CCR5 as a Treatment Target in Pulmonary Arterial Hypertension

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Background—Pulmonary arterial hypertension (PH), whether idiopathic or related to underlying diseases such as HIV infection, results from complex vessel remodeling involving both pulmonary artery smooth muscle cell (PA-SMC) proliferation and inflammation. CCR5, a coreceptor for cellular HIV-1 entry expressed on macrophages and vascular cells, may be involved in the pathogenesis of PH. Maraviroc is a new CCR5 antagonist designed to block HIV entry.

Methods and Results—Marked CCR5 expression was found in lungs from patients with idiopathic PH, in mice with hypoxia-induced PH, and in Simian immunodeficiency virus–infected macaques, in which it was localized chiefly in the PA-SMCs. To assess the role for CCR5 in experimental PH, we used both gene disruption and pharmacological CCR5 inactivation in mice. Because maraviroc does not bind to murine CCR5, we used human-CCR5ki mice for pharmacological and immunohistochemical studies. Compared with wild-type mice, CCR5−/− mice or human-CCR5ki mice treated with maraviroc exhibited decreased PA-SMC proliferation and recruitment of perivascular and alveolar macrophages during hypoxia exposure. CCR5−/− mice reconstituted with wild-type bone marrow cells and wild-type mice reconstituted with CCR5−/− bone marrow cells were protected against PH, suggesting CCR5-mediated effects on PA-SMCs and macrophage involvement. The CCR5 ligands CCL5 and the HIV-1 gp120 protein increased intracellular calcium and induced growth of human and human-CCR5ki mouse PA-SMCs; maraviroc inhibited both effects. Maraviroc also reduced the growth-promoting effects of conditioned media from CCL5-activated macrophages derived from human-CCR5ki mice on PA-SMCs from wild-type mice.

Conclusion—The CCL5-CCR5 pathway represents a new therapeutic target in PH associated with HIV or with other conditions. (Circulation. 2014;130:880-891.)

Key Words: hypertension, pulmonary ▪ inflammation ▪ receptors, CCR5 ▪ vascular smooth muscle

Pulmonary arterial hypertension (PH) develops either as an idiopathic condition or in association with various underlying diseases such as collagen vascular disease, portal hypertension, or HIV infection.1–3 The hallmark pathological feature of all forms of PH is structural remodeling of the small pulmonary vessels.4 Proliferation of vascular smooth muscle, accumulation of extracellular matrix, and perivascular infiltrates of inflammatory cells are the main components of the remodeling process.2,4

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Chronic inflammation is now considered an important feature of PH that contributes to the structural pulmonary vessel remodeling.5 Among immune mediators, chemokines, which control leukocyte trafficking and can activate resident vascular cells, are believed to play a major role.6,7 In PH, chemokines are secreted by inflammatory cells and by resident vascular cells, including pulmonary vascular endothelial and smooth muscle cells (SMCs).5 Alterations in the expression and production of several chemokines such as CCL2/monocyte chemotactrant protein 1,8 CX3CL1/fractalkine,9 and CCL5/
RANTES (regulated on activation, normal T-cell expressed and secreted)\textsuperscript{10} have been demonstrated in severe PH and shown to predominate within the pulmonary vascular lesions. Delineating the role and importance of chemokines involved in the pulmonary vascular remodeling process is therefore essential to the identification of specific therapeutic targets within the chemokine system. Because several chemokines may activate a single receptor, the role for chemokines is best assessed through their receptor-mediated effects.

The G-protein–coupled receptor CCR5 acts as a coreceptor required for HIV cell entry and is therefore a therapeutic target in HIV infection.\textsuperscript{11,12} New CCR5 antagonists have been developed.\textsuperscript{13,14} Whether these antagonists may slow the progression of HIV-associated PH and perhaps other forms of PH deserves evaluation. CCR5 is activated on stimulation by the CCR5 ligands CCL3 (macrophage inflammatory protein-1\textalpha{}), CCL4 (macrophage inflammatory protein-1\textbeta{}), and CCL5 (RANTES) and is strongly expressed on the principal cell types implicated in PH progression, including endothelial cells, SMCs, T cells, and macrophages.\textsuperscript{6,7,15,16} Moreover, CCL5 overexpression has been demonstrated within the pulmonary vascular wall of patients with idiopathic PH.\textsuperscript{10} That the CCR5 pathway plays an important role in atherosclerotic lesion formation is now well established, and several studies have documented protection against vascular lesions via inhibition of CCR5-mediated effects.\textsuperscript{6,17} Of note, several studies suggest a role in the pathogenesis of vascular injury for several HIV proteins, most notably HIV-1 gp120, which binds directly to CCR5.\textsuperscript{18–20}

The potential role for inflammatory processes in human PH, together with the importance of CCR5 in HIV infection and potentially in HIV-related PH or PH resulting from other causes, prompted us to investigate the CCR5 pathways in the pulmonary vascular remodeling process. To this end, we used several approaches: We evaluated CCR5 expression and localization in lung tissue from patients with PH and CCR5\textsuperscript{−/−} mice exposed to chronic hypoxia, and we assessed the effect of maraviroc-induced CCR5 inhibition on PH induced in mice by exposure to chronic hypoxia or SIV/HYPOXIA. For these studies, we used CCR5 knock-in mice expressing human CCR5 (h-CCR5ki) because maraviroc does not bind to murine CCR5,\textsuperscript{21–23} and we conducted cell studies in bone marrow (BM)–chimeric mice generated from CCR5\textsuperscript{−/−} and wild-type (WT) mice to investigate whether the CCL5-CCR5 pathway led to PH through direct effects on pulmonary vascular cells or through cross-talk between macrophages and pulmonary artery SMCs (PA-SMCs).

Methods

Patients

Human lung tissue was obtained from 6 patients with idiopathic pulmonary artery hypertension who underwent lung transplantation at the University Hospital of Leuven, Leuven, Belgium. The study protocol was approved by the Institutional Ethics Committee of the University Hospital of Leuven under agreement S51577, and written informed consent was obtained from each patient. Control lung tissue was obtained from 6 patients undergoing lung resection surgery for localized lung tumors at the Montsouris Mutualist Institute in Paris, France. The control subjects had a ratio of forced expiratory volume at the first second of expiration to forced vital capacity >70%; none of the patients or control subjects had chronic cardiovascular, hepatic, or renal disease or a history of cancer chemotherapy. This study was approved by the institutional review board of the Henri Mondor Teaching Hospital (Creteil, France). All control subjects signed an informed consent document before study inclusion. Pulmonary tissue was snap-frozen and then stored at −80°C until use. Lung tissue samples collected during surgery were used for in situ immunohistochemical studies and CCR5 protein level determinations. PA-SMCs were isolated from control lung tissues.

Macaque Tissue Analysis

Lung tissues were collected from Chinese rhesus macaques infected 1 year earlier with Simian immunodeficiency virus (SIV) Δ-B670. SIV-infected macaques reported previously to exhibit increased pulmonary artery pressure resulting from histopathological changes in the pulmonary vessels were used for CCR5 immunohistochemical analysis in comparison with controls.\textsuperscript{24}

Mouse Models

CCR5-deficient, h-CCR5ki, and WT C57Bl/6j mice are described in the Methods section of the online-only Data Supplement. BM-chimeric mice were generated (see the online-only Data Supplement Methods) and maintained under pathogen-free conditions at the functional investigations animal facility at the Pitié-Salpêtrière Teaching Hospital, Paris, France.\textsuperscript{24} The experiments were performed under agreement 94-28245 at a level 2 animal platform at the INSERM-Unit 955, Créteil, France.

Exposure to Chronic Hypoxia

Male mice 3 months of age were exposed to chronic hypoxia (9% O\textsubscript{2}) in a ventilated chamber (Biospherix, New York, NY).\textsuperscript{26} The hypoxic environment was a mixture of air and nitrogen. The chamber was opened daily for 1 hour if drug treatment was necessary and twice a week otherwise to replenish the food and water supplies and to clean the cages. Mice subjected to SU5416/hypoxia received an intraperitoneal injection of SU5416 20 mg/kg once a week during hypoxia exposure. Maraviroc obtained from Pfizer was administered daily by oral gavage (200 mg/kg).

Hemodynamics and Ventricular Weight Measurements

At the specified time points after hypoxia exposure, mice were anesthetized, and hemodynamic and ventricular weight measurements were performed as previously described.\textsuperscript{26}

Antibodies, Recombinant Proteins, and Reagents

Antibodies for immunohistochemistry, Western blot, and fluorescence-activated cell sorting; recombinant proteins (CCL5 and gp120); and SU5416 are described in the online-only Data Supplement Methods.

Mouse Lung Tissue Analysis

Lung tissue sections for immunostaining and immunolabeling were prepared as described in the online-only Data Supplement Methods. Total RNA and protein were extracted from the right lung of each animal. Total RNA was used for real-time polymerase chain reaction, and total protein was used for Western blot and ELISA. The immunostaining, Western blot, ELISA, and real-time polymerase chain reaction methods are described in the online-only Data Supplement Methods section.

Flow Cytometry

Lung and bronchoalveolar lavage (BAL) cell preparation and fluorescence-activated cell sorting analysis were performed as previously described.\textsuperscript{27,28} The methods are detailed in the data section in the online-only Data Supplement.
Cells Studies
Cultured human and mouse PA-SMCs were isolated by enzymatic digestion with the use of a mix of collagenase and elastase as previously described.\textsuperscript{28,29} Alveolar macrophages from BAL fluid were obtained and cultured as previously described.\textsuperscript{27} Serum-free conditioned media from BAL macrophages were collected after incubation for 24 hours.

Human PA-SMC proliferation was assessed by BrdU incorporation with the Cell Proliferation Elisa BrdU kit (Roche Diagnostics, Meylan, France) according to the manufacturer’s instructions. Mouse PA-SMC proliferation was assessed by MTT assay as previously described.\textsuperscript{29} Intracellular free calcium concentrations were measured in FURA-2–loaded cells using a 340/380 fluorescence ratio\textsuperscript{28,30} (see the online-only Data Supplement Methods section).

Assessment of Pulmonary Vascular Remodeling and Wall Thickness
Standard procedures were used to assess the morphological characteristics of pulmonary muscular arteries in lung tissue sections stained with hematoxylin and eosin. Vascular remodeling and wall thickness were assessed as previously described.\textsuperscript{26}

Statistical Analysis
Quantitative variables from animal studies are reported as median and individual values. These variables were compared by use of the nonparametric Kruskal-Wallis test followed by the Mann-Whitney post hoc test with the Bonferroni correction for multiple comparisons. Data from cell studies are reported as mean±SEM and compared by

![Figure 1](http://circ.ahajournals.org/)

**Figure 1.** Increased expression of CCR5 in human pulmonary arterial hypertension (PH). A, CCR5 protein level measured by Western blot in total lung protein extracts from patients P1 through P6 with idiopathic PH (iPH) and control subjects C1 through C6. Individual values and medians (black bar) from 6 patients and 6 age- and sex-matched control subject. \( \text{**P < 0.01.} \) B, Representative micrographs of lung tissue from patients and control subjects. CCR5 (red), \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA) for SMC staining, von Willebrand factor (vWF) for endothelial cell staining, CD68 for macrophage staining (green), or Dapi for nucleus staining (blue). No positive immunoreactivity was detected in sections incubated with the appropriate control IgG followed by secondary anti-rabbit and anti-mouse antibodies. Bar, 40 μm.
use of the Mann-Whitney test with the Bonferroni correction. For quantitative polymerase chain reaction data, the values were normalized, and a Wilcoxon rank-sum test was performed. Values of \( P < 0.05 \) were considered significant.

Results
Expression of CCR5 was increased in lungs from patients with idiopathic PH, mice with hypoxia-induced PH, and SIV-infected macaques. In explanted lungs from patients with idiopathic PH, we found marked increases in CCR5 protein levels compared with healthy donor lungs (Figure 1A). Immunofluorescence staining for CCR5 protein in lung tissues from the patients predominated in PA-SMCs from the hypertrophied media of pulmonary vessels, endothelial cells, and macrophages, as shown by double-immunofluorescence staining for CCR5 and \( \alpha \)-smooth muscle actin, von Willebrand factor, and CD68, respectively, of paraffin-embedded lung sections (Figure 1B–1D). CCR5 immunostaining was more marked in areas characterized by greater severity of medial hypertrophy. Immunostaining for CCR5, which was markedly increased in remodeled pulmonary vessels from SIV-infected macaques compared with controls, also predominated in the hypertrophied medial layer (Figure I in the online-only Data Supplement).

We then evaluated the expression of CCR5 and its ligands in lungs from WT mice developing hypoxic PH. Hypoxia exposure was followed by progressive increases in right ventricular systolic pressure and in the right ventricular hypertrophy index (right ventricular weight/left ventricle plus septum weight), which became significant on day 3 compared with control normoxic mice and then continued until day 18 (Figure 2A). Lung CCR5 mRNA levels were increased on day 3 and remained elevated until day 18 of hypoxia exposure (Figure 2B). CCL3, CCL4, and CCL5 mRNA levels were also elevated on day 3 (Figure 2B), and the corresponding protein levels increased from day 8 to 18 (Figure 2C).

CCR5 gene deficiency abrogates the development of hypoxic PH in mice. Our finding of CCR5 overexpression in PH prompted us to test whether CCR5 gene deletion altered PH development in mice. After hypoxia exposure for 18 days, right ventricular systolic pressure was significantly lower and right ventricular hypertrophy was less severe in CCR5 \(-/-\) mutants than in WT mice (Figure 3A). Furthermore, distal pulmonary vessels exhibited less muscularization in CCR5 \(-/-\) than in WT mice, with a smaller percentage of dividing Ki67-positive pulmonary vascular cells (Figure 3A). In both CCR5 \(-/-\) and WT mice, the total number of lung macrophages determined by either flow cytometry (Figure 3B) or immunohistochemical analysis (data not shown) remained unchanged after 18 days of hypoxia exposure and did not differ between mouse strains. However, the number of macrophages surrounding pulmonary vessels (Figure 3C) or present in BAL fluid (Figure 3D), which did not differ between CCR5 \(-/-\) and WT mice under normoxic conditions, increased markedly from normoxia to hypoxia in WT mice but not in CCR5 \(-/-\) mice. Consistent with decreased perivascular and alveolar macrophage recruitment, CCR5 \(-/-\) mice had lower counts of lung inflammatory (CCR2 hi) monocytes but not of constitutive (CX3CR1 hi) monocytes compared with WT mice during both normoxia and hypoxia (Figure II in the online-only Data Supplement).

Figure 2. Increased expression of CCR5 and its ligands in murine hypoxia-induced pulmonary arterial hypertension (PH). A, Effect of hypoxia on PH development. Graphs of right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (right ventricle/ left ventricle plus septum weight [RV/(LV+S)]) in wild-type mice exposed to hypoxia for various durations. Both individual and median values are shown. \( * P < 0.016 \), \( ** P < 0.0033 \) vs normoxia. B, Lung mRNA levels for CCR5 and its ligands CCL5, CCL3, and CCL4 measured with quantitative real-time polymerase chain reaction after hypoxia exposure for various durations. Both individual and median values are shown. \( * P < 0.025 \), \( ** P < 0.005 \) vs normoxia. C, Lung CCL5, CCL3, and CCL4 protein levels measured by ELISA. Both individual and median values are shown. \( * P < 0.025 \), \( ** P < 0.005 \) vs normoxia.
Maraviroc-induced CCR5 inhibition protects against PH development in h-CCR5ki mice. We hypothesized that pharmacological CCR5 inhibition with maraviroc altered PH development. Because maraviroc does not bind to murine CCR5,21–23 we used h-CCR5ki mice for pharmacological studies and immunohistochemical analyses of CCR5-expressing cells. After 18 days of hypoxia, the h-CCR5ki mice had a PH severity similar to that in WT mice and showed large increases in lung CCR5 protein levels (Figure 4A) and CCR5 staining in the PA-SMC layer of remodeled vessels (Figure 4B), whereas CCR5 staining in pulmonary artery endothelial cells and macrophages was less affected by PH development (Figure 4C and 4D). Maraviroc 200 mg·kg\(^{-1}\)·d\(^{-1}\) markedly attenuated but did not fully abrogate PH development as assessed by right ventricular systolic pressure, right ventricle/left ventricle plus septum weight, and distal pulmonary artery muscularization in chronically hypoxic h-CCR5ki mice (Figure 5A); this treatment had no effect in WT mice. The protective effect of maraviroc in h-CCR5ki mice coincided with a decrease in the percentage of Ki67-positive cells (Figure 5A). Consistent with our findings in WT and CCR5\(^{-/-}\) mice, we found that the total lung macrophage count remained unchanged after 18 days of hypoxia exposure, with no effect of maraviroc treatment (Figure 5B). In contrast, perivascular and BAL fluid macrophage numbers were substantially decreased by maraviroc treatment in hypoxic h-CCR5ki mice and unchanged in hypoxic WT mice (Figure 5C and 5D). In contrast to our finding in CCR5\(^{-/-}\) mice, lung monocyte subset counts were unaltered by maraviroc in h-CCR5ki mice (data not shown). Similar effects of maraviroc on PH severity were obtained in another model of PH induced in mice by simultaneous hypoxia exposure and treatment with the vascular endothelial growth factor receptor inhibitor SU5416 (Figure III in the online-only Data Supplement). In addition to its preventive effect, maraviroc from day 15 to 30 partially reversed PH in chronically hypoxic mice (Figure IV in the online-only Data Supplement).
BM cells did not differ in terms of PH parameters. However, WT mice reconstituted with CCR5−/− BM cells also benefited from some protection against PH compared with WT mice reconstituted with WT BM cells and exhibited a significant decrease in perivascular macrophages (Figure 6B).

CCR5 activation leads to proliferation of cultured human PA-SMCs, which is inhibited by maraviroc. To establish that cultured human PA-SMCs express CCR5 protein, we performed a double-immunostaining analysis for CCR5 and α-smooth muscle actin (Figure 7A). We found strong CCR5 immunostaining in all cultured PA-SMCs, which colocalized with the membrane-bound transient receptor potential channel 4 (Figure 7B). CCR5 expression increased in PA-SMCs exposed to hypoxia, and this effect was unaffected by maraviroc (Figure V in the online-only Data Supplement). We next assessed the ability of CCL5 to induce proliferation of human PA-SMCs. CCL5 added to human PA-SMCs cultured in serum-free media produced a concentration-dependent increase in BrdU incorporation (Figure 7C) that was not observed with similar concentrations of CCL3 or CCL4 (data not shown). Of note, mouse and human recombinant CCL5 produced similar levels of human PA-SMC stimulation (data not shown). Cell pretreatment with maraviroc (10 μmol/L) completely abolished PA-SMC growth induced by CCL5 but did not affect growth induced by platelet-derived growth factor (Figure 7D). Because SMC proliferation requires intracellular calcium mobilization, we then analyzed the ability of CCL5 to increase the PA-SMC cytoplasmic calcium concentration [Ca2+]i as a means of determining whether PA-SMC CCR5 was functionally coupled. CCL5 induced a transient [Ca2+]i increase in cells cultured in Ca2+-free medium (Figure 7E and 7H). Cell pretreatment with maraviroc (10 μmol/L) completely abolished [Ca2+]i changes induced by CCL5, whereas the transient [Ca2+]i increased by PDGF was not abrogated by maraviroc (Figure 7E, 7G, and 7H).

In addition, we found that treatment of human PA-SMCs with picomolar concentrations of the HIV-1 protein gp120 stimulated growth and increased intracellular calcium, both responses being inhibited by maraviroc (Figure 7D, 7F, and 7H). The heat-inactivated gp120 was without effects (Figure VI in the online-only Data Supplement).

Alveolar macrophages contribute to CCR5-mediated PA-SMC proliferation. We then examined the effects of CCL5 on mouse PA-SMCs. CCL5 stimulated the growth of PA-SMCs derived from WT and h-CCR5ki mice but, in contrast to PDGF, failed to alter PA-SMCs from CCR5−/− mice (Figure VII in the online-only Data Supplement). In h-CCR5ki and WT mice, CCL5 produced a concentration-dependent increase in cell proliferation that was not observed with a similar concentration of CCL3 or CCL4 (data not shown). Of note, mouse and human recombinant CCL5 stimulated h-CCR5ki PA-SMC cells similarly (data not shown). Cell pretreatment with maraviroc (10 μmol/L) completely abolished the growth-promoting effect of CCL5 on PA-SMCs derived from h-CCR5ki mice but not from WT mice (Figure VII in the online-only Data Supplement). Similarly, maraviroc abolished the migration of h-CCR5ki cells but not of WT cells stimulated by CCL5 (Figure VIII in the online-only Data Supplement).
To investigate whether lung macrophages contributed to PA-SMC growth, we assessed the effects of conditioned medium from cultured alveolar macrophages on PA-SMC growth. Compared with CCL5, supernatants of alveolar macrophages from WT mice induced greater stimulation of h-CCR5ki PA-SMC proliferation (Figure 8A). Maraviroc treatment abolished PA-SMC proliferation induced by CCL5 and attenuated that induced by macrophage conditioned media (Figure 8A).

The CCL5 level in macrophage conditioned media from hypoxic mice was increased compared with those from normoxic mice (Figure IX in the online-only Data Supplement). However, compared with normoxic macrophage conditioned medium, hypoxic macrophage conditioned medium did not induce greater cell proliferation (data not shown).

We then evaluated whether CCR5-mediated macrophage stimulation promoted the growth of cultured PA-SMCs. To this end, we used macrophages from h-CCR5ki mice and PA-SMCs from WT mice. As shown in Figure 8B, CCL5 potentiated the PA-SMC growth-promoting effects of alveolar macrophages, and this effect was slightly reduced by maraviroc. As expected, maraviroc did not affect CCL5-induced proliferation of PA-SMCs from WT mice.

**Discussion**

The role for chemokines and chemokine receptors in the progression of PH is receiving considerable attention. The recent addition of CCR5 receptor antagonists to the treatment armamentarium for HIV infection prompted us to evaluate...
the CCR5 receptor. To assess the role for CCR5 in experimental PH, we used both gene disruption and pharmacological CCR5 inactivation in mice. CCR5 receptor antagonists such as maraviroc do not bind to murine CCR5; therefore, we used h-CCR5ki mice for pharmacological studies and immunohistochemical analyses of CCR5-expressing cells. Using a combination of studies on human lung tissues and derived cultured cells, macaque lung tissues, transgenic mouse models, and mouse cells, we demonstrated that CCR5 activation in PA-SMCs and lung macrophages played a major role in PH progression.

Our findings provide strong support for CCR5 inhibition as a novel therapeutic approach in PH. Marked upregulation of the CCR5 receptor was found in our PH patients. As previously emphasized, inflammation is a common feature in human PH that is not targeted by any of the current treatments. An important point when considering chemokines as targets for PH treatment is that some ligand-receptor pairs are involved in activating not only inflammatory cells but also constitutive vascular cells directly involved in the remodeling process. We found that CCR5 in lungs from patients with PH was strongly expressed not only by lung macrophages but also by PA-SMCs and pulmonary artery endothelial cells, most notably at sites of intense vascular remodeling. Moreover, strong CCR5 immunostaining was detected in the media of pulmonary vessels from SIV-infected macaques exhibiting marked pathological changes. Although previous studies documented CCR5 expression by systemic vessels, a potential direct role for CCR5 in the pathogenesis and progression of PH had not been considered previously.

Figure 6. Hypoxic pulmonary arterial hypertension (PH) development is altered in bone marrow–chimeric mice generated from CCR5−/− and wild-type (WT) mice. A, Graphs of right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (right ventricle/left ventricle plus septum weight [RV/(LV+S)]), pulmonary vessel muscularization, and percentages of dividing Ki67-positive cells in WT mice and CCR5−/− mice under normoxia or hypoxia. Representative micrographs of pulmonary vessels stained for Ki67. No immunoreactivity was detected in sections incubated with rabbit IgG control and secondary anti-rabbit antibody. Bar, 50 μm. B, Graphs of F4/80-positive macrophage counts around lung microvessels detected with α-smooth muscle (α-SMA) actin staining. Micrographs showing stained lung vessels surrounded with macrophages in WT and CCR5−/− mice under hypoxia: F4/80 (red), α-SMA (green), and Dapi (blue). No positive immunoreactivity was detected in sections incubated with the appropriate IgG control followed by secondary anti-rabbit and anti-rat antibodies. For all graphs, both individual and median values are represented. Bar, 50 μm. *P<0.016, **P<0.0033.
Here, we used the well-known animal model of hypoxic PH in which inflammation is considered a potential contributor. In most species, hypoxia exposure immediately induces overexpression of inflammatory mediators such as CCL2, interleukin-6, and interleukin-1β, which usually precedes the development of hypoxic PH. In some species such as rats, which usually precedes the β-interleukin-6, and interleukin-1β overexpression of inflammatory mediators such as CCL2, were lower in CCR5−/− mice compared with WT mice and decreased with maraviroc treatment in h-CCR5ki mice, suggesting a contribution of CCR5 to macrophage recruitment during hypoxia exposure. Thus, pharmacological inhibition or genetic deletion of CCR5 produced similar effects, markedly diminishing pulmonary vascular remodeling and PA-SMC proliferation and decreasing perivascular and alveolar macrophage counts.

CCR5 exerted a strong functional effect on PA-SMCs when stimulated by CCR5 ligands in low physiological concentrations. Our finding that CCL5 was more potent than CCL3 or CCL4 for activating PA-SMCs is consistent with the differences in CCR5 ligand efficacy across cell types. PA-SMCs from h-CCR5ki and WT mice responded similarly to CCL5 on human PA-SMC proliferation measured by BrdU incorporation. Data are mean±SEM of 10 to 15 values from at least 3 different experiments. *P<0.0016, **P<0.0033 vs vehicle. D, Effect of platelet-derived growth factor (PDGF; 50 ng/mL), CCL5 (10 nmol/L), and gp120 (100 pmol/L) on intracellular calcium imaging in FURA-2–loaded cells in the presence of maraviroc (MVC; 10 μmol/L) or vehicle. Data are mean±SEM of 10 to 15 values. *P<0.05. G. Effects of CCL5 (10 nmol/L), PDGF (50 ng/mL), and gp120 (100 pmol/L) on intracellular calcium imaging in FURA-2–loaded cells in the presence of MVC (10 μmol/L; dotted line) or vehicle (solid line). Each curve represents the mean of 10 to 20 cells from the same experiment. The capacity of the agonist to trigger Ca2+ mobilization from intracellular stores was assessed in the absence of extracellular calcium (EGTA; 100 μmol/L), and Ca2+ influx through plasma membrane channels was assessed after the addition of extracellular Ca2+ (2 mmol/L). H, Bar graph comparing the intracellular calcium peak induced by PDGF, CCL5, and gp120 with or without MVC. Data are mean±SEM of 25 to 100 cells from at least 3 different experiments. *P<0.05, **P<0.001.

Both CCR5−/− mice and h-CCR5ki mice treated with maraviroc exhibit attenuations in PH severity with decreases in distal pulmonary artery muscularization and dividing Ki67-positive PA-SMCs. Interestingly, these effects were not associated with changes in total counts of lung macrophages or lung monocyte subsets. However, the macrophage counts around vessels and in BAL fluid, which increased during hypoxia exposure, were lower in CCR5−/− mice compared with WT mice and decreased with maraviroc treatment in h-CCR5ki mice, suggesting a contribution of CCR5 to macrophage recruitment during hypoxia exposure. Thus, pharmacological inhibition or genetic deletion of CCR5 produced similar effects, markedly diminishing pulmonary vascular remodeling and PA-SMC proliferation and decreasing perivascular and alveolar macrophage counts.
are consistent with a major role for the CCL5/CCR5 pathway in mediating pulmonary vascular remodeling through direct PA-SMC activation. In keeping with such a role, protection against PH was similar in CCR5−/− mice reconstituted with WT BM and in CCR5−/− mice reconstituted with CCR5−/− BM cells. These results are consistent with a prominent effect of CCR5-mediated signals on resident lung cells and, more specifically, on PA-SMCs, given the decrease perivascular and alveolar macrophage counts, supporting a role for BM-derived inflammatory cells possibly mediated by the CCL5/CCR5 pathway. Our finding that lung macrophages were the main cell type strongly expressing CCR5 in addition to vascular cells supports a contribution of these cells to PH through the CCL5/CCR5 pathway.

Conditioned medium from alveolar macrophages stimulated PA-SMC growth, and this effect was only partially inhibited by maraviroc, suggesting that macrophages released growth-promoting mediators other than CCL5.27 In addition, the growth of WT PA-SMCs exposed to conditioned media of CCL5-stimulated macrophages from h-CCR5ki mice was increased compared with vehicle-treated macrophages, indicating that the CCL5/CCR5 pathway contributed to activate the monocyte/macrophage lineage involved in pulmonary vascular remodeling during PH progression. However, this effect was only slightly altered by maraviroc treatment, a finding consistent with the fact that CCL5 activation of alveolar macrophages can be mediated by receptors other than CCR5.7 Taken together, these results indicate that the CCL5-CCR5 pathway plays a major role in the pulmonary vascular remodeling process through both direct PA-SMC activation and lung macrophage recruitment and activation.

These findings are highly relevant to the clinical management of patients with PH. That long-term maraviroc treatment markedly attenuated the development of hypoxic PH in h-CCR5ki mice but had no effect in WT mice indicates that maraviroc protected against PH by specifically binding to human CCR5 and was devoid of CCR5-independent effects. Moreover, we based the maraviroc dose for our in vivo studies in h-CCR5ki mice on the plasma levels achieved in humans. Thus, our results are consistent with a pharmacological action of maraviroc in humans, in whom the drug blocks the CCR5 receptor in micromolar plasma concentrations.13 These findings carry major implications for the treatment of HIV-related PH, which has a clinical presentation and an underlying pathology that are similar to those of idiopathic or associated PH and a prevalence in HIV-infected patients that is 1000-fold higher than the prevalence of idiopathic PH in the general population.3 Moreover, several studies of vascular injury pathogenesis in HIV-infected patients have implicated HIV proteins, most notably gp120, which binds to CCR5,19,20 Our results showing a growth-stimulating effect of gp120 on human PA-SMCs, and its inhibition by maraviroc may be of major clinical relevance. CCR5 inhibitors may therefore be of considerable clinical benefit in HIV-infected patients who develop PH. They should be considered for addition to other antiretroviral therapies such as HIV protease inhibitors, which we have previously shown to interfere with the pathophysiology of PH.25

The identification of specific therapeutic targets in the chemokine system is crucial to the development of treatments that counteract the progression of PH resulting from various causes. Our results also support the potential usefulness related to CCR5 inactivation. Consistent with this possibility, WT mice reconstituted with CCR5−/− BM cells showed substantial protection from PH, together with decreased perivascular macrophage counts, supporting a role for BM-derived inflammatory cells possibly mediated by the CCL5/CCR5 pathway.
of CCR5 inhibition in patients with other forms of PH. We characterized specific roles for the CCL5/CCR5 pathway in mediating pulmonary vascular remodeling using a CCR5 receptor antagonist designed to inhibit HIV cell entry. Thus, using available drugs to block the CCR5 signaling pathway might benefit patients with PH complicating HIV infection or with other forms of PH.

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Disclosures
None.

References
Inflammation is an important contributor to the pathogenesis of human pulmonary arterial hypertension (PH). Identifying specific therapeutic targets in the chemokine system is therefore crucial to develop new strategies against PH. The CCR5 receptor, which is expressed by vascular and inflammatory cells, acts as a coreceptor for HIV cell entry and is a therapeutic target in HIV infection. The addition of CCR5 receptor antagonists to the treatment armamentarium for HIV infection, together with the high prevalence of PH in HIV-infected patients, prompted us to investigate the role for CCR5 in the pathogenesis of PH. We found high CCR5 expression in lung vessels from patients with idiopathic PH, mice with hypoxia-induced PH, and Simian immunodeficiency virus–infected macaques. Pharmacological CCR5 inhibition by maraviroc or genetic CCR5 deletion markedly decreased hypoxia-induced PH and lung perivascular and alveolar macrophages in mice. Maraviroc, which does not bind to murine CCR5, was efficient in human-CCR5 knock-in mice but not in wild-type mice. Studies of bone marrow–chimeric mice and cultured cells demonstrated that CCR5 was involved in PH via stimulation of both smooth muscle cells and macrophages by CCR5 ligands, primarily CCL5. These results indicate a major role for the CCL5-CCR5 pathway in the pathogenesis of PH. Our results not only carry major implications for the treatment of HIV-related PH but also support the potential usefulness of CCR5 inhibition in patients with other forms of PH. Thus, using available drugs to block the CCR5 signaling pathway might benefit patients with PH complicating HIV infection or with other forms of PH.
CCR5 as a Treatment Target in Pulmonary Arterial Hypertension
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SUPPLEMENTAL DATA

CCR5 as a Treatment Target in Pulmonary Arterial Hypertension

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SUPPLEMENTAL METHODS

Animals. Mice (C57Bl/6j) were used according to institutional guidelines that complied with national and international regulations. All animal experiments were approved by the Institutional Animal Care and Use Committee of the French National Institute of Health and Medical Research (INSERM)-Unit 955, Créteil, France. Transgenic CCR5-deficient mice were produced and bred at the INSERM-Unit 945 as previously described. In addition, h-CCR5 ki mice were generated and provided by Pfizer. Briefly, the sequence encoding human CCR5 was cloned in a target vector and transfected to embryonic stem (ES) cells in order to obtain an ES clone containing the homologous recombination. Chimeric mice were then produced through aggregation to obtain heterozygous mice. First-generation of h-CCR5 ki mice were obtained by breeding two heterozygous mice. The h-CCR5 ki mice thus obtained were backcrossed to obtain a C57/BL6 background. To generate bone-marrow (BM) chimeras, we irradiated wild-type (WT) and CCR5 knock-out mice with a total dose of 10 Gy. Then, on the same day, each mouse received an intravenous injection of 5·10^6 total BM cells from WT and CCR5 donors. Animal experiments were approved by the institutional animal care and use committee of the Pitié-Salpêtrière School of Medicine, Paris, France.

Antibodies, recombinant proteins and reagents. Mouse monoclonal Ab (mAb) Zy12 against human CCR5 was obtained from TEBU-Bio, Santa Cruz Biotechnology, Santa Cruz, CA), rat mAb against F4/80 from Serotec (Düsseldorf, Germany), and mouse mAb against CD68 from Dako (Glostrup, Denmark). Polyclonal rabbit Abs against α-SMA, vWF, and Ki67 were purchased from Abcam (Abcam, Cambridge, MA). TRPC4 (#ACC-018) was obtained from Alomone Laboratories (Jerusalem, Israel) and mouse mAb against β-actin from Sigma (Saint Louis, MO). As negative controls, we used corresponding IgG antibodies (R&D Systems, Lille, France) in appropriate concentrations with the same protocol as for the anti-CCR5, anti-vWF, anti-α-SMA, anti-CD68 and anti-F4/80 antibodies. All Alexa secondary Abs were from Molecular Probes (Life Technologies, Carlsbad, CA). The mAbs used for flow cytometry were anti-CD11b (clone M1/70), anti-Ly-6G (clone 1A8), anti-NK1.1 (clone PK136), and anti-CD11c-PE (Clone HL3) from Becton Dickinson (Franklin Lakes, NJ); and anti-
neutrophil (clone 7/4) and anti-F4/80 (clone BM8) from Serotec. All cytokines, PDGF-BB, mouse recombinant CCL5, and human recombinant CCL5 were obtained from R&D Systems. Gp120 R5 tropic (97CN001 strain) was obtained from Interchim (Montluçon, France); heat-inactivated gp120 was obtained by heating at 90°C for 30min. SU5416 was obtained from SIGMA (Lyon, France).

**Tissue preparation and immunostaining.** The left lung from each animal was inflated with a 1:1 mixture of phosphate-buffered saline (PBS) and OCT embedding medium or 4% formalin (m/v). Formalin-inflated lungs were embedded in paraffin and cut into 5 µm-thick sections for hematoxylin and eosin staining and immunochemistry staining. In addition, OCT-inflated lungs were embedded in OCT then frozen at -80°C and cut into 10 µm-thick slices using a Leica Cryostat (Leica Microsystems, Wetzlar, Germany). The cryosections were used for immunofluorescence staining. Paraffin-embedded sections were deparaffinized using xylene and a graded series of ethanol dilutions then incubated in citrate buffer (0.01 M, pH 6) at 90°C for 20 minutes. Endogenous peroxidase activity was blocked with 3% H$_2$O$_2$ and 10% methanol in PBS for 10 minutes. Ki67 staining was preceded by a permeabilization step with 0.1% Triton X-100 in PBS for 10 min. Slides were incubated for 60 minutes in 1% bovine serum albumin and 5% goat serum in PBS then incubated overnight with the primary antibody. We used the ABC Vectastain kit (Vector Labs, Burlingame, CA) to mark the primary antibodies according to the user’s guide. The staining substrate was DAB (FastDAB, Sigma) and the sections were counterstained with hematoxylin.

Cryosections were defrosted, fixed with acetone at 4°C for 10 min, and dried for 10 min. After hydration in PBS, endogenous peroxidase activity was blocked with 3% H$_2$O$_2$ and 10% methanol in PBS for 10 minutes. For CCR5 staining, sections were saturated using the MOM kit according to the manufacturer’s instructions (Vector Labs) and incubated overnight at 4°C with the primary antibody (CCR5/α-SMA or CCR5/vWF or CCR5/Mac-3 or CCR5/CD68). We used α-SMA for SMC detection, vWF for endothelial cell (EC) detection, Mac-3 for mouse macrophage detection, and CD68 for human macrophage detection. On the next day, the sections were washed with PBS. Primary CCR5 was revealed using an amplification step and vWF/α-SMA or Mac-3 using a secondary antibody directly conjugated with Alexa (see Antibodies section). For CCR5 revelation, which required an amplification
step, we used the ABC Vectastain kit (Vector Labs) to mark the primary antibodies according to the user’s guide. Substrate staining was detected using fluorescent peroxidase according to the manufacturer’s instructions (PerkinElmer, Waltham, MA). All nuclei were visualized using DAPI (Sigma). Adobe Photoshop was used according to guidelines to construct multi-channel images, select specific regions of interest, and apply minor alterations to contrast and brightness uniformly across the entire figure panel.

**Flow cytometry.** The lung cells were prepared as follows. The lungs were isolated, minced, and digested with 400 units of collagenase D (Roche Diagnostics, Mannheim, Germany) for 30 minutes. Cell suspensions were filtered through a 70-µm cell strainer (BD Biosciences, Bedford, MA) and the leukocytes were collected using Ficoll-Paque (GE Healthcare, Cleveland, OH) and washed in phosphate buffer saline (PBS) prior to FACS staining. BAL fluid cells were collected as previously described. For FACS staining, lung or BAL fluid cells were labeled for flow cytometry using the appropriate Ab (see online supplemental methods section). Samples were acquired on a FACS Calibur cytometer (Becton Dickinson, Franklin LAKES, NJ) using Cell Quest Pro then analyzed using FlowJo (Tree Star, Ashland, OR) software. Absolute cell counts were computed using a fixed number of nonfluorescent 10-µm Polystrene® Carboxylate Microspheres (Polysciences, Niles, IL) as a reference.

**Alveolar macrophage isolation, culture, and conditioned media preparation.** Animals aged 3 months were anesthetized with ketamine/xylazine (60 mg/Kg and 10 mg/Kg, respectively). Macrophages from BAL fluid were obtained as previously described. For cell-culture experiments, BAL fluids were obtained as previously described. The fluorescence-activated cell-sorting (FACS) results showed that over 90% of the cells were macrophages (see flow cytometry section). To assess cell proliferation, macrophage-conditioned media were obtained as follows. Macrophages from BAL fluid were seeded onto 48-well plates in DMEM supplemented with 10% fetal calf serum (FCS), 10 mM L-glutamine, 100 IU penicillin, and 100 µg/mL streptomycin then left to grow for 4 hours. Cell viability as assessed by Trypan Blue exclusion was greater than 85% in all groups. To produce conditioned-medium cells, the medium was replaced by serum-free DMEM with or without murine...
recombinant CCL5 (2 ng/mL) and with or without CCR5 antagonist (maraviroc, 10 µM). For the PA-SMC proliferation assay, macrophage-conditioned media were diluted 2-fold with fresh serum-free medium and applied on PA-SMCs for 72 hours.

**Exposure to hypoxia.** For in vitro experiments, cells were placed in a hypoxic chamber containing 1% O₂ and 5% CO₂ at the indicated times.

**Cell migration assay.** The cell migration assay was performed as previously described. Briefly, PA-SMCs were subjected to growth arrest in FCS-free medium for 24 or 48 hours then resuspended at 40·10⁶ cells/mL in culture medium containing 15% FCS and 0.3% agarose. These cells were plated by forming an agarose spot in the center of each well of a 24-well tissue culture plate. The preparation was left at 4°C for 20 min to allow the agarose to gel. Then, 900 µL of medium was added to cover the drops. The preparation was incubated at 37°C in 5% CO₂ for 24 or 48 hours. Samples were fixed and stained using the Diff-Quick kit (Siemens Healthcare Diagnostics, Saint Denis, France). Images were imported into Image J analysis software (http://rsbweb.nih.gov/ij/) for calculation of cell migration under each condition.

**Measurement of intracellular free calcium concentrations** ([Ca²⁺]i). Human PA-SMCs were loaded with 2 µM Fura-2-AM for 45 min at 37°C then kept in serum-free medium for 30 min before the experiment. HEPES buffer (in 116 mM/L NaCl, 5.6 mM/L KCl, 1.2 mM/L MgCl₂, 5 mM/L NaHCO₃, 1 mM/L NaH₂PO₄, and 20 mM/L HEPES, pH 7.4) was used for the experiments. Single images of fluorescent emission at 510 nm under excitation at 340 and 380 nm were taken every 5 s. Changes in [Ca²⁺]i in response to the indicated agonist were calculated using the Fura-2 340/380 fluorescence ratio according to the equation of Grynkeiwicz or using the 380em/380em(basal) ratio.

**Cell immunostaining.** PA-SMCs were cultured on Labteck (10 000 cells/well), fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X100, and immunostained with the indicated
primary antibodies. Visualization was achieved using the appropriate Alexa Fluor-conjugated Ab (see Antibodies section).

**Fluorescence microscopy analysis.** Tissue sections were examined with an AxioCam microscope (Zeiss, Oberkochen, Germany). Immunocytochemistry slides were examined under a Zeiss LSM-510 confocal scanning laser microscope equipped with a 25 mW argon laser and a 1 mW helium-neon laser, using a Plan Apochromat 63X objective (NA 1.40, oil immersion). Green fluorescence was observed with a 505-550 nm band-pass emission filter under 488 nm laser illumination. Red fluorescence was observed with a 560 nm long-pass emission filter under 543 nm laser illumination. Pinholes were set at 1.0 Airy units. Stacks of images were collected every 0.7 µm along the z-axis. All settings were kept constant to allow comparisons. For double immunofluorescence, dual excitation using the multitrack mode (images taken sequentially) was achieved using the argon and He/Ne lasers. ImageJ software was used according to the guidelines to construct multi-channel images and Z-projection of serial slides.

**Protein extraction and ELISA on homogenized lung tissue.** Lung-tissue specimens each weighing 30 mg and stored at -80°C were defrosted and homogenized in a Tissue Lyser® homogenizer (Qiagen, Courtaboeuf, France) using 300 µL of T-PER® tissue-protein extraction reagent (Thermo Scientific Pierce, Illkirch, France). The homogenate was centrifuged at 10,000 g for 5 min to remove tissue debris, and the supernatant containing the total lung lysate was used for ELISA. The total protein level in the lung lysate was quantified using the Biorad DC protein assay (Biorad, Marnes-la-Coquette, France). Before each ELISA, various dilutions of protein lysates were tested to ensure that the detected level fit within the standard linear range. All ELISA kits were obtained from R&D Systems.

**Western-Blot.** Total proteins were extracted as described above. Immunoblots were carried out using the above-listed antibodies and detected using an enhanced chemiluminescence detection system (Millipore, Molsheim, France). Densitometric quantification was normalized for the β-actin level using Gene Tools software (Ozyme, Montigny le Bretonneux, France).
Real-time quantitative PCR (RT-qPCR). Total mRNA was extracted from pulmonary artery ECs using RNeasy Protect Mini Kit (Qiagen, ZA Courtaboeuf, France). First-strand cDNA was synthesized in reversed transcribed samples, as follows: 1 µg total RNA isolated from cells, 100 ng Random Primers, 0.4 mM mixed dNTP, 40 U RNaseOUT, and 200 U Superscript II. All reagents were obtained from Life Technology. Quantitative PCR was performed using an ABI PRISM 7000 detection system (Applied Biosystems, ZA Courtaboeuf, France), with SYBR Green from Life Technology. To normalize for cDNA input load, mouse 18s and human SF3A1 were used as endogenous standards. The analysis was performed using the standard ΔΔCt method. Specific primers were as follows:

Mouse CCR5
Fwd 5’-CGGTGTTCATTTTCCAGCAA-3’
Rev 5’-TCTCCTGTGGATCGGTATAGAC-3’;

Mouse CCL5
Fwd 5’-GCCACGTCAAGGAGTATTTCT-3’
Rev 5’-CAAACACGACTGCAAGATTGGA-3’

Mouse CCL3
Fwd 5’-GCCCTTGCTTCTTCTCTGT-3’
Rev 5’-GGCATTCAGTTCCAGGTCAGT-3’

Mouse CCL4
Fwd 5’-GCTCGTGCTGGCTTCTG-3’
Rev 5’-GAGGTGTAAGGAAACAGCAGGAAGT-3’

Human CCR5
Fwd 5’-GCTCTAACAGGTTGGACCAAGCT-3’
Rev 5’-TGATGCAGCAGTGCGTCAT-3’
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: CCR5 expression in SIV-infected macaques. Representative micrographs of lung tissue from an SIV-infected macaque with remodeled pulmonary vessels and uninfected, normal control macaque. CCR5 (Red), α-SMA for SMC staining or CD68 for macrophage staining (Green). No positive immunoreactivity was detected in sections incubated with the appropriate control IgG followed by secondary anti-rabbit and anti-mouse antibodies. Bar= 40 µm.

Supplemental Figure 2: Comparison of inflammatory and resident monocyte counts in lungs of wild-type (WT) and CCR5−/− mice under normoxia or hypoxia. Graph of the number of 7/4hi inflammatory monocytes (CCR5hi monocytes) and 7/4low resident monocytes (CX3CR1hi monocytes) in 1 mg of lung tissue, analyzed using FACS. Both individual and median values are shown.*P<0.025 and **P<0.005. Micrographs show representative FACS profiles of cells from hypoxiaWT and CCR5−/− mouse lung tissue.

Supplemental Figure 3: Effect of the pharmacological CCR5 inhibitor maraviroc (MVC) on PH induced by chronic hypoxia combined with SU5416. (A) Graphs of right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (RV/[left ventricle plus septum (LV+S)] weight), pulmonary vessel muscularization, and percentages of dividing Ki67-positive cells in h-CCR5ki mice subjected to 18 days of chronic hypoxia combined with SU5416, with daily MVC (CCR5ki SU+MVC) or vehicle (CCR5ki SU+Ve). Representative micrographs of pulmonary vessels stained for Ki67. Red arrows show Ki67-positive nuclei. No immunoreactivity was detected in sections incubated with rabbit IgG control and secondary anti-rabbit antibody. Bar=50 µm. (B) Medial wall thickness as a percentage of fully muscularized intraacinar arteries. Representative micrographs of pulmonary vessels stained with hematoxylin and eosin. Bar=50 µm. For all graphs, both individual and median values are shown. *P<0.025, **P<0.005.
Supplemental Figure 4: Reversal of pulmonary hypertension by maraviroc (MVC) treatment in h-CCR5ki mice. RVSP, RV/[LV+S] weight ratio, and pulmonary vessel muscularization in normoxic and chronically hypoxic mice studied on days 15 and 30 after hypoxia exposure. MVC 200 mg/Kg/day was given from day 15 to day 30. Both individual and median values are shown. *P<0.025. Representative micrographs of pulmonary vessels stained with hematoxylin eosin. Bar=50 µm

Supplemental Figure 5: Effect of hypoxia on CCR5 expression in human PA-SMCs treated with maraviroc (MVC) or vehicle. CCR5 mRNA levels were quantified by RT-qPCR after 4 h exposure to hypoxia. Data are mean±SEM of 9 values of three different experiments. *P<0.05 (Wilcoxon Rank test).

Supplemental Figure 6: Mobilization of intracellular calcium in hu-PA-SMC by native or heat-inactivated gp120. Intracellular calcium imaging in FURA-2 loaded cells. The curve represents the mean of three cells responding to native gp120 (100 pM) recorded in the same experiment. The capacity of gp120 to trigger the mobilization of Ca^{2+} from intracellular store though receptor activation was assessed by addition of heat-inactivated (90°C, 30 min) gp120 (100 pM) followed by addition of native gp120 (100 pM). Heat-inactivated gp120 had no effect on intracellular Ca^{2+}, whereas the addition of native gp120 triggered the calcium mobilization in three out of four cells.

Supplemental Figure 7: Effect of CCL5 (250 pM) on growth of PA-SMCs from h-CCR5ki, WT, and CCR5^{-/-} mice in the presence of maraviroc (MVC, 10 µM) or vehicle. Values are expressed as percent of control values in DMEM starvation medium. Data are mean±SEM of 8-12 values of three different experiments. *P<0.05.

Supplemental Figure 8: Effect of CCL5 (10 nM) on migration of PA-SMCs from h-CCR5ki, WT, and CCR5^{-/-} mice. Values are expressed as percent of control values in DMEM starvation medium. Data are mean ±SEM of 8 values. *P<0.05. Representative micrographs of agarose spots after migration for 48 hours.
Supplemental Figure 9: CCL5 levels measured by ELISA in media conditioned by BAL fluid macrophages from normoxic and hypoxic mice. Both individual and median values are shown. *P<0.05

SUPPLEMENTAL REFERENCES


Supplemental Figure 1

Smooth muscle cells

SIV

Control

Macrophages

SIV

Control
Supplemental Figure 2
Supplemental Figure 3

A

RvSP (mm Hg)

RV/LV+S (%)

Muscularized vessel (%)

Hypoxia Hypoxia Hypoxia +SU+Ve +SU+MVC

Hypoxia Hypoxia Hypoxia +SU+Ve +SU+MVC

NS

NS

B

Media thickness (%)

Hypoxia Hypoxia Hypoxia +SU+Ve +SU+MVC

Hypoxia Hypoxia Hypoxia +SU+Ve +SU+MVC

NS
Supplemental Figure 4

![Graphs showing RVSP, RV/LV+S, and Muscularized vessels (%)]

- **RVSP (mm Hg)**
- **RV/LV+S (%)**
- **Muscularized vessels (%)**

**Hypoxia**
- **MVC**
  - 15 days
  - 30 days

**Normoxia**
- Hypoxia
  - 15 days
  - 30 days + Ve
  - 30 days + MVC
Supplemental Figure 5

![Bar chart showing CCR5 vs SF3A1 fold change in different conditions: Normoxia, Hypoxia + Ve, and Hypoxia + MVC. Stars indicate significant differences.](chart.png)
Supplemental Figure 6
Supplemental Figure 7

**h-CCR5 ki**

**WT**

**CCR5−/−**
Supplemental Figure 8

Cell migration stimulation vs DMEM (%) for h-CCR5 ki, WT, and CCR5-/-

h-CCR5 ki cells

DMEM    SVF 5%    CCL5 + Ve    CCL5 + MVC

CIRCULATION/AHA/2014/010757R1
Supplemental Figure 9