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Inflammatory Macrophage Expansion in Pulmonary Hypertension Depends upon Mobilization of Blood-Borne Monocytes

Jonathan Florentin,* Emilie Coppin,* Sathish Babu Vasamsetti,* Jingsi Zhao,* Yi-Yin Tai,* Ying Tang,* Yingze Zhang,† Annie Watson,* John Sembrat,∗† Mauricio Rojas,∗§ Sara O. Vargas,§ Stephen Y. Chan,*† and Partha Dutta,*§,1

Pulmonary inflammation, which is characterized by the presence of perivascular macrophages, has been proposed as a key pathogenic driver of pulmonary hypertension (PH), a vascular disease with increasing global significance. However, the mechanisms of expansion of lung macrophages and the role of blood-borne monocytes in PH are poorly understood. Using multicolor flow cytometric analysis of blood in mouse and rat models of PH and patients with PH, an increase in blood monocytes was observed. In parallel, lung tissue displayed increased chemokine transcript expression, including those responsible for monocyte recruitment, such as Ccl2 and Cx3cl1, accompanied by an expansion of interstitial lung macrophages. These data indicate that blood monocytes are recruited to lung perivascular spaces and differentiate into inflammatory macrophages. Correspondingly, parabiosis between congenically different hypoxic mice demonstrated that most interstitial macrophages originated from blood monocytes. To define the actions of these cells in PH in vivo, we reduced blood monocyte numbers via genetic deficiency of cx3cr1 or cer2 in chronically hypoxic male mice and by pharmacologic inhibition of Cx3cl1 in monocrotaline-exposed rats. Both models exhibited decreased inflammatory blood monocytes, as well as interstitial macrophages, leading to a substantial decrease in arteriolar remodeling but with a less robust hemodynamic effect. This study defines a direct mechanism by which interstitial macrophages expand in PH. It also demonstrates a pathway for pulmonary vascular remodeling in PH that depends upon monocyte numbers via genetic deficiency of cx3cr1 or cer2 in chronically hypoxic male mice and by pharmacologic inhibition of Cx3cl1 in monocrotaline-exposed rats. Both models exhibited decreased inflammatory blood monocytes, as well as interstitial macrophages, leading to a substantial decrease in arteriolar remodeling but with a less robust hemodynamic effect. This study defines a direct mechanism by which interstitial macrophages expand in PH. It also demonstrates a pathway for pulmonary vascular remodeling in PH that depends upon interstitial macrophage-dependent inflammation yet is dissociated, at least in part, from hemodynamic consequences, thus offering guidance on future anti-inflammatory therapeutic strategies in this disease.

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Abbreviations used in this article: LV+S, left ventricle + septum; MHC-II, MH class II; MI, myocardial infarction; PAH, pulmonary arterial hypertension; PH, pulmonary hypertension; RV, right ventricle; RVSP, right ventricular systolic pressure; WT, wild-type.

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lung perivascular macrophages in PH has not been fully described. Recently, it was reported that activation and expansion of lung alveolar and interstitial macrophages are time and anatomic space dependent (21). Prior work suggested that expansion of lung macrophages in PH is dependent on circulating blood-borne progenitors (2). Moreover, a recent study demonstrated that TGF-β activation in the lungs by hematopoietic cell–derived thrombospondin-1 could trigger Schistosoma mansoni–induced PH (22). However, the precise dynamics, origin, and pathobiological roles of different lung macrophage subsets in PH have not been described, and, in particular, the exact identity of the blood-borne cells contributing to the lineage of lung macrophages in PH has not been defined.

Circulating monocytes represent a possible upstream progenitor of lung interstitial macrophages in PH, given their importance in contributing to the interstitial macrophage population at the steady-state (23). Prior lineage tracing-experiments have indicated that tissue-resident macrophages, including alveolar lung macrophages, are derived independently from yolk sac or fetal liver progenitors before birth (24). In general, hematopoietic cells, including macrophages, are produced by “primitive” and “definitive” hematopoiesis (25). During primitive hematopoiesis in the embryonic stage, yolk sac–derived progenitors give rise to primitive myeloid cells, including monocytes (26) and, later, tissue macrophages that can self-maintain in tissues by local proliferation (26). Monocytes are innate immune cells that specialize in phagocytosis, Ag presentation, and inflammation. In mice, monocytes fall into two main subsets: proinflammatory Ly6-6chigh CXCR1+ (27) and patrolling Ly-6chlow CX3CR1high (28). Humans have three distinct monocyte subsets: CD14high CD16low (inflammatory, classical), CD14high CD16high (intermediate, inflammatory), CD14low CD16low (patrolling, nonclassical) (29). In some inflammatory situations, classical monocytes have been shown to have proinflammatory features (30–32). Chemokine receptors, such as CX3CR1 and CCR2, help the egress of monocytes from the bone marrow (17, 28, 33–35). Once in the blood circulation, monocytes travel along a chemokine gradient and infiltrate into sites of injury. Deficiency in CCR2 expression results in decreased numbers of recruited monocytes at sites of inflammation (36).

In the current study, we aimed to determine the contribution of circulating monocytes to the expansion of perivascular macrophages, particularly interstitial macrophages, in PH and to define the role of circulating monocytes in PH pathogenesis. To do so, we used multicolor flow cytometric analysis, coupled with the availability of blood and lung tissues from multiple animal models and human patients of PH. Consequently, we delineated a direct mechanism by which interstitial macrophage expansion occurs in PH. By genetic and pharmacologic depletion of blood monocytes in rodent models of disease, we demonstrated that interstitial macrophages are derived from blood-borne monocytes and play a robust role in pulmonary vascular remodeling in PH, but they have a less prominent effect on hemodynamics. Thus, these results define the cellular origin of inflammatory macrophages in PH. They also demonstrate specific pathophenotypic actions of these cells in vivo and begin to delineate an inflammatory-mediated distinction between histologic and hemodynamic aspects of this disease.

**Materials and Methods**

**Human samples and cell storage**

PAH patient lung samples (n = 6) and control lung samples (n = 5) were collected at the University of Pittsburgh Medical Center and Boston Children’s Hospital. Lungs and peripheral blood from PAH patients and healthy donors were collected and processed as described below. A piece of lung was weighed, minced into small pieces, and digested in enzymatic mixture containing 450 U/ml Collagenase I, 125 U/ml Collagenase XI, 60 U/ml DNase I, and 60 U/ml hyaluronidase (Sigma-Aldrich) at 750 rpm for 1 h. Cells were passed through 40-μm cell strainer, washed in 10 ml of FACS buffer, and centrifuged (4°C, 370 × g, 7 min). Two to three milliliters of concentred EDTA-treated blood was collected and diluted twice. Leukocytes were separated from total blood by a Ficoll gradient. Briefly, the whole diluted blood was carefully added on top of the Ficoll. Then cells were centrifuged at 350 × g for 40 min at 4°C. The leukocyte ring was collected and washed into 10 ml of fresh DMEM and centrifuged at 350 × g for 10 min at 4°C. Lung and blood cells were counted using trypan blue (Cellgro; Mediatech), resuspended at 1 million per milliliter in freezing medium (70% DMEM, 20% FBS, and 10% DMSO v/v), aliquoted in cryovials, and stored in liquid nitrogen.

**Animals**

All animal experiments were conducted following National Institutes of Health guidelines under protocols approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. We chose to study the chronically hypoxic mouse as a first-line model of PH, because of its long-standing use in PH research and because of the well-established genetic tools and flow cytometric protocols in studying myeloid populations. Adult male C57BL/6 wild-type (WT), ccr2 Gfp/Gfp, ccr2-knockout, and ubiquitin-GFP mice (10–12 wk old) were obtained from The Jackson Laboratory and maintained under a standard light cycle (12 h light/dark). To induce PH, mice were put in an hypoxic chamber under normobaric 10% O2 for 3 wk. To track the cellular origin of interstitial macrophages, adult C57BL/6 WT and ubiquitin-GFP male mice were surgically joined from the hip to the shoulder for 3 wk. The blood chimera level was tested prior to putting the parabiosed mice in a hypoxic chamber for another 3 wk. Adult male Sprague-Dawley rats (8–12 wk old) were obtained from Charles River. To induce PH, rats were injected once with 60 mg/kg monocrotaline. Such monocrotaline-exposed rats tend to display pronounced proinflammatory features and have served for years as a long-standing and well-established model of experimental PH. Thus, this model served as an appropriate complement to the hypoxic mouse, because any molecular similarities found in both models would likely be broadly relevant across different subtypes of PH. To study the impact of monocyte recruitment into the lungs in the context of PH, rats were also injected daily with 10 μg of Cx3cl1 inhibitor for 3 wk (R&D Systems).

**Organ harvesting and flow cytometry**

Mice were euthanized and perfused thoroughly with 30 ml of ice-cold PBS through the left ventricle. The upper lobe of the right lungs was harvested, minced into small pieces, and digested in an enzymatic mixture containing 450 U/ml Collagenase I, 125 U/ml Collagenase XI, 60 U/ml DNase I, and 60 U/ml hyaluronidase (Sigma-Aldrich) at 750 rpm for 1 h at 37°C. Cells were passed through a 40-μm cell strainer, washed in 10 ml of FACS buffer, and centrifuged (4°C, 370 × g, 7 min). Peripheral blood was collected through retro-orbital bleeding. Erythrocytes were lysed using ACK lysis buffer for 3 min, and 3 ml of FACS buffer was added and centrifuged. Supernatant was carefully discarded, and the pellet was dissolved in FACS buffer. Total viable cell numbers were obtained from the above aliquots using trypan blue (Cellgro; Mediatech). Following the harvesting of single-cell suspensions, cells were washed with PBS containing 0.5% BSA. Leukocytes were washed and resuspended in FACS buffer. Total viable cell numbers were obtained from the above aliquots using trypan blue (Cellgro; Mediatech). To study the impact of monocyte recruitment into the lungs in the context of PH, rats were also injected daily with 10 μg of Cx3cl1 inhibitor for 3 wk (R&D Systems).
dry ice. For immunofluorescence staining, 29-μm sections were rehydrated with PBS and permeabilized with 0.1% (v/v) Triton X-100 for 1 h at room temperature. Following blockade with blocking solution (PBS + 2% BSA) for 1 h, sections were incubated with anti-Cd68 (clone EPR1344; Abcam) and anti-Sma–Cy3 (Millipore) for 1 h, washed with PBS + 0.5% BSA, and incubated with anti-mouse secondary Ab conjugated with FITC. The sections were counterstained with VECTASHIELD mounting medium with DAPI to visualize nuclei, and images were taken using a confocal laser scanning immunofluorescence microscope. Image analysis was done using ImageJ software (Fiji). Human lungs from control donors or from PH patients were processed in the same way as mouse lungs. Following blockade, sections were incubated with anti-Cd68 (clone EPR1344; Abcam), anti-Sma–Cy3 (Millipore), and anti-cd14 for 1 h. Rat lung sections were stained with anti-Cd68 (clone E1; Bio-Rad), anti-Sma–Cy3 (Millipore), and anti-cd31 (clone H3; Santa Cruz Biotechnology) for 1 h. Small pulmonary vessels (10 vessels per section) that were not associated with bronchial airways were selected for analysis (n > 5 animals per group). The intensity of staining was quantified using ImageJ software (Fiji). The degree of pulmonary arteriolar muscularization was assessed in paraffin-embedded lung sections stained for α-SMA by the calculation of the proportion of fully and partially muscularized peripheral (<100 mm in diameter) pulmonary arterioles among total peripheral pulmonary arterioles, as previously described (29). Medial thickness was also measured in α-Sma–stained vessels (<100 mm in diameter) using ImageJ software (Fiji) and expressed as arbitrary units. All measurements were performed blinded to condition.

Quantitative RT-PCR

Following collection, lungs were cut into small pieces and flash frozen in liquid nitrogen. Total mRNA was extracted using an RNeasy RNA Isolation Kit (Qiagen), and cdna was prepared from 100 ng of mrna with a High-Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative RT-PCR was performed using SYBR Green primers (IDT), and the results were expressed as Ct values normalized to the housekeeping gene β-actin (with the control set as 1).

Right heart catheterization and tissue harvest

On day 21 of hypoxia, right ventricular catheterization was performed, followed by tissue and blood collection. The mice were anesthetized with ketamine/xylazine and ventilated through a tracheal catheter. The abdominal and thoracic cavities were opened, and a 1-Fr pressure-volume catheter (Millar PVR-1035; Millar Instruments) was placed through the right ventricle (RV) apex to transduce the pressure. The heart was flushed with 10 ml of PBS and removed, followed by dissection and weighing of the RV and the left ventricle + septum (LV+S). Organs were then harvested for histological preparation or flow cytometric experiments or were flash frozen in liquid N2 for subsequent homogenization and extraction of RNA and/or protein.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance between groups was assessed using a two-tailed Student t test or ANOVA, depending on the data set. Results were considered statistically significant when p < 0.05.

Study approval

All animal experiments were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. All experimental procedures involving the use of human lung tissue included the relevant receipt of written informed consent and were approved by the Committee for Oversight of Research and Clinical Training Involving Decedents No. 101 at the University of Pittsburgh, as well as the Institutional Review Board of the University of Pittsburgh No. REN17020169/IRB0208180, and the Institutional Review Board at Boston Children’s Hospital. All experimental procedures involving the use of human peripheral blood included the relevant receipt of written informed consent and were approved by the Institutional Review Board of the University of Pittsburgh No. REN16070123/PR011070366 and No. REN17030011/IRB0306040. Ethical approval for this study and informed consent conformed to the standards of the Declaration of Helsinki.

Results

Interstitial macrophage and monocyte populations expand in the lungs of hypoxic mice, monocrotaline-injected rats, and PH patients

To quantify the alterations in specific subsets of lung and pulmonary vascular macrophages in PH, we studied multiple animal and human models of PH, including mice with hypoxia-induced PH, rats with monocrotaline-induced PH, and human PH patients. In the lungs of hypoxic mice with PH and normoxic mice (hemodynamics and remodeling score are shown in Supplemental Fig. 1A and 1B respectively), we enumerated alveolar and interstitial macrophages by multicolor flow cytometry (Supplemental Fig. 1C) at different time points of hypoxia exposure. In hypoxic mice, we found a gradual loss of alveolar macrophages and a significantly increased number of interstitial macrophages (Fig. 1A, 1B). Next, we investigated the mechanisms of the disappearance of alveolar macrophages. Flow cytometry analysis of the cell cycle status of alveolar macrophages did not reveal any change in proliferation (Supplemental Fig. 1D). We observed increased expression of cleaved caspase 3 in alveolar macrophages of hypoxic mice, indicating augmented apoptosis of these macrophages under hypoxic conditions (Fig. 1C, Supplemental Fig. 1E). To determine the mechanisms of increased apoptosis of alveolar macrophages in hypoxic mice, we assessed the levels of gm-csf, which is important for alveolar macrophage maintenance (37), and m-csf, which is necessary for tissue-resident macrophage survival in the lungs (38). The level of gm-csf was significantly decreased in hypoxic mice; however, m-csf expression was not altered in hypoxic mice compared with normoxic control (Fig. 1D).

In parallel, perivascular lung macrophages were observed in monocrotaline-exposed PH rats (hemodynamics shown in Supplemental Fig. 1F), a well-established model of PAH with significantly increased inflammation in the lungs (39). Because of the lack of well-characterized anti-rat Abs for flow cytometry, confocal imaging of lung sections of monocrotaline- versus PBS-injected rats was performed, and the expansion of CD68+ macrophages, specifically in the pulmonary arteriolar compartment, was confirmed (Fig. 1E, 1F). Finally, we quantified lung macrophages from human PH patients (clinical demographics and hemodynamics are shown in Supplemental Table I). Notably, alterations in lung macrophage subsets of PH patients were similar to those seen in hypoxic mice (Fig. 1G, 1H, Supplemental Fig. 1G).

Given the direct differentiation of macrophages from inflammatory monocytes in other contexts of inflammation (40), we also enumerated monocytes in the lungs of hypoxic mice (Supplemental Fig. 2A). We confirmed that the number and percentage of monocytes were gradually increased in the lungs of mice at different time points after hypoxia exposure (Fig. 2A, 2B). Similarly, we observed a significant increase in the number and percentage of nonclassical lung monocytes in PH patients (Fig. 2C, 2D, Supplemental Fig. 2B). Confocal imaging confirmed the accumulation of CD14+ CD68+ monocytes, as well as CD14+ CD68+ interstitial macrophages, in the arteriolar compartment and interstitium in the patients (control 1 ± 0.48, PH 3.8 ± 0.64) (Fig. 2E, 2F). Taken together, these data demonstrate a parallel expansion of monocytes and perivascular interstitial macrophages across multiple instances of PH lung tissue in rodents and humans. Additionally, we found decreased alveolar macrophage numbers in the lung of hypoxic mice and PH patients.

Inflammatory monocytes are increased in the blood of hypoxic PH mice and human PAH patients

Given the increase in macrophages and monocytes across diseased PH lungs, we also assessed blood-borne monocyte populations in hypoxic PH mice and human PH patients. In hypoxic mice, the percentage (Fig. 3A) and the number (Fig. 3B) of circulating monocytes, including proinflammatory Ly-6C+hig monocytes, increased at different time points after hypoxic exposure (41). We also quantified blood monocyte populations in human PH patients (demographics and hemodynamics are shown in Supplemental Table I). Consistent with the monocytosis in hypoxic mice, we
found that these patients displayed a higher number of circulating monocytes (control $3.9 \times 10^4 \pm 2.3 \times 10^4$, PH $3.2 \times 10^5 \pm 1.2 \times 10^5$) (Fig. 3C, 3D). We wanted to assess the possible discrepancies in monocyte number and frequency between PAH scleroderma and PAH idiopathic patients. No significant difference in monocyte number or frequency was observed (Supplemental Fig. 2C).
Notably, due to the scarcity of available human lung tissue and blood from human PH patients and the logistical difficulties in precisely timing appropriate sample procurement, it has been exceedingly challenging to analyze matched blood and lung tissue from the same PH patient, thus revealing only an indirect association between blood monocytosis and lung macrophage expansion in humans. Stemming from the tremendous generosity of a single PH patient who donated blood and lung tissue immediately after death, we enumerated monocyte and interstitial macrophage populations in blood and lungs. Consistent with our findings in rodents and humans, the increased frequency of nonclassical monocytes in the blood and lungs (Fig. 3E) correlated with increased monocytes and interstitial macrophages in the pulmonary vascular compartment (Fig. 3F). Thus, in aggregate, hypoxic PH mice and PH patients display an inflammatory monocytosis in blood and lung tissue that correlates with increased interstitial macrophages in the lungs and pulmonary vascular compartment.

Monocyte and macrophage expansion in PH lungs promotes a proinflammatory milieu

To determine the extent of an inflammatory environment promoted by monocyte and macrophage expansion, we quantified chemokine and cytokine production in the lungs of PH patients based on known inflammatory signaling in other contexts (summarized in Fig. 4A). The lungs of hypoxic mice displayed increased levels of chemokines and proinflammatory cytokines, such as Cx3c11, Ccl2, Il1b, Il6, Il18, and Tnfα (Fig. 4B) (28, 41). Lungs of PH patients exhibited a similar pattern of inflammatory gene expression (Fig. 4C). Additionally, we found increased levels of chemokines, such as CCL1, CCL2, CCL3, CCL4, and CX3CL1, in the lungs of PH patients (Fig. 4C, Supplemental Fig. 2D); these factors are known to induce migration of monocytes (42). To assess the change in the phenotype of circulating monocytes in PH, we sorted these cells from the blood of PH patients using FACS. Importantly, circulatory monocytes in PH patients displayed augmented expression of corresponding chemokine receptors, such as CCR1, CCR2, CCR5, and CX3CR1 (Fig. 4D, Supplemental Fig. 2E). Interestingly, the phenotype of blood monocytes isolated from PH patients mirrored the lung inflammatory microenvironment (Fig. 4D). To ascertain the progressive change in the phenotype of blood monocytes after hypoxia exposure, we sorted these cells from the blood of hypoxic mice. Monocytes sorted at days 3 and 12 of hypoxia exhibited a gradual increase in the expression of the chemokine receptors CCR2 and CX3CR1 (Supplemental Fig. 2F). Concomitantly, the levels of the proinflammatory cytokines Il1b, Il6, and Tnfα progressively increased in

![Figure 2](http://www.jimmunol.org/)

**Figure 2.** The number of inflammatory lung monocytes is elevated in hypoxic mice and PAH patients. C57BL/6 mice were placed in a 10% O2 hypoxic chamber (n = 4 normoxic controls and 6 hypoxic mice) for 3 wk to induce PH. (A) Flow cytometric plots show the proportion of Ly-6Chigh and Ly-6Clow monocytes in hypoxic and normoxic mouse lung samples. (B) Quantitation of Ly-6Chigh and Ly-6Clow monocytes at 21 d, and total monocytes at various time points after hypoxia initiation. (C) PAH patient lung samples (n = 6) or control (CTL) lung samples (n = 5) were collected. Flow cytometric plots show the proportion of nonclassical monocytes in the lung of CTL and PAH patients. (D) Quantitation of classical, intermediate, and nonclassical monocytes in CTL and PAH lung samples. (E) Confocal imaging of patient lung sections stained for CD68, SMA, and CD14. (F) Quantification of monocytes and macrophages in CTL and PAH lung samples. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.005.
sorted monocytes. These data indicate their active recruitment into the lungs, as well as their conversion from the noninflammatory to the proinflammatory phenotype. Next, we investigated the mechanisms of egress of bone marrow monocytes immediately after hypoxia exposure. Because bone marrow niche cells, such as endothelial cells, mesenchymal stem cells, and osteoblasts, secrete factors (e.g., ccl1, ccl2, and cx3cl1) that retain monocytes in the bone marrow at the steady-state (43), we sorted these niche cells and quantified these retention factors using quantitative RT-PCR. Our analysis demonstrated significant decreases in these retentions factors in the niche cells sorted from hypoxic mice compared with normoxic controls (Supplemental Fig. 2G). Collectively, these data demonstrate a proinflammatory monocyte and macrophage milieu in PH that is reflected in blood and lung tissue. More specifically, the elevation of chemokines in the lungs and the increased chemokine receptor expression in blood monocytes indicate that there are specific signals for the mobilization of monocytes from the blood to the lungs.

**Interstitial macrophages, but not alveolar macrophages, are derived from infiltrating blood monocytes in the lungs of hypoxic mice**

To directly test the hypothesis that inflammatory blood monocytes are the source of pulmonary perivascular interstitial macrophages in PH, we studied a model of parabiosis wherein the circulatory systems of two congenically distinct mice were joined surgically. Three weeks after surgical parabiosis, when the chimera levels reached ∼30% (Fig. 5A, Supplemental Fig. 3A), we placed the parabionts in a hypoxic chamber for 3 wk and quantified the chimera levels in monocytes and alveolar and interstitial macrophages in the lungs (Fig. 5B). Hemodynamic and histologic manifestations of hypoxia-induced PH were observed in both parabionts (Supplemental Fig. 3B). In that setting, the level of chimera for interstitial macrophages reached ∼70%, whereas the level of chimera for alveolar macrophages was only 13% (Fig. 5C), consistent with a report describing the embryonic origin of alveolar macrophages (37). Thus, these data demonstrate that...
more than two thirds of lung interstitial macrophages are derived from blood monocytes (Fig. 5C). Moreover, to explore the origin of interstitial macrophages located in the perivascular spaces, we performed immunofluorescence of lung sections of hypoxic mice. We observed the presence of parabiont-derived perivascular macrophages in the lungs of hypoxic mice (Fig. 5D). As a result, we conclude that, at least in the context of hypoxic PH, interstitial perivascular lung macrophages are derived directly from circulating blood monocytes.

Furthermore, these data indicate that resident interstitial macrophages are replaced by infiltrating monocyte-derived interstitial macrophages. To investigate the fate of resident interstitial macrophages in hypoxia, we needed to distinguish these two subsets of interstitial macrophages. Consistent with published reports (16, 23, 44), our parabiosis experiments revealed that host-derived (resident) interstitial macrophages expressed higher levels of MHC class II (MHC-II) compared with parabiont (monocyte)-derived interstitial macrophages (Supplemental Fig. 3C). In fact, ∼4% of parabiont-derived interstitial macrophages were MHC-II+, whereas >96% of host-derived interstitial macrophages were MHC-II+ (Supplemental Fig. 3D). Enumeration of these two subsets of interstitial macrophages by flow cytometry at different time points after hypoxia initiation revealed that the numbers of both cell populations diminished significantly at days 3 and 12 of hypoxia. However, only the monocyte-derived interstitial macrophage numbers increased significantly at day 21 compared with normoxic controls (Fig. 5E). To check alterations in the resident interstitial macrophage phenotype, we sorted resident interstitial macrophages and observed decreased RNA levels of ccl2 and cx3cl1 (chemokines involved in monocyte recruitment) and il1b, il6, and tnf-a (inflammatory cytokines) (Supplemental Fig. 3E).

CX3CR1 or CCR2 deficiency in rodents results in reduced pulmonary inflammation and diminished remodeling of lung vasculature

Numerous studies have proposed a role for monocytes in various diseases (27, 41); however, the role of monocytes in the exacerbation of inflammation and tissue remodeling in PH is not known. Monocytes express high levels of the chemokine receptor Ccr2 (45). This receptor is essential for the egress of monocytes from the bone marrow and their recruitment at sites of inflammation (33, 43). Monocytes also express high levels of Cx3cr1, which is involved in their retention and survival (46, 47).

Thus, cx3cr1- and ccr2-deficient mice and WT mice were exposed to chronic hypoxia and used to explore the role of monocytes in hypoxic PH. First, we enumerated monocyte subsets and interstitial macrophages in the lungs (Fig. 6A, 6B) and monocyte subsets in the blood (Fig. 6C) of hypoxic mice. In the blood, cx3cr1- and ccr2-deficient hypoxic mice exhibited a decreased number of inflammatory myeloid cells compared with hypoxic WT mice (Fig. 6C). A similar reduction in the number of monocytes and interstitial macrophages was observed in the lungs

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**FIGURE 4.** Hypoxic mice and patients with PH had increased levels of cytokines, chemokines, and chemokine receptors. C57BL/6 mice were placed in a 10% O2 hypoxic chamber (n = 5 per group) for 3 wk to induce PH. (A) Schematic diagram shows that lung chemokines trigger the active recruitment of blood monocytes to the lungs. This scheme was drawn using pictures from Servier Medical Art under a Creative Commons Attribution 3.0 Unported License. (B) Quantitation of cx3cl1, ccl2, il1b, il6, il18, and tnfα mRNA expression in the lungs of hypoxic and normoxic mice. Blood and lung samples from PAH patients (n = 9) and healthy donors (n = 7) were collected. (C) Quantitation of CX3CL1, CCL2, IL1B, IL6, IL18, and TNFA expression in the lungs of PH patients and healthy controls. (D) Quantitation of chemokine receptors and pro-inflammatory cytokine mRNA expression (CX3CR1, CCR2, IL1B, IL6, IL18, and TNFA) by monocytes sorted from the blood of PH patients and healthy controls. Data are mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
of hypoxic \textit{cx3cr1}- and \textit{ccr2}-deficient mice compared with WT mice (Fig. 6B). The frequency of cells followed the same pattern across all of the conditions in blood (Supplemental Fig. 3F) and lungs (Supplemental Fig. 3G) of hypoxic mice versus normoxic mice. The expression of genes encoding inflammatory cytokines and chemokines in \textit{cx3cr1}- and \textit{ccr2}-deficient mice diminished significantly compared with hypoxic WT mice (Fig. 6D). Via in situ staining of vascular smooth muscle, pulmonary vascular remodeling, as quantified by arteriolar wall thickness, in \textit{cx3cr1}- or \textit{ccr2}-deficient mice was significantly decreased compared with WT mice under hypoxic conditions (Fig. 6E). Conversely, \textit{cx3cr1} and \textit{ccr2} deficiency did not significantly alter the hemodynamic manifestation of PH, as reflected by right ventricular systolic pressure (RVSP) (Fig. 6F).

**FIGURE 5.** Parabiosis between hypoxic mice demonstrated the monocytic origin of interstitial macrophages in the lungs. (A) A CD45.1 C57BL/6 mouse and a CD45.2 GFP C57BL/6 mouse were surgically joined. Three weeks later, they were placed in a hypoxic chamber for 3 wk. (B) Flow cytometric plots show the chimera levels in blood monocytes and lung macrophages of hypoxic mice. (C) Percentage of CD45.2\textsuperscript{+} parabiont-derived cells in blood monocytes, interstitial macrophages, and alveolar macrophages in CD45.1 hypoxic mice. (D) Confocal imaging of lung sections of the CD45.1\textsuperscript{+} parabions shows GFP\textsuperscript{+} interstitial perivascular macrophages. The red arrows show CD68\textsuperscript{+} macrophages and the white arrows show GFP\textsuperscript{+} monocytes and monocyte-derived macrophages. (E) Flow cytometric enumeration of total, monocyte-derived, and resident interstitial macrophages in the lungs of hypoxic mice and normoxic controls. \textit{n} = 4 mice per group. Data are mean ± SEM. *\textit{p} < 0.05, **\textit{p} < 0.01, ***\textit{p} < 0.001.
neutralizing Ab for Cx3cl1 or IgG isotype control (48). By echocardiography, such treatment did not affect left ventricular function or structure (Supplemental Fig. 3H); however, Cx3cl1 neutralization significantly reduced monocyte and macrophage expansion in the lungs (Fig. 7A). As with genetic deficiency in mice, Cx3cl1 neutralization reduced pulmonary arteriolar remodelling, as assessed by in situ quantitation of α-SMA (Fig. 7B).

However, similar to cx3cr1−/− mice, Cx3cl1 neutralization resulted in only subtle trends toward improvement of RVSP (Fig. 7C) and right ventricular remodeling (RV mass/LV+S mass) (Fig. 7C). In aggregate, these findings demonstrate that Cx3cl1 and Ccr2 are crucial mediators of recruitment of lung-infiltrating monocytes in PH and that lung perivascular macrophages derived from blood monocytes actively participate in pulmonary vascular remodeling across various models of PH. Importantly, in contrast to the more robust effects on pulmonary vascular remodeling, hemodynamic manifestations of PH are more subtly altered by such inflammatory mediators.

Discussion
Collectively, via the study of animal and human examples of PH, our data reveal the mechanisms of interstitial lung macrophage expansion in this complex disease. We have found that elevated levels of chemokines in the lungs and increased chemokine receptor expression in blood monocytes initiate mobilization of inflammatory monocytes to the lungs in PH. These monocytes differentiate into interstitial perivascular macrophages, which secrete proinflammatory cytokines and contribute to vascular remodeling (Fig. 7D). These newly generated monocyte-derived interstitial macrophages replace resident interstitial macrophages. Importantly, via genetic and pharmacologic means in multiple rodent models of PH, we report that strategies to inhibit

![FIGURE 6. CCR2- and CX3CR1-deficient mice exhibited attenuated lung remodeling. ccr2−/− and cx3cr1−/− mice were put in a hypoxic chamber for 3 wk. n = 5 normoxic WT mice, 5 hypoxic WT mice, 5 hypoxic cx3cr1−/− mice, and 5 ccr2−/− hypoxic mice. (A) Flow plots show the proportions of interstitial macrophages and monocytes in WT, ccr2−/−, and cx3cr1−/− mice. (B) Bar graphs of the number of total monocytes, Ly-6Chigh monocytes, and Ly-6Clow monocytes in the lungs. (C) Bar graphs of the number of interstitial macrophages, Ly-6Chigh monocytes, and Ly-6Clow monocytes in the blood of WT, cx3cr1−/−, and ccr2−/− mice. (D) Quantitation of mRNA levels of chemokines and proinflammatory cytokines in the lungs. (E) Confocal images of lung sections stained for CD31 and SMA. (F) Bar graphs of RVSP. Data are mean ± SEM. *p < 0.05, ***p < 0.001, ****p < 0.0001.](http://www.jimmunol.org/)}
FIGURE 7. Cx3cl1 inhibitor limited the recruitment of monocytes in monocrotaline-injected rats. Adult male Sprague-Dawley rats were injected once with monocrotaline to induce PH and with Cx3cl1 inhibitor or isotype Ab for 3 wk. (A) Confocal imaging of lung sections stained for CD68 and SMA (left panels). Quantification of lung perivascular macrophages (right panel). The arrows show CD68+ macrophages. (B) Confocal images of lung sections stained for SMA show lung vasculature remodeling (left panels). The remodeling score was quantified (right panel). (C) Bar graphs of RVSP (left panel) and RV mass/LV+S mass ratio (right panel) in isotype control– and Cx3cl1 inhibitor–injected rats. n = 5 mice per group. (D) Schematic diagram depicting monocyte recruitment to the lungs and expansion of interstitial macrophages in PH. This scheme was drawn using pictures from Servier Medical Art under a Creative Commons Attribution 3.0 Unported License. CX3CR1+ CCR2+ monocytes egress from the bone marrow into the blood stream. The production of chemokines, such as CX3CL1 and CCL2, in the lungs and increased expression of the receptors of these chemokines on circulatory monocytes mediate their recruitment into the lungs in the context of PH. These newly recruited monocytes differentiate into interstitial perivascular macrophages. Data are mean ± SEM. **p < 0.01, ****p < 0.001.
CX3C1/CX3cr1 signaling and interstitial macrophage expansion potently reduce pulmonary vascular remodeling and inflammation, but with less substantial hemodynamic amelioration. Such a distinct macrophage-dependent control of primarily histologic, rather than hemodynamic, parameters of this disease offers insight into the fundamental biology of these inflammatory cells in the pulmonary vasculature, as well as guidance about how to translate antimacrophage therapies into clinical PH practice.

A seminal finding of our work includes direct lineage tracing of hematopoietic-derived blood-borne monocytes to interstitial lung macrophages in PH. In other inflammatory diseases, it has been reported that monocytes are generated from hematopoietic progenitor cells in the bone marrow (27) and spleen (49). In PH as well, there has been an increasing focus on the importance of bone marrow cells in pathogenesis (20, 50, 51). For example, hematopoietic cell–derived thrombospondin-1 was found to activate lung TGF-β as a seminal event driving PH due to schistosomiasis infection (22). Additionally, prior work demonstrated that BMPR2 deficiency can drive macrophage expansion in this disease (1), thus linking the established genetic connection of BMPR2 haploinsufficiency to bone marrow–derived cells, rather than simply pulmonary vascular cell types or cardiomyocytes. A more recent study reported that reconstitution with bmpr2-deficient bone marrow in WT mice led to aggravated lung remodeling and inflammation in PH (20), more directly implicating bone marrow mobilization in this process. In light of our current findings linking those hematopoietic processes to the interstitial macrophage via the blood-borne monocyte, it remains to be seen at what stage of myeloid development BMPR2 signaling exerts its primary influence. Furthermore, given the known temporal dependence of human myeloid development from early in development to adulthood, it is possible that myeloid hematopoiesis may be a key determining event in the variable penetrance and genetic anticipation reported in BMPR2-specific cases of hereditary PAH (52). Our prior work has also demonstrated that, in contexts beyond PH, such as myocardial infarction (MI), monocyte expansion is triggered through extramedullary myelopoiesis and accelerates atherosclerosis (42). Similarly, it is possible that myeloid mobilization in PH depends upon other extramedullary sources. Given the known association of PH development with splenectomy (53–55), future work will likely link the specific actions of the spleen in PH pathogenesis, potentially via myeloid development. Finally, the upstream triggers that signal to myeloid cells, especially monocytes, for expansion in PH are not known. Different mechanisms of hematopoietic progenitor differentiation into myeloid cells, such as sympathetic activation (56, 57) and danger-associated molecular patterns (58), have been proposed in other disease contexts. These or other processes that control myeloid mobilization could factor substantially into the underlying known genetic or environmental triggers of PH, which have been poorly characterized at the molecular level.

In addition to directly identifying blood monocytes as the source of interstitial macrophages, we observed an increased mobilization of monocyte subsets in the blood and lung in PH. Yet, the exact role of each of these monocyte populations in PH has yet to be fully described. Monocyte recruitment plays an important role in various sterile inflammatory conditions, such as atherosclerosis (59, 60). During the progression of this disease, Ly-6Chigh and Ly-6Clow monocytes are recruited to atherosclerotic plaques. CCR2, CCR5, and CX3CR1 assist in Ly-6Chigh monocyte recruitment into the lesions (61–63), and this monocyte subset is known to differentiate into inflammatory macrophages (64). Ly-6Clow monocytes have phagocytic and proangiogenic functions. Although their direct role in disease progression is poorly described (65), their behavior in PH may mimic that described recently in coronary artery disease. MI triggers an acute inflammatory response, with an early peak in circulating inflammatory classical CD14+ CD16+ monocytes, followed by an increase in circulating nonclassical CD14dim CD16+ monocytes (66). The same pattern of monocyte response has been observed in a mouse model of MI (67). This temporal difference in the recruitment of monocyte subsets to the infarcted myocardium allows for the removal of dead cardiac myocytes in the first phase and triggers the resolution of inflammation and tissue healing in the second phase (68). To define the recruitment pattern and functions of monocyte subsets in PH, future studies will be required for specific depletion of each monocyte subset in vivo (e.g., starting with nonclassical monocytes via deletion of the Nr4a1 superenhancer subdomain) (69).

Prior to our work, particularly in humans, the dynamics of the distinct subsets of lung macrophages in PH, primarily alveolar and interstitial, had not been defined. Although the importance of interstitial macrophages has been described in PH (21, 70), the contribution of alveolar macrophages to its pathogenesis is unclear. In contrast to the progressive expansion of interstitial macrophages, we found that alveolar macrophages were decreased in rodent and human cases of PH. To our knowledge, alveolar macrophages have not been quantified in depth in human PH, but other groups have reported their increase (71) and activation (72) in chronically hypoxic mice. These discrepancies may be explained by a recent report delineating a spatiotemporal program of, at times, divergent alveolar and interstitial macrophage activation in hypoxic lungs of mice (21). It remains unclear whether such complex cellular programming of alveolar macrophages exists in the development of human PH as well. Nonetheless, the functional consequences of the decrease in alveolar macrophages observed in our study are not fully understood. Because alveolar macrophages are known to participate in local immune homeostasis (14, 15) and offer protection from viral infections, such as influenza (73), it would be intriguing to determine whether such changes may predispose to the development of certain types of PH that rely upon infectious (i.e., HIV infection) or autoimmune (i.e., connective tissue disease) etiologies.

Although substantial evidence is mounting regarding the pathogenic importance of interstitial macrophage expansion and inflammation in PH, prior studies have failed to define the exact downstream parameters through which these cells exert their influence on the pulmonary vasculature. Pulmonary vascular hemodynamics and histopathology are thought to actively contribute directly to disease pathogenesis, and these indices can be simultaneously affected by macrophage disruption (22, 71), particularly during severe end-stage disease. In contrast, our results demonstrate that, in multiple separate examples of rodent PH, pulmonary vascular remodeling, rather than hemodynamic dysfunction, is under more robust macrophage-dependent control. Namely, we found that inhibition of Cx3cr1/Ccr2 signaling and the consequent decrease in interstitial macrophage expansion potently reduced pulmonary vascular remodeling and inflammation, with a less robust hemodynamic amelioration, in two rodent models of PH. Consistent with our findings, previous work has suggested that macrophage depletion via pharmacologic means in hypoxic mice can improve vascular remodeling without mention of any hemodynamic improvements (2). Furthermore, more recent evidence suggests other contexts in which inflammatory injury may display such dissociation (74). The molecular etiologies of this dissociation remain unclear, but this observation appears to emphasize the distinct immunologic cues controlling vascular hyperproliferation rather than vasomotor tone. Given this observation, coupled with the distinct spatiotemporal cues that govern macrophage...
expansion in PH (21), it may also be possible that dissociation is more prominent when comparing early- versus late-stage PH or when comparing specific anatomic compartments of the pulmonary vascular tree. Future delineation of this hypothesis will necessitate an ability to follow simultaneous tissue remodeling with hemodynamics over the course of disease, technology that is not available for human study but potentially could be envisioned in rodents when coupling molecular imaging with hemodynamic telemetry. This dissociation also has fundamental implications regarding the immunologic nature of vascular lesions in PH. For example, pulmonary vascular remodeling in the absence of increased pulmonary arterial pressures was reversible when induced by inhalation of Stachybotrys chartarum spores (75). Such reversibility, if applicable to the macrophage-dependent vascular alterations reported in this article, gives credence to the potential efficacy of anti-inflammatory agents for this disease. In contrast, these data suggest that any anti-inflammatory agents tested in humans should be combined with existing vasodilators or another drug with complementary vasomotor efficacy to attain truly durable histologic and hemodynamic improvement.

Our findings also serve as a platform for future translational development in myeloid-based diagnostics and therapeutics. Diagnostically, identification of inflammatory monocytes, especially nonclassical monocytes, could aid in the prognostic evaluation of PH patients and serve as a method to identify patients appropriate for emerging immunologic therapy. Whether these cells could be quantified as a measure of timing or severity of disease will depend on future studies defining a timeframe of activation and expansion of proinflammatory monocytes and their recruitment to the lungs. Consequent therapeutic targeting of these cells or molecules, such as Cx3CL1, that control their expansion could decrease lung inflammation and pathogenic tissue remodeling in PH patients. More sophisticated drug development would rely on a better understanding of the monocyte-to-lung perivascular macrophage transition, because targeting the precursor monocyte subset before infiltration may be a more effective strategy for completely preventing tissue inflammation. Moreover, because hemodynamics alone may not fully reflect the potency of a macrophage-depleting agent, our data support the notion of using an independent index of disease progression to evaluate the efficacy of any anti-inflammatory agent in future clinical trials.

Our study reveals that chemokine receptors, such as CX3CR1 and CCR2, are important in the recruitment of monocytes in the setting of PH. Other groups also highlighted the relevance of these two chemokines in the context of PH. Amsellem et al. (71) showed that lung vasculature remodeling, as well as hemodynamics, in ccl2-deficient hypoxic mice was comparable to that in WT hypoxic mice, which contrasts with our observation of improved pulmonary remodeling in hypoxic ccr2-deficient mice. This discrepancy may be explained by the known compensatory mechanisms of other CCR2 ligands, such as CCL7. Additionally, this group did not find a decrease in the number of monocytes in cx3cr1-deficient mice (71), despite well-established literature reporting the crucial role of CX3CRI in monocyte recruitment and survival (76, 77). Interestingly, Yu et al. (78) found increased pulmonary artery muscularization in ccr2−/− mice that was due to dysregulation of Nox7 signaling. Yet, in line with our findings, another group reported that ccr2 deficiency alleviates angiotensin II–induced vascular remodeling by suppressing blood monocyte–macrophage transition, may hold great promise in this disease.

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Disclosures

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