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A role for the CXCL12 receptor, CXCR7, in the pathogenesis of human pulmonary vascular disease

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ABSTRACT

Rationale: Given the critical role that endothelial cell dysfunction plays in the pathogenesis of pulmonary hypertensive diseases, we set out to establish if CXCR7, a receptor for the pro-angiogenic ligand, CXCL12, is expressed in the vasculature of human lung diseases and examine its role in mediating CXCL12 induced responses in primary pulmonary human microvascular endothelial cells.

Methods: Receptor and ligand expression was examined in control and explanted human hypertensive lungs, in human plasma, and in hypoxic rodent lungs, by ELISA and immunohistochemical studies. Functional *in vitro* experiments examined the role of CXCR7 in CXCL12-induced lung microvascular endothelial cell proliferation, migration, and wound regeneration and repair.

Results: CXCR7 is elevated in the endothelium of explanted human hypertensive lungs and circulating CXCL12 concentrations are significantly elevated in disease. We demonstrate that alveolar hypoxia similar to that found in lung disease increases CXCR7 expression in the pulmonary endothelium. Furthermore, CXCR7 is the receptor through which endothelial cell regeneration and repair, and proliferation, is mediated, whereas signaling via CXCR4 is essential for chemotactic cell migration.

Conclusions: **Our findings demonstrates that CXCR7 has a critical but previously unrecognized role to play in endothelial cell proliferation, suggesting that CXCR7-mediated signaling may be functionally important in pulmonary vascular diseases.**

Keywords: CXCR7, pulmonary vasculature, hypoxia, CXCR4, CXCL12.

INTRODUCTION

Pulmonary hypertension (PH) occurs in many forms, which are classified into different categories that reflect the diverse causes and sites of injury associated with disease (1). Irrespective of the initiating event, the underlying etiology is characterised by abnormal vascular remodeling, persistent vasoconstriction and increased pulmonary vascular resistance. The pulmonary endothelium is increasingly viewed as being critically involved in abnormal vascular remodeling and vasoconstriction in disease. For example, several rodent studies show that vascular endothelial growth factor (VEGF) receptor blockade, either alone or in combination with hypoxia, culminates in severe PH and the development of vascular lesions similar to those observed in patients with pulmonary arterial hypertension (PAH) (2-4). The conventional model of pulmonary endothelial dysfunction in PAH includes resident endothelial cell damage, loss of barrier integrity due to failure of repair and the development of characteristic vascular lesions in the small pulmonary arterioles (5, 6). More recently, a role for circulating endothelial progenitor cells in the pathology of vascular lesions in human PAH has been suggested (7).

In a recent study designed to identify genes selectively regulated in primary human pulmonary microvascular endothelial cells in response to hypoxia, we showed that chemokine (C-X-C motif) receptor 7 (CXCR7) was up-regulated in these cells *in vitro*, and in hypoxic murine lungs *in vivo* (8). CXCR7 was recently identified as a receptor for the chemokine ligand, CXCL12, which also signals via a second G-protein coupled receptor, CXCR4 (9, 10). Initial studies established that CXCR7 was an atypical chemokine receptor, with no detectable activation of heterotrimeric G-proteins upon ligand binding (9, 10). However, several modes of action for CXCR7 have now been described in different cells; developmental studies in zebrafish showed that CXCR7 acts as a scavenger receptor generating CXCL12 gradients along which primordial germ cells migrate (11-13), CXCR7 can act as a co-receptor for

CXCR4 in transiently transfected cells (14, 15), and CXCR7 has been shown to signal independently of CXCR4 via β-arrestin recruitment (16-18). Signaling via CXCL12 is now recognised to be important in the development of PH; recent studies show that CXCR4 is expressed on the human endothelium in patients with PAH, and blockade of this receptor in animal models prevents the development of hypoxic PH (7, 19). However, expression of CXCR7 has not been examined in the human endothelium nor has its role in CXCL12-induced responses in PH been defined. Therefore, the objectives of the present study were to establish if CXCR7 is expressed in human pulmonary hypertensive diseases and examine its role in mediating pulmonary microvascular endothelial cell responses to CXCL12.

MATERIALS AND METHODS

Additional details on Methods are provided in the on-line supplementary material.

Human studies

Immunohistochemistry on explanted lung tissue obtained at the time of lung transplantation from IPAH patients and patients with usual interstitial pneumonia with pulmonary hypertension (UIP-PH) was carried out as previously described (8). Histologically normal tissue was obtained from lung tissue resected during cancer surgery at a site remote from the tumour. CXCL12 levels were measured in plasma from non-smoking controls (n=15; mean age 47.1 yrs, 13F) with no history of underlying lung disease, and age and sex matched IPAH patients (n=15; mean age 49.7 yrs, 12F), by ELISA (DSA00, R&D Systems). All experiments with human material were approved by the Ethics Committees at the Mater Misericordiae University Hospital and the David Geffen School of Medicine at UCLA.

Animal studies

All animal experiments were approved by the UCD animal research ethics sub-committee and carried out under license from the Department of Health. Chronic hypoxia was induced by housing adult male C57Bl6 mice or Sprague-Dawley rats in 10% inspired oxygen for 2 days or 3 weeks respectively, while control animals were housed in normoxic conditions (inspired oxygen 21%) in the same room. Processing of lung samples for ELISA (MCX120, R&D Systems) and immunohistochemical analysis is described in the online supplement. The extent of protein staining was determined by quantitative stereological techniques as previously described (8).

Cell culture studies

The separate role of CXCR7 and CXCR4 in modulating endothelial wound healing and repair (scratch assay), proliferation (cell count) and migratory (modified Boyden chamber) responses of primary human lung microvascular endothelial cells to CXCL12 was investigated as outlined in the on-line supplement. Cell experiments were carried out n=6 independent times using concentrations of specific inhibitors of CXCR7 (CCX771 and CCX773, ChemoCentryx) and CXCR4 (AMD3100, Sigma-Aldrich) previously shown to effectively block these receptors (17).

Statistical Analyses

Statistical analyses were performed with SPSS 12.0 statistics software. For normally distributed data, values are reported as mean ± standard error of the mean (SEM). Comparison of means in two group experiments was carried out using a t-test. For non-normally distributed data, statistical comparisons were made using Mann-Whitney U test. Corrections of multiple comparisons of means were carried out using the Holm-Sidak step-down post-hoc test. Values of $p<0.05$ were considered to be statistically significant.

RESULTS

CXCR7 is highly expressed in human pulmonary hypertensive diseases compared to control lungs

We found that CXCR7 expression was low or frequently absent in the vasculature of control subjects (figure 1a) but was widespread in the lungs of IPAH patients, with strong positive staining observed in the thickened intimal layer of remodeled blood vessels (figure 1b). CXCR4 and CXCL12 were also more highly expressed in the microvasculature of IPAH (figure 1e and 1h respectively) compared to histologically normal lungs (figure 1d and 1g respectively). We also show for the first time that infiltrating cells, clearly observed in the alveolar spaces of IPAH lungs, were stained for CXCR7 (figure 2b) whereas only faint CXCR7 staining was observed in these cells in normal lungs (figure 2a). This marked increase in protein expression in infiltrating cells in IPAH was also observed with CXCR4 (figure 2e) compared to control tissue (figure 2d). Likewise, CXCL12 expression was also increased in infiltrating cells in pulmonary hypertensive lungs (figure 1h) compared to histologically normal lungs (figure 1g).Lungs from a second pulmonary hypertensive patient group, usual interstitial pneumonia with pulmonary hypertension (UIP-PH), also showed marked staining of all three proteins in the endothelium of blood vessels and infiltrating inflammatory cells (representative images are shown in supplemental figures S1 and S2). No positive staining was observed with appropriate irrelevant IgG isotype controls for the three proteins of interest (figures 1, 2, S1 and S2 - panels c, f and i).

CXCL12 is significantly elevated in patients with IPAH

ELISA analysis of CXCL12 concentrations in plasma from IPAH patients indicated that CXCL12 concentrations were significantly elevated in the patient group (n=15) when compared to non-diseased age and gender-matched control subjects (n=15) (figure 3).

CXCR7, CXCR4 and CXCL12 are elevated in hypoxic rodent lungs

Immunohistochemical staining showed that CXCR7 protein was expressed basally in the normoxic lung (figure 4a) and increased substantially after 2 days in hypoxia (10% O_2) (figure 4b). CXCR7 immunoreactivity was clearly observed in the alveoli walls and in the vessel endothelium. Quantification of the volume of CXCR7-stained tissue per unit volume of alveolar wall in the lung showed that CXCR7 protein was significantly higher in hypoxic lungs than in normoxic lungs (figure 4d). Similarly, basal CXCR4 expression observed in normoxic murine lungs (figure 4e) increased significantly with exposure to hypoxia (figure 4f), quantification of protein staining as described above showed that CXCR4 protein was also significantly higher in hypoxia when compared to normoxia (figure 4h). The specificity of CXCR7 and CXCR4 staining was confirmed with appropriate irrelevant IgG controls (figures 4c and 4g).

We next examined CXCL12 concentrations and protein localisation in hypoxic murine lung. ELISA analysis indicated that CXCL12 concentration in whole lung homogenates from hypoxic mice was significantly elevated when compared to normoxic lungs (figure 5A). In a separate cohort of mice, immunohistochemical staining (figure 5B) with anti-CXCL12 antibody showed low levels of CXCL12 staining in normoxic endothelial cells (panel a). In contrast, the endothelium of the lung microvasculature was highly positive for CXCL12 when exposed to hypoxia (panel b). The specificity of positive staining was established with an irrelevant rabbit polyclonal IgG (panel c).

It is well documented that rats develop marked pulmonary vascular remodelling, similar to humans and other species, after chronic hypoxia exposure, in contrast to mice, where relatively minor remodelling is observed even though they develop sustained pulmonary hypertension (20). Thus we also examined receptor expression in a rat model of hypoxic pulmonary hypertension (Sprague-Dawley rats exposed to 10% O_2 for 3 weeks). Immunohistochemical analysis showed low levels of CXCR7 protein in normoxic lung (figure S3a) whereas strong CXCR7 staining was observed in hypoxic lung tissue, particularly in the vascular endothelium (figure S3b). Stereological quantification of CXCR7 immunoreactivity confirmed that the extent of CXCR7 protein expression was significantly higher in hypoxia (figure S3d). Likewise, basal CXCR4 expression observed in normoxic murine lungs (figure S3e) increased significantly with exposure to hypoxia (figure S3f). Stereological quantification of the extent of protein staining showed that CXCR4 was also significantly higher in hypoxia when compared to normoxia (figure S3h). The specificity of positive immunohistochemical staining was confirmed with appropriate irrelevant IgG controls (figures S3c and g).

Inhibition of CXCR7 blocks CXCL12 induced monolayer regeneration and repair

In order to establish the potential roles of CXCR7 and CXCR4 in mediating CXCL12-induced responses in primary human lung microvascular endothelial cells, we first confirmed that both receptors were expressed by these cells (figure 6A). We next examined the effect of receptor inhibition on the regeneration and repair of endothelial cell monolayers; preliminary concentration-response experiments using our *in vitro* wound healing model showed that recombinant CXCL12 (10nM) elicited maximum wound-healing (data not shown). We next determined if a small molecule inhibitor of CXCR7, namely CCX771, altered the CXCL12 induced wound healing response. As shown in figure 6B, dose-response experiments with

CCX771 demonstrated that maximal inhibition of wound healing was observed with 1μM of CCX771. A second CXCR7 inhibitor, CCX773, also inhibited CXCL12-induced wound healing (figure S4A). In contrast, increasing concentrations of the CXCR4 receptor antagonist, AMD3100, (0.01-1μM) had no effect on CXCL12 induced wound healing (figure 6C).

Inhibition of CXCR7 blocks proliferation but not migration of human pulmonary microvascular endothelial cells *in vitro*

To further examine the wound healing response, we next established that CXCL12 (10nM) induces proliferation of microvascular lung endothelial cells, a proliferative response that was inhibited by CCX771 (1μM) (figure 7A). The second CXCR7 inhibitor (CCX773, 1μM) was also capable of inhibiting cell proliferation (figure S4B). In contrast, inhibition of CXCR4 with the CXCR4 receptor antagonist, AMD3100 (1μM) had no effect on microvascular lung endothelial cell proliferation (figure 7B). These results suggest that CXCL12 enhances proliferation of endothelial cells through interaction with CXCR7, whereas CXCR4 is not required in this process.

Inhibition of CXCR7 has no effect on migration of human pulmonary microvascular endothelial cells *in vitro*

In a Boyden chamber assay, cell migration towards vehicle alone was low (figure 8A - panel a), which was in marked contrast to the chemotactic response observed in the presence of CXCL12 (10nM) (figure 8A - panel b). Subsequent experiments showed that the CXCL12-induced increase in microvascular lung endothelial cell migration was unaffected by CXCR7 inhibition (CCX771, 1μM, figure 8B), a similar effect was observed with the second CXCR7

inhibitor, CCX773 (figure S4C). In contrast, endothelial cell migration was significantly inhibited by CXCR4 blockade (AMD3100, 1μM, figure 8C). These results suggest that, of the two CXCL12 receptors, CXCR4 is primarily required for the chemotactic response of endothelial cells to CXCL12.

DISCUSSION

Given the critical role that endothelial cell dysfunction plays in the pathogenesis of pulmonary hypertensive diseases, we set out to establish if CXCR7, a receptor for the potent pro-angiogenic ligand, CXCL12, was expressed in human pulmonary hypertensive diseases (IPAH and UIP-PH) and in the setting of hypoxia-induced PH. We show for the first time that CXCR7 is highly expressed in the pulmonary vascular endothelium of explanted human hypertensive lungs and circulating concentrations of its ligand are significantly elevated in the plasma of IPAH patients. Our study is the first demonstration that hypoxia increases CXCR7 expression in the pulmonary endothelium *in vivo*. Furthermore, CXCR7 has a distinct role to play in the CXCL12-induced responses of primary pulmonary human endothelial cells from that mediated by CXCR4 i.e. our data suggest that CXCR7 is the receptor through which endothelial cell proliferation, and regeneration and repair of endothelial cell monolayers, is mediated, whereas signaling via CXCR4 is essential for chemotactic cell migration. These results, taken together with marked staining of CXCR7 in vascular lesions in human lung disease, suggest CXCR7-mediated signaling may be functionally important in pulmonary hypertensive disease.

In a previous microarray study, we originally showed that CXCR7 mRNA was selectively upregulated in the hypoxic murine lung *in vivo* while remaining unchanged in systemic organs (8). In humans, CXCR7 was previously reported not to be expressed in endothelium of normal systemic organs *in vivo* but highly expressed in tumour associated vasculature or in endothelial cells during human renal allograft rejection (21, 22). We now show that, contrary to the vasculature of systemic organs, CXCR7 is present on the pulmonary endothelium under normal circumstances *in vivo*. Furthermore, alveolar hypoxia similar to that found in lung disease results in increased CXCR7 expression by the pulmonary microvascular endothelium.

In support of this contention that CXCR7 expression is different in the pulmonary circulation from that in the systemic circulation, we also show that CXCR7 is expressed by normal lung endothelial cells *in vitro* whereas previous reports suggest that CXCR7 is only expressed by endothelial cells which are first activated by pro-inflammatory cytokines (i.e. TNFα and IL-1β) or in tumour or transformed cell lines (10, 17).

We show for the first time that CXCR7 is highly expressed in the grossly remodeled microvasculature of human hypertensive diseases such as IPAH and UIP-PH whereas CXCR7 expression is low or frequently absent in the vasculature of control subjects, suggesting that this pathway may be involved in disease pathogenesis. We also observed that CXCR4 and CXCL12 were more highly expressed in remodeled vessels in both hypertensive diseases compared to controls, supporting a recent report that these two latter proteins were expressed in concentric and plexiform lesions characteristically seen in IPAH (7). Since the initial description of CXCR7 as a second receptor for CXCL12, several studies have established an important physiological function of CXCR7 in vascular development and disease (10, 23). For example, morpholino-mediated knockdown of CXCR7 in zebrafish results in disrupted vascular formation (21). Furthermore, the majority of $CXCR7^{-/-}$ mice, or mice conditionally deficient for CXCR7 in the endothelium, die at birth due to ventricular septum and heart valve malformation; in wild type animals CXCR7 is expressed in the microvasculature of these sites (15). Both these studies suggest a critical role for CXCR7 in normal vascular homeostasis during development. Given that we now show that CXCL12 and both receptors are expressed by the lung endothelium, and circulating CXCL12 concentrations are significantly elevated in IPAH, suggests that autocrine/paracrine modes of actions may be at play in the setting of lung disease. In contrast to our finding, Montani *et al* showed that circulating levels of CXCL12 were not significantly elevated in IPAH (24). The cause of this discrepancy is not

immediately apparent but differences in patient populations between the two studies may be a factor. For example, a recent paper demonstrated that CXCL12 concentrations are age dependent (25); in our study we closely age-matched our study populations so this was not a factor in significantly elevated CXCL12 measured in our patients. Additional studies in larger IPAH cohorts and other hypertensive patient groups are now warranted but nevertheless, our finding of elevated CXCL12 in IPAH plasma, together with elevated expression of both the ligand and its receptors in explanted human hypertensive lungs, are compatible with an important role for this biological axis in PAH.

In addition to demonstrating that CXCR7 is elevated in the grossly remodelled microvasculature of human hypertensive diseases, we now demonstrate that CXCR7 is highly expressed on infiltrating cells in diseased lungs, as was CXCR4. We have yet to elucidate the exact consequence of receptor expression on these cells in IPAH and UIP-PH; however, CXCL12 is a potent chemoattractant of T-lymphocytes and monocytes *in vitro* and CXCL12 upregulation has been shown to precede accumulation of mononuclear cells in vessel walls in a murine model of hypoxic PH *in vivo* (26, 27). Once in the lung, these cells are purported to contribute to disease pathogenesis primarily via the local release of cytokines and growth factors which modulate changes in resident endothelial, smooth muscle or adventitial cells. Indeed elevated levels of circulating pro-inflammatory cytokines and inflammatory infiltrates in vascular lesions in PAH are now well described (5, 6, 28).

The exact role of CXCR7 signaling in hypoxia-induced lung disease *in vivo* is as yet unclear. The fundamental principle that CXCL12 is important in hypoxic PH is well established by animal studies showing that a reduction in CXCL12 signaling via CXCR4 alleviated hypoxia-induced PH in mice (19, 29, 30). A recent study also suggest that CXCR7 blockade may

reduce hypoxia-induced perivascular accumulation of c-kit$^+$ haematopoietic cells in remodeled vessels (19). Our *in vivo* immunohistochemical studies show that the extent of CXCR7 protein is significantly elevated in the alveolar walls and in the vascular endothelium of small pulmonary vessels in both the hypoxic murine lung (10% O_2 for 2 days) and in a rat model of hypoxic PH (10% O_2 for 3 weeks) but further studies are required to establish the precise role of CXCR7 signaling in hypoxia-induced disease pathogenesis.

In the present study, we demonstrate a functional outcome of CXCR7 signalling in lung endothelial cells. It is now well established that CXCR7 (unlike CXCR4) does not couple G proteins, with several studies reporting the absence of GTP hydrolysis, calcium mobilization and chemotaxis upon ligand binding to CXCR7 (10, 14). In fact, the CXCR7-mediated cellular responses to CXC12 are not yet fully elucidated, but it is now well established that ligand binding triggers β-arrestin-2 association with CXCR7 and ligand/receptor internalization *in vitro* (16-18). CXCR7 expression on the endothelium is involved in various biologic processes such as transendothelial migration of tumour cells and human renal progenitors *in vitro* (17, 31) and primordial germ cell migration during zebrafish development *in vivo* (11-13). Furthermore, CXCR7 signaling has also been shown to promote tumour growth *in vivo* (10, 21, 32) and exerts pro-survival and anti-apoptotic effects *in vitro* (10, 31). Our experiments now show a further important action of CXCR7 in pulmonary endothelial cells, namely a role in cell proliferation. In our *in vitro* cell experiments, blockade of CXCR7 significantly reduced the ability of CXCL12-induced pulmonary endothelial cells to proliferate, and repair and regenerate a wounded endothelial cell monolayer, whereas CXCL12-induced pulmonary vascular endothelial cell migration was unaffected by CXCR7 inhibition, but significantly inhibited by CXCR4 blockade. Distinct roles for receptor mediated signaling have previously been reported in the homing of human renal progenitor

cells where CXCL12-induced migration was blocked by CXCR4 inhibition whereas cell survival was blocked by CXCR7 inhibition (31). Interestingly, although CXCL12-CXCR4 interaction was responsible for renal progenitor cell recruitment, both receptors were required for transendothelial migration underlining a complex interplay between CXCR7 and CXCR4 signaling (31). Since coordinated proliferation and migration is required for normal endothelial repair and angiogenesis, alterations in the normal balance of these receptors could be important in the pathogenesis of PH, given that dysregulated endothelial cell proliferation, resulting in intimal hyperplasia is a pathological hallmark of hypertensive lung disease (5, 6).

It is interesting that CXCR4 inhibition blocked CXCL12 stimulated migration of endothelial cells in the Boyden chamber experiment but had no effect on wound repair in the scratch assay. However, there is a growing understanding that chemotaxis (directed individual cell motility along a chemical gradient, as measured in Boyden chamber experiments) and chemokinesis (non-directional motility of cells due to the presence of a chemical substance, as occurs during coordinated wound closure by the cell monolayer) represent two distinct forms of migration. For example, the coordinated EGF-stimulated wound closure of mouse colonic epithelial cell monolayers requires PI 3-kinase, PKC, and JNK/SAPK activity, whereas chemotaxis (Boyden chamber) to EGF did not (33). These findings indicate that the specific signalling pathways necessary for chemotaxis are different from those required for chemokinesis during wound healing. Our findings now provide evidence of separate pathways regulating CXCL12-induced chemotaxis and the process of chemokinesis required for pulmonary microvascular endothelial wound healing.

In summary, we show that the predominant cells expressing CXCR7 in the lung *in vivo* are endothelial cells and that CXCL12 is elevated in the plasma of human IPAH. We also show

that CXCL12 induces proliferation, migration and wound healing of human microvascular endothelial cells *in vitro.* CXCR7 and CXCR4 appear to have distinct roles in these latter processes; in particular, we report a previously unrecognized function for CXCR7 in endothelial cell proliferation and wound repair, suggesting that this protein may have an important role to play in the vascular lesions observed in disease. We propose that signaling via CXCR7 plays a key role in pulmonary endothelial cell responses to CXCL12 in disease, suggesting that therapeutic manipulation of this pathway may present novel treatment opportunities.

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AUTHOR CONTRIBUTION

CM., PMcL, JAB, MPK and SG designed research, CMC, BMcC, KH and MS performed research; CMC analysed data, BMcC, JAB, MPK and SG recruited patients and obtained clinical tissue specimens. All authors contributed to writing the paper and reviewed the final manuscript.

FIGURE LEGENDS

Figure 1. Immunostaining in the vasculature of control and idiopathic pulmonary arterial hypertensive patient (IPAH) lungs

CXCR7 (panels a & b), CXCR4 (panels d & e) and CXCL12 (panels g & h) protein is expressed (brown colour) in the endothelium of the pulmonary vasculature of control lungs and in IPAH. Staining was absent when isotype matched antibodies were tested on the tissue sections (IgG_{2A} for CXCR7 (panel c), IgG_{2B} for CXCR4 (panel f), IgG_1 for CXCL12 (panel i). Representative image from three control and three IPAH patients are shown. Arrows indicate endothelium of vessels, scale bar = 20μm, x 100 objective.

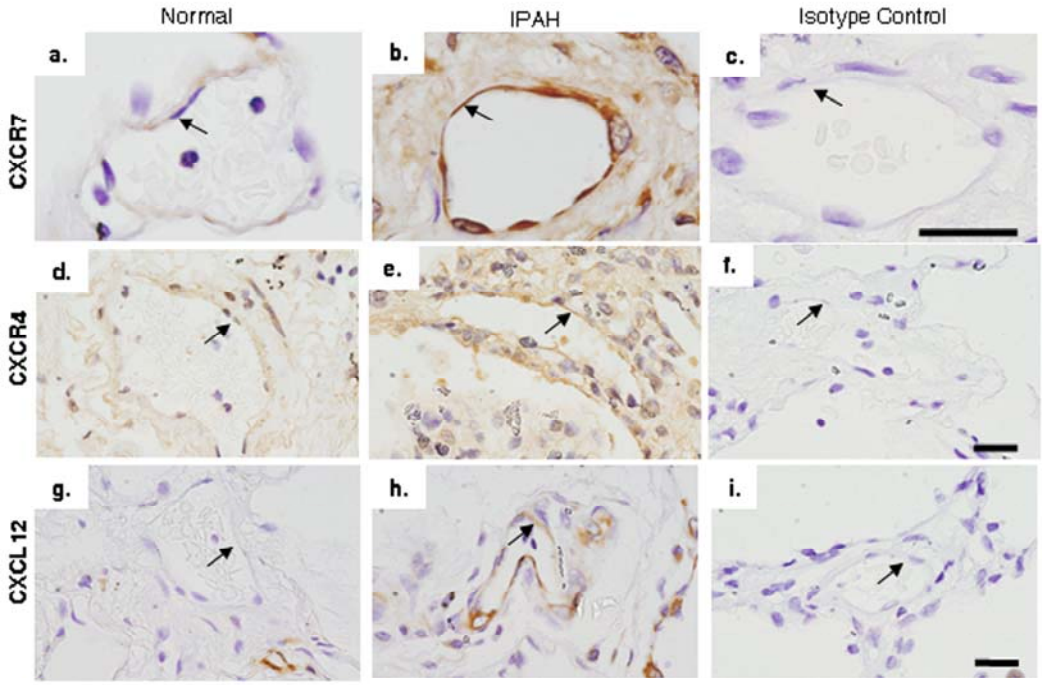

Figure 1.

Figure 2. Immunostaining of infiltrating inflammatory cells in control and idiopathic pulmonary arterial hypertensive (IPAH) patient lungs

Representative images of infiltrating cells in the lung in normal controls showed that basal positive CXCR7 staining (panel a) increased in IPAH patients (panel b). A similar pattern of increased positive staining in disease was observed with CXCR4 (Control (d) vs. IPAH (e)) and CXCL12 (Control (g) vs. IPAH (h)). No staining was observed in these cells with appropriate isotype controls (IgG_{2A} for CXCR7 (panel c), IgG_{2B} for CXCR4 (panel f), IgG_1 for CXCL12 (panel i)). Representative image from three control and three IPAH patients are shown, scale bar = 20μm, x 100 objective.

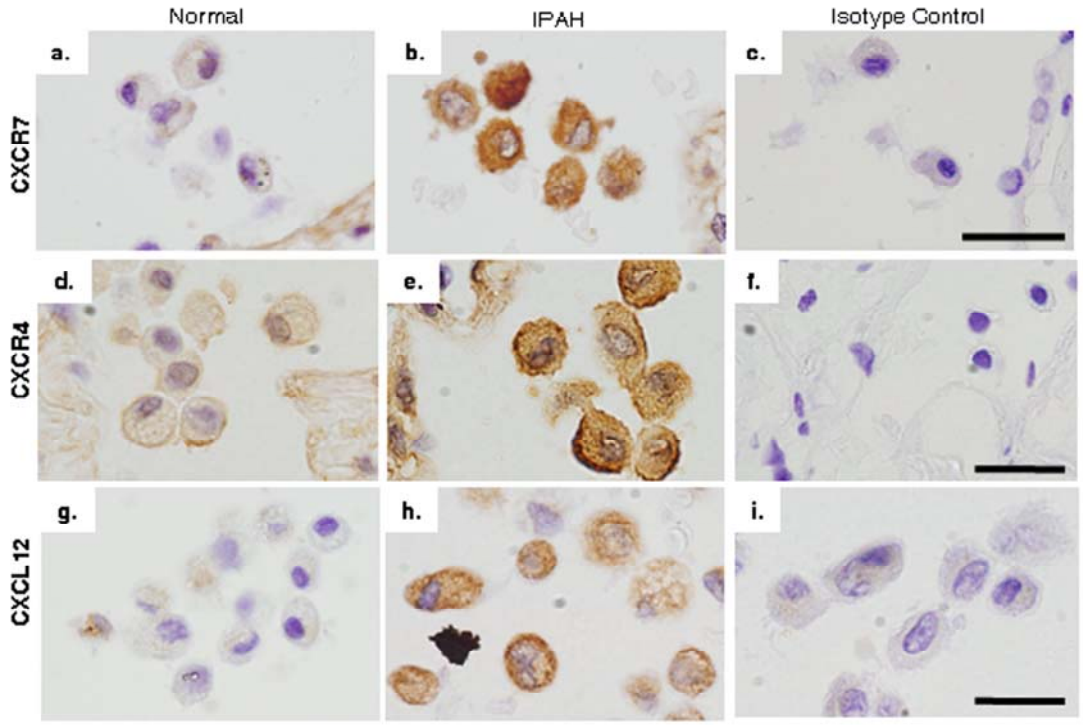

Figure 2.

Figure 3. CXCL12 determination by ELISA in human plasma

CXCL12 levels in plasma samples from controls (n=15) and idiopathic pulmonary arterial hypertensive patients (n=15). Protein levels of this potent pro-angiogenic cytokine were significantly increased in the diseased population (# = $p<0.05$). Horizontal bars indicate median values.

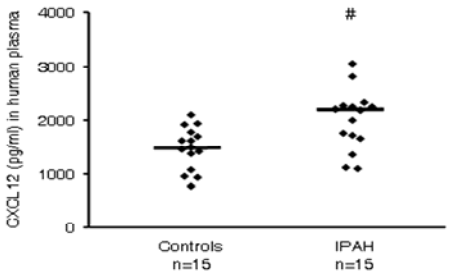

Figure 3.

Figure 4. Immunohistochemical localization of CXCR7 and CXCR4 in normoxic and hypoxic murine lungs

Immunohistochemical staining (brown color) indicated that CXCR7 protein was expressed basally in normoxic lung tissue (panel a) and increased with exposure to hypoxia (panel b). Using stereology, the volume of cells within the alveolar wall that were CXCR7-positive was expressed as a fraction of total alveolar wall volume (volume fraction) and was significantly

higher in hypoxic lungs (panel d). Similarly, the volume fraction of CXCR4-stained tissue in normoxic (panel e) and hypoxic (panel f) lungs showed that CXCR4 protein was significantly higher in the hypoxic lungs (panel h).

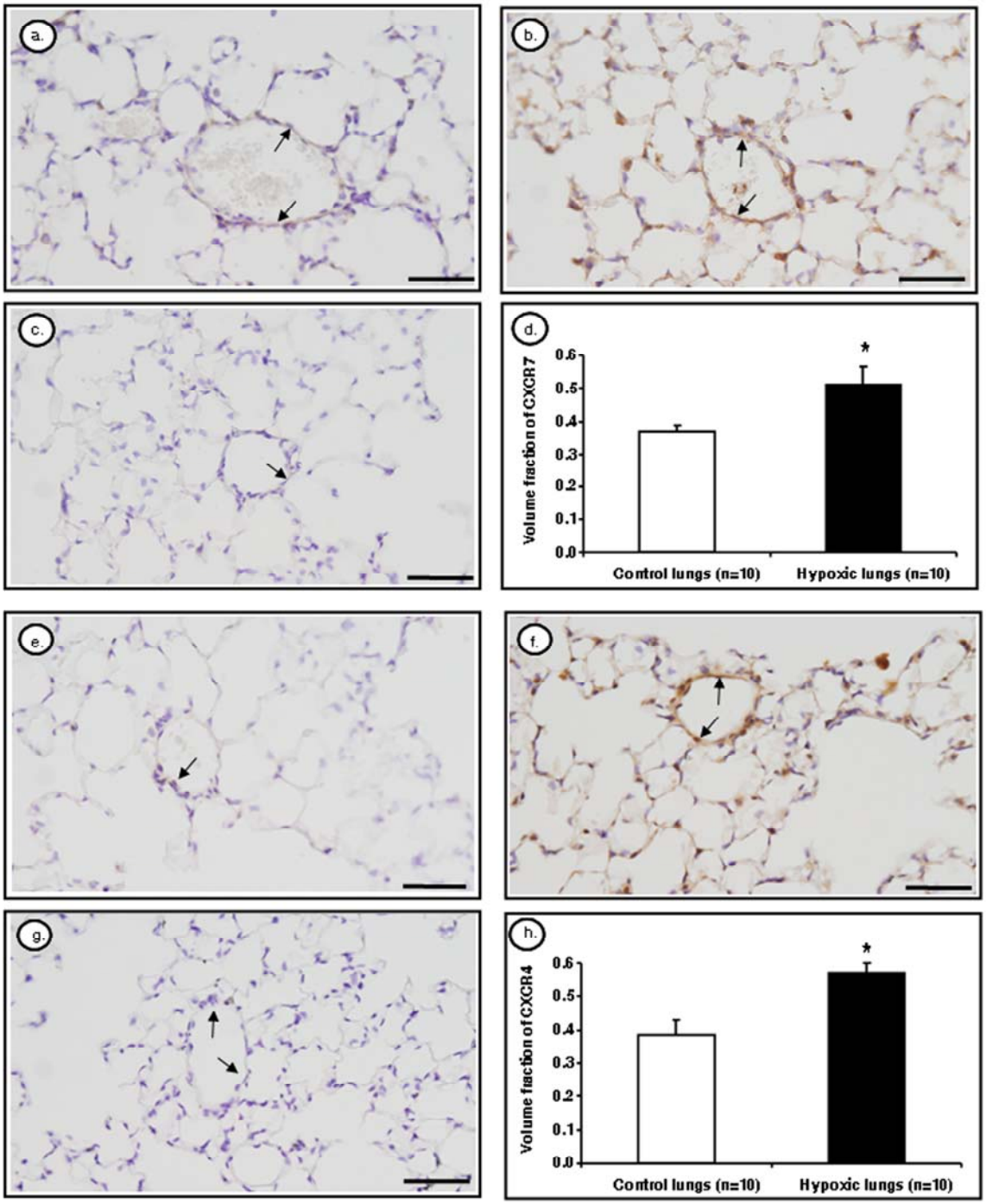

Figure 4.

Figure 5. CXCL12 quantification and localisation in murine lungs

(A)CXCL12 protein was significantly higher in hypoxic lung homogenates (n=10) compared to normoxia (n=9) by ELISA (* = $p<0.05$). (B) Immunohistochemical staining indicated that a low basal level of CXCL12 was observed in the endothelium of normoxic blood vessels (panel a) in the gas exchange region, which increased with exposure to hypoxia (panel b). No staining was observed with irrelevant rabbit polyclonal IgG (panel c). Arrows indicate vessels, x 40 objective, scale bar = 50μm.

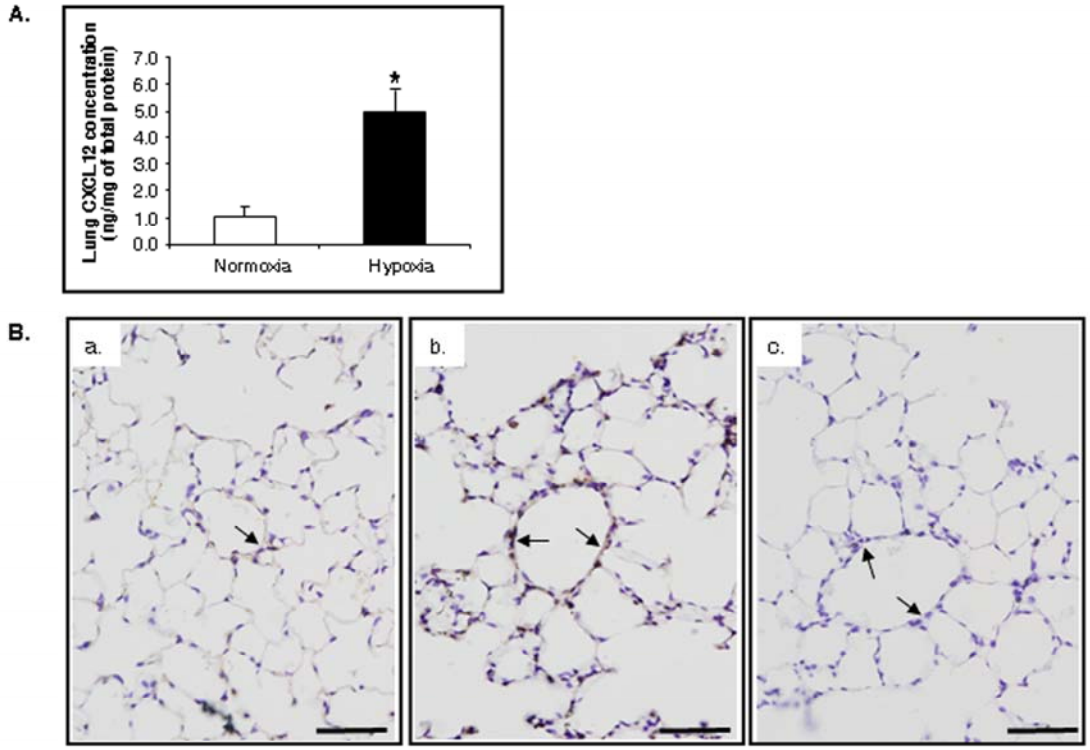

Figure 5.

Figure 6. CXCL12 induced wound healing of human pulmonary microvascular endothelial cells was blocked by CXCR7 inhibition

(A) Western blot analysis confirmed that CXCR4 and CXCR7 protein were expressed by human pulmonary microvascular endothelial cells. Cell pellets from three independent experiments are shown. (B) The specific CXCR7 inhibitor, CCX771, inhibited CXCL12 (10nM) induced primary human pulmonary microvascular endothelial cell wound healing. (C) The CXCR4 inhibitor, AMD3100, had no effect on wound healing in the same concentration range (0.01-1.0μM). All experiments were carried out n=6 independent times; * indicates significant difference between control and CXCL12; # indicates significant difference from all other groups ($p<0.05$).

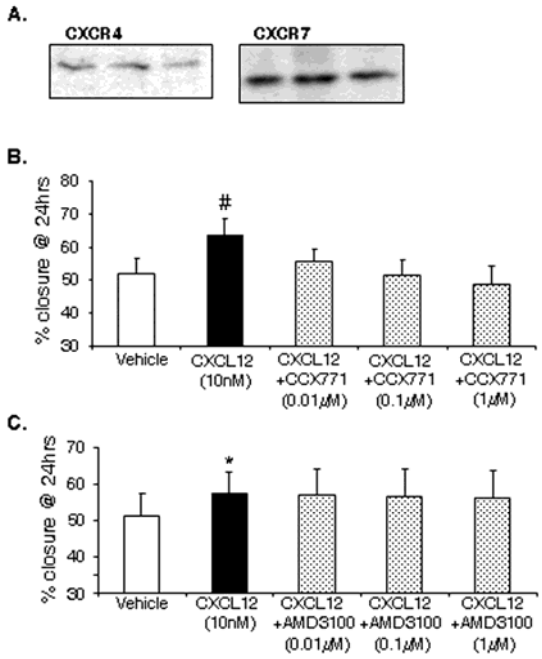

Figure 6.

Figure 7. CXCR7 inhibition blocked CXCL12 induced proliferation of human pulmonary microvascular endothelial cells

(A) CXCL12 (10nM) induced primary human pulmonary microvascular endothelial cell proliferation was blocked by CXCR7 inhibition (CCX771, 1.0μM) whereas (B) CXCR4 blockade (AMD3100, 1.0μM) did not significantly alter the CXCL12-induced proliferative

effect. All experiments were carried out n=6 independent times; * indicates significant difference between control and CXCL12; # indicates significant difference from all other groups ($p<0.05$).

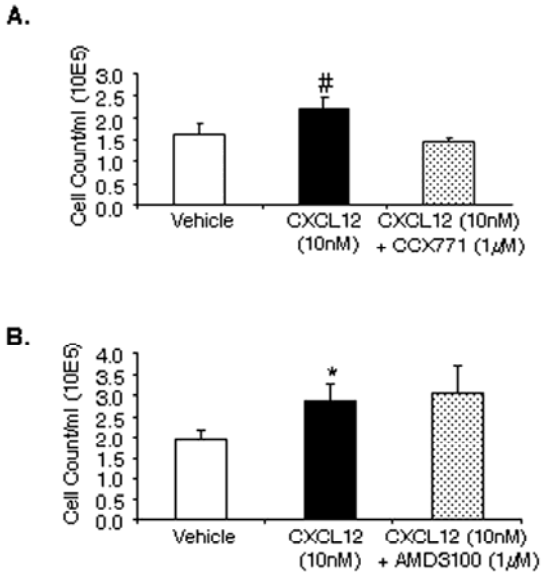

Figure 7.

Figure 8. CXCR4 inhibition blocked CXCL12 induced migration of human pulmonary microvascular endothelial cells

(A) Representative images of pulmonary endothelial cell migration with cells treated with vehicle alone (panel a) or with CXCL12 (10nM) (panel b) are shown. (B) CXCL12 (10nM) significantly induced cell migration, an effect that was unaltered by inhibition of CXCR7 (CCX771, 1μM). (C) In contrast, cell migration was blocked by CXCR4 inhibition (AMD3100, 1μM). All experiments were carried out n=6 independent times; * indicates significant difference between control and CXCL12; # indicates significant difference from all other groups ($p<0.05$).

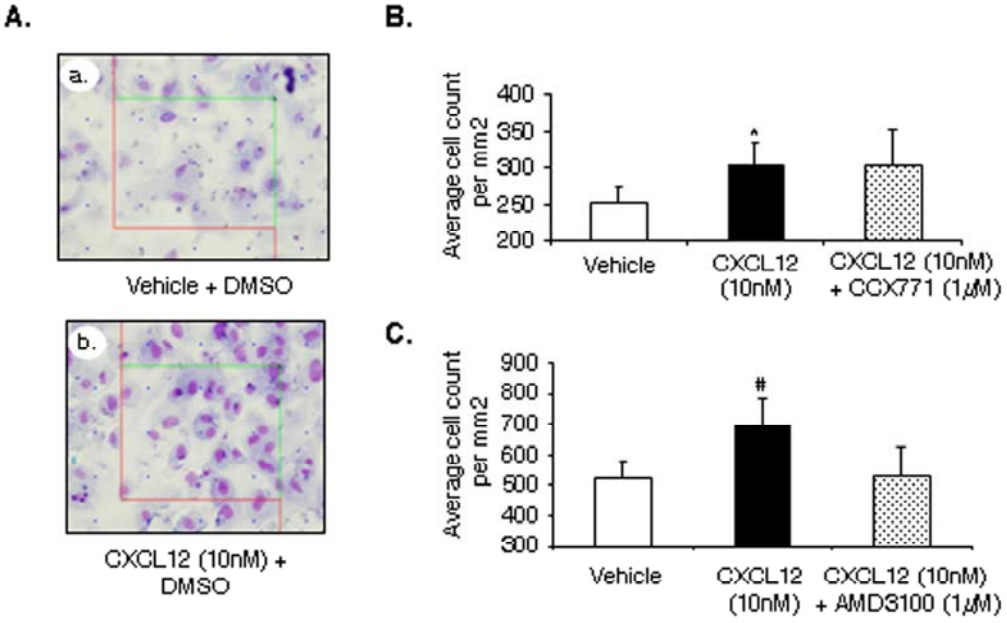
A.
a.
Vehicle + DMSO
b.
CXCL12 (10nM) + DMSO
B.
Average cell count per mm2
400
350
300
250
200
*
Vehicle
CXCL12 (10nM)
CXCL12 (10nM) + CCX771 (1μM)
C.
Average cell count per mm2
900
800
700
600
500
400
300
#
Vehicle
CXCL12 (10nM)
CXCL12 (10nM) + AMD3100 (1μM)

Figure 8.