Supporting Information

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SI Materials and Methods

Isolation and Preparation of Murine Alveolar Macrophages. BALF was obtained through the intratracheal instillation of three 0.9-mL aliquots of PBS, which were collected and filtered through a 40-μm cell strainer to exclude contaminating epithelial cells, which appeared in clumps. The percentage of the macrophage lineage ($CD45^+CD11c^+F4/80^+$) cells in the living cells isolated from BALF was 83.7 ± 0.7% by flow cytometry analysis as shown in Fig. S2. For cell-culture experiments, primary mouse alveolar macrophages were obtained by bronchoalveolar lavage (BAL) and were cultured in DMEM containing 2% FBS. The cells were seeded at 1.5×10^5 cells per well in 24-well plates containing supplemented DMEM (10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin) and were incubated at 37 °C for 4 h; then the medium was replaced with serum-free DMEM. The cells were serum-starved overnight and then were stimulated with mouse recombinant IL-21 (20 ng/mL), IL-6 (100 ng/mL), and/or sIL-6R (100 ng/mL) (R&D Systems) for 18 h.

PASMC Proliferation Assay. HPASMCs were purchased from Lonza and used within the first six passages. HPASMCs were cultured in 96-well tissue-culture plates (2×10^3 cells per well) in 100 μL of smooth muscle cell basal medium (SmBM; Lonza) with 5% FBS and SingleQuot supplement (Lonza). Two days before the assay, the medium was replaced with SmBM supplemented with 0.1% FBS, 10 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. For the macrophage-conditioned medium experiment, normoxic primary mouse alveolar macrophages were obtained by BAL, pooled, washed by centrifugation, counted, and added to 96-well tissue culture plates at 2×10^5 cells per well in supplemented DMEM (10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin). The macrophages were incubated at 37 °C for 4 h, followed by medium replacement with serum-free DMEM. The macrophages were serum-starved overnight, stimulated with mouse recombinant IL-21 (20 ng/mL) for 18 h, washed with PBS, and cultured again in DMEM alone. After 24 h the culture medium was collected, diluted twofold with fresh DMEM supplemented with 0.2% FBS, and used as conditioned medium. The conditioned medium was applied to HPASMCs, and the cultures were incubated for an additional 3 d. When indicated, the HPASMCs were treated with the conditioned medium supplemented with a CXCR4 chemical inhibitor, AMD3100 (1 μg/μL) (Sigma), or vehicle (PBS). The HPASMCs were stimulated with human recombinant IL-21 (10–100 ng/mL) (Life Technologies), IL-6 (100 ng/mL), and/or sIL-6R (100 ng/mL) (R&D Systems) for 24 h. HPASMCs treated with 5% FBS served as a positive control. Cell proliferation was assessed using the cell proliferation reagent from the Cell Titer 96 kit (Promega). The Cell Titer 96 reagent (20 μL) was added to each well (containing 100 μL of medium), followed by a 2-h incubation at 37 °C, and then the absorbance at 490 nm was determined.

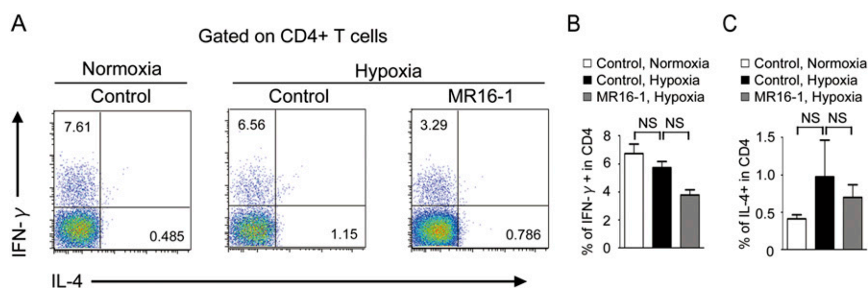

Fig. S1. IL-6 blockade does not significantly affect Th1 and Th2 cell accumulation in mouse lungs after hypoxia exposure. (*A*) Flow cytometry analysis of IFN-γ–expressing cells and IL-4–expressing cells in the $CD4^+$-gated T-cell population isolated from the lungs of mice treated with control antibody or MR16-1 after exposure to hypoxia or normoxia for 3 d. Representative results from three independent experiments are shown ($n = 3$ per group). (*B* and *C*) Percentage of IFN-γ–expressing (*B*) and IL-4–expressing (*C*) cells in the $CD4^+$ population. Values shown are the mean ± SEM for three independent experiments. NS, not significant.

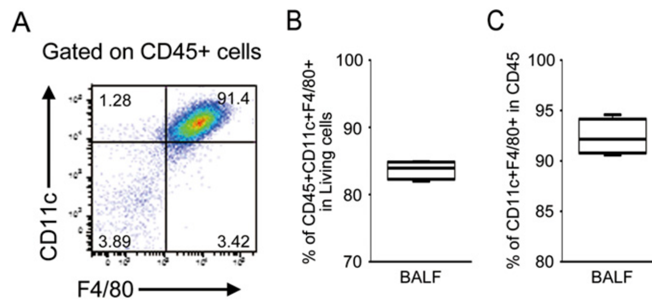

Fig. S2. Cells isolated from BALF are mainly alveolar macrophages. (*A*) Flow cytometry analysis of F4/80$^+$ and CD11c$^+$ cells in the CD45$^+$-gated cell population isolated by BALF in mice ($n = 4$). (*B*) The percentage of alveolar macrophages in living cells isolated by BALF. (*C*) The percentage of F4/80$^+$ and CD11c$^+$ alveolar macrophages within the CD45$^+$ population. Values shown are the mean ± SEM from two independent experiments.

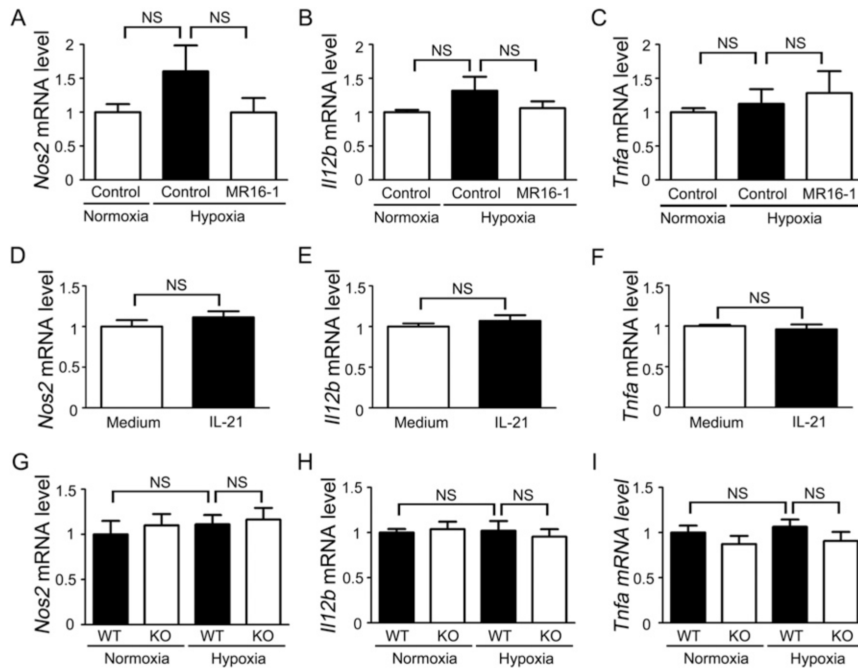

Fig. S3. Neither IL-6 nor IL-21 is involved in the gene expression of M1 macrophage markers. (*A–C*) IL-6 blockade does not affect M1 macrophage activation in mouse lungs. qRT-PCR analysis of M1 macrophage marker genes including *Nos2* (*A*), *Il-12β* (*B*), and *Tnf-α* (*C*) in the alveolar macrophages isolated from mice treated with control antibody or MR16-1 after exposure to hypoxia or normoxia for 4 d ($n = 6$ in each group). Values shown are the mean ± SEM from three independent experiments. (*D–F*) IL-21 does not affect the expression of M1-related macrophage marker genes in primary alveolar macrophages. qRT-PCR analysis of M1 macrophage marker genes including *Nos2* (*D*), *Il-12β* (*E*), and *Tnf-α* (*F*) in primary mouse alveolar macrophages treated with IL-21 (20 ng/mL). The results shown are the pooled data from three independent experiments with six total wells per group. Values shown are the mean ± SEM. (*G–I*) IL-21 blockade does not affect M1 macrophage polarization in mouse lungs. qRT-PCR analysis of M1 macrophage marker genes including *Nos2* (*G*), *Il-12β* (*H*), and *Tnf-α* (*I*) in the alveolar macrophages isolated from WT or IL-21RKO (KO) mice after exposure to hypoxia or normoxia for 4 d ($n = 6$ in each group). Values shown are the mean ± SEM from three independent experiments. NS, not significant.

Fig. S4. qRT-PCR analysis of M2-related (*A*–*E*) and M1-related (*F*–*H*) macrophage marker genes in primary mouse alveolar macrophages treated with IL-6, sIL-6R, or both (each at 100 ng/mL) shows that IL-6 does not directly affect the expression of either M2-related or M1-related macrophage marker genes in primary alveolar macrophages. The results shown here are the pooled data from three independent experiments, each with six wells per group. Values shown are the mean ± SEM. NS, not significant.

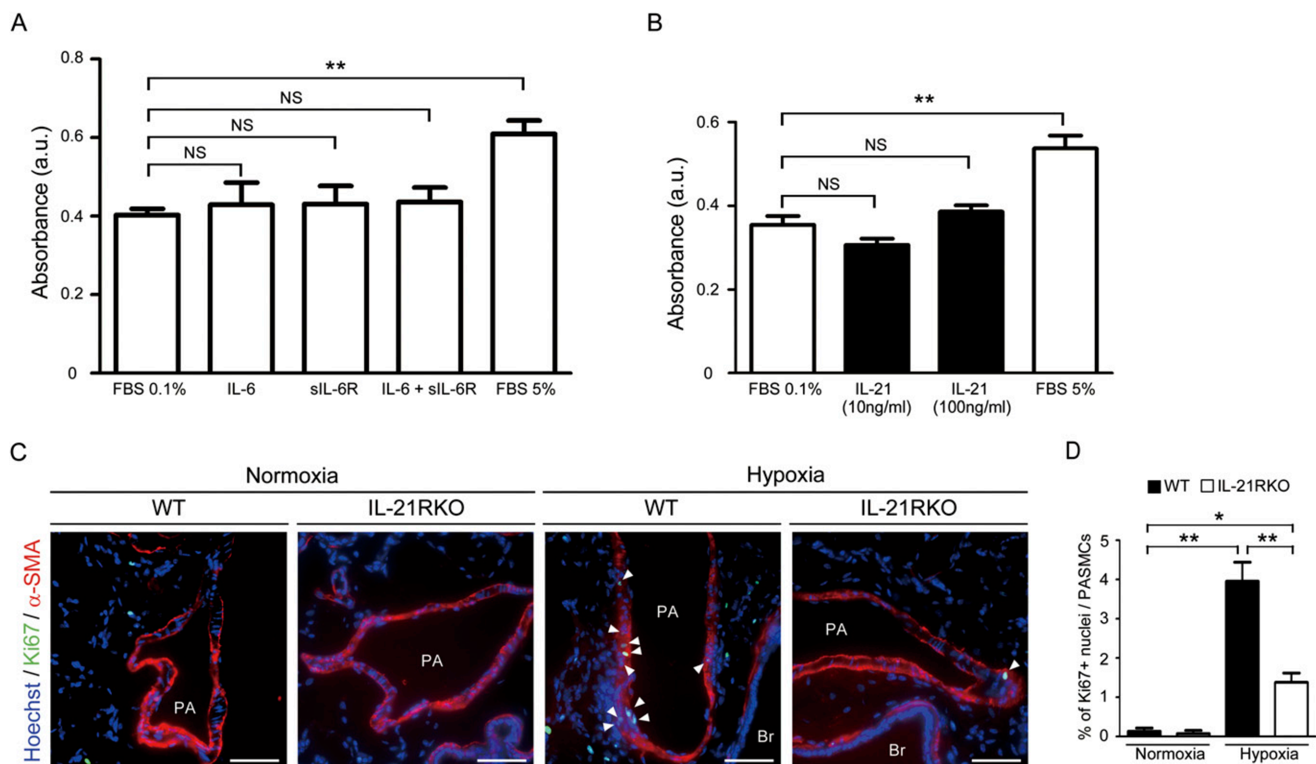

Fig. S5. Hypoxia-induced proliferation of PASMCs is mediated indirectly through IL-21–dependent signaling. (*A* and *B*) Treatment with IL-6, sIL-6R, or both, or with IL-21 does not promote the proliferation of HPASMCs. Cell proliferation analysis of HPASMCs treated with IL-6, sIL-6R, or both (each at 100 ng/mL) (*A*) or with IL-21 (10 or 100 ng/mL) (*B*) in the presence of 0.1% FBS. Stimulation of HPASMC proliferation by 5% FBS was used as a positive control. The results shown are pooled data from three independent experiments with 15 wells per group. Values shown are the mean ± SEM; $**P < 0.01$ compared with control medium containing 0.1% FBS. (*C* and *D*) IL-21R deletion inhibits the hypoxia-induced proliferation of PASMCs. (*C*) Ki67 (green) and α-SMA (red) immunostaining of lung sections from WT and IL-21RKO mice after exposure to hypoxia for 1 wk ($n = 5$). Arrowheads indicate $Ki67^+$ nuclei. Br, bronchus; PA, pulmonary artery. (Scale bars: 40 μm.) (*D*) Percentage of $Ki67^+$ nuclei in the PASMCs. Values shown are the mean ± SEM; $*P < 0.05$, $**P < 0.01$ calculated using ANOVA. NS, not significant.

Table S1. Physiological profiles of the four experimental groups exposed to normoxic and hypoxic conditions and treated with control antibody or MR16-1

	Normoxia		4-wk hypoxia	
	Control	MR16-1	Control	MR16-1
Final body weight, g	25.5 ± 0.8	25.4 ± 1.7	22.4 ± 1.2**	22.1 ± 1.0**
RV/BW, mg/g	0.74 ± 0.09	0.79 ± 0.08	0.96 ± 0.09**	0.84 ± 0.09#
LV/BW, mg/g	3.02 ± 0.30	3.22 ± 0.30	2.85 ± 0.20	3.11 ± 0.44
Systolic BP, mm Hg	98.9 ± 7.9	100.7 ± 6.8	101.2 ± 10.5	103.0 ± 7.9
HR, bpm	588 ± 12	591 ± 9	575 ± 7	572 ± 8

Values shown are the mean ± SEM (n = 8–12 for each group). BP, blood pressure; BW, body weight; HR, heart rate; LV, left ventricle; RV, right ventricle.

$**P < 0.01$ compared with mice treated with control antibody under normoxia for 4 wk.

$^{\#}P < 0.05$ compared with mice treated with control antibody under hypoxia for 4 wk.

Table S2. Physiological profiles of the four experimental groups comprised of WT and IL-21RKO mice exposed to normoxic or hypoxic conditions

	Normoxia		4-wk hypoxia	
	WT	IL-21RKO	WT	IL-21RKO
Final body weight, g	24.5 ± 0.7	23.5 ± 0.8	21.2 ± 0.6*	21.5 ± 0.7*
RV/BW, mg/g	0.81 ± 0.04	0.82 ± 0.06	1.13 ± 0.05**	0.93 ± 0.03[#]
LV/BW, mg/g	3.43 ± 0.10	3.38 ± 0.15	3.20 ± 0.11	3.27 ± 0.13
systolic BP, mm Hg	101.5 ± 2.1	101.8 ± 0.9	100.6 ± 1.3	102.8 ± 0.9
HR, bpm	571 ± 17	606 ± 16	601 ± 11	590 ± 6

Values shown are the mean ± SEM (n = 5–10 for each group). BP, blood pressure; BW, body weight; HR, heart rate; LV, left ventricle; RV, right ventricle.

*$P < 0.05$ compared with WT mice under normoxia for 4 wk.

**$P < 0.01$ compared with WT mice under normoxia for 4 wk.

[#]$P < 0.05$ compared with WT mice under hypoxia for 4 wk.

Table S3. Patient characteristics in the immunohistochemical study

Patient	Age, y	Sex	Condition	WHO class	Heath–Edwards grade
1	48	F	IPAH	3	3
2	44	F	IPAH	3	4
3	13	M	IPAH	3	3
4	35	M	IPAH	3	4
5	46	F	Control	NA	NA

Characteristics of the four IPAH patients and one control patient who provided lung tissue for immunohistochemical analyses with anti–IL-21, anti–Arg-1, and anti-MRC1 antibodies. F, female; M, male; NA, not applicable; WHO, World Health Organization.