Early Macrophage Recruitment and Alternative Activation Are Critical for the Later Development of Hypoxia-Induced Pulmonary Hypertension

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Background—Lung inflammation precedes the development of hypoxia-induced pulmonary hypertension (HPH); however, its role in the pathogenesis of HPH is poorly understood. We sought to characterize the hypoxic inflammatory response and to elucidate its role in the development of HPH. We also aimed to investigate the mechanisms by which heme oxygenase-1, an anti-inflammatory enzyme, is protective in HPH.

Methods and Results—We generated bitransgenic mice that overexpress human heme oxygenase-1 under doxycycline control in an inducible, lung-specific manner. Hypoxic exposure of mice in the absence of doxycycline resulted in early transient accumulation of monocytes/macrophages in the bronchoalveolar lavage. Alveolar macrophages acquired an alternatively activated phenotype (M2) in response to hypoxia, characterized by the expression of found in inflammatory zone-1, arginase-1, and chitinase-3-like-3. A brief 2-day pulse of doxycycline delayed, but did not prevent, the peak of hypoxic inflammation, and could not protect against HPH. In contrast, a 7-day doxycycline treatment sustained high heme oxygenase-1 levels during the entire period of hypoxic inflammation, inhibited macrophage accumulation and activation, induced macrophage interleukin-10 expression, and prevented the development of HPH. Supernatants from hypoxic M2 macrophages promoted the proliferation of pulmonary artery smooth muscle cells, whereas treatment with carbon monoxide, a heme oxygenase-1 enzymatic product, abrogated this effect.

Conclusions—Early recruitment and alternative activation of macrophages in hypoxic lungs are critical for the later development of HPH. Heme oxygenase-1 may confer protection from HPH by effectively modifying the macrophage activation state in hypoxia. (Circulation. 2011;123:1986-1995.)

Key Words: heme oxygenase-1 | hypertension, pulmonary | hypoxia | macrophage activation | inflammation

Pulmonary arterial hypertension (PAH) is a devastating disease characterized by vasoconstriction and vascular wall remodeling with resultant right ventricular hypertrophy and eventual failure. Despite significant progress in this field, the mechanisms underlying the development of PAH are still obscure. Recently, in an increasing number of studies, lung inflammation has been implicated as a potential maladaptation underlying the development of PAH. Infiltrates of leukocytes and inflammatory mediators have been detected in patients with PAH, and have been reported to contribute to pulmonary vascular remodeling in animal models of disease.4–6 Tissue hypoxia, a well-known stimulus for pulmonary hypertension, has also been demonstrated by our group and others to induce an inflammatory response that precedes the development of hypoxia-induced pulmonary hypertension (HPH).7–9

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Among the inflammatory cells implicated in PAH, those of the monocyte/macrophage lineage have been correlated with disease more often.1,2,8–10 However, macrophages efficiently respond to environmental signals with remarkable plasticity and undergo different forms of polarized activation that can be roughly categorized as classically activated (M1), alternatively activated (M2), and anti-inflammatory (regulatory) macrophages.11,12 Classically activated macrophages are effector phagocytes activated by interferon-γ and tumor necrosis factor (TNF-α). They produce inducible nitric oxide synthase and interleukin (IL)-12 and exhibit enhanced microbicidal or tumoricidal capacity.11 On the other hand, M2-polarized macrophages are activated mostly by IL-4 or IL-13, and, as recently discovered, by CCL2 and IL-6, and they...
expression arginase-1 (Arg-1), found in inflammatory zone-1 (Fizz1), chitinase-3-like-3 (Ym1), and mannose receptor, C type lectin-1.11,12,14 M2 macrophages have been implicated in the pathogenesis of lung and other disorders via their ability to promote trophic, profibrotic, and angiogenic functions.15,16 The major characteristic of the third population, regulatory macrophages, is the production of high IL-10 and low IL-12 levels and the promotion of immunosuppression.11,14 In the case of PAH, the activation state of the recruited macrophages and their contribution to disease has remained unclear until now.

Heme oxygenase-1 (HO-1) is a major antioxidant and cytotoxic enzyme that catalyzes the degradation of heme to 3 enzymatic end products: carbon monoxide (CO), free Fe2+, and biliverdin.17 Heme oxygenase-1 and its enzymatic product, CO, have been reported by our group and others to be protective in HPH.7,18–20 This protection, up to now, has been attributed mainly to the relaxation of vascular tone and inhibition of vascular smooth muscle cell proliferation by CO.21 However, it has been demonstrated that HO-17,17,22,23 and CO24,25 have potent anti-inflammatory properties, some of which may be exerted via the upregulation of the anti-inflammatory cytokine IL-10.25,26 Moreover, HO-1− deficient mice develop a chronic oxidative inflammatory state that progresses with age27 and have a maladaptive response to hypoxia with right ventricular dilation, fibrosis, and inflammation.18,28 Therefore, we hypothesized that immunomodulation is a key mechanism of HO-1 protection in PAH.

To characterize in detail the lung inflammatory response caused by hypoxia, to assess its role in pulmonary hypertension, and to investigate the protective properties of HO-1 in this context, we generated a bitransgenic mouse model with doxycycline-inducible, lung-specific expression of HO-1. We report here that hypoxic exposure in the absence of doxycycline provoked a significant monocytic/macrophage accumulation in the bronchoalveolar lavage fluid (BALF) that manifested a phenotype consistent with alternative activation, with upregulated expression of Fizz1, Arg1, Ym1, and mannose receptor, C type lectin-1. Heme oxygenase-1 overexpression by doxycycline treatment inhibited hypoxic macrophage recruitment and activation and resulted in upregulation of IL-10 in macrophages. Supernatants from hypoxic cultures of M2 macrophages promoted proliferation of pulmonary artery smooth muscle cells, whereas CO treatment abrogated this effect. By modulating the timing and duration of HO-1 expression with doxycycline, we were able to either delay or suppress lung inflammation and macrophage activation and, in the latter case, abolish HPH.

**Methods**

Bitransgenic mice were generated by crossing Balb/c transgenic mice that harbor the tetracycline transcriptional activator (tetON system) under the control of the Clara cell secretory protein promoter with FVB transgenic mice that carry the human HO-1 (hHO-1) transgene under the control of the tetracycline response element (Figure 1A). Expression of hHO-1 in the lung was achieved by the addition of 1 mg/mL doxycycline to the drinking water. The CCTA mouse line that lacks the hHO-1 transgene was treated with doxycycline and served as control to eliminate any potential effects imparted by doxycycline itself independently of hHO-1. All animal procedures were approved by the Children’s Hospital Boston Animal Care and Use Committee. An expanded Methods section is available in the online-only Data Supplement.

**Statistical Analysis**

All values are expressed as mean±SD. Comparison of results between different groups was performed by 1-way ANOVA or Mann-Whitney U test when appropriate with GraphPad InStat (GraphPad Software, San Diego, CA). Values of P<0.05 were considered significant.

**Results**

**Lung-Specific, Inducible Expression of Human Heme Oxygenase-1**

Based on the design of the bitransgenic model (designated CC77; Figure 1A), the hHO-1 transgene is under the control of both doxycycline and the Clara cell secretory protein promoter and therefore is inducibly expressed in the lung epithelium. Semiquantitative polymerase chain reaction analysis on total lung RNA with hHO1-specific primers indicates that hHO-1 levels were upregulated with doxycycline treat-
Hypoxia exposure and before the development of hyperten-

sion, animals were exposed to hypoxia, and a temporal profile of

the cell content in BALF was performed. More than 95% of the isolated

BALF cells were CD45-positive leukocytes (Figure IA in the online-only Data

Supplement). The cells expressing the macrophage-specific cell

surface antigens F4/80 and CD11c remained the predominant

population (>98%) regardless of hypoxic exposure

or doxycycline treatment (Figure IB in the online-only Data

Supplement and Figure 3A). Under these conditions, only a

subtle increase in BALF neutrophils and T lymphocytes was

observed (data not shown). When hypoxic exposure was

begun, the numbers of monocytes/macrophages were signif-

icantly increased in the BALF of control hypoxic mice,

reaching a peak at 2 days of hypoxia and dropping signifi-

cantly by 7 days but remaining slightly elevated compared

with normoxic animals (Figure 3B). Doxycycline administra-

tion had a suppressive effect on the accumulation of cells at

all time intervals investigated (Figure 3B). Doxycycline

treatment of the control mice (CCTA) had no impact on

monocyte/macrophage accumulation, establishing that inhi-

bition of cell infiltrate is due specifically to HO-1 overex-

pression and is not an artifact of doxycycline treatment

(Figure 3C).

To identify the specific enzymatic product of HO-1, CO,

and/or biliverdin that is responsible for suppressing the

peak of inflammation at 2 days of hypoxia, we exposed

animals to intermittent inhalation of CO (250 ppm for 1

hour twice a day) and/or biliverdin injections (50 μmol/kg

IP twice a day). Intraperitoneal PBS injections served as

control. Inhaled CO or CO plus biliverdin, but not biliv-

erdin alone, was effective in inhibiting the inflammatory

cell infiltrate in the BALF at levels comparable to doxy-

cycline (Figure 3C).

In addition to the accumulation of macrophages, several

cytokines/chemokines were upregulated in the BALF of

hypoxic mice (Figure II in the online-only Data Supplement).

In as early as 2 and 4 days of hypoxia, upregulation of

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In as early as 2 and 4 days of hypoxia, upregulation of

fibroblast growth factor β, IL-1β, macrophage inflammatory

protein-1α, IL-17, and IL-2, as well as of Th2-related cytokines IL-13 and IL-4, was observed. The Th1-related cytokines, IL-12, and TNF-α remained unaffected (Figure II in the online-only Data Supplement), and interferon-γ levels were undetectable (not shown). Doxycycline administration effectively suppressed fibroblast growth factor-2, IL-1β, macrophage inflammatory protein-1α, and IL-2 at both 2 and 4 days of hypoxia and suppressed IL-17, IL-13, and IL-4 only after 4 days of continuous administration (Figure II in the online-only Data Supplement).

Interestingly, a striking alteration of macrophage morphology in cytospin preparations of BALF-isolated macrophages was observed at 4 days of hypoxia, characterized by cytoplasmic enlargement in a population of cells. This phenotype is consistent with activation and was not detected in any of the macrophages isolated from doxycycline-treated mice (Figure 3D).

**Hypoxia Induces Alternative Activation of Macrophages: Suppressive Effect of Heme Oxygenase-1**

The observation that macrophage morphology was altered in response to hypoxia led us to further investigate the potential activation state of hypoxic macrophages. Quantitative polymerase chain reaction analysis of BALF-isolated alveolar macrophages from CC77 bitransgenic mice revealed an induction of well-defined markers of M2 macrophages in hypoxic mice, including Arg1, Fizz1, Ym1 (Figure 4A), and mannose receptor, C type lectin-1 (data not shown). The peak expression occurred at 4 days of hypoxia and remained upregulated for at least 14 days. In contrast, there was no change in the mRNA levels of markers of M1 macrophage phenotype such as inducible nitric oxide synthase, TNF-α, and IL-12 (IL-12p40) or the costimulatory molecules, CD80/86, essential in the process of antigen presentation (Figure III in the online-only Data Supplement). Urea production, indicative of arginase activity, was also upregulated in in vivo hypoxic alveolar macrophages (Figure 4C); this increase in enzymatic activity was due to Arg1 because Arg1 mRNA levels were induced 9.1 ± 3.4-fold after 4 days of hypoxic exposure, whereas Arg2 mRNA levels were 0.6 ± 0.1 of their normoxic value at this time point. Inducible nitric oxide synthase activity, as assessed by nitrite and nitrate production in the BALF, remained unchanged (Figure 4D). Doxycycline administration effectively suppressed all markers of alternative activation (Figure 4A through 4C), whereas these markers were not suppressed in the CCTA line treated with doxycycline (Figure 4B and 4C and Figure IVA in the online-only Data Supplement). Immunofluorescent staining confirmed the localization of Fizz1 and the absence of inducible nitric oxide synthase in the cytoplasm of hypoxic macrophages (Figure 5B and 5C).
Apart from the slight elevation of the Th2 cytokines IL-13 and IL-4 in the BALF of hypoxic mice, we investigated the potential presence of other noncanonical inducers of M2 polarization. Thus, we assessed the mRNA levels of CCL2 and IL-6 in total lung extracts by quantitative polymerase chain reaction. CCL2 and IL-6 mRNA was robustly upregulated soon after hypoxic exposure but was significantly suppressed in the presence of doxycycline (Figure VA in the online-only Data Supplement). In the CCTA control line lacking the HO-1 transgene, doxycycline treatment did not suppress CCL2 and IL-6 levels (Figure VB in the online-only Data Supplement). Interestingly, primary alveolar macrophages cultured in vitro under hypoxic conditions (0.5% O₂) also manifested the M2 phenotype with increased levels of Ym1, but not IL-12 and TNF-α (Figure VI in the online-only Data Supplement), suggesting that M2 polarization induced by hypoxia is a cell autonomous phenomenon.

Heme Oxygenase-1 Promotes the Expression of Interleukin-10 in Alveolar Macrophages

In an effort to further evaluate the effect of HO-1 on macrophage phenotype, mRNA and protein levels of IL-10, a well-documented anti-inflammatory mediator, were directly assessed in freshly isolated alveolar macrophages. Interleukin-10 was significantly elevated in alveolar macrophages derived from hypoxic mice treated with doxycycline (Figure 6). Doxycycline treatment in the CCTA line failed to upregulate IL-10 (Figure IVB in the online-only Data Supplement), establishing that the observed effect is HO-1 dependent. Furthermore, the number of regulatory macrophages expressing IL-10 (CD11c, IL-10) under hypoxia was assessed by flow cytometry to be increased 4- to 9-fold with doxycycline treatment, comprising slightly <10% of the total macrophage population in BALF (data not shown).

Early Monocyte/Macrophage Accumulation Is Critical for the Later Development of Pulmonary Hypertension

To determine whether this early inflammatory response is essential for the later development of HPH and whether inducible expression of HO-1 at defined intervals during this process modulates the disease, we exposed the bitransgenic mice to doxycycline for various time periods. In addition to

Figure 4. Hypoxia induces alternatively activated macrophages: the suppressive effect of heme oxygenase-1 (HO-1). A, Quantitative polymerase chain reaction analysis of hypoxic alveolar macrophage mRNA isolated from bitransgenic mice (CC77) revealed increased found in inflammatory zone-1 (Fizz1), arginase-1 (Arg1), and chitinase-3-like-3 (Ym1) levels that were suppressed with doxycycline (dox). B, Western blot analysis for Fizz1 on bronchoalveolar lavage fluid (BALF) from normoxic mice (Nrm) and mice exposed to hypoxia for 4 days–dox (Hyp-dox) or with dox treatment (Hyp-dox); IgA served as internal control. C, Arginase activity (U/L) was assessed by urea formation in alveolar macrophages from normoxic and hypoxic animals. D, Inducible nitric oxide synthase activity was estimated by the levels of nitrate and nitrite in the BALF of hypoxic mice. Supernatants from RAW 264.7 macrophages stimulated with 100 μg/mL lipopolysaccharide (LPS) Escherichia coli and 100 U/mL interferon-γ (INF-γ) for 48 hours served as positive controls. Mean±SD is depicted for n=6 mice per group. *P<0.05, **P<0.01, ***P<0.001 relative to normoxia; #P<0.05, ##P<0.01, ###P<0.001 relative to hypoxia-dox.

Figure 5. M2 and M1 expression profile of hypoxic alveolar macrophages. Found in inflammatory zone-1 (Fizz1) expression in alveolar macrophages from normoxic mice or mice exposed to hypoxia in the absence or presence of doxycycline (dox) was assessed by flow cytometry (A) and immunofluorescence (B; FITC). C, Inducible nitric oxide synthase (iNOS; FITC) staining in alveolar macrophages. Primary alveolar macrophages stimulated with 100 μg/mL lipopolysaccharide Escherichia coli and 100 U/mL interferon-γ (INF-γ) for 48 hours served as positive controls. Nuclei were counterstained with DAPI. Scale bar=25 μm.
control animals exposed to hypoxia for 3 weeks in the absence of doxycycline, 1 group of littermates received doxycycline continuously for 3 weeks, and in 2 other groups, doxycycline was removed from the drinking water at 2 or 7 days of hypoxia. In all groups, animals remained in hypoxia for the entire 3-week period. On removal of doxycycline, hHO-1 mRNA levels returned to baseline within 3 days (Figure 7A and 7B).

A short 2-day pulse of doxycycline at the onset of hypoxia caused a delay in the peak of macrophage recruitment in the BALF from 2 to 7 days, a time when HO-1 levels were reduced to baseline (Figure 7A). In the same group of animals, development of pulmonary hypertension, as assessed by right ventricular systolic pressure, the Fulton Index, the medial wall thickness index, and histology of vascular remodeling, was not prevented (Figure 7C through 7F). In the case of a more prolonged, yet still transient, administration of doxycycline for 7 days, there was no macrophage influx even when HO-1 had reached baseline low levels, and pulmonary hypertension was completely prevented at 3 weeks (Figure 7B through 7F).

Alternative Macrophage Activation Is Associated With the Development of Hypoxia-Induced Pulmonary Hypertension In Vivo and Enhances Pulmonary Artery Smooth Muscle Cell Proliferation In Vitro

In agreement with macrophage numbers, M2 markers followed a similar pattern (Figure 8A). In the group in which doxycycline was removed after 2 days of hypoxic exposure and HPH was not prevented, mRNA levels of Fizz1, Arg1, and Ym1 increased 4 days after HO-1 levels had fallen to baseline (Figure 8A). However, in the group that received doxycycline for the first 7 days of hypoxia and the later development of hypertension was prevented, levels of Fizz1, Arg1, and Ym1 remained suppressed for all time periods examined (Figure 8B). Interestingly, in only the 7-day doxycycline treatment group, the anti-inflammatory marker IL-10 remained sustainably elevated above baseline (Figure 8A and 8B).
Interleukin-10 mRNA levels were also upregulated in primary alveolar macrophages cultured in vitro under hypoxic conditions (0.5% O₂) and treated with CO (500 ppm; Figure 8C). This suggests that CO release on HO-1 induction in this system is the trigger for IL-10 induction in macrophages.

Supernatants of primary alveolar macrophages that were cultured in vitro under hypoxic conditions (0.5% oxygen) or normoxic macrophages treated with IL-4 (20 ng/mL) had high levels of Fizz1 and low levels of IL-10, and were able to stimulate pulmonary artery smooth muscle cell proliferation (Figure 8D). However, supernatants from CO-treated hypoxic macrophages had reduced levels of Fizz1 and elevated IL-10 and had no proliferative effect on pulmonary artery smooth muscle cells (Figure 8D). Exogenous administration of IL-10 on pulmonary artery smooth muscle cells treated with hypoxic macrophage supernatants had no direct suppressive effect on their proliferation (Figure 8D). Exogenous administration of IL-10 on pulmonary artery smooth muscle cells treated with hypoxic macrophage supernatants had no direct suppressive effect on their proliferation (Figure 8D).

Discussion

In our bitransgenic mouse model, we demonstrate that hypoxia provokes an accumulation of alternatively activated alveolar macrophages that precedes the development of pulmonary hypertension and appears to play a critical role in the pathogenesis of disease. Overexpression of HO-1 induced a switch in macrophage polarity toward an anti-inflammatory phenotype, and this effect was associated with protection from HPH.

Hypoxia resulted in alveolar inflammation that consisted predominantly of macrophages. These findings correlate with the fact that macrophages tend to accumulate in poorly vascularized areas with low oxygen tension, and correlates with previous studies in HPH that highlighted the predominant role of the monocyte/macrophage lineage in modulating vascular remodeling. Additionally, we found that hypoxia in vivo and in vitro polarized the population of alveolar macrophages toward the M2 phenotype. Hypoxic microenvironment is also a hallmark feature of tumors, and similar to the
hypoxic macrophages in our model, the tumor-associated macrophages exhibit an M2-like phenotype.\textsuperscript{11,20} The cell-autonomous M2 polarization in in vitro hypoxic conditions, the upregulation in vivo of mRNA levels of 2 recently recognized noncanonical inducers of M2 polarization, CCL2 and IL-6,\textsuperscript{13} and the increased IL-13 and IL-4 cytokine levels in the BALF of hypoxic mice support the M2-like activation in our hypoxic model. Furthermore, Fizz1, an M2-specific marker, is a hypoxia-inducible molecule, also designated hypoxia-induced mitogenic factor.\textsuperscript{30} However, contrary to our findings, 2 previous studies reported upregulation of TNF-\(\alpha\),\textsuperscript{31} IL-12, and interferon-\(\gamma\)\textsuperscript{12} in hypoxic macrophages. We believe that our in vivo studies most closely approximate the disease physiology because the first of the 2 previous studies was performed only in vitro using isolated rat alveolar macrophages in a sepsis-induced hypoxia model and the second study was conducted on peritoneal macrophages.

Our finding that the presence of M2 macrophages is associated with the development of HPH in vivo and pulmonary artery smooth muscle cell proliferation in vitro suggests that these polarized trophic macrophages may play a significant role in the later development of pulmonary hypertension. Indeed, enhanced polyamine and \(\gamma\)-proline synthesis caused by Arg1 has been shown to contribute to vascular damage and remodeling, and elevated Arg1 in lungs of hypoxic mice has been associated with increased severity of PAH.\textsuperscript{33,34} Fizz1 has been recently reported to have mitogenic, angiogenic, and vasoconstrictive properties that are associated with pulmonary vascular remodeling.\textsuperscript{5,30,35} Its human homolog, resistin-like molecule \(\beta\), has also been detected in patients with scleroderma-associated pulmonary hypertension.\textsuperscript{36} However, to the best of our knowledge, this is the first study to propose a link between alternatively activated macrophages and the development of HPH. One potential mechanism of action of alternatively activated macrophages may be the secretion of Fizz1, the overexpression of which has been reported to lead to PAH.\textsuperscript{5} Further studies are required to determine the specific mediators from the secretome of M2 macrophages that may be contributing factors in the signaling cascade leading to HPH. In agreement with our findings, previous studies have shown that Th2 cytokines, as well as IL-6 and CCL2, induce M2 and have been implicated in human pulmonary disease and animal models of PAH.\textsuperscript{3,6,37}

Overexpression of HO-1 in our bitransgenic model provoked a robust anti-inflammatory effect. It suppressed macrophage accumulation, M2 activation, and cytokine production in the lungs and prevented the subsequent development of pulmonary hypertension. Carbon monoxide appeared to be the key HO-1 effector inhibiting macrophage accumulation in the BALF and suppressing the expression of M2 markers in vitro. This observation is in agreement with previous studies in which HO-1 and CO were reported to have potent anti-inflammatory effects.\textsuperscript{7,25}

Endogenous HO-1 is upregulated in hypoxia as a compensatory mechanism,\textsuperscript{19} but its brief upregulation is not adequate to prevent hypoxia-induced inflammation and HPH, and only a more sustained enhancement of HO-1 expression can be protective in HPH.\textsuperscript{7} For this reason, it is not surprising that a 2-day upregulation of HO-1 with doxycycline only postponed inflammation and did not protect from the development of HPH. Interestingly, HO-1 induction for 7 days, covering the entire period of hypoxia-induced inflammation, was sufficient to prevent the later development of HPH. Indeed, 7-day upregulation of HO-1 suppressed the inflammatory response even 2 weeks after the return of HO-1 to baseline levels, despite continuous hypoxia.

Because the switch in macrophage phenotype occurred within the first 4 days of hypoxia, we hypothesize that the enhancement of HO-1 during this critical period may act as a pivot to shift the balance of immune response from proinflammatory toward immunosuppressive. In support of this, lung HO-1 overexpression upregulated IL-10 in hypoxic macrophages and increased the number of IL-10--expressing regulatory macrophages in the BALF. Because CO could upregulate IL-10 in the in vitro cultured hypoxic macrophages, it seems to be a major effector molecule of HO-1 immunomodulation. In agreement with our findings, HO-1 and exogenous CO have previously been reported to increase IL-10 expression in macrophages in vivo and in vitro.\textsuperscript{25,38–40}

Interestingly, IL-10 remained elevated even 2 weeks after HO-1 expression returned to baseline levels. These findings point to a switch in immunomodulation triggered by HO-1 during an early critical period, and whose presence was no longer essential, at least for the subsequent 2 weeks of hypoxia. Elevated IL-10 levels were also associated with protection from HPH, and IL-10 expression has been reported to protect from monocrotaline-induced PAH in rats.\textsuperscript{4} We show that macrophages were the source of IL-10, but it remains unclear whether they are also a target of this cytokine. Because IL-10 is a pleiotropic cytokine, it may act in an autocrine and paracrine manner to affect many different cell types besides macrophages. However, IL-10 did not have direct antiproliferative effects on pulmonary artery smooth muscle cells in our in vitro model, indicating that HO-1 and CO may also have anti-inflammatory functions independently of IL-10. Further studies are required to decipher the role of IL-10 pathway in the protection from HPH development and the mechanism of sustained protection from HPH conferred by a transient immunomodulatory event.

**Conclusions**

The present study demonstrates a link between macrophage accumulation and M2 activation and the promotion of HPH. On the basis of our findings, targeting the elimination or inactivation of this subset of macrophages may ameliorate the outcome of disease and improve the long-term prognosis of PAH. Importantly, M2 activation may serve as a biomarker to identify a therapeutic window for anti-inflammatory treatments and obviate the need for long-term therapies.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

Pulmonary arterial hypertension is a devastating disease with molecular and cellular underpinnings that remain poorly understood, despite substantial progress in the field. Recent studies from several groups, including ours, have documented an important role for inflammation in the development of pulmonary hypertension, but the specific inflammatory cell types and mediators leading to lung vascular remodeling have yet to be characterized. In this report, using a murine model of hypoxia-induced pulmonary hypertension, we show that hypoxia leads to early accumulation of macrophages in the lung that acquire an activated M2 phenotype characterized by overexpression found in inflammatory zone-1 and arginase-1. These M2 markers have been recognized as mitogenic, angiogenic, and profibrotic factors, and their induction by hypoxia in our model is associated with the later development of pulmonary arterial hypertension. Using transgenic mice with lung-specific, inducible expression of heme oxygenase-1, we demonstrated suppression of macrophage accumulation and M2 activation by hypoxia and a shift toward an anti-inflammatory macrophage phenotype that was associated with prevention of pulmonary arterial hypertension. Activated lung macrophages may thus be a significant source of mitogenic and trophic factors that contribute to the remodeling observed in pulmonary arterial hypertension. Importantly, M2 activation may serve as a biomarker of disease progression to identify a critical window for treatment aimed at promoting a switch toward the anti-inflammatory macrophage phenotype that, according to our findings, has antiproliferative and antihypertensive effects on the lung vasculature.
Early Macrophage Recruitment and Alternative Activation Are Critical for the Later Development of Hypoxia-Induced Pulmonary Hypertension

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Supplemental Methods

**Bitransgenic mice**

Bitransgenic mice were generated by the crossing of Balb/c transgenic mice that harbor the reverse tetracycline transcriptional activator (rtTA, tetON system) under the control of the Clara Cell secreted protein (CCSP) promoter (CCTA line: a kind gift from Dr. J.A Whitsett) with FVB transgenic mice that carry the human HO-1 transgene under the control of the tetracycline response element (TH77 line: CCSP-rTTA x TRE-hHO1). The latter was generated by microinjection of a (TetO)7-CMV-human HO-1 transgene that consists of seven copies of the tet operator linked to a minimal CMV promoter, the human HO-1 cDNA, and SV40 polyadenylation signals. Animals were maintained in the pathogen-free Children’s Hospital Animal Care facility and all animal experiments were approved by the Children’s Hospital Boston Animal Care and Use Committee.

**Hypoxic mouse model of PAH**

Expression of human HO-1 in the lung was achieved by the addition of 1 mg/ml doxycycline (dox) (Sigma-Aldrich, Inc., St. Louis, MO) in the drinking water. After two days pretreatment with dox, animals were introduced to normobaric hypoxia (8.5% O2) inside a chamber where oxygen was tightly regulated by an Oxycycler controller (Biospherix, Ltd., Lacona, NY). Nitrogen was automatically introduced as required to maintain the proper FiO2 and ventilation was adjusted to keep CO2 levels less than 8,000 ppm (0.8%). Ammonia was removed by charcoal filtration using an electric air purifier. Dox administration was either continued for the entire duration of the hypoxic exposure or terminated at two days or after seven days of hypoxia. Age and sex-matched littermates were exposed to identical conditions in hypoxia or normoxia and served as controls. The CCTA mouse line that lacks
the human HO-1 transgene, treated with dox, served as control to eliminate any potential
effects imparted by dox itself, independent of human HO-1 expression.

CO treated mice inhaled the gas intermittently: 250 ppm for 1 hour prior to hypoxic
exposure and then received 250 ppm for 1 hour twice daily, inside the hypoxic chamber for a
total of 48 hours. A group of mice underwent i.p injections of 50 μmol/kg biliverdin IX
hydrochloride (Frontier Scientific, Inc., Logan, UT) as previously reported, prior to the onset
of hypoxia and twice daily thereafter. Finally, a third group received both CO and biliverdin
as above. Control mice were injected i.p with the same volume of PBS and inhaled room air
or hypoxic air (8.5% O₂) without CO.

**Hemodynamic and ventricular weight measurements**

After hypoxic exposure at the indicated time periods, mice were anesthetized and
hemodynamic and ventricular weight measurements were performed. Right ventricular
systolic pressure (RVSP) was measured through a trans-thoracic route: a pressure
transducer (ADI Instruments, Inc., Colorado Springs, CO) attached to a 23G needle was
used and data were collected and analyzed using the PowerLab Software (ADI Instruments,
Inc., Colorado Springs, CO). Right ventricular (RV) hypertrophy was assessed by
harvesting hearts, removing atria, dissecting the RV and deriving Fulton’s Index, i.e the
weight ratio of (right ventricle)/(left ventricle and septum) [(RV)/(LV+S)].

**Immunohistochemical analysis**

Lungs were initially perfused with PBS through the right ventricle. The perfusion flow was
kept at approximately 1ml/min by the use of a peristaltic pump with Platinum L/S 13
Masterflex silicone tubing. Lungs were then intratracheally inflated with 4%
paraformaldehyde, fixed overnight at 4°C, then stored in 70% ethanol before embedding in
paraffin. Lung tissue sections were deparaffinized and rehydrated. Immunohistochemical
assessment of vascular remodeling was performed by staining for alpha-smooth muscle
actin (anti-α-SMA antibody, Sigma-Aldrich, Inc., St. Louis, MO), a marker of smooth muscle

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Endogenous peroxidase activity was inhibited with 3% H$_2$O$_2$ (Sigma-Aldrich, Inc., St. Louis, MO) in methanol. Next, the sections were incubated with a biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA), treated with the avidin-biotin complex (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA), and stained with 3,3′-diaminobenzidine substrate (KPL, Inc., Gaithersburg, MD). Slides were counterstained with 1% Methyl Green (Sigma-Aldrich, Inc., St. Louis, MO).

**Morphometric analysis**

Alveolar/distal pulmonary arterioles of 50-100 μm in diameter, not associated with bronchi, from lung sections immunostained with Ú-SMA (as described above), were captured with light microscopy. At least 10 representative pulmonary arterioles were chosen from three different sections from each animal. Morphometric analysis of medial vessel wall thickness was performed using the software package Metamorph v.6.2r (Universal Imaging, Downingtown, PA). The entire vessel area including the lumen was identified as “Total Area,” and the area of brown-color (Ú-SMA stained) that represents the medial smooth muscular layer was labeled “Threshold Area.” Medial Wall Thickness Index was determined by using the quotient of Threshold Area x 100 over Total Area [(%Threshold Area x 100)/ total Area].

**Isolation of alveolar macrophages**

Animals were anesthetized with 2,2,2-tribromoethanol (avertin, Sigma-Aldrich, Inc., St. Louis, MO) after exposure for the indicated time periods in hypoxia. Bronchoalveolar lavage fluid (BALF) was obtained through intratracheal instillation of 4 x 0.85 ml PBS and filtered via a 35 μm cell strainer to exclude contamination from epithelial cells that appeared in clusters. Red blood cells were lysed using ammonium chloride lysis buffer (Sigma-Aldrich, Inc., St. Louis, MO). More than ninety percent of the cells isolated this way appeared to be of the monocyte/macrophage lineage and this was confirmed by cell-specific markers in flow cytometry (below). Isolated cells were used for RNA extraction, flow cytometry, or immunocytochemistry.
**Flow cytometry**

Total white blood cell (WBC) counts of BALF isolated cells were accessed by hemocytometer counting using Kimura stain, and reconfirmed by flow cytometry analysis by the use of FITC-anti mouse CD45 antibody (BD Biosciences, Franklin Lakes, NJ) and flow cytometry absolute count standard beads (Bangs Laboratories, Fishers, IN). Differential WBC analysis was performed using APC-anti mouse F4/80 (eBioscience, Inc., San Diego, CA), PE-anti-mouse Ly-6G/Ly-6C (BD Biosciences, Franklin Lakes, NJ), and Pacific Blue-anti-mouse CD3 (eBioscience, Inc., San Diego, CA) antibodies specific for macrophages, neutrophils, and T cells, respectively. Expression profile of BALF isolated alveolar macrophages was assessed by APC-anti mouse F4/80 (eBioscience, Inc., San Diego, CA), FITC-anti-mouse CD11c, and PE-anti-mouse CD45 (BD Biosciences, Franklin Lakes, NJ) in separate analyses. Fizz1 expression was assessed by performing fixation with parafomdeldehyde, intracellular permeabilization and staining with primary rabbit anti-mouse-Fizz1 antibody (Abcam, Cambridge, MA) followed by secondary rabbit FITC-conjugated anti-rabbit antibody (BD Biosciences, Franklin Lakes, NJ). In order to evaluate the IL-10-expressing alveolar macrophages, BALF-isolated cells were incubated with Golgi inhibitor (monensin, BD Biosciences, Franklin Lakes, NJ) fixed and permeabilized, and stained with APC-conjugated monoclonal antibody against murine IL-10 (BD Biosciences, Franklin Lakes, NJ). The proper isotype controls were used in each case. The flow cytometry events were acquired in a MoFlo Legacy Cell Sorter (Beckman Coulter, Inc., Brea, CA) and analyzed with the use of Summit Software (Summit Software, Inc., Fort Wayne, IN).

**Cytospin preparation and Immunocytochemistry**

BAL was performed and the cell suspension was cytocentrifuged at 300g for 5 min using the Shandon Cytospin 4 (Thermo Fisher Scientific, Inc., Waltham, MA). Slides were air-dried overnight, stained with Hema stain set (Fisher Diagnostics, Middletown, VA), and evaluated
under light microscope. Immunocytochemistry for Fizz1 or iNOS was performed by immersion of the slides in 2% paraformaldehyde, incubation with blocking serum, followed by incubation at 4°C overnight with rabbit polyclonal anti-mouse-Fizz1 antibody (Abcam, Cambridge, MA), or rabbit polyclonal anti-mouse iNOS antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Goat biotinylated anti-rabbit secondary antibody (Cell Signaling Technology, Inc., Boston, MA) and FITC-avidin conjugate (Vector Laboratories, Inc., Burlingame, CA) were further used. Primary alveolar macrophages stimulated with 100 μg/ml LPS E.coli and 100 U/ml INF-γ for 48 hours served as positive controls. Nuclei were counterstained with DAPI (Thermo Fisher Scientific, Inc., Waltham, MA) and samples were observed with fluorescent microscopy.

Quantitative PCR

RNA from total lung or from alveolar macrophages was isolated using the Qiagen RNAeasy mini and micro extraction kit, respectively (Qiagen, Hilden, Germany). One μg of total DNA-digested RNA was used for cDNA synthesis (Superscript III oligo dT primer kit, Invitrogen Corporation, Carlsbad, CA). The following primers were used in the PCR reaction: human HO-1; fwd: 5' GCAGTCAGGCAGAGGGTGATA-3' rev: 5' AGCCTGGGAGCGGGTGTTGAG-3' Ym1; fwd: 5' GCAGAAGCTCTCCAGAAGCAATCCTG-3' rev: 5' AGCCTGGGAGCGGGTGTTGAG-3' Fizz1; fwd: 5' GCTGATGGTCCCAGTGAATAC-3' rev: 5' CCAGTAGCAGTCATCCCAGC-3' Arginase-1; fwd: 5' CAGAAGAATGGAAGAGTCAG-3' rev: 5' CAGAAGAATGGAAGAGTCAG-3' Arginase-2; fwd: 5' CACGGGCAATTTCTTTGCCTTCCGTCCT-3' rev: 5' GGTTGGCAAGGCCACTGAACG-3' Mannose Receptor, C type 1 (MR); fwd: 5' TTTCCATCGAGACTGCTGC-3' rev: 5' CTGCAGGGAACACATGCCAC-3' iNOS; fwd: 5' TCCTCCACGGGCCCGGTACTC-3' IL12b; fwd: 5' GGAGGGGCTGTAACCCAAGAGGTGC-3' rev: 5' CCTGCAGGGAACACATGCCAC-3' TNFa; fwd: 5' GCCCAGTCGTAGCAAAACCACC-3' rev: 5' CGGGGCAGCCTTTGCTCCCTTGG-3' CCL2 (MCP-1); fwd: 5'
GGCTGGAGCATCCACGTGTTGG-3\ö rev: 5\öTTGGGTCAGCACAGACCTCTCTC-3\ö IL-6;
fwd: 5\öCAAAGCCAGAGTCCTCAGAG-3\ö rev: 5\öCACTCCTCTTGTGACTCCAGC-3\ö IL-
10;  fwd: 5\ö GCGCTGTATCGATTTCTCCCCTG-3\ö rev: 5\ö
GGCCTTGTAGACACCTTGGTCTTG-3\ö PDGF-BB: fwd: 5\ö
GGGAGCAGCGAGCCAAGACG-3\ö CD80 (B7-1);
fwd: 5\öGGGAAAAACCCCCAGAAG-3\ö rev: 5\ö CCCGAAGGTAAGGCTGTTG-3\ö CD86;
fwd: 5\ö CAGCCTAGCAGGCCAGG-3\ö rev: 5\ö GGCTCTCACTGCCTTCACTC-3\ö Ribosomal
Protein S9 (Rps9) with forward primer 5\öGCTAGACGAGAAGGATCCCC-3\ö and reverse
primer 5\ö. CAGGCCCAGCTTAAAGACCT -3\ö served as housekeeping gene. Annealing was
carried out at 60\öC for 30 sec, extension at 72\öC for 30 sec, and denaturation at 95\öC for 30
sec for 40 cycles. Analysis of the fold change was performed based on the Pfaffl method.6

**BAL fluid cytokine profile**

The BALF supernatant was analyzed using a multiplex mouse cytokine kit (FGFβ, MIP-1Î, IL-1Î, IL-17, IL-2, IL-13, IL-4, TNF-Î, IL-12, INF-Î) (Invitrogen Corporation, Carlsbad, CA) in
the Luminex 200È System (Luminex Corporation, Austin, TX). BALF supernatant samples
from animals treated with either dox or regular water in normoxia versus two and four days in
hypoxia were analyzed in duplicate. Standard Luminex protocol was followed as suggested
by the manufacturer.

**Western blot analysis**

Protein concentration from BALF or total lung was determined by the Bradford assay.
BALF samples were concentrated with 20% trichloroacetic acid (TCA, Sigma-Aldrich, Inc., St.
Louis, MO) overnight, washed with ice-cold acetone and resuspended in SDS-containing
loading dye. Twenty ëg of protein was electrophoresed on 13.3% denaturing polyacrylamide
gel prior to wet transfer to 0.2 ìm PVDF membrane (Bio-Rad Laboratories, Hercules, CA).
Briefly, after blocking with 5% bovine serum albumin (BSA) in phosphate buffered saline (pH
7.4) containing 0.1% Tween 20 (PBST) for an hour at room temperature, the membranes
were incubated with rabbit polyclonal anti-mouse Fizz1 antibody (Abcam, Cambridge, MA), rabbit polyclonal anti-human and anti-mouse HO-1 antibody (Enzo Life Sciences International, Inc., Plymouth Meeting, PA), rabbit polyclonal anti-mouse HO-2 antibody (Enzo Life Sciences International, Inc., Plymouth Meeting, PA) or goat polyclonal anti-mouse IgA Ab (Millipore, Billerica, MA) at 4°C overnight. The membranes were then incubated with 40 ng/ml of peroxidase-conjugated anti-rabbit or anti-goat secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), respectively, for 30 min at room temperature followed by reaction with Lumi-Light ECL substrate (Thermo Fisher Scientific, Inc., Waltham, MA).

For IL-10 detection in alveolar macrophages, cells were collected from BALF and centrifuged at 400g for 5 min. Cells were washed with PBS prior to lysis in 20 ul of RIPA buffer supplemented with protease inhibitor cocktail. Ten ug protein per lane was loaded on 13.3% polyacrylamide gel, wet transfer was performed at 200 mAmp for 2-3 hrs, followed by blocking with 2.5% BSA in PBST (0.1% Tween 20) for 30 min, and incubation with rat anti-mouse IL-10 antibody (Abcam, Cambridge, MA) overnight at 4°C with continuous shaking. Anti-mouse β-actin monoclonal antibody (R&D Systems, Minneapolis, MN) was used as internal control and densitometric analysis was performed with the NIH ImageJ program.

**Griess reaction and arginase activity assay**

In the Griess reaction, nitrite and nitrate concentration in BALF supernatant were measured by the Total NO/Nitrite/Nitrate Assay (R&D Systems, Minneapolis, MN). Proteins were removed before analysis with ultrafiltration using 10,000 molecular weight (MW) cut-off filters (Amicon Ultra; Millipore, Billerica, MA). Supernatants from RAW 264.7 macrophages stimulated with 100 μg/ml LPS or 100 U/ml INF-γ for 48 hours served as positive controls.

Arginase activity in BALF-isolated alveolar macrophages was assessed utilizing the Quantichrom arginase assay kit (Bioassay Systems, Hayward, CA). Briefly, 10^5 cells per sample were harvested, washed, and lysed with 10 mM Tris.HCl (pH 7.4) containing 0.4%
(w/v) Triton X-100 and protease inhibitor cocktail (Complete; Roche Diagnostics, Mannheim, Germany). Cell lysate samples were analyzed for arginase activity in duplicate.

**Primary alveolar macrophage culture**

For cell culture experiments, BALF was obtained through intratracheal instillation of 5 x 1 ml Hank’s Balanced Salt Solution (without calcium and magnesium) supplemented with 10 mM EDTA and 1 mM HEPES and filtered twice via a 35 μm cell strainer to exclude contamination of epithelial cells. 3.5x10^5 macrophages per well were seeded in 48-well tissue-culture plates in a volume of 0.25 ml macrophage complete medium (DMEM/10: Dulbecco’s Modified Eagle Medium) (GIBCO, Invitrogen Corporation, Carlsbad, CA) supplemented with 10% (v/v) FBS, 10 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin). Cells were incubated at 37°C for 4 hours and then their medium was replaced with serum-free DMEM prior to hypoxic exposure or IL-4 stimulation.

**Macrophage activation and CO treatment**

Macrophages were cultured for 48 hours at 0.5-1% O₂ (pO₂ in the media was 14-18 torr) in a hypoxic work station (in Vivo2, Ruskinn Technology, Ltd., Bridgend, UK) and/or Billups chambers that were flushed with a mix of 0.5% O₂ and 5% CO₂ (N₂ balance). Alternatively, macrophages were stimulated with 20 ng/ml murine recombinant interleukin-4 (IL-4) (R&D Systems, Minneapolis, MN) in order to be polarized towards M2.9,10 CO treatment was performed in Billups chambers that were flushed with a mix of 0.5% O₂, 5% CO₂, 500 ppm CO (N₂ balance). Cells were pretreated with CO for 1 hour in normoxia prior to hypoxic exposure. Cell viability was greater than 80% in all groups as assessed by trypan blue exclusion.

**PASMC proliferation assay**

Mouse primary pulmonary artery smooth muscle cells (PASMCs) were cultured (2x10^3 cells per well) in a volume of 100 μl of DMEM (GIBCO, Invitrogen Corporation, Carlsbad,
CA) supplemented with 10% FBS, 10 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin, using 96-well tissue culture plates. Two days prior to proliferation assay, the medium was replaced with DMEM supplemented with 0.1% FBS, 10 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. Macrophage-conditioned media, diluted two-fold with fresh low-serum media, were then applied to PASMCs and the cultures were incubated for an additional three days. Cell proliferation was assessed by cell proliferation reagent WST-1 (Roche Diagnostics, Mannheim, Germany) by applying 10 µl of WST-1 reagent to each well and measuring OD440-OD690 after two hours of incubation at 37°C. Treatment of PASMCs with 25 ng/ml PDGF-BB served as a positive control. Fresh cell culture medium (DMEM) or medium equilibrated in 0.5% Oxygen for 48 hours were used as negative controls. Mouse recombinant IL-10 (R&D Systems, Minneapolis, MN) was used in the range of 1-100 ng/ml.
Supplemental Figures and Figure Legends

Supplemental Figure 1. Analysis of BALF cell content in hypoxic mice. [A] More than 95% of the cells isolated (gated for Side and Forward Scatter) were CD45(+) i.e white blood cells. [B] More than 98% of CD45 (+) cells expressed the macrophage cell surface antigens F4/80 and CD11c, irrespective of treatment.
Supplemental Figure 2. Chemokine and cytokine profile in the BALF of hypoxic mice.

Chemokine/cytokine profile in the BALF of mice exposed in hypoxia for two and four days in the absence (-) or presence (+) of dox. Upregulation of FGFβ, IL-1β, MIP-1α, IL-13, IL-4, IL-17 and IL-2 in hypoxia and the suppressive effect of HO-1 expression (+dox). Note that the Th1 related cytokines, TNF-α and IL-12, were not upregulated in hypoxia as compared to normoxic mice. (Numbers represent mean +/- SD, with a minimum of 6 animals per time point or treatment group). *: relative to normoxia; *p<0.05, **p<0.01, ***p<0.001. #: relative to hypoxia ÷ dox; #p<0.05, ###p<0.001.
Supplemental Figure 3. Expression levels of M1 markers in hypoxic alveolar macrophages. mRNA levels of M1 markers, iNOS, TNFα, IL-12β, CD80, and CD86, were assessed through qPCR in alveolar macrophages isolated from normoxic animals or animals exposed to hypoxia for four days with or without dox treatment. Values are shown relative to normoxia.
Supplemental Figure 4. The anti-inflammatory effects of dox-treatment are hHO-1 dependent. The CCTA transgenic line that harbors the tetracycline transactivator but lacks the human HO-1 transgene was used to assess potential biologic effects of dox treatment. [A] Markers of alternative activation, Fizz1, Arg1, and Ym1, were not suppressed in hypoxic CCTA animals treated with dox. [B] Concordantly, the anti-inflammatory mediator, IL-10, was not upregulated in dox-treated CCTA animals. *: relative to normoxia; *p<0.05.
Supplemental Figure 5. Suppression of CCL2 and IL-6 levels in the hypoxic lung is HO-1 dependent [A] CC77 animals. The hypoxic induction of CCL2 and IL-6 mRNA levels in the lung was suppressed by dox treatment. [B] CCTA animals. CCL2 and IL-6 mRNA levels were not suppressed in hypoxic CCTA animals lacking the hHO-1 transgene. Numbers represent mean +/-SD, with a minimum of 6 mice per group. *: relative to normoxia, ***p<0.001. #:relative to hypoxia/dox; #:p<0.05, ##p<0.01.
Supplemental Figure 6. Cytokine and growth factor profile of *in vitro* stimulated primary alveolar macrophages. [A] mRNA levels of the M2 marker, Ym1, but not the growth factor, PDGF-BB, were upregulated in primary alveolar macrophages stimulated with 20 ng/ml IL-4 or hypoxia (0.5% O₂) and suppressed upon CO treatment. [B] The mRNA levels of M1 specific markers, TNF-α and IL-12p40 subunit, were downregulated in alveolar macrophages stimulated with 20 ng/ml IL-4 or hypoxia (0.5% O₂) with or without 250 ppm CO. Numbers represent mean +/- SD, at least 5 mice donors per group. *: relative to normoxia; *p<0.05, **p<0.01, ***p<0.001. #: relative to hypoxia or normoxia + IL-4; *p<0.05.
Supplemental Figure 7. IL-10 is not sufficient to block hypoxic macrophage-derived signals for PASMC proliferation. PASMC cultures were incubated with media conditioned by either normoxic [Normoxia] or hypoxic [Hypoxia] alveolar macrophages in the presence or absence of IL-10 at the indicated final concentrations, and their proliferation rate assessed. Stimulation of PASMC proliferation by 25 ng/ml PDGF-BB served as a control. *: relative to normoxia; *p<0.05, **p<0.01, ***p<0.001.
Supplemental References


