substantiates the renewed interest in the hypothesis that macrophages and monocytes contribute to lung scarring (20). To investigate the contribution of the ExM and Ly-6C⁺high monocyte subpopulations to fibrogenesis, we used double-transgenic DTR⁺CCR2-deficient mice in which the accrual of these cells within the lung is impaired (24, 25, 38). As expected, CCR2 deficiency completely abrogated the accumulation of ExM and Ly-6C⁺high monocytes in response to alveolar injury but had no effect on AM or Ly-6Clow monocytes. The significance of this observation is underscored by our finding that CCR2-deficient mice are substantially protected from fibrosis and mortality in this model. Our observation that DT-treated DTR⁺:CCR2⁻/⁻ mice lost weight relative to untreated mice and DT-treated WT mice implies that the protection against fibrosis and mortality was not attributable to an inhibition in lung injury. Collectively, these results suggest that the nonresident ExM and Ly-6C⁺high monocytes are the subsets most likely to contribute to aberrant fibrotic responses in the injured lung. This assertion is supported by a preliminary analysis of IPF patients enrolled in the Evaluating the Effectiveness of Prednisone, Azathioprine, and N-acetylcysteine in People With Idiopathic Pulmonary Fibrosis (PANTHER-IPF) trial and the Prednisone, Azathioprine, and N-acetylcysteine in People With Idiopathic Pulmonary Fibrosis (PANTHER-IPF) trial (39). These findings additionally complement those of Gibbons et al. (15), who reported that monocyte depletion reduced lung fibrosis in mice in response to bleomycin administration or TGF-β overexpression and that fibrosis was exacerbated by the adoptive transfer of Ly-6C⁺high monocytes.

The molecular mechanisms by which inflammatory cells contribute to fibrosis are of considerable interest and debate. One increasingly popular hypothesis suggests that lung leukocytes indirectly promote lung fibrogenesis through their effects on local epithelial and mesenchymal cells. Our prior study was the first to demonstrate that ExM were a source of PAI-1 in mice with DT-mediated type II AEC injury. PAI-1 has been implicated in numerous profibrotic processes, and we showed that, in the absence of PAI-1, mice were protected from lung fibrosis in this model. In addition to PAI-1, in the current study we show that ExM express mRNA for Arginase-1 and Galactin-3, as well as an elevated Arginase/iNOS ratio, a pattern consistent with alternative activation. This phenotype, increasingly reported in fibrotic tissues, may serve as a biomarker of scarring (15–17, 19, 31). Furthermore, arginase itself may participate in the fibrotic cascade (40). ExM and their precursors, Ly-6C⁺high monocytes, also express mRNA for the profibrotic cytokines IL-13 and TGF-β, which could activate local mesenchymal cells in a paracrine manner.

A second and more controversial hypothesis suggests that lung leukocytes directly contribute to fibrosis by their production and deposition of collagen. Central to the development of this hypothesis has been the identification of fibrocytes as bone marrow–derived cells displaying evidence of collagen production within injured tissues, including lung, which develop fibrosis. The results of our studies raise two important questions relevant to this hypothesis. First, are ExM and/or Ly-6C⁺high monocytes fibrocytes? Second, what is the relative contribution of monocytes and macrophages to collagen deposition? Most broadly defined, fibrocytes are bone marrow–derived cells identified by the expression of CD45 and collagen, as assessed by staining for intracellular collagen and/or the presence of collagen gene expression. Fibrocyte expression of MHC class II, CD80, CD11b, CCR2, and CXCR4 and their CCR2-dependent accumulation within injured tissues were reported (23, 36, 37, 41). Based on the above criteria, our data indicate that subsets of both ExM and Ly-6C⁺high monocytes in the lungs of mice with type II AEC injury qualify as fibrocytes. These findings complement those of Niedermeier et al. (42), who demonstrated that splenic fibrocytes are derived from CD11b⁺GR-1⁺ (comparable to Ly-6C⁺high) monocytes and that fibrocyte development was influenced by CD4⁺ T cells and a panel of T cell–associated cytokines, including IL-2, TNF-α, IFN-γ, and IL-4. Our additional phenotypic characterization provides the first data, to our knowledge, that ExM and Ly-6C⁺high monocytes in the lungs of mice that develop fibrosis express the immunomodulatory costimulatory molecules PD-L1 and PD-L2. Thus, an intriguing direction of future studies will be to determine whether cell–cell interactions between fibrocytes and T cells might influence lung fibrogenesis in this model. Collectively, our results strongly suggest that the term “fibrocyte,” as broadly defined, consists of a heterogeneous population of cells including subsets of ExM and Ly-6C⁺high monocytes that may promote fibrosis through growth factor, cytokine, and/or collagen production.

With respect to the second question outlined above, the relative contribution of bone marrow–derived cells to lung fibrosis through direct collagen synthesis cannot be answered by our current results. However, our data provide some important insights through the enumeration of CD45⁺Col1⁺ cells and CD45⁺Col1⁺ cells in the lungs of DT-treated WT mice, DTR⁺mice, and DTR⁺:CCR2⁻/⁻ mice (Fig. 6A). Using this approach, we observed that the number of CD45⁺Col1⁺ cells in DTR⁺mice increased significantly relative to their numbers in WT mice; a similar increase was not observed in the DTR⁺:CCR2⁻/⁻ mice, which are protected from fibrosis. At the time of peak inflammation (day 14), the total number of CD45⁺Col1⁺ cells in the DTR⁺mice was approximately half of the
CD45+ Col1+ population in the same animals. Thus, the CD45+ Col1+ population of cells, although fewer in number than the CD45− Col1+ cells, still represented a sizable fraction (approximately one third) of Col1+ cells. The phenotype of the CD45+ Col1+ cells in this model is unknown, and we acknowledge that identification of intracellular collagen staining does not reliably account for potential differences in the quantity or rate with which various cell types might deposit matrix constituents in the lungs of these mice. Thus, future studies will be required to more rigorously investigate the relative contribution of the various subsets of cells to collagen production.

In summary, we established that targeted type II AEC injury induces a modest inflammatory response that is nonetheless significantly enriched for alternatively activated and profibrotic ExM and Ly-6Ghigh monocytes. The accumulation of these nonresident cells and the development of pulmonary fibrosis in response to targeted alveolar injury is CCR2 dependent. We believe that this model system recapitulates many features of pulmonary fibrosis in humans, thereby implicating ExM and Ly-6Ghigh monocytes and the CCR2/CCR2−ligand axis as potential therapeutic targets to prevent or treat fibrotic lung disease.

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