Hypoxia-induced endothelial CX3CL1 triggers lung smooth muscle cell phenotypic switching and proliferative expansion

Jianliang Zhang,1 Hanbo Hu,1 Nadia L. Palma,1 Jeffrey K. Harrison,2 Kamal K. Mubarak,1 Robin D. Carrie,1 Hassan Alnuaimat H, Shen X, Luo D, Patel JM. Hypoxia-induced endothelial CX3CL1 triggers lung smooth muscle cell phenotypic switching and proliferative expansion. Am J Physiol Lung Cell Mol Physiol 303: L912–L922, 2012. First published September 18, 2012; doi:10.1152/ajplung.00014.2012.—Distal arterial remodeling is a hallmark of pulmonary hypertension induced by oxygen deficiency that is present in people living at high-altitude regions and patients with obstructive lung diseases. However, the molecular events that trigger alveolar hypoxia-induced peripheral endothelial modulation of vessel wall smooth muscle cell (SMC) proliferation and filling of nonmuscular areas are unclear. Here, we investigated the role of CX3CL1/CX3CR1 system in endothelial-SMC cross talk in response to hypoxia. Human lung microvascular endothelial cells responded to alveolar oxygen deficiency by overproduction of the chemokine CX3CL1. The CX3CL1 receptor CX3CR1 is expressed by SMCs that are adjacent to the distal endothelium. Hypoxic release of endothelial CX3CL1 induced SMC phenotypic switching from the contractile to the proliferative state. Inhibition of CX3CR1 prevented CX3CL1 stimulation of SMC proliferation and monolayer expansion. Furthermore, CX3CR1 deficiency attenuated spiral muscle expansion, distal vessel muscularization, and pressure elevation in response to hypoxia. Our findings indicate that the capillary endothelium relies on the CX3CL1/CX3CR1 axis to sense alveolar hypoxia and promote peripheral vessel muscularization. These results have clinical significance in the development of novel therapeutics that target mechanisms of distal arterial remodeling associated with pulmonary hypertension induced by oxygen deficiency that is present in people living at high altitudes and patients with obstructive lung diseases.

endothelial release of substances including vasoactivators, chemokines, and growth factors into the circulation contributes to hypoxic pulmonary vasoconstriction and microvascular remodeling in coordination with pathophysiological alterations (1, 10, 18, 46, 64).

CX3CL1/fractalkine is anchored to the endothelial surface for capturing circulating cells that express its sole receptor, CX3CR1, while shed/released CX3CL1, acts as a soluble chemoattractant. The CX3CL1/CX3CR1 axis has been linked to inflammatory cell infiltration, vascular repair, and inflammatory PH induced by monocrotaline (40, 45, 61). Furthermore, CX3CL1-elevated conditions, such as scleroderma and chronic obstructive pulmonary diseases (COPD), often complicate with PH (12, 55). Hypoxia and reoxygenation promote endothelial CX3CL1 expression (58) and also activate ADAM17 (13). ADAM17 cleaves CX3CL1 from the cell surface (19, 29). CX3CL1 expression was upregulated in the rats that were chronically exposed to hypoxia (14). In addition, plasma CX3CL1 levels are increased in PH patients (4, 28).

CX3CL1 modulates CX3CR1+ cells such as bone marrow-derived cells, lymphocytes, fibroblasts, and SMC (27, 43, 61). Circulating mesenchymal precursors and fibroblasts contribute to remodeling of lung artery adventitia in neonatal rat and calf models of hypoxic PH (16, 21, 48). This indicates that hypoxic recruitment of inflammatory/progenitor cells can contribute to the appearance of newly formed α-smooth muscle actin-positive (α-SMA+) cells in the vessel wall through their differentiation into SMC (17). However, blocking antigen-driven Th2 immune responses does not prevent pulmonary arterial pressure elevation (15). The observations are consistent with the fact that PH does not always respond to immunosuppression (33). Thus, CX3CL1 may modulate SMC growth in hypoxic vascular remodeling and PH. Indeed, SMCs express CX3CR1, and soluble CX3CL1 stimulates SMC proliferation (40). Endothelial CX3CL1 modulation of SMC growth may be the key event in hypoxic vessel remodeling since normal contractile SMCs show a growth inhibitory response to hypoxia (5, 38, 49). SMCs respond to stimuli through alterations of contractility-related gene expression such as α-SMA, smooth muscle myosin heavy chain (MYH11), vimentin, calponin, transgelin (SM22α), and desmin (7–8, 64). These observations support the notion that CX3CL1 may act as the endothelium-specific mediator that transmits hypoxic stimulation in the lung alveoli to promote the shift of the SMC state from the contractile to the synthetic/proliferative phenotype.

Although extensive investigations have led to the characterization of new α-SMA+ cells in the vessel wall and adventitia, the molecular event that triggers these cells into the proliferative phenotype in the hypoxic microvasculature is unclear.
Particularly, how the peripheral endothelium respond to alveolar hypoxia by modulating SMC to fill the nonmuscular areas remains the puzzling aspect of hypoxic PH. We hypothesized that the microvascular endothelium relies on the CX3CL1-CX3CR1 axis to sense alveolar oxygen deficiency and promote the peripheral vessel remodeling in the pathophysiology of hypoxic PH. Our findings include that 1) hypoxic exposure of MVEC leads to increased CX3CL1 expression and shedding, 2) soluble CX3CL1 triggers SMC phenotypic switching and proliferation, and 3) CX3CR1 deficiency prevents hypoxia-induced distal vessel muscularization.

MATERIALS AND METHODS

Cell culture. Human pulmonary MVEC, pulmonary artery endothelial cells, and pulmonary artery SMC were purchased from Lonza and propagated in monolayer cultures as described by Zhang et al. (59, 62). Cells that were subcultured < 5 times in postconfluent monolayers and maintained in EGM-2 MV or SmGM medium (Lonza) were used for all experiments.

Hypoxic exposure of human lung endothelial cells. Oxygen levels < 5% are hypoxic to lung endothelial cells. Hence, the cultures of serum-starved human lung endothelial cells (Lonza) in endothelial basal medium-2 (EBM-2) containing 1% FBS were exposed to room air containing 5% CO2 or a gas mixture containing 1% or 3% oxygen, and balanced N2 at 37°C in an incubator chamber (Billups-Rothenberg) for 12, 24, or 48 h (60).

Chronic exposure of mice to hypoxia. Homozygous CX3CR1-deficient CX3CR1<sup>GFP/GFP</sup> mice backcrossed to the C57BL/6 background were obtained from JAX Laboratories. A breeding colony was maintained at the University of Florida (UF). Animal experimental protocols were approved by the UF-Institutional Animal Care and Use Committee (IACUC) and Veterans Affairs Medical Center-IACUC.

Table 1. Healthy volunteer and patient information

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<th>mRAP, mmHg</th>
<th>PCWP, mmHg</th>
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Healthy volunteers (C) and patients (P) who were scheduled for a right heart catheterization, male/female, and those in relatively stable clinical status for the past 6 wk and able to provide consent were asked to participate in the study. WHO FC, World Health Organization Functional Class; O₂SAT, oxygen saturation level; mPAP, mean pulmonary arterial pressure; mRAP, mean right atrial pressure; PCWP, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; CTEPH, chronic thromboembolic pulmonary hypertension; CHD, congenital heart disease; APAH, associated pulmonary arterial hypertension; IPAH, idiopathic pulmonary arterial hypertension; ILD, interstitial lung disease; RA, room air; 3L, 3 liters of oxygen; 4L, 4 liters of oxygen.
mRNAs. The values of relative quantities were normalized to the relative quantity value of the control.

Assessment of CX3CL1 levels. The levels of soluble CX3CL1 in endothelial cell-conditioned medium and control/patient plasma were assessed using the quantitative sandwich immunoassay following the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Samples were added to the anti-CX3CL1 antibody-coated 96-well plate. After being washed, horseradish peroxidase-conjugated anti-CX3CL1 antibody was placed in the wells. The bound enzymatic activity was quantified by using a peroxidase substrate. Western blot analysis was performed to assess the CX3CL1 levels in peripheral and capillary wedge blood as previously described (60). The goat anti-CX3CL1 antibody was used (cat. no. SC-7225; Santa Cruz Biotechnology, Santa Cruz, CA). The band intensities were determined using a densitometer (Bio-Rad, Hercules, CA).

Fluorescence immunohistochemical examinations. To assess the effects of soluble CX3CL1 on SMC phenotypes, the medium conditioned by MVEC was collected. The conditioned medium, plasma derived from control or PH patients, and rCX3CL1 was used to treat cells. The cells were fixed in cold methanol, immunostained with the Cy3-conjugated antibody recognizing α-SMA, a common SMC marker, and examined under a fluorescence microscope.

The mouse lungs were fixed in 4% formaldehyde, embedded in optimal cutting temperature (OCT) compounds, and sectioned (5-μm). The sections were immunostained with the Cy3-conjugated anti-α-SMA antibody. Some lung transverse sections were coimmunostained with the antibodies recognizing mouse endothelium (MECA-32) and SMC (α-SMA). The sections were examined under a fluorescence microscope using 552/570 nm wavelength for Cy3, 488/575 nm for phycocerythrin (PE), and 488/509 nm for green fluorescent protein.

Assessment of SMC proliferation. Soluble CX3CL1-stimulated SMC proliferation was assessed using [4-[3-(4-lodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate] (WST-1) as previously described (42). SMC were incubated in HBSS containing WST-1 at 37°C for 10 min. The OD values at 450 (detection) and 590 (reference) nm wavelengths were assessed, respectively.

CX3CR1 siRNA transfection of SMC. SMC were transfected with siRNA using Amaxa nucleofector kits and following the manufacturer’s instructions (Lonza). SMC (10^6 cells) were mixed with CX3CR1 siRNA (200 nM, SC-39904, Santa Cruz Biotechnology) in 100 μl of nucleofector solution. The SMC/siRNA mixture in a certified cuvette was electroporated on a nucleofector device. The siRNA-transfected cells were electroporated and cultured in a CO2 incubator at 37°C, and then incubated with the medium containing 10% FBS for 48 h. The cells were harvested for quantification of CX3CL1 and CX3CR1 expression.

**Fig. 1. Hypoxic modulation of endothelial CX3CL1 expression.** A: cultures of human microvascular endothelial cells (MVEC; Lonza) in endothelial basal medium-2 (EBM-2) containing 1% FBS were exposed to room air containing 5% CO2 (Air) or a gas mixture containing 1% or 3% oxygen, 5% CO2, and balanced N2 at 37°C in an incubator chamber (Billups-Rothenberg) for 24 h. Total RNA was extracted and converted to cDNA as described in MATERIALS AND METHODS. The relative levels of CX3CL1 mRNA were assessed by using quantitative (q)RT-PCR with β-2-microglobulin (B2M) primers in a multiplexed reaction. The relative quantity (RQ) values of hypoxia-treated cells were normalized to the RQ value of the normoxic samples (Air). *P < 0.01, 1-way ANOVA/t-test, n = 8. B: Human MVEC were exposed to room air (N12-N48) or 3% oxygen (H12-H48) for 12 to 48 h. The levels of CX3CL1 mRNA were assessed and normalized to the RQ values of N12. *P < 0.01, 1-way ANOVA, n = 4. C: Male C57BL/6 mice were exposed to room air (N1-N4) or hypoxia (H1-H4, 10% O2 with balanced N2) in a chamber (Coy Laboratory Products) for 1–4 wk. The relative levels of CX3CL1 mRNA in the lung lysates were assessed using qRT-PCR. *P < 0.05 vs. N, t-test, n = 4–8. D: circulating endothelial-like cells were isolated from peripheral blood derived from volunteers (C; control; n = 5) and pulmonary hypertension patients (PH; n = 21). An anti-human endothelium antibody and secondary antibody-conjugated magnetic microbeads (cat. no. M-450 Dyna beads) were used for the separation of endothelial-like cells. RNA was extracted, converted to cDNA, and assessed for the CX3CL1 levels using qRT-PCR analysis. *P < 0.05 vs. C, t-test.
cells were stimulated by rCX3CL1 and assessed for proliferation using WST-1.

Cell expansion assay. Oris cell seeding stoppers were used to restrict cell seeding to the outer annular regions of the wells in a 96-well plate (Platypus Technologies). Removal of the stoppers revealed unseeded/detection areas in the center of each well. Serum-starved SMC were stimulated with rCX3CL1 (0–2 ng/ml; R&D systems) for 24 h. The Oris detection mask was used to restrict the visualization to the detection regions. SMC were labeled by calcine AM and examined under a fluorescence microscope. The fluorescence intensities of the cells in the unseeded areas were assessed under a fluorescence microplate reader. The wavelengths of excitation and emission were 485 and 530 nm, respectively.

Identification of muscular segments adjacent to spiral muscles and diameter assessments. To determine hypoxic muscularization of previously partial muscular segments, we identified and measured the interface between the muscular and partially muscular segments. After the blood was drawn from the heart, the murine lungs were flushed via the main pulmonary artery with heparinized saline solution at 20 cm H2O pressure until the pulmonary venous effluent was cleared of blood. Then, the mouse lungs were flushed and fixed in 4% formaldehyde at the same pressure and embedded in OCT compounds. The fixed lung was serially sectioned at the thickness of 5 μm for nine times. The sections were immunostained with the Cy3-conjugated anti-αSMA antibody and examined under a fluorescence microscope.

The cross section of the muscular vessel shows a complete circle. Thus, the muscular segment adjacent to the spiral muscle was identified, i.e., the same vessel showed complete and incomplete circles on one and the next slides, respectively. The diameters of the muscular segments adjacent to the partially muscular vessels were measured from the top to bottom and from the left to right of the vessel. Average pixels were used.

Assessments of muscular vessel numbers. The numbers of α-SMA+ distal vessels were measured using immunohistochemical examinations. After normoxic/hypoxic exposure, the mouse lungs were fixed in OCT and sectioned (5 μm). The PE-conjugated anti-mouse endothelium (MECA-32) antibody and the Cy3-conjugated anti-α-SMA antibody were used to immunostain the mouse lung sections. Four areas per slide were randomly picked and examined under a fluorescence microscope. Four microphotographic images per slide were taken. The numbers of α-SMA+ vessels (10–100 μm) per image were counted and normalized to the viewed area. Results were shown as numbers of α-SMA+ vessels per mm2.

Statistical analysis. Significance of the hypoxic effects on CX3CL1/CX3CR1/ADAM17 expression, SMC proliferation, and lung distal vessel muscularization were determined by analysis of standard errors and t-test/ANOVA using the data analysis tools of Microsoft Excel and Prism (GraphPad Prism, GraphPad Software, CA) (57).

Declaration of ethical approval for experiments. All human and animal studies were approved by the UF/Va-IRB and UF/Va-IACUC, respectively. The written informed consent was received from participants prior to inclusion in the study. Participants were only identified by number, not by name.

RESULTS

Hypoxic stimulation of lung endothelial CX3CL1 expression. The reactions of the alveolar barrier endothelium to low levels of oxygen are of particular significance (37–38). To determine whether hypoxia induces endothelial CX3CL1 expression, human lung MVECs were exposed to room air (21% oxygen), 1%, or 3% oxygen for 12, 24, or 48 h. Hypoxia (1–3% oxygen) stimulated CX3CL1 expression in MVEC (Fig. 1A). Exposure of human MVEC to hypoxia (3% oxygen) led to increased levels of CX3CL1 mRNA in a time-dependent manner (Fig. 1B). Next, we evaluated the effects of chronic hypoxia on CX3CL1 expression in the lungs. Mice were exposed to hypoxia (10% oxygen) for 1–4 wk as described in MATERIALS AND METHODS, which increased the relative levels of CX3CL1 mRNA in the mouse lungs (Fig. 1C). PH is a complex disease that can be caused by many factors, including hypoxia. Hypoxic exposure often results in the development of PH, which is correlated with CX3CL1 elevation (22, 50, 52, 63). To determine whether endothelial CX3CL1 elevation was correlated with PH, circulating endothelial-like cells were isolated from PH and control patients. The relative level of CX3CL1 mRNA in the cells derived from PH patients was higher than that from control subjects (Fig. 1D). The observations demonstrate that the vascular endothelium responds to hypoxic stimulation by overexpression of CX3CL1 in vitro and in vivo.

PH-related elevation of peripheral and lung capillary CX3CL1 levels. CX3CL1 shed from the endothelium can be released to the circulation. Indeed, the level of plasma CX3CL1 in patients with or without PH was 0.69 or 0.25 ng/ml, respectively (Fig. 2A). Next, we examined whether lung microvascular beds contributed to PH-associated elevation of plasma CX3CL1. Lung capillary wedge blood samples were collected from patients who underwent pulmonary artery cath-
acterization. Interestingly, CX3CL1 levels in peripheral blood were lower than those in capillary wedge blood derived from PH patients, implying the lung microvasculature contributed to circulating CX3CL1 (Fig. 2B).

**Hypoxia-increased ADAM17 expression.** To determine whether hypoxia could specifically promote elevation of circulating CX3CL1 in vivo, mice were exposed to hypoxia or normoxia. The levels of plasma CX3CL1 protein in the blood derived from hypoxic mice were higher than those from control animals (Fig. 3A). This implies that hypoxia can be one of the factors that contribute to the elevation of plasma CX3CL1 levels in PH patients.

We subsequently tested whether hypoxia could induce CX3CL1 release from the endothelium. The medium conditioned by either hypoxic or normoxic human pulmonary artery endothelial cells was assessed for soluble CX3CL1 levels. Hypoxia increased the levels of soluble CX3CL1 in the conditioned medium in a time-dependent manner (Fig. 3B). Medium conditioned by the human lung endothelial cells exposed to hypoxia for 48 h had a CX3CL1 concentration of 1.9 ng/ml, while the normoxic cell-conditioned medium had a concentration of 0.25 ng/ml.

To determine whether hypoxia modulated ADAM17 expression in the microvasculature, human MVEC were exposed to hypoxia and assessed for the levels of ADAM17 mRNA and protein. The level of ADAM17 mRNA measured in the cells exposed to hypoxia was higher than that in the control cells (Fig. 3C). Additionally, hypoxic elevation of ADAM17 mRNA was correlated with increased levels of ADAM17 protein (Fig. 3D).

**Soluble CX3CL1 stimulation of SMC phenotypic switching.** SMC respond to stimuli through altered expression of contractility-related genes such as α-SMA. Interestingly, recombinant CX3CL1 (rCX3CL1) altered α-SMA expression (reduced/defused presence) in SMC (the first column of Fig. 4A). WST-1 was used to assess SMC proliferation. The levels of WST-1 in the SMC treated with rCX3CL1 were higher than those in the cells treated with vehicles (Fig. 4B). Although CX3CL1 stimulated SMC phenotypic switching toward proliferation, it was still unclear whether CX3CL1 released from the endothelium could promote SMC growth. To address this question, we stimulated SMCs using the hypoxic MVEC-conditioned medium (HMCM). The conditioned medium altered α-SMA expression and increased SMC proliferation (the second column of Fig. 4A, the third and fourth bars of Fig. 4B). Furthermore, plasma derived from PH patients stimulated SMC phenotypic switching and proliferation (Fig. 4A, column 3; Fig. 4B, bars 5 and 6). The medium and plasma consist of other substances that may also contribute to the phenotype switch.

To determine whether CX3CL1-promoted SMC proliferation was mediated through CX3CR1, siRNA was used to silence CX3CR1 expression in SMCs. CX3CR1 deficiency attenuated HMCM-induced SMC proliferation (Fig. 4C). The data indicate that hypoxia-increased CX3CL1 levels in the circulation can stimulate CX3CR1-dependent SMC phenotypic switching, which may contribute to lung vascular muscle hyperplasia in PH.

**CX3CL1-stimulated SMC proliferation expands the cell monolayer.** CX3CL1 does not promote SMC migration but rather proliferation (Fig. 4) (40). To examine whether CX3CL1-stimulated SMC proliferation resulted in the extension of the SMC monolayer, we used the Oris assay to specifically detect cells in the uncovered areas (Fig. 5A). CX3CL1 promoted SMC expansion to uncovered areas (Fig. 5B). The fluorescent intensities of the cells in the previously

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**Fig. 3.** Hypoxia (H) increased ADAM17 expression. A: mice were exposed to normoxia (N) or hypoxia for 3 wk. Plasma CX3CL1 levels were assessed using Western blot analysis (insets). *P < 0.05 vs. C, t-test, n = 3. B: Soluble CX3CL1 in the human pulmonary artery endothelial cell-conditioned medium was assessed using the quantitative sandwich immunoassay. *P < 0.05 1-way ANOVA, n = 3. C: human MVEC were exposed to room air (normoxia) or 3% oxygen (hypoxia) for 24 h. The relative levels of ADAM17 mRNA were assessed using qRT-PCR. *P < 0.01 vs. N, t-test, n = 4. D: lysates derived from normoxic/hypoxic human MVEC were assessed for the relative levels of ADAM17 protein using Western blot analysis (insets). Densities of the bands were quantified using a densitometer (Bio-Rad). *P < 0.05 vs. C, t-test, n = 6.

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uncovered areas were increased in the wells containing CX3CL1 in a dose-dependent manner (Fig. 5C). The observations demonstrate that CX3CL1 triggers SMC proliferation, resulting in filling the unoccupied areas.

CX3CR1 deficiency attenuated hypoxic spiral muscle expansion. Hypoxic distal vessel remodeling involves spiral muscle expansion to the nonmuscular areas via SMC proliferation. To determine whether CX3CL1/CX3CR1 signaling modulates spiral muscle expansion, C57BL/6 (WT) and CX3CR1 knockout (KO) mice were exposed to room air (normoxia) or 10% oxygen (hypoxia) for 4 wk. We utilized a new method to assess spiral muscle expansion. The serial sections of the mouse lung were immunostained using the anti-α-SMA antibody. The interface region between the muscular and partially muscular segments (Fig. 6A) was identified by examination of the serial lung sections; and the diameters of the muscular segments adjacent to the spiral muscles were measured (Fig. 6B). Hypoxia-decreased diameters of the adjacent muscular segments suggest expansion of the muscular segment toward the previously spiral muscle region; this was attenuated in the CX3CR1 KO mice (Fig. 6C). These results suggest that the CX3CL1-CX3CR1 axis is involved in hypoxic stimulation of muscle expansion in the lung distal segments.
CX3CR1 deficiency attenuated hypoxia-induced distal vessel muscularization. Hypoxic PH is often associated with severe muscularization of the precapillary arterioles through new growth (2, 51, 53). Indeed, immunohistochemical examinations (Fig. 7A) revealed that the numbers of \( \alpha \)-SMA \(^+ \) vessels in the lungs of hypoxia-exposed WT mice were higher than those in room air-exposed WT animals (Fig. 7B). However, the numbers of \( \alpha \)-SMA \(^+ \) vessels in hypoxic CX3CR1 KO and normoxic mice were comparable (Fig. 7B). This indicates that inhibition of CX3CL1/CX3CR1-mediated muscle expansion (Fig. 6) attenuates distal vessel muscularization (Fig. 7B). The findings suggest a vital role of the CX3CL1-CX3CR1 axis in hypoxic microvascular remodeling.

**CX3CR1 deficiency decreased hypoxia-induced RV pressure and hypertrophy.** To determine whether CX3CL1-CX3CR1 signaling contributed to hypoxia-induced lung vascular hemodynamic changes, we exposed WT and CX3CR1 KO mice to 10% oxygen for 5 wk. The levels of RVSP in CX3CR1 KO mice were significantly lower than those in the WT animals (Fig. 8A). This was correlated with decreased RV/WH ratios due to CX3CR1 deficiency (Fig. 8B). Histological analysis revealed that CX3CR1 deficiency decreased hypoxia-induced muscularization, compared with hypoxic WT animals (Fig. 8C). The results indicated that CX3CL1/CX3CR1 signaling contributed to hypoxic PH in mice.

**DISCUSSION**

The lung microvasculature often compensates alveolar hypoxia by enlarging the gas exchange area and prolonging the diffusion time through increased capillary volume and length (23). Our findings demonstrate that the lung microvascular endothelium responds to hypoxic stimulation by overproduction of CX3CL1, which triggers SMC phenotypic switching, proliferation, and muscle expansion (Fig. 9).

CX3CL1 levels were increased in plasma and circulating endothelial cells derived from PH patients. Vascular smooth muscles express CX3CR1, implying that CX3CL1 elevation may contribute to muscle abnormality in hypoxic PH. Indeed, blocking CX3CL1-triggered stimulation by deleting the receptor CX3CR1 prevented hypoxia expansion of smooth muscles into smaller vessel segments of the mouse lungs. In addition, CX3CR1 deficiency attenuated hypoxia-increased numbers of muscular distal vessels. The observations support the notion that the CX3CL1-CX3CR1 axis contributes to hypoxic distal vessel remodeling. Our discovery has significant translational value for the development of novel therapeutic interventions to block abnormal CX3CL1 stimulation of distal vessel remodeling. For instance, blocking CX3CR1-dependent SMC overgrowth may prevent the development of PH in people living at high altitudes. COPD patients who live in the mountain states within the United States are more likely to die than those patients living in the other states (34). In addition, PH is the common complication of hypoxemic COPD (32, 56). Thus, CX3CR1 antagonists would be predicted to help prolong the lives of patients with COPD.

CX3CL1 triggered SMC phenotypic switching and proliferation, which could serve as a signal event to relay hypoxic stimulation to smooth muscle growth in the lung microvasculature. This notion is supported by in vitro and in vivo studies. First, hypoxic MVEC-conditioned medium and plasma derived from PH patients induced a contractile-to-synthetic shift of the SMC phenotype. Mediators released from endothelial cells modulated SMC states (9). Second, the conditioned medium and plasma-induced SMC proliferation was mimicked by rCX3CL1. It was reported that soluble CX3CL1 stimulated SMC proliferation in monocrotaline-induced PH (11). Third, siRNA-mediated specific inhibition of CX3CR1 expression attenuated rCX3CL1 stimulation of SMC proliferation.

The capillary endothelium is located in the gas exchanges regions in the lungs. The endothelial responses to alveolar hypoxia by releasing mediators play a critical role in arteriolar...
remodeling. Interestingly, our results indicated that hypoxia stimulated CX3CL1 expression in human lung MVEC. Human coronary artery and umbilical vein endothelial cells had a similar response to hypoxia/reoxygenation (58). However, early studies suggested that hypoxia inhibited cytokine-stimulated CX3CL1 expression in human umbilical vein endothelial cells (26). The reasons for the differences regarding the effects of hypoxia on umbilical vein endothelial CX3CL1 expression are unclear. Endothelial cells derived from different vascular beds may respond to oxygen deficiency in a different manner regarding to modulation of CX3CL1 expression. For example, hypoxia increased CX3CL1 expression in coronary/pulmonary artery endothelial cells (58) but inhibited CX3CL1 expression in the umbilical vein endothelium (26). Furthermore, MVEC are the first in the vascular system to respond to alveolar hypoxia, which may be different to the response of umbilical vein endothelial cells to hypoxemia in modulation of CX3CL1 expression. This may imply a possible explanation for the differential vasoactive responses of pulmonary arteries (constriction) and umbilical veins (dilation) to hypoxic stimulation.

ADAM17 is required for inducible shedding/release of CX3CL1. We observed that hypoxia upregulated ADAM17 expression in MVEC. Exposure of synovial cells to low levels of oxygen led to increased ADAM17 mRNA levels and activity (13). Our studies indicated that ADAM17 activation was correlated with increased soluble CX3CL1 levels in the MVEC-conditioned medium. The observations imply that endothelial CX3CL1 expression and ADAM17-mediated shedding can contribute to PH-associated elevation of plasma CX3CL1.

Chronic hypoxia-induced muscularization of previously nonmuscular segments is the most characteristic lung vascular structural changes in the development of PH. Experimental data indicate multiorigins of SMC in hypoxia-induced lung vascular smooth muscle hypertrophy. For example, hypoxia promoted differentiation of circulating progenitor cells into SMC (6). Furthermore, local endothelial cells can transdifferentiate into SMC (65). Our finding revealed the state shift of local SMC under hypoxic conditions. The CX3CL1-CX3CR1 axis could transmit hypoxic stimulation of the lung microvascular endothelium to SMC proliferation of distal vessels. Specifically, the distal endothelium responds to the drop of alveolar oxygen levels by overproduction of CX3CL1, which triggers lung vascular SMC phenotypic switching and growth. In addition, endothelial release of CX3CL1 can contribute to hypoxic arterial constriction through Rho kinase-associated pathways (28). Defining the novel function of the chemokine CX3CL1 as a mediator in hypoxic stimulation of lung distal SMC hyperplasia will help us better understand how hypoxia promotes smooth muscle expansion in the lung microvasculature. This can set a stage for the development of novel therapeutic interventions to treat hypoxic PH caused by conditions such as COPD and high-altitude oxygen deficiency.

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DISCLOSURES
No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS
Fig. 8. CX3CR1 deficiency led to reduced vascular pressure and hypertrophy. WT and CX3CR1 KO mice were exposed to hypoxia for 5 wk. A: assessments of pulmonary artery pressure. Right ventricular systolic pressure (RVSP) was measured using the Seldinger technique. *P < 0.05, t-test, n = 5. B: measurements of right ventricle (RV) hypertrophy. The RV was separated from the heart and weighted. The RV/WH weight ratios were calculated. WH, whole heart. *P < 0.05, t-test, n = 5. C: histological microimaging of distal pulmonary artery. Lung tissues were fixed and sliced. The slides were stained using hematoxylin and eosin and examined using a microscope.


REFERENCES

Fig. 9. CX3CL1-CX3CR1 mechanisms of hypoxic distal vessel muscularization. Hypoxia-induced overproduction/shedding of endothelial CX3CL1 triggers SMC phenotypic switching in a CX3CR1-dependent manner. SMC proliferative migration fills nonmuscular areas. This leads to extension of the muscular segment to previously partial muscular regions.


