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Adventitial Fibroblasts Induce a Distinct Proinflammatory/Profibrotic Macrophage Phenotype in Pulmonary Hypertension

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Macrophage accumulation is not only a characteristic hallmark but is also a critical component of pulmonary artery remodeling associated with pulmonary hypertension (PH). However, the cellular and molecular mechanisms that drive vascular macrophage activation and their functional phenotype remain poorly defined. Using multiple levels of in vivo (bovine and rat models of hypoxia-induced PH, together with human tissue samples) and in vitro (primary mouse, rat, and bovine macrophages, human monocytes, and primary human and bovine fibroblasts) approaches, we observed that adventitial fibroblasts derived from hypertensive pulmonary arteries (bovine and human) regulate macrophage activation. These fibroblasts activate macrophages through paracrine IL-6 and STAT3, HIF1, and C/EBPβ signaling to drive expression of genes previously implicated in chronic inflammation, tissue remodeling, and PH. This distinct fibroblast-activated macrophage phenotype was independent of IL-4/IL-13–STAT6 and TLR–MyD88 signaling. We found that genetic STAT3 haploinsufficiency in macrophages attenuated macrophage activation, complete STAT3 deficiency increased macrophage activation through compensatory upregulation of STAT1 signaling, and deficiency in C/EBPβ or HIF1α attenuated fibroblast-driven macrophage activation. These findings challenge the current paradigm of IL-4/IL-13–STAT6–mediated alternative macrophage activation as the sole driver of vascular remodeling in PH, and uncover a cross-talk between adventitial fibroblasts and macrophages in which paracrine IL-6–activated STAT3, HIF1α, and C/EBPβ signaling are critical for macrophage activation and polarization. Thus, targeting IL-6 signaling in macrophages by completely inhibiting C/EBPβ or HIF1α or by partially inhibiting STAT3 may hold therapeutic value for treatment of PH and other inflammatory conditions characterized by increased IL-6 and absent IL-4/IL-13 signaling.

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K.C.E.K. and K.R.S. conceived the study, delineated the hypotheses, designed the study, analyzed and interpreted data, and prepared the manuscript; S.R.R., H.K.E., A.L.A., M.G.F., M.L., S.S.P., S.C.P., R.S., M.A.N., M.A.F., and J.M.P. acquired data, performed experiments, and provided reagents and technical assistance; and B.B.G., R.M.T., J.E.F. and R.J.S. were substantially involved in the preparation of the manuscript.

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Abbreviations used in this article: AAM, alternative activation of macrophages; BMDM, bone marrow–derived macrophage; CM, conditioned medium (conditioned media); CO-Fib, fibroblast isolated from human or bovine control; CT, threshold cycle; dPA, distal pulmonary artery; iPAH, idiopathic pulmonary arterial hypertension; MCT, monocrotaline; PA, pulmonary artery; PH, pulmonary arterial hypertension; PB, barometric pressure; PBGD, porphobilinogen deaminase; PH-Pbs, adventitial fibroblast isolated from calves with PH or humans with iPAH; siRNA, small interfering RNA; WT, wild-type.

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fibroblasts (termed “PH-Fibs” in this article: adventitial fibroblast isolated from calves with PH or humans with iPAH) with a hyper-proliferative, apoptosis-resistant, and proinflammatory phenotype (the last defined by increased generation of IL-6, IL-1β, CCL2/MCP1, CCL12/SDF1, VCAM1, osteopontin) that are involved in macrophage recruitment, retention, and activation (10, 12–17). We further demonstrated that the proinflammatory phenotype of PH-Fibs remains persistent ex vivo over numerous passages in culture and is regulated through epigenetic mechanisms involving alterations in histone deacetylase activity and microRNA expression (16, 17). In line with this paradigm, we found that PH-Fibs recruit, retain, and activate naïve macrophages (17). However, neither the exact phenotype induced nor the signaling pathways involved in the polarization of macrophages in sterile forms of PH have been identified.

Tissue remodeling and fibrotic-angiogenic responses in chronic inflammatory conditions, including PH, have long been associated with alternative activation of macrophages (AAM) (12, 18–20). The current paradigm holds that IL-4/IL-13 signaling and STAT6—with alternative activation of macrophages (AAM) (12, 18–20)—is pivotal (27–34). Further, recent studies strongly support a spectrum of inflammatory conditions, including PH, have long been associated with chronic hypoxia, are characterized by reduced IL-4/IL-13 signaling and STAT6-regulated AAM, and are important in the vascular remodeling process in some forms of PH and in other fibrotic/tissue-remodeling conditions in which Th2 responses are prominent (21–26). However, recent reports documented that STAT3 signaling also can play a key role in chronic inflammatory diseases in which tissue remodeling is prominent (27–34). Further, recent studies strongly support a spectrum model of macrophage activation in which macrophage phenotype is dependent on specific signals present in the inflammatory microenvironment, rather than simply classic “M1” versus “M2” (35).

IL-6 has been identified as a major activator of STAT3 signaling in macrophages, and IL-6−/−STAT3 signaling promotes an activation profile distinct from that in IL-4−/−IL-13−/−AAM (36–38). IL-6 signaling recently was shown to drive fibrosis and unresolved inflammation in the peritoneum in the absence of IL-4, IL-13, and TGFβ (39). Further, many reports documented increased serum and lung concentrations of IL-6 in patients with PH (31, 40, 41). A pathogenic role for IL-6 in certain forms of PH is supported by observations that IL-6−/−knockout mice are protected from PH (32), whereas IL-6−transgenic mice develop spontaneous PH and vascular remodeling (30).

Thus, we sought to examine the hypothesis that certain forms of PH, including those associated with chronic hypoxia, are characterized by the presence of a fibroblast-activated macrophage that exhibits a unique phenotype dependent on IL-6 and STAT3 signaling and distinct from the canonical alternatively (IL-4/IL-13) activated macrophage phenotype. Our approach was to examine the phenotype of macrophages both in vivo using models of severe hypoxia-induced PH and patients with PAH and in vitro by examining the effects of fibroblasts derived from animals and humans with PH on macrophage phenotype signaling. In this article, we show that fibroblast-derived paracrine IL-6 and macrophage STAT3 in conjunction with HIF1α and C/EBPβ signaling, and not IL-4/IL-13−STAT6, are pivotal in mediating activation of macrophages by adventitial fibroblasts from the pulmonary hypertensive vessel. Further, these fibroblast-activated macrophages express genes previously implicated in the pathogenesis of PH and associated with chronic nonresolving and fibrosing tissue responses. Thus, fibroblast-mediated macrophage activation and STAT3, HIF1α, and C/EBPβ signaling downstream of IL-6 may be important elements underlying certain forms of PH.

Materials and Methods

Immunohistological/immunofluorescence staining and laser-capture microdissection

Frozen OCT-embedded sections of human lung specimens from control subjects (n = 4) and patients with idiopathic PAH (iPAH; n = 5) were provided by Dr. Barbara Meyrick (Transplant Procurement Center, Vanderbilt University, Nashville, TN) via the Pulmonary Hypertension Breakthrough Initiative funded by the Cardiovascular Medical Research Education Fund. Additional paraffin-embedded, human tissue (n = 5 donor controls and n = 5 iPAH) was provided by S.S.P. Patient descriptions for this tissue were provided previously (16). Immunostaining was performed according to standard protocols.

For laser-capture microdissection, human lung tissue specimens from subjects with iPAH (n = 8) or donor controls (n = 7), obtained during lung transplantation, were used. Tissue specimens were the same ones used in our previous study (16). Intrapulmonary arteries (50–100 μm diameter) were microdissected under optical control using the Laser microdissection device LMD6000 (Leica, Wetzlar, Germany) and collected in Eppendorf tubes containing RNA lysis buffer. Total mRNA was extracted from human microdissected vessels using RNeasy Micro Kits (Qiagen, Hilden, Germany). RT-PCR was performed using the iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany). Real-time PCR was performed using qSYBR Green Supermix, according to the manufacturer’s instructions (Bio-Rad), and with an Mx3000P (Stratagene, Heidelberg, Germany). Intron-spanning primers were designed using sequence information from the National Center for Biotechnology Information database. Threshold cycle (Ct) values were normalized to the endogenous control (porphobilinogen deaminase [PBGD]).

The study protocol for tissue donation was approved by the Ethics Committee (Ethik Kommission am Fachbereich Humanmedizin der Justus Liebig Universita¨t Giessen) of the University Hospital Giessen (Giessen, Germany), in accordance with National Law and with Good Clinical Practice/International Conference on Harmonization guidelines. Written informed consent was obtained from each patient or his/her next of kin (AZ 31/93).

Animals

One-day-old calves were exposed to hypobaric hypoxia (barometric pressure [PB] = 445 mm Hg) for 2 wk (n = 7); age-matched controls (n = 7) were kept at ambient altitude (PB = 640 mm Hg). Wistar-Kyoto rats (8 wk old) were exposed to hypobaric hypoxia (PB = 380 mm Hg) for 4 wk (n = 9); age-matched controls (n = 9) were kept at ambient altitude. Monocrotaline treatment of rats (experimental group, n = 12, controls, n = 12) were performed as described elsewhere (17). Standard veterinary care was according to institutional guidelines in compliance with Institutional Animal Care and Use Committee-approved protocols.

Bone marrow–derived macrophages

Mouse bone marrow–derived macrophages (BMDMs) were generated from Il4ra−/−IL13−/−, IL4ra−/−Stat6−/−, Myd88−/−, C/EBPβ−/−, Stat3−/−, Tie2cre, Stat3fl/fl, Tie2cre, and Stat3−/−;Tie2cre mice (a gift from Dr. P. Murray, St. Jude Children’s Research Hospital, Memphis, TN; Hif1α−/−LysMcre were bred in-house. C57BL/6 and BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were on a C57BL/6 background, with the exception of Il4ra−/− mice (BALB/c). Rat BMDMs were generated from Sprague-Dawley rats. Bovine BMDMs were isolated from healthy calves and grown in complete Iscove’s DMEM with 20% FBS and human M-CSF (50 ng/ml). When bovine BMDM cultures were initiated from frozen bone marrow cells, live cells were obtained using negative column selection (Dead Cell Removal Kit; Miltenyi Biotec, Auburn, CA) and incubated for 24 h with human (due to limited availability of bovine-specific) cytokines, stem cell factor (50 ng/ml), IL-3 (20 ng/ml), and IL-6 (50 ng/ml) before exposure to M-CSF. All BMDMs were cultured in murine or bovine rM-CSF (100 ng/ml) for 7 d prior to use.

Adventitial fibroblasts

Adventitial fibroblasts from distal PAs (dPAs) of calves (control or with PH) were isolated by explant culture, as described (16, 17). Consistent with our previous observations (17), fibroblasts from chronically hypoxic hypertensive calves (PH-Fibs) were significantly smaller in size than were those from controls (CO-Fibs) and proliferated at markedly higher rates, as shown previously (16). The PH-Fibs used for study exhibited modest α-smooth muscle actin immunoreactivity, were negative for smooth muscle myosin, and were HSP-47–positive and vimentin-positive. At the time of study, these cells lacked expression of CD34, CD14, and CD68. Human adventitial fibroblasts were a kind gift from Dr. N.W. Morrell (University of Cambridge, London, U.K.). HFL-1 human fetal lung fibroblasts were purchased from American Type Culture Collection (Manassas, VA). Experiments were performed on cells at passages 4–10. Conditioned medium (CM) was collected from confluent fibroblast cul-
tures and used to treat BMDMs or the THP-1 monocyte cell line (American Type Culture Collection).

For Transwell (Corning, Tewksbury, MA) experiments, distal pulmonary arteries (1–3 mm outer diameter) were isolated from either control or pulmonary hypertensive calf lungs and left whole or were separated into medial and adventitial layers prior to dissecting into 3-mm² pieces for incubation in the upper chamber, above naive mouse BMDMs, for 16 h. Adjacent tissue pieces were used for whole and separated tissue. Three tissue pieces from each animal were used per experiment.

All cytokines were purchased from BD Biosciences (San Jose, CA), Cell Signaling (Danvers, MA), Miltenyi Biotec (San Diego, CA), or Kingfisher Biotech (St Paul, MN). Ultrapure LPS was purchased from Sigma (St. Louis, MO).

**Immunoblotting**

Cells were lysed in M-PER Mammalian Protein Extract Reagent containing protease and phosphatase inhibitors (Thermo-Fisher, Waltham, MA). Protein lysates were separated by 4–15% gradient SDS-PAGE in Tris-HCl buffer and transferred to nitrocellulose membranes. Membranes were blocked in 3% milk in TBS containing 0.05% Tween. Abs included mouse monoclonal anti-ARG1 Ab (1:2000, BD Biosciences); rabbit polyclonal anti-total STAT1, p-STAT1, total STAT3, p-STAT3, total STAT6, p-STAT6 (1:1000; all from Cell Signaling Technology); and mouse monoclonal anti-GAPDH (1:1000, Santa Cruz, Dallas, TX).

**IL-6 protein detection**

Human IL-6 was quantified in cell culture supernatant using a human IL-6 multiplex sandwich ELISA with electrochemiluminescence detection on the MSD Platform, as per the manufacturer’s protocol, on a SECTOR Imager 2400A (both from MESO SCALE DISCOVERY, Rockville, MD). Quantitative detection range for IL-6 in our analysis was 0.6 to 9871.4 pg/ml. Bovine IL-6 was quantified using an ELISA development kit (Kingfisher Biotech), according to the manufacturer’s instructions and standard ELISA procedures. Bovine rIL-6 was used as a standard.

**Quantitative RT-PCR**

RNA was isolated using TRizol reagent (Invitrogen, Grand Island, NY) or the Qiagen RNeasy Kit (Valencia, CA), according to the manufacturer’s instructions. First-strand cDNA synthesis was performed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative RT-PCR was performed using TaqMan probes and reagents (Applied Biosystems, Grand Island, NY), according to the manufacturer’s instructions. Gene expression was calculated after normalization to Hprt1 using the ∆∆ Ct method.

**Statistical analysis**

GraphPad Prism software was used to determine significance. Student t test was used to compare two groups. One-way ANOVA and Bonferroni correction for multiple comparisons were used to compare more than two groups.

**Results**

**Expression of the STAT3-regulated proteins CD163 and CD206 on macrophages is observed in vivo in humans and animals with PH**

We first sought to define the adventitial macrophage phenotype in situ in humans, as well as in several animal models of PH. Macrophage CD163 and CD206 expression was reported in various chronic inflammatory/fibrotic tissue responses (42, 43). CD163 is a STAT3-regulated gene (44), and CD206 can be activated through either IL-6–STAT3 or IL-4–STAT6 (Supplemental Fig. 1A). Thus, we chose to examine in situ expression of CD163 and CD206 on adventitial macrophages. Consistent with previous reports, we detected pronounced adventitial accumulation of macrophages (defined as cells positive for the pan-macrophage marker CD68) in the PA of patients with iPAH, calves with hypoxia-induced PH, and rats with hypoxia- or monocrotaline (MCT)-induced PH (Supplemental Fig. 1B, 1C) (3–6). Furthermore, the PAs of humans, calves, and rats with PH revealed markedly increased numbers of CD163+ cells, which were specifically localized to the adventitia (Fig. 1A, Supplemental Fig. 1C). To attribute CD163 and CD206 staining to macrophages, the PA adventitia from calves with hypoxia-induced PH was stained with the typical macrophage marker MHC class II. Approximately 90% of MHC II⁺ cells coexpressed the macrophage-specific marker CD68, as detected by double staining and confocal microscopy. In addition, the majority (75%) of CD163⁺ and CD206⁺ cells also coexpressed MHC class II (Supplemental Fig. 1D). CD163⁺ cells in the PA adventitia from calves with hypoxia-induced PH also expressed the canonical STAT3-regulated protein SOCS3 (Supplemental Fig. 1E). Moreover, we detected increased adventitial protein expression of p-STAT3 in PAs from patients with iPAH (Fig. 1B), which was paralleled by the detection of increased mRNA (obtained by laser-capture microdissection) of the canonical STAT3-regulated genes SOCS3 and IL4Ra (Fig. 1C), together with increased protein and mRNA expression of the STAT3-activating cytokine IL-6 (Fig. 1B, 1C). Notably, we did not detect mRNA for the STAT6-activating cytokines IL4 or IL13 in PAs from calves, humans, or rats with PH (data not shown).

These results demonstrate that, in various species and multiple models of PH, macrophages consistently accumulate within the PA adventitia, the site of active vascular remodeling, and stereotypically express surface markers associated with a proremodeling phenotype, consistent with activation through IL-6–STAT3 signaling.

**Adventitial fibroblasts mediate macrophage activation**

We next sought to determine the cell type(s) and mechanism(s) responsible for macrophage activation toward this phenotype, hypothesizing that the PA adventitia was the vascular compartment most capable of activating these macrophages. Exposure of naive primary BMDMs in vitro to intact whole PA explants from calves with hypoxia-induced PH significantly increased transcription of Cd163, Cd206, Il4ra, and Socs3 (Fig. 2A). Removal of adventitia from the PA explant resulted in a marked decrease in mRNA expression in BMDMs. However, the adventitia alone induced an identical response to that observed with whole PA explants (Fig. 2A). These findings were confirmed in a separate experiment using bovine BMDMs (data not shown). These results demonstrate that during vascular remodeling in PH, cells within the remodeled PA adventitia produce soluble factors that can activate primary naive macrophages toward a gene expression phenotype that is identical to the one observed on adventitial macrophages in situ.

To test whether proinflammatory PA adventitial fibroblasts (16, 17) were the cellular source of soluble factors inducing this macrophage phenotype, we exposed naive BMDMs to CM generated by ex vivo–cultured human (from patients with iPAH) or bovine (from calves with hypoxic PH) adventitial fibroblasts (PH-Fibs). Remarkably, CM from both bovine and human PH-Fibs significantly increased transcription of Cd163, Cd206, Socs3, and Il4ra in naive macrophages (mouse and bovine) and THP-1 monocytes in comparison with that induced by CM from CO-Fibs. Additionally, CM from bovine PH-Fibs induced similar gene expression in mouse and rat (data not shown) naive macrophages, indicating that this signaling mechanism is effective across species (Fig. 2B, Supplemental Fig. 2). Thus, within the remodeled PA adventitia of animals and humans with PH, activated fibroblasts provide the soluble factors that induce this macrophage phenotype.

Fibroblast-activated adventitial macrophages do not exhibit a canonical alternatively activated phenotype

Macrophage expression of CD163 and CD206 in fibrosis and tissue remodeling is thought to reflect a canonical AAM phenotype (42, 45, 46), as defined by IL-4/IL-13/IL-4Rα/STAT6-dependent signaling (47). Therefore, we sought to determine whether the functional programming of adventitial macrophages by the PA adventitia and PA adventitial fibroblasts involved IL-4/IL-13/IL-4Rα/STAT6 signaling.

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Accordingly, we examined transcription of canonical STAT6-regulated genes whose combined expression in macrophages has traditionally been viewed to reflect the AAM phenotype (Arg1 encoding Arginase1, Chi3l3 encoding chitinase-3-like protein 3/YM1, and Retnla encoding resistin-like molecule Relmα/FIZZ1) (19, 47). We discovered that soluble factors released by intact whole PA explants or by isolated PA adventitia (both containing fibroblasts) derived from calves with chronic hypoxia–induced PH significantly increased transcription of Arg1, but not of Chi3l3 and Retnla, in mouse macrophages. PA explants from which the adventitia was removed (absence of fibroblasts) failed to induce significant expression of any gene (Fig. 3A). In addition, CM produced by PH-Fibs replicated this gene expression pattern in mouse, rat, and bovine BMDMs (Fig. 3B, 3C, Supplemental Fig. 3A). The absence of Chi3l3 and Retnla mRNA induction in mouse macrophages was not due to an inability to respond to IL-4 and also was not limited to a particular time point (Supplemental Fig. 3B).

Furthermore, compared with CO-Fibs, neither human nor bovine PH-Fibs had increased transcription for IL4 or IL13 and neither human nor bovine PH-Fib CM contained detectable amounts of IL-4 or IL-13 protein (data not shown). In addition, neither bovine nor human PH-Fib CM induced transcription of another canonical STAT6-regulated AAM gene, TGM2/Tgm2 (data not shown). Moreover, we discovered that PH-Fib CM induced Arginase1 protein expression in mouse, rat, and bovine macrophages in the absence of STAT6 phosphorylation but in the presence of STAT3 phosphorylation (Supplemental Fig. 3C–E). Additionally, Arg1 mRNA expression in PH-Fib CM–activated macrophages did not require paracrine or autocrine IL4/IL-13 signaling, because expression of Arg1 was similar in macrophages from IL-4/IL-13 double-deficient mice (unresponsive to autocrine IL-4/IL-13), Il4Ra<sup>−/−</sup> mice (unresponsive to paracrine and autocrine IL-4 and IL-13), and STAT6-deficient mice (unable to upregulate Arg1 in response to IL-4/IL-13) in response to bovine PH-Fib CM (Fig. 3D). Because Arg1 also can be induced in response to TLR–MyD88 signaling (48) and C/EBPb signaling (36, 48, 49), we exposed naive mouse macrophages from wild-type (WT), Myd88<sup>−/−</sup>, and C/ebpb<sup>−/−</sup> mice to bovine PH-Fib CM (Fig. 3E). Because Arg1 expression was similar in WT and MyD88<sup>−/−</sup> macrophages (Fig. 3E), C/ebpb<sup>−/−</sup> macrophages showed dramatically reduced Arg1 expression (Fig. 3F). These results demonstrate that PH-Fib–activated macrophages were not alternatively activated through the IL-4/IL-13–STAT6 pathway and that neither the IL-4/IL-13/IL-4R/STAT6 pathway nor the MyD88 (microbe- and danger-associated signals and IL1) pathway underlies Arg1 expression; instead, STAT3 and C/EBPb play a critical role.

PH-Fibs activate macrophages through paracrine IL-6

Because IL-6 activates STAT3 signaling in macrophages, which, in turn, can activate C/EBPb (36, 37), we next examined whether

![FIGURE 1.](http://www.jimmunol.org/)

In various forms of PH, adventitial macrophages express CD163. (A) Accumulation of CD163<sup>+</sup> (human, calf) and ED2<sup>+</sup> (CD163 analog in rats) cells (red fluorescence) in human, calf, and rat PAs. Note localization to the PA adventitia (Adv, shown by arrows). All panels show DAPI counterstain (cell nuclei, blue) and autofluorescence of vascular elastic lamellae (green) defining borders of vascular media. Cryosections of intralobar PAs were immunostained. Scale bars, 100 μm. (B) Immunostaining for p-STAT3 (brown) and IL-6 (pink) in formalin-fixed tissue from patients with iPAH, and donor(s) as controls. Note adventitial staining for p-STAT3 and IL-6 in PA from patients with iPAH (arrowheads). Images are representative of eight patients and five donors. Scale bars, 20 μm. (C) Gene expression of canonical macrophage STAT3-regulated genes IL4Ra and SOCS3, as well as the STAT3 inducer IL6, in laser-capture microdissected PA tissue from humans with iPAH and controls (Donor; both n = 8), expressed as ΔCt values normalized to expression of PBGD. *p < 0.05, paired two-tailed Student t test. Control human donors or normoxic animals; iPAH, idiopathic pulmonary arterial hypertension; M, media of PA; MCT, MCT-induced PH.
Mature mRNA levels are presented as mean $\pm$ SEM of PCR triplicates, where $\times$ indicates a significant difference from basal expression. *p < 0.05, one-way ANOVA.

**FIGURE 2.** Adventitial fibroblasts activate macrophages. (A) Transwell experiment depicting mRNA expression in mouse BMDMs exposed to soluble factors generated by dPA explants from calves with chronic hypoxia-induced PH. dPAs were isolated, and as depicted in the diagram, an intact piece of PA tissue (intact dPA), a PA tissue piece from which the adventitia had been removed (dPA w/o adv.), and the removed adventitia piece itself (dPA adventitia) were incubated in the upper chamber of a 0.4-μm Transwell in the presence of naive mouse BMDMs (lower chamber) for 16 h prior to RNA isolation and quantitative RT-PCR. Displayed is the fold induction (normalized to basal expression) of a representative PCR triplicate (average $\pm$ SEM) from one of two calves. Three dPA segments were tested from each animal. *p < 0.05, one-way ANOVA. (B) Gene expression in BMDMs or monocytes exposed to CM from dPA adventitial fibroblasts (PH-Fibs or CO-Fibs). CM from ex vivo–cultured bovine PH-Fibs (PH-Fib CM), and not from controls (CO-Fib CM), promotes gene expression of Cd163 (upper left panels) and C2d06 (lower left panels) in naive mouse and bovine BMDMs. CM from bovine and human PH-Fibs induce expression of C2d163 (upper right panels) and C2d06 (lower right panels) in naive human THP-1 monocytes after 16 h of exposure. Relative mRNA levels are presented as mean $\pm$ SEM of PCR triplicates after normalization to Hprt1 expression and relative to gene expression in untreated macrophages/monocytes (basal) and are representative of at least three PH-Fib and CO-Fib populations isolated from at least three animals/patients and using BMDMs from at least three animals. *p < 0.05, unpaired two-tailed Student t test.

Paracrine, fibroblast-derived IL-6 could be responsible for the macrophage activation. Increased IL-6 and p-STAT3 protein expression in the PAs from patients (Fig. 1B), along with evidence of STAT3 signaling in adventitial macrophages (Supplemental Fig. 1E [SOCS3 staining], Supplemental Fig. 4), supported the idea that IL-6 was the most likely paracrine cytokine to activate STAT3 signaling in fibroblast-activated macrophages. We found that adventitial fibroblasts from calves with PH generated IL-6 protein and IL6 mRNA (Fig. 4A). Small interfering RNA (siRNA) knockdown of IL6 expression in bovine PH-Fibs led to a reduction in IL-6 protein in the CM (Fig. 4B), which resulted in significant attenuation of the CM-induced transcriptional upregulation of STAT3-regulated genes in naive mouse macrophages (Fig. 4C). In addition, expression of these genes was not reduced in IL-6–deficient macrophages in response to PH-Fib CM (Fig. 4D), ruling out any potential contribution of autocrine IL-6 signaling to the activation of STAT3 signaling in fibroblast-activated macrophages.

**PIM1 and NFATC2 signaling is promoted in fibroblast-activated macrophages**

We next tested whether this IL-6–STAT3–regulated fibroblast-activated macrophage phenotype would also include expression of additional functional STAT3-regulated genes that have previously been associated with the pathogenesis of PH, such as Pim1, and Nfatc2 (33, 34). Gene expression analysis using laser-capture microdissection of PAs from patients with iPAH revealed upregulation of mRNA for PIM1 and NFATC2 (Fig. 5A). In addition, intact whole PA and PA adventitia from PH calves induced transcription of Pim1 and Nfatc2 in naive mouse macrophages (Fig. 5B). Moreover, bovine PH-Fib CM induced Pim1 and Nfatc2 expression in naive bovine (Fig. 5C), mouse (Fig. 5D), and rat (Fig. 5E) macrophages. Human PH-Fib CM also was capable of inducing transcription of PIM1, but not NFATC2, in human monocytes (data not shown). PH-Fib CM–induced expression of Pim1 and Nfatc2 was dependent on paracrine IL-6 but not autocrine IL-6 (Fig. 5F, 5G). Finally, transcriptional induction of Pim1, Nfatc2, Soxs3, and Il4ra mRNA in mouse macrophages activated by bovine PH-Fib CM was independent of STAT6 or MyD88 (experiments performed in Stat6$^{-/-}$ and MyD88$^{-/-}$ BMDMs) (data not shown).

**PH-Fib–activated macrophages demonstrate upregulated Hif1a and Vegfa mRNA**

HIF1 can be induced through STAT3 (50). Gene expression analysis of PAs from patients with iPAH revealed upregulation of HIF1A mRNA (Fig. 6A). Mouse macrophages exposed to whole intact PA and PA adventitia from calves with hypoxia-induced PH showed increased Hif1a mRNA expression (Fig. 6B). Bovine PH-Fib CM also induced transcription of Hif1a in naive mouse, rat, and...
bovine macrophages (Fig. 6C–E). Human PH-Fib CM also induced HIF1A mRNA in naive human monocytes (Fig. 6F). Notably, in mouse macrophages, this response was independent of autocrine IL-6, STAT6, and MyD88 (experiments performed in Il62/2, Stat62/2, and MyD882/2 BMDMs), as well as hypoxia (experiments conducted under normoxic conditions) (data not shown).

HIF1 regulates expression of VEGFA, which has been implicated in the pathogenesis of PH. Mouse macrophages exposed to whole intact PA and PA adventitia from calves with hypoxia-induced PH showed increased Vegfa mRNA expression (Fig. 7A). Bovine PH-Fib CM also induced transcription of Vegfa in naive mouse, rat, and bovine macrophages (Fig. 7B–D). Human PH-Fib CM also induced VEGFA mRNA in naive human monocytes (Fig. 7E).

STAT3, HIF1α, and C/EBPβ are critical regulators of PH-Fib–mediated macrophage activation

The above results suggested that STAT3, C/EBPβ, and HIF1 are central regulators of the fibroblast-activated and IL-6–activated macrophage phenotype. Therefore, we examined their role in fibroblast and IL-6–mediated macrophage activation by using a genetic approach. We first examined the role of STAT3 and used BMDMs from mice with conditional alleles of Stat3 (Stat3fl/fl [complete knockout] and Stat3fl/+ [haplodeficiency, incomplete knockout]) crossed onto the Tie2cre background [mice lacking Stat3 globally are embryo lethal (51)]. As expected, BMDMs with haplodeficiency in STAT3 (from Stat3fl/+; Tie2Cre mice) displayed attenuated activation, as reflected by ∼50% reduction in gene expression of STAT3 target genes in response to PH-Fib CM and bovine rIL-6 (Fig. 8A) and mouse rIL-6 (data not shown). Unexpectedly, expression of STAT3 target genes was not attenuated in BMDMs with complete absence of STAT3; instead, it was increased in response to bovine PH-Fib CM (Fig. 8A). As reported previously, IL-6 stimulates STAT1 signaling in murine fibroblasts with complete genetic absence of STAT3 (52). Thus, we determined STAT1 and STAT3 signaling in WT BMDMs, in response to either IL-6 or PH-Fib CM, and found that STAT1 was weakly activated.
animals of each genotype. exposed for 16 h to bovine PH-Fib CM. One representative experiment with CM from one of three PH-Fib populations was tested on BMDMs from three

**FIGURE 4.** PH-Fibs activate macrophages through paracrine IL-6. (A) IL-6 protein amounts (left panel) in CM from two bovine PH-Fib populations; mean ± SEM of technical triplicates of individual biological samples (two CO-Fibs and two PH-Fibs populations) are shown. *p < 0.05 versus CO-Fib CM. Student t test. Relative *IL6* mRNA (right panel) in bovine CO-Fibs (normalized to 1, n = 5) and in PH-Fibs (n = 4). *p < 0.05 versus CO-Fibs. Student t test. (B) siRNA-mediated knock down of *IL6* transcription in bovine CO-Fibs and PH-Fibs (left panel) and IL-6 protein amounts (ELISA assay, right panel) in CM from untreated (basal), scrambled (Scr), and IL-6 siRNA–treated PH-Fibs (n = 3 each) tested in triplicate; mean ± SEM is shown from analysis of triplicate samples and is representative of two separate experiments. *p < 0.05. (C) siRNA-mediated suppression of *IL6* gene transcription in bovine PH-Fibs limits the ability of CM to induce transcription of STAT3-regulated genes in WT mouse BMDMs. Gene expression is normalized to expression of *Hprt1* and relative to that in macrophages exposed to CM from CO-Fibs treated with control siRNA. Data are mean ± SEM from triplicate determinations and are representative of two separate experiments. *p < 0.05. (D) Expression of STAT3-regulated genes in WT and *Il6*−/− BMDMs exposed for 16 h to bovine PH-Fib CM. One representative experiment with CM from one of three PH-Fib populations was tested on BMDMs from three animals of each genotype.

phosphorylated and, as expected, STAT3 was strongly phosphorylated, demonstrating that IL-6R can initiate both STAT3 and STAT1 signaling (Supplemental Fig. 4A). Consistent with this finding and the previously reported data in mouse *Stat3*−/− fibroblasts, we found that, in BMDMs with complete genetic absence of *Stat3*, both IL-6 and PH-Fib CM induced increased and prolonged STAT1 phosphorylation in the absence of STAT3 phosphorylation (Supplemental Fig. 4A). Moreover, PH-Fib CM induced significantly higher transcript levels of the canonical STAT1-regulated genes *Ip10* and *Irf1* in BMDMs with complete STAT3 deficiency compared with WT macrophages (Fig. 8B). In addition, in BMDMs with complete STAT3 deficiency, expression of *Ip10* and *Irf1* mRNA was increased in response to both IL-6 and IFN-γ (Fig. 8C), demonstrating that, in the absence of STAT3, upregulation of STAT1 signaling was not restricted to activation by IL-6R. Moreover, we found that the anti-inflammatory properties of IL-10 in suppressing LPS-mediated induction of IL-1β were conserved in STAT3-haplodeficient BMDMs, whereas these properties were abrogated in BMDMs with complete STAT3 deficiency (Fig. 8D). These results indicated that STAT3 plays a critical role in the fibroblast-activated macrophage and that incomplete inhibition attenuates macrophage activation by IL-6 and PH-Fib CM and conserves responsiveness to anti-inflammatory IL-10; however, complete deletion of STAT3 promotes macrophage activation in response to IL-6 through increased STAT1 signaling and abrogates responsiveness to anti-inflammatory IL-10.

Based on the attenuated *Arg1* expression found in PH-Fib CM–activated C/ebpβ-deficient macrophages (Fig. 3F), we next examined the role of C/EBPβ in this STAT3-activated macrophage phenotype. *Cebpb* mRNA was robustly expressed in mouse, rat, and bovine macrophages after exposure to bovine and human PH-Fib CM (Supplemental Fig. 4B). Importantly, PH-Fib–mediated expression of STAT3-regulated genes was significantly attenuated in macrophages with genetic deletion of *Cebpb* (Fig. 8E).

Finally, we determined the role of HIF1 signaling in IL-6–mediated STAT3-regulated gene expression. For this purpose, we used WT BMDMs (derived from *Hif1α*+/+: LysMcre) and *Hif1α*−/− BMDMs (derived from *Hif1α*Δm; LysMcre mice) and measured gene expression of the canonical STAT3-regulated gene *Arg1* in response to IL-6. We found that IL-6–induced expression of *Arg1* was significantly increased in the presence of HIF1α stabilization with dimethyloxalogsilic, 2 μM, demonstrating that HIF1α can directly promote STAT3-regulated gene expression. Genetic absence of HIF1α significantly reduced IL-6–induced *Arg1* expression in both the presence and absence of HIF1α stabilization (Fig. 8F), demonstrating a critical role for HIF1α in regulating IL-6–mediated *Arg1* expression.

**Discussion**

Our studies provide evidence that, in multiple animal models and humans with PH/IPAH, macrophages accumulate specifically within the PA adventitia (a site of active vascular remodeling) (12);
these adventitial macrophages stereotypically express CD163 and CD206; the adventitial compartment is responsible for macrophage activation to this phenotype; adventitial fibroblasts are the responsible adventitial cell that mediates macrophage activation toward a previously unrecognized and distinct alternative activation phenotype; the mechanism of macrophage activation involves paracrine fibroblast-derived IL-6 and macrophage STAT3, C/EBPβ, and HIF1α signaling in the absence of IL-4/IL-13–STAT6, as well as TLR–MyD88; and targeting of C/EBPβ and HIF1α and attenuation of, but not annihilation of, STAT3, reduces macrophage activation. Based on these findings, we propose that fibroblast-mediated activation of macrophages through IL-6–STAT3 signaling toward a proremodeling phenotype (42, 43, 45, 46) is a stereotypic response pattern in vascular remodeling associated with various forms of PH. Furthermore, cooperative signaling among STAT3, HIF1α, and C/EBPβ in response to IL-6 shapes a distinct macrophage phenotype.

Considering the reported pivotal role of macrophages in PA vascular remodeling in PH (8, 24, 53), our study suggests that adventitial macrophages expressing CD163/CD206 through STAT3 signaling are critical regulators of the vascular remodeling process in PH. To our knowledge, our study is the first to show that, in vascular remodeling associated with PH, adventitial macrophages express CD163 and CD206 and that this phenotype can be induced by soluble factors generated by adventitial fibroblasts. Being that, to our knowledge, our study is the first to associate this macrophage phenotype with vascular remodeling and considering that studies examining the role of this macrophage...
phenotype functionally in fibrosing diseases are rare, we can only speculate with regard to its specific role in vascular remodeling. Christmann et al. (23) showed that PBMCs from patients with scleroderma-PH express higher levels of CD206 than do those from patients with scleroderma without PH and healthy controls; they correlated CD206 expression with mean PA pressures and mortality. Nakayama et al. (54) found that, of patients with scleroderma and elevated serum soluble CD163 levels, 70% had an elevated right ventricular systolic pressure on echocardiography. Nakayama et al. (54) found that, of patients with scleroderma without PH and healthy controls; they correlated CD206 expression with mean PA pressures and mortality. Nakayama et al. (54) found that, of patients with scleroderma and elevated serum soluble CD163 levels, 70% had an elevated right ventricular systolic pressure on echocardiography. Nakayama et al. (54) found that, of patients with scleroderma without PH and healthy controls; they correlated CD206 expression with mean PA pressures and mortality. Nakayama et al. (54) found that, of patients with scleroderma and elevated serum soluble CD163 levels, 70% had an elevated right ventricular systolic pressure on echocardiography. Nakayama et al. (54) found that, of patients with scleroderma without PH and healthy controls; they correlated CD206 expression with mean PA pressures and mortality. Nakayama et al. (54) found that, of patients with scleroderma and elevated serum soluble CD163 levels, 70% had an elevated right ventricular systolic pressure on echocardiography. Nakayama et al. (54) found that, of patients with scleroderma without PH and healthy controls; they correlated CD206 expression with mean PA pressures and mortality. Nakayama et al. (54) found that, of patients with scleroderma and elevated serum soluble CD163 levels, 70% had an elevated right ventricular systolic pressure on echocardiography. Nakayama et al. (54) found that, of patients with scleroderma without PH and healthy controls; they correlated CD206 expression with mean PA pressures and mortality. Nakayama et al. (54) found that, of patients with scleroderma and elevated serum soluble CD163 levels, 70% had an elevated right ventricular systolic pressure on echocardiography. Nakayama et al. (54) found that, of patients with scleroderma without PH and healthy controls; they correlated CD206 expression with mean PA pressures and mortality. Nakayama et al. (54) found that, of patients with scleroderma and elevated serum soluble CD163 levels, 70% had an elevated right ventricular systolic pressure on echocardiography. Nakayama et al. (54) found that, of patients with scleroderma without PH and healthy controls; they correlated CD206 expression with mean PA pressures and mortality. Nakayama et al. (54) found that, of patients with scleroderma and elevated serum soluble CD163 levels, 70% had an elevated right ventricular systolic pressure on echocardiography. Nakayama et al. (54) found that, of patients with scleroderma without PH and healthy controls; they correlated CD206 expression with mean PA pressures and mortality. Nakayama et al. (54) found that, of patients with scleroderma and elevated serum soluble CD163 levels, 70% had an elevated right ventricular systolic pressure on echocardiography. Nakayama et al. (54) found that, of patients with scleroderma without PH and healthy controls; they correlated CD206 expression with mean PA pressures and mortality. Nakayama et al. (54) found that, of patients with scleroderma and elevated serum soluble CD163 levels, 70% had an elevated right ventricular systolic pressure on echocardiography. Nakayama et al. (54) found that, of patients with scleroderma without PH and healthy controls; they correlated CD206 expression with mean PA pressures and mortality. Nakayama et al. (54) found that, of patients with scleroderma and elevated serum soluble CD163 levels, 70% had an elevated right ventricular systolic pressure on echocardiography. Nakayama et al. (54) found that, of patients with scleroderma without PH and healthy controls; they correlated CD206 expression with mean PA pressures and mortality. Nakayama et al. (54) found that, of patients with scleroderma and elevated serum soluble CD163 levels, 70% had an elevated right ventricular systolic pressure on echocardiography.
obtained 16 h after stimulation. *Human CO-Fib CM. One representative experiment with CM from CO-Fibs and PH-Fibs isolated from at least three patients/controls is shown. Data were protected from PH (30, 32).

Inflammatory vascular remodeling, whereas mice genetically deficient in IL-6 display severe hypoxia-induced PH accompanied by inflammatory gene expression. These are representative of three experiments with CM from three CO-Fib and three PH-Fib cell populations on BMDMs from three animals. **VEGFA gene expression in human THP-1 monocytes exposed to human PH-Fib CM compared with those exposed to human CO-Fib CM. One representative experiment with CM from CO-Fibs and PH-Fibs isolated from at least three patients/controls is shown. Data were obtained 16 h after stimulation. *p < 0.05, ANOVA.

Recent studies reported that src-induced STAT3–PIM1–NFACTc2 signaling enhances proliferation and resistance to apoptosis in smooth muscle cells during PH-associated vascular remodeling, although the role of IL-6 was not addressed (33, 34). In this study, we found that, consistent with the known role of IL-6 in activating STAT3 in macrophages (37), STAT3 signals in PH can be regulated through IL-6 and that PIM1 and NFACTc2 expression in macrophages can be mediated by fibroblast-derived IL-6. The incomplete attenuation of Pim1 and Nfatc2 expression in macrophages in response to CM from siRNA–IL-6–treated PH-Fibs raises the possibility that intracellular signaling pathways through src contribute to IL-6–induced STAT3 signaling in macrophages and that enhanced proliferation and apoptosis resistance in the vessel wall also are controlled by fibroblast-derived IL-6 in any IL-6–responsive cell, including fibroblasts, macrophages, endothelial cells, and smooth muscle cells. We (12) and other investigators (9) proposed that intercellular signaling pathways originating in the PA adventitia may be essential in promoting medial and intimal changes in the vascular remodeling process. The observation that expression of IL-6, STAT3, and IL-4Rx was not limited to the PA adventitia supports IL-6–STAT3 signaling as a mechanism of cross-talk between the different compartments of the vascular wall.

PH-Fib CM–activated macrophages also expressed Hif1a and Vegfa, factors critically involved in PH tissue remodeling and vascularization (61, 62). This finding led us to hypothesize that HIF1α plays a critical role in IL-6–STAT3–mediated gene expression. Thus, we tested the isolated effect of IL-6 on expression of Arginase1 in the presence or absence of HIF1α. We made the novel observation that HIF1α plays a critical role in regulating expression of Arginase1 in response to IL-6. Thus, HIF1α may fine-tune IL-6–STAT3 signaling–regulated gene expression in macrophages, as well as other cells within the vessel wall that are responsive to IL-6. Thus, because HIFα is downstream of STAT3, IL-6–induced STAT3 might serve as a central hub for the coordination of gene expression patterns that are involved in inflammation (Hif1a, Arg1, Socs3), remodeling (Arg1), metabolism (Hif1a), proliferation and apoptosis (Pim1, Nfatc2), and vascularization (Vegfa). Furthermore, this hypothesis is supported by the recent observation that transcription of Vegf can be cooperatively regulated by STAT3 and HIF1α (63, 64). Moreover, analysis of the STAT3 promoter revealed HIF1α binding sites (J.M. Poth, unpublished observations); thus, a feed-forward signaling loop between HIF1α and STAT3 might drive persistent activation of STAT3-regulated genes in response to IL-6.

Therefore, we hypothesized that genetic deficiency of STAT3 would attenuate the IL-6– and PH-Fib–activated macrophage phenotype, which would be consistent with the proposed role of STAT3 inhibition as a therapeutic approach in treating PH (33, 34, 65). We observed that activation of BMDMs with genetic haplodeficiency in Stat3 in response to PH-Fib CM and rIL-6 was attenuated, consistent with a critical role for STAT3 in promoting this macrophage phenotype. However, we also made the important observation that complete genetic blockade of STAT3 signaling in IL-6– and PH-Fib–stimulated macrophages resulted in increased activation (increase in expression of STAT3-regulated genes), which was associated with increased and prolonged STAT1.
phosphorylation and increased expression of canonical STAT1-regulated proinflammatory genes. These findings prompted the question of how STAT3-regulated genes can be upregulated in the absence of STAT3 protein. A previous study demonstrated that genetic deletion of STAT3 in IL-6–stimulated mouse fibroblasts also resulted in increased and prolonged p-STAT1 signaling and upregulation of STAT1 and STAT3 target genes (Arginase1) (52). These findings, together with our results, demonstrate that, in the complete absence of STAT3, IL-6 signaling enhances expression of both STAT1- and STAT3-regulated genes in fibroblasts and macrophages. Similarly, a previous study demonstrated that, in the genetic absence of STAT1, macrophages express increased amounts of STAT3-regulated genes (66). These studies, together with our findings, highlight that STAT1 and STAT3 can cross-regulate gene expression. In addition, our data also demonstrate that anti-inflammatory IL-10 signaling is abrogated in the complete absence of STAT3 but is fully preserved in the incomplete inhibition of STAT3, findings that are consistent with the reported nonredundant role of STAT3 in mediating the IL-10–regulated anti-inflammatory response in macrophages and the principal role for macrophages in mediating the IL-10–STAT3 anti-inflammatory signaling (67). Thus, our data are consistent with reported STAT3-inhibition studies that show attenuation of PH in animal models, but they also suggest that complete STAT3 blockade may render any IL-6-responsive cell within the vessel wall, including fibroblasts, refractory to anti-inflammatory IL-10 and promote persistent proinflammatory STAT1-mediated activation in response to paracrine IL-6 (33, 34). Thus, it remains to be determined whether STAT3 blockade is a safe approach for treatment of PH.

We next focused on signaling pathways downstream of STAT3 that were involved in promoting this macrophage phenotype and, thus, could be additional candidate therapeutic targets. We found that genetic absence of C/EBPβ or HIF1α (both transcription factors associated with proinflammatory activation of macrophages and both STAT3 target genes) attenuated macrophage activation in response to IL-6 and PH-Fib CM, which was paralleled by attenuated transcription of STAT3 target genes. This finding is consistent with the reported synergistic function of C/EBPβ and HIF1α with STATs (49, 63, 64, 68), as well as with studies in-
indicating a role for C/EBPβ in regulating macrophage activation in obesity, wound healing, and ischemic heart injury (36, 48, 49–69–71). Importantly, unlike the consequences of completely blocking STAT3, the IL-10–mediated anti-inflammatory response is fully functional in the absence of C/EBPβ and HIF1α (K.C. El Kasmí and P.J. Murray, unpublished observations; P.J. Murray, personal communication). Thus, targeting C/EBPβ and/or HIF1α may effectively mitigate IL-6–mediated remodeling in various pathologies, such as PH and rheumatoid arthritis. Analysis of the therapeutic potential of STAT3 versus C/EBPβ or HIF1α inhibition on vascular remodeling awaits the examination of animal models with cell-type–specific ablation of STAT3, HIF1α, and C/EBPβ.

Collectively, our data identify an intercellular signaling mechanism in the PA adventitia that is controlled by fibroblast-mediated signaling in various species and forms of PH and that shapes macrophage differentiation toward a distinct phenotype critically regulated through IL-6, STAT3, HIF1α, and C/EBPβ in the absence of pathogens, danger signals, and Th2 cytokines. Based on the independence of this macrophage phenotype from TLR–MyD88 and STAT6 signaling and considering the critical role of STAT3 signaling, together with the role of fibroblast-generated paracrine signals, chiefly IL-6, in controlling this phenotype, we conclude that these STAT3-activated macrophages portray a distinct functional phenotype representing its own category of macrophage activation that is crucial in pathological tissue remodeling. This intercellular signaling axis in IL-6–, fibroblast–, and STAT3–activated macrophages might be important in other chronic fibrotic inflammatory conditions that occur in the absence of Th2-derived cytokines and pathogens, such as scleroderma, rheumatoid arthritis, acute respiratory distress syndrome, and fibrotic lung disease.

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Disclosures
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