CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Reduced Supply of Monocyte-Derived Macrophages Leads to a Transition from Nodular to Diffuse Lesions and Tissue Cell Activation in Silica-Induced Pulmonary Fibrosis in Mice

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Pulmonary fibrosis (PF) is an intractable disorder with a poor prognosis. Lung macrophages have been reported to regulate both progression and remission of bleomycin-induced diffuse PF. However, it remains unclear how macrophages contribute to silica-induced progressive nodular PF and the associated tissue cell responses *in vivo*. We found that lack of monocyte-derived macrophages results in the formation of diffuse PF after silica instillation. We found that the proportion and the number of monocyte-derived macrophages were persistently higher in silica-induced progressive PF compared with bleomycin-induced PF. Surprisingly, in *Ccr2*^{-/-} mice, in which monocyte-derived macrophage infiltration is impaired, silica administration induced diffuse PF with loose nodule formation and greater activation of tissue cells. In the diffuse lesions, the distribution of epithelial cells, distribution of myofibroblasts, and architecture of the basement membrane were disrupted. Consistent with the development of diffuse lesions, genes that were differentially expressed in CD45⁺ tissue cells from the lung of wild-type and *Ccr2*^{-/-} mice were highly enriched in human diffuse, progressive PF. In gene ontology network analyses, many of these genes were associated with tissue remodeling and included genes not previously associated with PF, such as *Mmp14*, *Thbs2*, and *Fgf4*. Overall, these results indicate that monocyte-derived macrophages prevent transition from nodular to diffuse silica-induced PF, potentially by regulating tissue cell responses. (*Am J Pathol* 2015, 185: 2923–2938; <http://dx.doi.org/10.1016/j.ajpath.2015.07.013>)

Pulmonary fibrosis (PF) is a progressive lung disease that is characterized by the large-scale formation of fibrous connective tissue.¹ PF is often associated with chronic interstitial lung diseases (ILDs), such as idiopathic pulmonary fibrosis (IPF), silicosis, and fibrotic nonspecific interstitial pneumonia (NSIP).² In patients with chronic ILD-associated PF, the irreversible deposition of extracellular matrix (ECM) components, together with hyperplasia of alveolar epithelial cells (AECs) and fibroblasts, lead to destructive remodeling of the lung tissue and subsequent respiratory failure.^{2,3} Although this persistent remodeling is an attractive

therapeutic target against progressive PF, the mechanisms that regulate this process remain poorly understood.

Silicosis is a chronic human ILD that manifests with progressive PF and one of the most important environmental and occupational diseases.⁴ Most forms of silicosis manifest as

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nodular PF and gradually develop during 30 to 40 years of silica inhalation,⁴ whereas others (eg, acute silicosis) manifest as diffuse PF, of which fibrotic lesions have an usual interstitial pneumonia pattern,^{5,6} one of the common features of diffuse, progressive PF.² The diffuse form of silicosis is often associated with worse prognosis than nodular silicosis.^{4,6} However, it remains largely unclear what regulates the development of nodular and diffuse PF in silicosis.

Tissue-resident alveolar macrophages (AMs) and monocyte-derived macrophages (MMs) are the dominant macrophage subsets in the lung. MMs are recruited from the bone marrow (BM) to the lung in response to inflammation in a C-C chemokine receptor type 2 (CCR2)-dependent manner.⁷ These macrophages have been variously found to ameliorate and exacerbate PF in mice.^{1,8–12} In the bleomycin-induced acute model, AMs and MMs promote lung inflammation, the disruption of lung architecture, and activation of tissue cells in the early stage through the secretion of proinflammatory mediators and matrix metalloproteinases (MMPs).^{8,13,14} On the other hand, AMs and MMs also suppress excessive damage and ECM deposition to the lungs by the secretion of anti-inflammatory mediators,^{15–17} the clearance of fibrous connective tissue, and possibly the clearance of proinflammatory extracellular debris, such as apoptotic cells.^{8,10,18}

AMs have also been found to regulate the pathologic mechanisms of the silica-induced progressive nodular PF model. AMs engulf inhaled silica particles and produce proinflammatory mediators, such as IL-1 β and tumor necrosis factor- α .¹⁹ In addition, IL-1 α activates phagocytotic effects of AMs and prevents alveolar proteinosis in the silica model.²⁰ However, the role of MMs in the progression phase of silica-induced PF, in particular how these cells influence the formation of fibrotic lesions and the activation pattern of tissue cell subsets in the lung, remains poorly understood. We examined the role of macrophages in the progression of silica-induced PF using a mouse model of silicosis.

Materials and Methods

Mice

C57BL/6J (B6) mice were purchased from Japan SLC (Shizuoka, Japan). ColGFP mice (backcrossed to B6 mice for >10 generations) were generated in a previous study.²¹ *Ccr2*^{-/-} mice (B6.129S4-*Ccr2*^{tm1Ifc/J}) were purchased from Jackson Laboratories (Bar Harbor, ME) and backcrossed to B6 mice for >10 generations. All mice were bred under specific pathogen-free conditions at the University of Tokyo and were 7 to 9 weeks old at the commencement of experiments. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of the University of Tokyo.

Administration of Silica or Bleomycin into Mouse Lungs

Mice were administered with a single dose of crystalline silica particles [400 mg/kg in 50 μ L of sterile saline;

MIN-U-SIL5, US Silica, Frederick, MD; mass-median-diameter (d_{50}) = 1.4 μ m] or bleomycin sulfate (1.25 mg/kg in 50 μ L of sterile saline; Toronto Research Chemical, Toronto, ON, Canada) by oropharyngeal aspiration.^{22,23} Silica particles were pretreated as described previously.²⁴ In brief, 3% w/v silica particles were boiled at 105°C in 1.0 M HCl for 1 hour, washed with dH₂O, and dried overnight at 110°C. The particles were then weighed, heat sterilized at 200°C for 2 hours, and suspended in sterile saline. Silica suspensions were sonicated for 30 minutes with a Bioruptor (Cosmo Bio USA Inc, Carlsbad, CA) immediately before use.

Measurement of Hydroxyproline Content

Lung hydroxyproline content was measured as described previously.²² In brief, the right lung was harvested, minced into small pieces, and suspended in 1600 μ L of ultrapure water. Then, 1600 μ L of 12 M HCl (Wako, Osaka, Japan) was added, and samples were heated for 24 hours at 110°C. After filtration, 25 μ L of the resulting acid hydrolysate was transferred to a new tube. Next, 25 μ L of 6 M NaOH (Wako), 50 μ L of citric acetate buffer [5% citric acid (Wako), 7.24% sodium acetate (Wako), 3.4% NaOH (Wako), 1.2% glacial acetic acid (Wako), pH 6.0], and 400 μ L of chloramine T solution (564 mg of chloramine T [Tokyo Chemical Industry, Tokyo, Japan], 4 mL of H₂O, 4 mL of *n*-propanol [Wako], and 32 mL of citric acetate buffer) were added sequentially, and the mixture was incubated for 20 minutes at room temperature. Next, 400 μ L of Ehrlich's solution [4.5 g 4-dimethylaminobenzaldehyde (Sigma-Aldrich, St Louis, MO), 18.6 mL *n*-propanol (Wako), and 7.8 mL 70% perchloric acid (Wako)] was added, and the mixture was incubated for 15 minutes at 65°C before the absorbance (optical density of 550) of the solution was measured.

Lung Cell Preparation and Flow Cytometry

Lung single-cell suspension was measured as described previously.²² In brief, mice were perfused with phosphate-buffered saline via the right ventricle until lungs became whitish. Perfused lungs were cut into small pieces with a razor blade and digested with 0.2% crude collagenase (Wako), 0.96 mg/mL of Dispase II (Roche, Basel, Switzerland), and 20 kU/mL DNase I (Sigma) in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan) and 10 mmol/L HEPES for 60 minutes at 37°C. The suspension was gently agitated by pipetting every 20 minutes throughout the digestion. After filtration through a 70- μ m cell strainer (BD Biosciences, San Jose, CA), cells were washed and resuspended in Dulbecco's modified Eagle's medium (Sigma) supplemented with 5% fetal bovine serum and 10 mmol/L HEPES. Erythrocytes and silica particles were removed by Percoll gradient centrifugation (GE Healthcare, Buckinghamshire, UK) for 20 minutes at room

temperature ($1000 \times g$). The single-cell suspensions were blocked with Fc block (anti-CD16/32, clone: 2.4G2, Bio X Cell, Lebanon, NH) and stained with appropriate antibody mixtures diluted with Isoflow (Beckman Coulter, Brea, CA) supplemented with 2% fetal bovine serum. Data were collected on a Gallios flow cytometer (Beckman Coulter) and analyzed using FlowJo software (FlowJo LLC, Ashland OR).

Antibodies

Detailed lists of the antibodies and streptavidin used for flow cytometry and immunohistochemistry are provided in the [Tables 1](#) and [2](#).

In Vivo BrdU Labeling

Long-term continuous BrdU labeling was performed as described previously.²⁵ In brief, for long-term continuous labeling, mice were provided with 0.8 mg/mL of BrdU (Sigma)—supplemented drinking water immediately after oropharyngeal aspiration of silica particles. BrdU-containing water was protected from light and replaced every 2 to 3 days. BrdU incorporation was detected using a BrdU Flow kit (BD Biosciences) according to the manufacturer's protocol.

Masson's Trichrome and Immunofluorescence Staining

Frozen lung sections (6- μ m thick) were isolated as described previously²² and subjected to Masson's trichrome staining or immunofluorescent staining. In brief, for isolation of fixed frozen section, isolated lungs were fixed by immersion in 4% paraformaldehyde-PBS for 4 hours at 4°C, dehydrated with 30% sucrose for 48 hours at 4°C, then embedded in optimal cutting temperature compound (Tissue-Tek; Sakura Finetek Japan, Tokyo, Japan) before snap-freezing with liquid nitrogen. For isolation of freshly frozen section, isolated lungs were infused with 400 μ L of optimal cutting temperature compound, then embedded in optimal cutting temperature compound before snap-freezing with liquid nitrogen. Frozen sections (6- μ m thick) were subjected to Masson's trichrome staining or immunofluorescent staining. For Masson's trichrome staining, sections were fixed with Bouin's solution (Sigma) for 60 minutes at 56°C and then washed in running tap water until the yellowish color disappeared from the sections. The sections were stained with Weigert's iron hematoxylin (Wako) for 10 minutes, then stained with acid fuchsin/Biebrich scarlet solution (Sigma) for 30 minutes, and differentiated in a phosphomolybdic/phosphotungstic acid solution (Sigma) for 5 minutes. Finally, the sections were stained with aniline blue solution (Sigma) for 1 hour and differentiated in 1% acetic acid solution for 5 minutes. For immunofluorescent staining, the sections were blocked with Blocking One (Nacalai Tesque, Kyoto, Japan), stained with primary antibodies overnight at 4°C, then stained with Alexa Fluor—conjugated secondary

antibodies for 30 minutes at 37°C (Life Technologies, Carlsbad, CA). Masson's trichrome—stained sections were mounted with Entellan New (Merck, Kenilworth, NJ). Immunofluorescently stained sections were mounted with Prolong Gold (Life Technologies). Immunofluorescent images were acquired with a SP-5 confocal microscope (Leica Microsystems, Wetzlar, Germany). Masson's trichrome images were acquired with a NanoZoomer 2.0-HT C9600 digital slide scanner (Hamamatsu Photonics, Sizuoka, Japan) at 20 $\times$ magnification or a SP-5 confocal microscope. Images were processed and quantified by using Photoshop CS6 Extended (Adobe Systems Inc, San Jose, CA) and ImageJ software version 1.48v (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). For the quantification of the CD68⁺ macrophage density, the cross-section of each nodule was split into four areas from the inner to outer boundary, and the CD68⁺ pixel area was determined using Photoshop CS6 Extended software (Adobe, San Jose, CA). Then, the percentage of CD68⁺ pixels in each split area per total area was quantified using ImageJ software. For the quantification of collagen fiber area, bronchial wall and peribronchial scaffold fibers were excluded manually, and the areas of blue pixels (representing collagen fibers) were determined using Photoshop CS6 Extended software. Total and collagen-positive areas in whole lung sections were then quantified using ImageJ software.

Generation of BM Chimeric Mice

Recipient ColGFP mice were lethally irradiated (9 Gy, in two separate doses) before transplant with 3×10^6 B6 or *Ccr2*^{-/-} whole BM cells on the following day. Mice were not administered with silica suspension until at least 6 weeks after transplantation.

Magnetic Separation, Total RNA Preparation, and Microarray Raw Data Collection

Lung CD45⁻ tissue cells were isolated by magnetically labeling total lung cells with APC-Cy7 anti-CD45 (clone 30-F11; BioLegend, San Diego, CA) and APC-Cy7 anti-Ter-119 (clone TER-119; BioLegend), followed by incubation with anti-APC microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Labeled cells were magnetically separated using an AutoMACS cell separator (Miltenyi Biotech). Purity of isolated CD45⁻ tissue cells among live cells was >95% for all experiments. Total RNA was isolated from magnetically sorted CD45⁻ tissue using TRIzol Reagent (Life Technologies) and RNeasy Mini Kits (Qiagen, Venlo, The Netherlands). RNA purity and integrity were confirmed based on its optical density 260/280 ratio before analysis using a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Each total RNA sample pooled from three biological replicates (one experimental replicate) was labeled and hybridized according to the Agilent One-Color Microarray-Based Gene Expression

Table 1 List of Antibodies Used for Flow Cytometry

Antigen	Clone/catalog no.	Isotype	Manufacturer	Fluorochrome
CD3ε	500A5	Hamster IgG1, κ	BD Biosciences	BD Horizon V500
CD4	RM4-5	Rat IgG2a, κ	BioLegend	PE-Cy7
CD8α	53-6.7	Rat IgG2a, κ	BD Biosciences	APC
CD11b	M1/70	Rat IgG2b, κ	BD Biosciences	FITC
				BD Horizon PE-CF594
			BioLegend	Pacific blue
				PE-Cy7
CD11c	N418	Hamster IgG	eBioscience	APC-eFluor 780
	HL3	Hamster IgG1, λ	BD Biosciences	PE-Cy7
CD31	390	Rat IgG2a, κ	BioLegend	PerCP-Cy5.5
			BD Biosciences	PE-Cy7
CD45	30-F11	Rat IgG2a, κ	BioLegend	Pacific blue
				APC-Cy7
CD45.2	104	Mouse IgG2a, κ	BD Biosciences	BD Horizon V500
B220	RA3-6B2	Rat IgG2a, κ	BioLegend	PE
CD64	X54-5/7.1.1	Mouse IgG1, κ	BD Biosciences	PE
			BioLegend	APC
CD103	2E7	Hamster IgG	BioLegend	APC
EpCAM	G8.8	Rat IgG2a, κ	BioLegend	PE
				PerCP-Cy5.5
Ly6C	HK1.4	Rat IgG2c, κ	BioLegend	Pacific blue
	AL21	Rat IgM, κ	BD Biosciences	APC-Cy7
Ly6G	1A8	Rat IgG2a, κ	BD Biosciences	BD Horizon PE-CF594
				Alexa Fluor 700
Gr-1	RB6-8C5	Rat IgG2a, κ	BioLegend	PE
NK1.1	PK136	Mouse IgG2a, κ	BioLegend	Pacific blue
Ter-119	TER-119	Rat IgG2b, κ	BioLegend	APC-Cy7
Siglec-F	E50-2440	Rat IgG2a, κ	BD Biosciences	PE
I-A/I-E	M5/114.15.2	Rat IgG2a, κ	BioLegend	Pacific blue
				PerCP-Cy5.5
TCR γδ-chain	GL3	Hamster IgG2, λ	BD Biosciences	PE
α-SMA	1A4	Mouse IgG2a	R&D systems	APC
BrdU	Bu20a	Mouse IgG1, κ	BioLegend	APC
Cleaved caspase-3	#9661S	Rabbit immunoglobulin polyclonal	Cell Signaling Technology Japan	Purified
Rabbit IgG	A-31573	Donkey immunoglobulin polyclonal	Life Technologies	Alexa Fluor 647

α-SMA, α-smooth muscle actin; APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, R-phycoerythrin; PerCP, peridinin chlorophyll; TCR, T cell receptor.

Analysis version 6.5 protocol (Agilent Technologies). In brief, 200 ng of total RNA from each sample was linearly amplified, labeled with Cy3-dCTP, and purified by using RNeasy Mini Kits (Qiagen). RNA concentration and dye load were measured using a NanoDrop ND-1000 (Thermo Fisher Scientific). Labeled RNA (600 ng) was fragmented by heating for 30 minutes at 60°C after addition of 5 μL of 10× blocking agent and 1 μL of 25× fragmentation buffer. Finally, the RNA solutions were diluted with GE hybridization buffer, dispensed into the gasket slide, and assembled onto the SurePrint G3 Mouse GE 8 × 60K Microarray (Agilent Technologies). The hybridized array was scanned immediately using an Agilent Microarray Scanner (Agilent Technologies). Raw data were extracted using Agilent Feature Extraction Software software version 11.0.1.1 (Agilent Technologies). The raw data for the same genes were then summarized automatically via the Agilent feature extraction protocol to generate raw data text files that contain expression data for each gene probed on the array. Array probes that displayed flag A in samples were

filtered out. The purity of isolated CD45⁺ tissue cells among live cells was >95% for all experiments. Raw data from these experiments have been deposited in the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>; accession number GSE61709).

Clustering of Microarray Expression Data and Preprocess of the Public Transcriptome Data

After averaging the expression values of duplicated probes, all expression values for the probes were log₂ transformed and normalized between groups using the quantile method. Then, probes for which the mean raw expression values across all experiments were <30 were filtered out. Next, we selected probes that had a fold-change of ≥1.5 between *Ccr2*^{-/-} → ColGFP and wild-type (WT) → ColGFP chimeras on days 28, 40, or both days. The 2406 probes selected were Z-scaled, clustered by the CLICK method,²⁶ and visualized by using R software version 3.0.2 (www.r-project.org; last

Table 2 List of Antibodies and Streptavidin Used for Immunohistochemistry

Antigen	Clone/catalog no.	Isotype	Manufacturer	Fluorochrome
CD11c	N418	Hamster IgG	BioLegend	Purified
CD68	FA-11	Rat IgG2a, κ	BioLegend	Purified
EpCAM	G8.8	Rat IgG2a, κ	BioLegend	Biotin
Ly6G	RB6-8C5	Rat IgG2a, κ	BD Biosciences	Biotin
GFP	GF090R	Rat IgG2a	Nacalai Tesque	Purified
α -SMA	1A4	Mouse IgG2a	R&D Systems	APC
Podoplanin	8.1.1	Syrian Hamster IgG	BioLegend	Purified
GFP	598	Rabbit immunoglobulin polyclonal	Medical & Biological Laboratories	Purified
CD31	AF3628	Goat immunoglobulin polyclonal	R&D systems	Purified
Collagen 4	LSL-LB-1403	Rabbit antisera	LSL	Unlabeled
Rat IgG (H+L)	A-21208	Donkey immunoglobulin polyclonal	Life Technologies	Alexa Fluor 488
Rabbit IgG (H+L)	A-21206	Donkey immunoglobulin polyclonal	Life Technologies	Alexa Fluor 488
Goat IgG (H+L)	A-21432	Donkey immunoglobulin polyclonal	Life Technologies	Alexa Fluor 555
Rat IgG (H+L)	A-11081	Goat immunoglobulin polyclonal	Life Technologies	Alexa Fluor 546
Rabbit IgG (H+L)	A-11010	Goat immunoglobulin polyclonal	Life Technologies	Alexa Fluor 546
Hamster IgG (H+L)	A-21111	Goat immunoglobulin polyclonal	Life Technologies	Alexa Fluor 546
Hamster IgG (H+L)	A-21541	Goat immunoglobulin polyclonal	Life Technologies	Alexa Fluor 647
Rat IgG (H+L)	A-21247	Goat immunoglobulin polyclonal	Life Technologies	Alexa Fluor 647
Rabbit IgG	A-31573	Donkey immunoglobulin polyclonal	Life Technologies	Alexa Fluor 647
Rabbit IgG	A-21308	Goat immunoglobulin polyclonal	Life Technologies	Alexa Fluor 700

α -SMA, α -smooth muscle actin; APC, allophycocyanin; GFP, green fluorescent protein.

accessed June 16, 2015). Public transcriptome data for human clinical lung samples were downloaded from the ArrayExpress (<http://www.ebi.ac.uk/arrayexpress/experiments>; last accessed June 16, 2015) and preprocessed by using the limma package²⁷ (for reanalyzing E-GEOD-47460) and the affy/vsn package^{28,29} (for reanalyzing E-GEOD-32539) in R software version 3.0.2. The public microarray data used in this study were as follows: IPF, fibrotic NSIP, and healthy whole human lungs (E-GEOD-47460³⁰ obtained from Lung Tissue Research Consortium and E-GEOD-32539³¹). After exclusion of background, all the expression values for the probes were $\log_2$ transformed and normalized between arrays. Next, the expression values were normalized between groups using the quantile method, and the values of duplicated probes were averaged. Then, probes for which the mean raw expression values across all samples were <60 were filtered out.

Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA)³² was performed using each clustered gene list and human lung expression data (E-GEOD-47460) as gene sets and expression data sets, respectively. For comparison of IPF and fibrotic NSIP versus healthy lung, metrics of gene rankings were determined using tTest mode, the permutation type was gene set, and other calculation settings were set to defaults.

Gene Ontology Network Analysis and Identification of the Diffuse, Progressive PF-Associated Tissue Remodeling-Related Genes

Gene ontology (GO) network analysis was performed using Cytoscape version 3.1.0 with the ClueGO plugin software

version 2.1.1.^{33,34} Significantly enriched GO terms³⁵ (GO biological process; GO levels: 3 to 7) and KEGG pathway terms³⁶ in the clusters E2 to E4 were explored (minimum contained gene number, 10; percentage of minimum contained gene, 12%) and grouped, and a term network was constructed based on the overlap of their elements (κ score = 0.3). Leading terms within each group were defined as the most significantly enriched term in each group. Terms not connected with any other term were excluded. The genes in the clusters E2 to E4 belonging to the tissue remodeling-related groups and leading edge genes of IPF, which were defined by the GO network and GSEA, were identified as the diffuse, progressive PF-associated genes. We used versions of the GO term database and KEGG pathway term database that were current on January 6, 2015.

Real-Time Quantitative PCR Analysis

The mRNA isolated for microarray and a portion of the mRNA of sorted MMs were reverse transcribed to cDNA using a ReverTra Ace real-time quantitative PCR (qPCR) kit (Toyobo, Osaka, Japan). qPCR analysis was performed by using Thunderbird SYBR qPCR Mix (Toyobo) on an ABI7500 real-time PCR system (Life Technologies). The expression levels of all target mRNAs were normalized against the expression level of Actb in each sample. Sequences of the primers used for qPCR were as follows: Thbs2 forward 5'-TCGGACCTCAAGTATGAGTGC-3', Thbs2 reverse 5'-ATCTAAGAAGGGGTGTTTGCAG-3', Loxl2 forward 5'-TGACTGCCAGTGGATAGACATC-3', Loxl2 reverse 5'-GTTGGGGTTAATGACAACCTG-3', Mmp2 forward 5'-TCTTTGATGGCATCGCTCAGA-3', Mmp2 reverse 5'-GTACTCATTCCCTGCGAAGAACA-3',

Mmp14 forward 5'-GAGAACTTCGTGTTGCCTGA-3',
 Mmp14 reverse 5'-CTTTGTGGGTGACCCCTGACT-3',
 Grem1 forward 5'-GACCCACGGAAGTGACAGA-3',
 Grem1 reverse 5'-CCCTCAGCTGTTGGCAGTAG-3', Actb
 forward 5'-CTAAGGCCAACCGTGAAAAG-3', Tnc forward
 5'-GGGCTATAGAACACCGATGC-3', Tnc reverse 5'-
 CATTAAAGTTTCCAATTTCAGGTTTC-3', Actb reverse 5'-
 ACCAGAGGCATACAGGGACA-3'.

Statistical Analyses

Statistical analyses (for all except microarray data) were performed using two-way analysis of variance with Tukey-Kramer's multiple comparison post hoc test (for more than three groups) or two-tailed unpaired *t*-test (between two groups except gene expression data) or two-tailed unpaired Welch's *t*-test (between two groups of gene expression data) in GraphPad Prism version 5.0 software (GraphPad Software, La Jolla, CA) and R software version 3.0.2. The significance and false discovery rate of overenrichment of each cluster in ILD lung or healthy lung were analyzed using GSEA software version 2.2.0. The significance of enrichment of each GO term was analyzed using Cytoscape version 3.1.0 with the ClueGO plugin version 2.1.1. Correction for the multiple comparisons was performed using the Benjamini-Hochberg method. The gene expression of human ILD lungs (E-GEOD-32539) was log₂ transformed, and the significance of their changes was analyzed by using Kruskal-Wallis test with Dunn's multiple comparison post hoc test in GraphPad Prism version 5.0 software. *P* < 0.05 was considered to be statistically significant.

Results

Identification of the Features of Lung Macrophage Kinetics in Silica-Induced Progressive Nodular PF

To determine the progression phase in silica-induced progressive PF, we compared collagen deposition, fibroblast activation, and leukocyte infiltration between silica-induced progressive PF and bleomycin-induced acute lung injury and PF. We used *Col1a2*-reporter mice (ColGFP mice) to identify lung fibroblasts.^{21,22} Hydroxyproline content in the lung increased on day 28 in both models. In contrast, after day 28, their elevation was maintained in the silica model but decreased in the bleomycin model up to day 63 (Figure 1A), which is consistent with previous reports.^{16,24} Furthermore, flow cytometric analysis revealed that total fibroblast number increased to peak on day 7 in the silica model but did not significantly change after day 28 in both models compared with the steady-state level. The frequency of α -smooth muscle actin (α -SMA)-positive activated fibroblasts (myofibroblasts)¹⁸ and the total leukocyte number increased to peak on day 7, then decreased moderately to day 28 in both models. After day 28, these cell

populations remained elevated in the silica model but decreased in the bleomycin model up to day 63 (Figure 1, B and C, and Supplemental Figure S1A). On the basis of these kinetics of total leukocyte number (a measure of inflammation) and fibroblast activation in the two models, we defined the boundary between the initiation and progression phases of the disease at day 28.

Next, to characterize the macrophage response in silica-induced PF, we compared changes in lung macrophage subpopulations over time in the silica and bleomycin models (Supplemental Figure S1B).³⁷ Despite their proposed role in the removal of silica particles,^{4,9} the number of AMs did not significantly change in either model (Figure 1D). In contrast, the number of MMs, Ly6C^{lo} monocytes, and interstitial macrophages (IMs) increased in both models, peaking on day 7. Notably, the number of these cells remained elevated in the silica model but returned to normal levels by day 63 in the bleomycin model (Figure 1D). Moreover, the proportion of MMs among total leukocytes was higher in the silica model than the bleomycin model from day 7 onward. On the other hand, the difference in the frequency of IMs between the two models was only marginal (Figure 1E). The differences in the kinetics of MMs between silica-induced and bleomycin-induced PF prompted us to further investigate the roles of these cells in silica-induced progressive PF, including their roles in the formation of nodular fibrosis and/or its irreversibility.

Non-AM Macrophages Selectively Accumulate at the Periphery of Silica-Induced Fibrotic Nodules Colocalizing with Hyperplastic AECs and Myofibroblasts

To investigate the link between the localization of non-AM macrophages and the pathologic features of silica-induced PF, we next assessed the localization of these cells by immunofluorescent analysis of lung sections. MMs, IMs, and Ly6C^{lo} monocytes, which were all identified as CD68⁺ CD11c⁻ cells, selectively accumulated within the fibrotic nodules (Figure 2A). Moreover, on day 63 the density of CD68⁺ cells was higher at the periphery of the lesions rather than at the center (Figure 2B). Interestingly, hyperplastic AECs, a feature of progressive fibrosis,^{2,3} were also colocalized with CD68⁺ CD11c⁻ cells at the periphery (Figure 2C). α -SMA⁺ ColGFP⁺ myofibroblasts also began to accumulate at the periphery as early as day 14 (Figure 2D). Taken together, these findings suggest that non-AM macrophages, particularly MMs, could regulate fibroblast activation and AEC hyperplasia at the periphery of silica-induced fibrotic nodules.

Impairment of Monocyte-Derived Macrophage Infiltration into the Silica-Treated Lung Results in Diffuse PF

Next, we investigated whether MMs contribute to the development of silica-induced PF. To specifically delete

Figure 1 Characterization of lung macrophage kinetics in silica-induced progressive nodular pulmonary fibrosis (PF). **A**: Quantification of hydroxyproline content in the whole right lung of silica-, bleomycin-, or saline-treated (control) B6 mice. **B–E**: Quantification of lung leukocyte and fibroblast populations throughout silica- and bleomycin-induced PF. The number of cells in each population was quantified by flow cytometry. **B**: Numbers of ColGFP⁺ fibroblasts and frequencies of green fluorescent protein (GFP)⁺ α -smooth muscle actin (α -SMA)⁺ myofibroblasts among total GFP⁺ fibroblasts. Representative flow cytometry plots for day 0 (untreated) and day 14/day 63 (silica) are shown. **C–E**: Number of CD45⁺ total leukocytes (**C**) and monocyte/macrophage subsets (**D**), as well as frequency of lung macrophage subsets among total CD45⁺ leukocytes (**E**), at each time point. Gray shaded areas in **D** and **E** indicate the progression phase of the silica model. Data are expressed as means $\pm$ SEM (**A–E**) from two (**A** and **B**) and three (**C–E**) independent experiments. $n = 6$ to 8 (**A**); $n = 6$ (**B**); $n = 8$ to 10 (**C–E**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared between silica and bleomycin at the same time point; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.001$ compared with control at the same time point by two-way analysis of variance with Tukey-Kramer's multiple comparison post hoc test. AMs, alveolar macrophages; IMs, interstitial macrophages; MMs, monocyte-derived macrophages. See also [Supplemental Figure S1](#), A and B.

Ccr2 expression in BM-derived cells and address fibroblast responses, we used *Ccr2*^{−/−} BM chimeric mice generated using ColGFP recipient mice. ColGFP mice were lethally irradiated and transplanted with *Ccr2*^{−/−} or WT BM cells, generating *Ccr2*^{−/−}→ColGFP or WT→ColGFP chimeras, respectively ([Figure 3A](#)). *Ccr2* deficiency leads to defective Ly6C^{hi} monocyte infiltration into peripheral tissues.³⁸ As

expected, MM number in *Ccr2*^{−/−}→ColGFP chimeras was substantially lower than in WT→ColGFP chimeras on day 40 after aspiration of silica ([Supplemental Figure S2A](#)). The numbers of Ly6C^{lo} monocytes, IMs, and CD11b⁺ DCs in *Ccr2*^{−/−}→ColGFP chimeras were also lower than in WT→ColGFP chimeras but to a lesser extent than observed for MMs. However, the cell numbers within nonmonocyte

Figure 2 Nonalveolar macrophages (non-AMs) selectively accumulate and are co-localized with hyperplastic alveolar epithelial cells (AECs) and myofibroblasts at the periphery of silica-induced fibrotic nodules. **A:** Immunofluorescent staining for CD11c (green), CD68 (magenta), and ColGFP (cyan) in silica- or saline (control)-treated left lung sections on day 28 after aspiration. Note that CD68⁺CD11c[−] non-AMs accumulated at the periphery of the fibrotic lesions to a greater extent than CD68⁺CD11c⁺ AMs (orange arrows) and CD68[−]CD11c⁺ DCs (white arrows). **B:** Quantification of CD68⁺ macrophage density in the silicotic nodules on day 63. The cross-section of each nodule is split into four areas from the inner to outer boundary. The percentage of CD68⁺ pixels in each split area per whole area was quantified. **C:** Immunofluorescent staining for CD68 (magenta), EpCAM (green), and ColGFP (cyan) in silica-treated left lung sections on days 28 and 63. Hyperplastic AECs and fibroblasts cluster selectively at the periphery of fibrotic lesions. **D:** Immunofluorescent staining for α-smooth muscle actin (α-SMA) (magenta) and ColGFP (green) in silica-treated left lung sections on days 14 and 63. α-SMA⁺ green fluorescent protein⁺ myofibroblasts (white arrows) selectively localize to the periphery of fibrotic nodules. Enlarged images of boxed regions are shown in the adjacent panel. Data are expressed as means ± SEM (**B**) pooled from two independent experiments. $n = 12$ (**B**); $n = 8$ to 10 (**A**, **C**, and **D**). $**P < 0.01$, $***P < 0.001$ compared with 0-25; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with 25-50 by one-way analysis of variance with Tukey-Kramer's multiple comparison post hoc test. Scale bars: 250 μm (**A–D**, not enlarged); 50 μm (**A**, **C**, and **D**, enlarged). Br, bronchioli; C, center; F, fibrotic lesion; P, periphery.

or macrophage leukocyte populations were comparable between the two chimeras, indicating that the accumulation of monocytes and macrophages, particularly MMs, was selectively impaired in *Ccr2*^{−/−}→ColGFP chimera (Supplemental Figure S1, C and D, and Supplemental Figure S2, A–C).

Surprisingly, *Ccr2*^{−/−}→ColGFP chimeras developed diffuse PF with occasional formation of loose silicotic nodules (Figure 3B). Dissemination of PF lesions was evidenced by a greater area of collagen deposition than in

WT→ColGFP chimera (Figure 3, B and C). We observed similarly altered pathologic features in nonchimeric *Ccr2*^{−/−} mice (Supplemental Figure S3A). Despite the expansion of the fibrotic area, the total hydroxyproline content of the silica-treated lungs was not different between nonchimeric *Ccr2*^{−/−} and WT mice (Supplemental Figure S3B).

Within the regions that contained diffuse fibrotic lesions, interstitial fibrosis was expanded in the alveolar septa and intra-alveolar area with the disruption of alveolar architecture (Figure 3B). Dense fibrotic lesions were occasionally

Figure 3 Impairment of monocyte-derived macrophage infiltration into the silica-treated lung results in diffuse rather than nodular pulmonary fibrosis. **A:** Experimental model. Lethally irradiated ColGFP recipient mice received bone marrow cells from *Ccr2*^{-/-} or wild-type (WT) donors. At least 6 weeks later, the chimeras were treated with silica or saline. **B:** Masson's trichrome staining of silica-treated left lung sections from WT→ColGFP and *Ccr2*^{-/-}→ColGFP chimeras on day 40 after aspiration. Blue collagen fibers accumulate in the interstitium of alveolar walls (orange arrows) and intraalveolar area (orange arrowheads) in diffuse fibrotic lesions of *Ccr2*^{-/-}→ColGFP chimera. Dense collagen fibers accumulate in the dense fibrotic lesions (red arrowheads) in *Ccr2*^{-/-}→ColGFP chimeras. **C:** Percentage of the areas of collagen fiber in whole lung sections stained by Masson's trichrome staining. **D:** Immunofluorescent staining for CD31 (magenta), EpCAM (cyan), and ColGFP (green). Hyperplastic EpCAM⁺ alveolar epithelial cell (orange arrowheads) and CD31⁺ endothelial cell clusters (white arrowheads) are present only in dense silicotic nodules. Enlarged images of the boxed regions are shown on the right side of the overviews. Data are expressed as means ± SEM from two independent experiments (C). *n* = 7 to 8. *P < 0.05 by *t*-test (C). Scale bars: 4 mm (B, overview); 100 μm (B, enlarged); 250 μm (B, control; D, not enlarged); 50 μm (D, enlarged). Br, bronchioli; F, fibrotic lesion. See also Supplemental Figures S1–S5.

present close to the diffuse fibrotic lesions (Figure 3B). Collagen deposition in the dense lesions appeared scattered, whereas extensive collagen deposition was observed mainly in the center of the nodules in WT→ColGFP chimeras (Figure 3B). In addition, the collagen in the dense lesions appeared darker than in the diffuse lesions, suggesting a higher density of collagen fibers in the dense lesions (Figure 3B). The histological heterogeneity of the fibrotic lesions in *Ccr2*^{-/-}→ColGFP chimeras, the presence of dense and diffuse lesions and the various densities of

collagen deposition were similar to histological features of the usual interstitial pneumonia pattern.^{3,39} On the other hand, consistent with previous reports,^{11,13} such histopathological changes of fibrotic lesions were not observed between chimeric/non-chimeric *Ccr2*^{-/-} and WT mice on day 21 in the bleomycin model (Supplemental Figure S4, A and B). Moreover, hyperplastic AECs, endothelial cells, and myofibroblasts were scattered in the diffuse lesions of the *Ccr2*^{-/-}→ColGFP lung, whereas these cells displayed distinct clustering in the nodules of the WT→ColGFP

Figure 4 Monocyte-derived macrophages suppress diffuse, progressive pulmonary fibrosis (PF)—associated activation of the tissue cells of silica-treated lung. **A:** Experimental scheme. Wild-type (WT) $\rightarrow$ ColGFP and $Ccr2^{-/-} \rightarrow$ ColGFP chimeras were treated with silica or saline, and CD45⁺ lung leukocytes were magnetically depleted from total lung cells. Microarray analysis was performed against the CD45⁺ lung tissue cells from untreated (day 0) and silica-treated WT $\rightarrow$ ColGFP and $Ccr2^{-/-} \rightarrow$ ColGFP chimeras on days 28 and 40. Flow cytometry plots indicate the purity of CD45⁺ lung tissue cell and magnetically depleted CD45⁺ leukocyte fraction of silica-treated $Ccr2^{-/-} \rightarrow$ ColGFP chimera on day 40 after aspiration. **B** and **C:** Transcriptomic profile of CD45⁺ lung tissue cells from silica-treated WT $\rightarrow$ ColGFP and $Ccr2^{-/-} \rightarrow$ ColGFP chimeras on days 28 and 40 and untreated mice on day 0. **B:** Heat map representation of differentially expressed genes based on CLICK clustering. Each column represents group and time point, whereas each row represents an individual gene. **C:** Z-score of gene clusters that were persistently up- or down-regulated along with PF progression. **D** and **E:** Gene set enrichment analysis of $Ccr2^{-/-}$ biased clusters for the samples from idiopathic pulmonary fibrosis (IPF) or fibrotic nonspecific interstitial pneumonia (NSIP) (E-GEOD-47460). **D:** Gene clusters E1 to E6 are plotted by their normalized enrichment score and *P* value. The shaded areas indicate significant enrichment. **E:** Enrichment plots for clusters E2 to E4. Black bars represent the gene positions on the ranked gene list. Data are expressed as means $\pm$ SD (**C**). FDR, false discovery rate; MACS, magnetic cell separation. See also [Supplemental Figures S6 and S7](#).

Figure 5 Monocyte-derived macrophages inhibit changes in the expression of tissue remodeling-related genes in silica-treated lung tissue cells. **A:** Diagram of functional term network construction. Biological events associated with the *Ccr2*^{-/-}-biased clusters E2 to E4 were investigated. Overenriched gene ontology terms and KEGG pathway terms were explored, and a term network was mapped using Cytoscape version 3.1.0 with the ClueGO plugin. **B:** Network of significantly enriched terms ($P < 0.05$). Each node and its size represent functional term and enrichment significance, respectively. Group of nodes and their leading terms are shown in the same color. Multicolored nodes belong to multiple groups. The protein digestion and absorption group is detailed. **C:** The enrichment significance of the leading terms (the most significantly enriched term of each group). P values were calculated for each term by using two-sided hypergeometric test with Benjamini-Hochberg correction. ECM, extracellular matrix.

lung (Figure 3D and Supplemental Figure S5A). In addition, the basement membrane was denuded and the network of type 1 AECs was disrupted in the diffuse lesions (Supplemental Figure S5, B and C), similar to the characteristics of fibroblastic foci in the usual interstitial pneumonia pattern.^{40,41} These results indicate that MMs selectively regulate the histopathologic features of silica-induced PF, preserving nodular PF without affecting its irreversibility or the total amount of collagen deposition.

Monocyte-Derived Macrophages Suppress Diffuse, Progressive PF Associated Activation and Turnover of the Tissue Cells in Silica-Treated Lung

Next, we investigated how impairment of MMs affects the activation state of lung tissue cells in silica-induced PF.

First, we measured the turnover of the tissue cells (Supplemental Figure S6A) because the turnover of fibroblasts is associated with their focal activation in the bleomycin model.²² Continuous BrdU-labeling revealed that the turnover of fibroblasts, epithelial cells, and endothelial cells, but not their total number, was increased in *Ccr2*^{-/-} → ColGFP chimeras compared with their WT → ColGFP counterparts on day 40 in the silica model (Supplemental Figure S6, B and C).

To further determine what activation changes occur in the tissue cells of silica-treated *Ccr2*^{-/-} → ColGFP chimeras, we measured the transcriptomic profile of CD45⁺ lung tissue cells on days 0, 28, and 40 after aspiration (Figure 4A). Microarray-based genome-wide transcriptome analysis identified 2406 genes that were differentially expressed with a fold-change of ≥ 1.5 between *Ccr2*^{-/-} → ColGFP and WT → ColGFP chimeras on

Figure 6 Loss of monocyte-derived macrophages promotes the expression of tissue remodeling-related genes. **A:** Diagram of the identification of the diffuse, progressive pulmonary fibrosis (PF)-associated tissue remodeling-related genes. **B:** Heat map representation of the identified tissue remodeling-related genes. **C:** Expression of the identified tissue remodeling-related genes in idiopathic pulmonary fibrosis (IPF) and healthy human lung (E-GEOD-47460). **D:** Validation of gene expression by real-time quantitative PCR. Gene expression in CD45⁺ tissue cells of silica- or saline-treated wild-type (WT)→ColGFP and *Ccr2*^{-/-}→ColGFP chimera lung on day 40. Expression levels were normalized to *Actb*. **E:** Gene expression in human interstitial lung disease lungs (E-GEOD-32539). Signal intensity of genes in each sample was plotted. Data are expressed as means ± SEM pooled from two independent experiments (**D** and **E**). *n* = 7 to 8 (silica-treated) (**C**); *n* = 4 to 6 (control) (**C**); *n* = 50 (healthy group) (**D**); *n* = 119 (IPF group) (**D**); *n* = 17 (fibrotic nonspecific interstitial pneumonia [NSIP] group) (**D**). *P < 0.05, **P < 0.01, and ***P < 0.001 by Welch's *t*-test; †*P* < 0.05, ††*P* < 0.01, and †††*P* < 0.001 by Kruskal-Wallis test with Dunn's multiple comparison post hoc test. GSEA, gene set enrichment analysis.

one or both of day 28 and day 40. These genes were clustered based on their expression kinetics using the CLICK method²⁶ (Figure 4, B and C, Supplemental Figure S7, and Supplemental Table S1). Within the six major gene clusters identified (including >100 genes each), changes in gene expression followed several distinct patterns: i) E3: stepwise up-regulation throughout PF progression in *Ccr2*^{-/-}→ColGFP chimeras; ii) E2 and E4: stepwise down-regulation throughout PF progression in *Ccr2*^{-/-}→ColGFP chimeras; and iii) E1, E5, and E6: no clear association with PF progression in either chimera (Figure 4, B and C). Notably, changes in the expression levels of genes in groups i and ii were greater in

Ccr2^{-/-}→ColGFP chimeras than in WT→ColGFP chimera on both day 28 and day 40, suggesting that MMs may modulate the expression of genes in the corresponding clusters (Figure 4C).

Next, to assess the correlation between silica-induced diffuse, progressive PF in *Ccr2*^{-/-}→ColGFP chimeras and human diffuse, progressive PF (IPF and fibrotic NSIP),³ we performed GSEA³² on public transcriptome data based on clinical lung samples from ILD patients.³⁰ Although the gene cluster E3 was significantly enriched in IPF and fibrotic NSIP (ie, most genes in this cluster were expressed more highly in IPF and fibrotic NSIP than in healthy lungs), gene clusters E2 and E4 were

significantly enriched in healthy lungs (Figure 4, D and E). Other gene clusters did not show a common pattern of enrichment in IPF or fibrotic NSIP versus healthy lungs (Figure 4D). Thus, the absence of MMs resulted in up-regulation of genes associated with human diffuse, progressive PF but down-regulation of genes associated with the healthy condition. These data demonstrate that MMs limit diffuse, progressive PF—associated activation of tissue cells in silica-induced PF.

In Silica-Treated Lung Tissue Cells, Monocyte-Derived Macrophages Inhibit Changes in the Expression of Tissue Remodeling—Related Genes That Also Change in Clinical IPF

To clarify the biological functions associated with the gene clusters E2 to E4, we constructed a functional term network from the genes pooled from these clusters (Figure 5A). Groups of tissue remodeling—related terms (blood vessel morphogenesis or development, protein digestion and absorption, morphogenesis of a branching epithelium or structure, cellular response to [fibroblast] growth factor stimulus, epithelial proliferation, and lung or endothelium development) were highly enriched within the network (Figure 5, B and C). Among the tissue remodeling—related genes in the clusters E2 to E4, we found 86 genes that were also differentially expressed in the IPF lungs (46 up-regulated and 40 down-regulated) (Figure 6, A–C). The up-regulated genes contained many PF-associated genes, including activation markers of fibroblasts (*Spp1* and *Tnc*),^{22,42} exacerbating factors of PF (*Mmp2*, *Grem1*, *Serpinf1*, and *Postn*),^{43–46} a collagen crosslinking enzyme and IPF progression marker (*Lox12*),⁴⁷ and transforming growth factor- β pathway—related genes (*Sfrp1*, *Sulf1*, and *Bmp7*).^{48–50} The down-regulated genes contained potentially protective factors, such as a negative regulator of transforming growth factor- β signaling (*Smad6*)⁵¹ and a negative regulator of lung fibroblast activation *in vitro* (*Foxf1*).⁵² The differentially expressed genes also contained membrane type 1 MMP (*MMP14*, *Mmp14*), thrombospondin 2 (*TSP-2*, *Thbs2*), and fibroblast growth factor receptor 4 (*FGFR4*, *Fgfr4*) (Figure 6B). Remarkably, none of these genes have been found previously to play a role in PF, despite their proposed roles in fibrosis of other organs.^{53–55} Consistent with our microarray data, qPCR analysis revealed that lung tissue cells from silica-treated *Ccr2*^{-/-}→ColGFP chimeras expressed *Mmp2*, *Grem1*, *Mmp14*, *Thbs2*, *Lox12*, and *Tnc* at higher levels than the corresponding cells from WT chimeras (Figure 6D). In addition, these genes were expressed significantly higher in IPF lungs than in healthy lungs in another public transcriptome data set³¹ (Figure 6E). Taken together, these results indicate that MMs repress changes in the expression of IPF-associated tissue

remodeling—related genes in the tissue cells of silica-treated lung.

Discussion

In this study, we report that impaired MM recruitment resulted in diffuse PF induction. In the progression phase of the silica model, the absence of MMs promoted transcriptomic changes in the lung tissue cells, which correlated with the changes observed in human diffuse, progressive PF. These results suggest that MMs limit the dissemination of fibrotic lesions and associated activation of lung tissue cells in silica-induced progressive PF.

It has been reported that MMs regulate the early phase of PF in a context-dependent manner in murine models.^{10–13} Several groups report that *Ccr2* deficiency partially attenuates bleomycin-induced PF.^{11–13} However, deficiency of monocyte chemoattractant protein-1/CCL2, the major ligand of CCR2 and the major inducer of MM infiltration, does not attenuate fluorescein isothiocyanate—induced PF.⁵⁶ In addition, Liang et al¹⁰ reported that lung-specific overexpression of CCL2 in mice increases CCR2-dependent infiltration of macrophages into the bronchoalveolar lavage and attenuates bleomycin-induced PF and its mortality in a CCR2-dependent manner. Although in a different experimental setting, the finding of Liang et al is consistent with our results that CCR2-dependent MMs inhibit the development of IPF-like diffuse PF in the progression phase of silica-induced PF. These reports and our findings suggest that MMs may have variable roles in the regulation of PF in a phase- and a model-dependent manner.

The variable roles of MMs could be explained by the differences in fibrotic stimuli and the actions of innate immune signals. Differences in stimulus and exposure time lead to the development of different activation states in macrophages.^{57,58} Thus, because different fibrotic stimulants, such as bleomycin and silica/fluorescein isothiocyanate, have different targets and half-lives,⁵⁹ they may also lead to the differential activation of MMs. On the other hand, recent findings suggest the NLRP3/MyD88/IL-1 cascade exerts profibrotic effects in the bleomycin model,^{60,61} whereas it acts protectively against diffuse PF induction in silica model.⁶² Given the importance of the NLRP3/MyD88/IL-1 cascade in macrophage responses,^{63,64} these different outcomes may explain the different roles of MMs in the bleomycin-induced acute injury and fibrosis model and the silica-induced progressive model.

The variable roles of MMs could also be explained by the prevalence of MMs in the lung. Blood monocytes have a cell-to-cell, contact-dependent, suppressive effect against the contractility of fibroblasts *in vitro* when the ratio of monocytes and fibroblasts is >0.5:1 but not when it is <0.1:1.⁶⁵ It is noteworthy that the ratio of MMs and fibroblasts in the lung differed between the silica and bleomycin models in our experiments, where it was approximately 1:1 and approximately 0.1:1, respectively. Because non-AM

macrophages and myofibroblasts colocalized in silicotic nodules, the requirement for a certain ratio to be effective raises the possibility that MMs may not be able to exert protective role in PF progression if their absolute number is below a certain threshold.

We identified three genes that are also differentially expressed in the lung of IPF and fibrotic NSIP patients (*Mmp14* and *Thbs2* were up-regulated; *Fgfr4* was down-regulated). MMP-14 (also called membrane type 1 MMP) is essential for fibroblast invasion *in vitro*,⁶⁶ and heterozygous deletion of MMP14 expression attenuates cardiac fibrosis.⁵⁵ Because MMP14 activates various MMPs,⁶⁷ including profibrotic MMP2⁴⁴; turnover of ECM and invasion potential of fibroblasts may increase in silica-treated *Ccr2*^{-/-} → ColGFP lungs. This hypothesis could explain our observation that the absence of MMs leads to the development of diffuse PF without affecting total collagen amount. This idea is partly supported by our observation that some of the fibroblast activation marker genes that encode ECM components, such as *Spp1* and *Tnc*, were up-regulated by the absence of MMs. In addition, thrombospondin 2 promotes expression of α -SMA, fibronectin, and other fibroblast activation markers and worsens chronic renal fibrosis.⁵³ Fibroblast growth factor receptor 4 represses excessive α -SMA expression and elastogenesis of fibroblasts during lung development coordinating with fibroblast growth factor receptor 3,⁶⁸ adding to the protective role in CCl₄-induced resolvable liver fibrosis.⁵⁴ Modulation of *Thbs2* and *Fgfr4* expression by the absence of MMs might also support the above hypothesis.

Colocalization of MMs with myofibroblasts and hyperplastic AECs at the periphery of fibrotic nodules suggests that these cells interact functionally to regulate the progression of silicosis. This idea is supported by our observation that the IPF-associated gene expression signature in tissue cells of silica-treated lung manifests in the absence of MMs. On the basis of these findings, we propose that MMs in silica-treated lung contain a population of regulatory MMs that limit the spread of the fibrotic lesion by yet to be characterized mechanism(s). The concept of regulatory MMs may help explain why long-term exposure to silica particles causes nodular fibrotic lesions, whereas many other fibrogenic exposures (eg, asbestos and coal mine dusts) cause diffuse fibrotic lesions, which remains unresolved.⁶ In support of this hypothesis, a paucity of MMs also leads to disrupted organization of the granuloma in *Mycobacterium tuberculosis*-infected mouse lungs.⁶⁹ Further studies examining the responses of MMs in the context of PF may be helpful to clarify the molecular mechanism(s) that mediate pathologic diversity in this disease. The concept of regulatory MMs also draws attention to the potential adverse effects of MM-targeting PF therapies, including anti-CCL2 therapy. Global attenuation of MM recruitment to the lung may increase the disease activity of silicosis or other forms of PF.

One candidate lung macrophage subpopulation that may contain regulatory MMs is the lung counterpart of Ly6C^{lo} restorative macrophages, which have been proposed to play a

role in CCl₄-induced hepatic fibrosis and resolution.⁷⁰ These restorative macrophages differentiate from Ly6C^{hi} monocyte-derived macrophages, and selective depletion of restorative macrophages delays the resolution of CCl₄-induced hepatic fibrosis.⁷⁰ Of note, resolution is also delayed in *Ccr2*^{-/-} mice.⁷¹ In support of a regulatory role for monocyte-derived Ly6C^{lo} macrophages in the lung, Ly6C^{lo} monocyte numbers were decreased in silica-treated *Ccr2*^{-/-} → ColGFP chimeras compared with WT → ColGFP chimeras.

In the context of tumor immunity, lung monocytic myeloid-derived suppressor cells, which express the surface marker pattern CD11b⁺ Ly6G⁻ F4/80⁺ Gr-1^{int} Ly6C⁺, exert anti-inflammatory effect through production of IL-10 and arginase-1 and suppress production of profibrotic cytokine IL-13¹ from lung conventional dendritic cells.⁷² The markers of lung monocytic myeloid-derived suppressor cells are similar to that of MMs,^{37,73} and the accumulation of myeloid-derived suppressor cells is impaired in *MyD88*-deficient mice, in which diffuse silicosis is induced.⁶² Thus, MMs may regulate silica-induced PF through controlling the expression of IL-10, arginase-1, and IL-13. Further characterization of regulatory MMs and the regulatory mechanisms for their differentiation and function will pave the way for the fine-tuning of profibrotic and antifibrotic effectors of diffuse, progressive PF.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.07.013>.

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