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

Christine M. Costello[1#], Brian McCullagh[1], Katherine Howell[1], Michelle Sands[1], John A Belperio[2], Michael P. Keane[1], Sean Gaine[3], Paul McLoughlin[1]

[1]UCD School of Medicine and Medical Science, Conway Institute, Dublin, Ireland; [2]Division of Pulmonary and Critical Care Medicine, Department of Medicine, David Geffen School of Medicine at UCLA, CA, USA; [3]UCD Department of Respiratory Medicine, Mater Misericordiae University Hospital, Dublin, Ireland.

ONLINE DATA SUPPLEMENT

ONLINE METHODS

Immunohistochemical Analysis in Human Tissues

Immunohistochemical analysis was carried out as previously described (8). Paraffin-embedded, formalin-fixed lung tissue samples from histologically normal tissue (obtained from lung tissue resected during cancer surgery at a site remote from the tumour), patients with idiopathic pulmonary arterial hypertension (IPAH) or usual interstitial pneumonia with pulmonary hypertension (UIP-PH) were incubated with monoclonal antibodies to CXCL12 (MAB350; final concentration: 10μg/ml), CXCR4 (MAB172; final concentration: 2.5μg/ml) and CXCR7 (MAB4227; final concentration: 2.5μg/ml). Appropriate isotype controls were IgG_1 (MAB002) for CXCL12, IgG_{2B} (MAB004) for CXCR4 and IgG_{2A} (MAB003) for CXCR7; all from R&D Systems, Minneapolis, MN.

ELISA Analysis of CXCL12 Levels in Human Pulmonary Hypertensive Disease

For human studies, venous blood from non-diseased age and gender-matched control subjects (n=15; mean age: 47.1yrs; 13F) or IPAH patients (n=15; mean age: 49.7yrs; 12F) was collected in Li-Heparin LH monovettes (Sarstedt) by venipuncture. Immediately after collection, the tube was gently inverted 5 times to mix the clot activator with the blood. Samples were processed within 30 minutes of taking by centrifugation at 2,500 rpm for 10 minutes. CXCL12 levels in undiluted plasma samples were measured using an ELISA kit according to manufacturer's instructions (DSA00 - R&D Systems, Minneapolis, MN).

Immunohistochemical Analysis in Murine Lungs

To obtain mouse lung sections, male C57BL/6 mice (n = 10) were maintained in normoxia or exposed to 10% oxygen for two days. Mice were anaesthetized (sodium pentobarbitone 60mg/kg, i.p.) and anti-coagulated (1000 units/kg heparin) and killed by exsanguination. The heart and lungs were removed en-bloc and lungs were fully inflated via the trachea with standard pressures (25 cm H_2O) in fixative (4% paraformaldehyde) overnight and then embedded in paraffin as previously described (8). Immunohistochemistry was performed as previously described with rabbit polyclonal anti-CXCL12 (ab25117; final concentration: 11.11μg/ml) and anti-CXCR4 (ab2074; final concentration: 8.33μg/ml). An irrelevant rabbit polyclonal IgG (ab27478) was used as a control; all from Abcam, Cambridge, England. Immunohistochemistry with monoclonal anti-CXCR7 (Clone 11G8, R&D Systems, Minneapolis, MN) was essentially as previously described (8) but with an additional blocking step to eliminate background staining due to the binding of secondary anti-mouse antibody to murine tissue. Endogenous mouse IgG blocking was achieved by first incubating lung sections with unconjugated affinity purified Fab fragment goat anti-mouse IgG (H+L) overnight at RT (Jackson ImmunoResearch Labs, Product code: 115-007-003, final concentration: 0.13mg/ml). Monoclonal anti-CXCR7 (Clone 11G8) was then added to the lung sections and incubated overnight at 4ºC (MAB42273; final concentration of 50μg/ml). Finally, sections were incubated with a biotin-SP-conjugated affinity purified Fab fragment goat anti-mouse IgG (H+L) secondary antibody for 1hr at RT (Jackson ImmunoResearch Labs, Product code: 115-065-062, final concentration: 3μg/ml). Slides were washed in PBS and incubated in immunoperoxidase solution (ABC Vectastain Kit, Vector Laboratories) for 1 hour. Negative control slides were exclusion of primary or secondary antibody. After allowing the diaminobenzidine reaction product to develop for 20 min, sections were washed extensively in PBS and counterstained with haematoxylin (BDH Laboratory, UK) before being examined microscopically on a BX-61 microscope (Olympus, UK) (x 40 objective).

Immunohistochemical Analysis in Rat Lungs

Hypoxic pulmonary hypertension was induced in adult male specific pathogen free (SPF) Sprague Dawley rats (Harlan, Bicester, UK) by exposure to chronic hypoxia (10% O_2) for 21 days as previously described (34). Control animals were maintained in the same room under normoxic conditions for the same period of time and lung fixation is as described (34). Immunohistochemistry was carried out essentially as described with the murine lungs with the following changes: rabbit polyclonal anti-CXCR4 (ab2074) was used at a final concentration of 6.67mg/ml, and the additional blocking step described above was not required for the CXCR7 rat immunohistochemistry expt.

Quantification of CXCR7 and CXCR4 staining

The extent of CXCR7 and CXCR4 staining was determined by quantitative stereological techniques as previously described (8) using a computer-based analysis system (CAST; VisioPharm, Hørsholm, Denmark) combined with light microscopy (Leica, Laboratory Instruments). Random fields of view were acquired (x40 objective) from all sections of each lung using a systematic random sampling strategy, digitized, and displayed on screen. A point-counting grid was digitally superimposed on these images to determine the fraction of the alveolar walls that was occupied by positively stained cells.

CXCL12 ELISA Analysis in Murine Samples

Male C57BL/6 mice were maintained in normoxia or exposed to 10% oxygen for 2 days. Mice were then killed by exsanguination under general anaesthesia as described above. Lungs were removed and protein was extracted by first crushing the lungs to a fine powder

using a mortar and pestle, weighed and homogenized in 5X volume of PBS plus a protease inhibitor cocktail tablet, according to manufacturer's instructions (Roche, West Sussex, UK). Total protein was determined by BCA Bradford Assay (Pierce) and CXCL12 levels determined by ELISA (MCX120, R&D Systems, Minneapolis, MN). In a separate cohort of animals, heparinised blood was immediately spun at 4,000rpm for 15mins and CXCL12 levels in undiluted plasma samples were measured by ELISA according to manufacturer's instructions (DSA00 - R&D Systems, Minneapolis, MN).

Western Blotting in Human Microvascular Endothelial Cells

Human microvascular endothelial cells (CC-2527: Lonza Bioscience, UK) were grown to confluency on tissue culture dishes. Adherent cells were washed with ice-cold PBS and scrapped off the dish with a rubber policeman. Cells were centrifuged at 14,000rpm for 5 mins, the supernatant carefully removed and ice-cold radio-immunoprecipitation (RIPA) lysis buffer ((Millipore, Billerica, MA), with PMSF (1mM) and protease (P8340) and phosphatase inhibitor cocktail (P5726) added, all from Sigma-Aldrich, St. Louis, MN) added to the pelleted cells. After thorough homogenization (30 sec sonication bursts for 5-10 min), cellular debris was removed by centrifugation at 14,000 rpm for 15 minutes at 4ºC. Protein concentration was determined using the BCA Bradford Assay (Pierce Chemicals, Rockford, IL). Protein samples (40 µg each) were separated by standard SDS-polyacrylamide gel electrophoresis and proteins transferred onto polyvinylidene difluoride membranes (Sigma-Aldrich, St. Louis, MN). Membranes were blocked for 2hrs in 5% Marvel/TBST and probed with either monoclonal 11G8-CXCR7 (kind gift from ChemoCentryx; final concentration: 7.8µg/ml) or ab2074-CXCR4 (final concentration: 0.1µg/ml, Abcam, Cambridge, England). The peroxidase reaction products were visualized by ECL substrate (Pierce Chemicals, Rockford, IL).

Scratch Assay

The scratch assay was carried out as previously described with minor modifications (8). In brief, primary human lung microvascular endothelial cells (CC-2527: Lonza Bioscience, UK) were allowed grow into a confluent monolayer on 12 well plates in EGM-2MV medium (25ml FBS, 0.2ml hydrocortisone, 2ml hFGF-B, 0.5ml VEGF, 0.5ml R3-IGF-1, 0.5ml ascorbic acid, 0.5ml hEGF and 0.5ml GA-1000 made up in 500ml of the basal medium). The night before experiment start, the medium was changed to serum-depleted medium (3% FBS) with no VEGF added. The following morning, a single vertical scratch was applied to each well; cells were washed in the same medium to remove wound-derived cells and allowed to settle for 2-3 hrs before addition of experimental treatments. The scratch was visualized using phase contrast (x10 objective) and the width of the wound measured at 0hr and 24 hrs (AxioVision 4.4 software, Zeiss, Jena, Germany). The investigator was blinded to the well contents when measuring scratch widths. Each treatment was carried out in duplicate on a plate and each experiment was carried out a minimum of n=6 independent times.

Proliferation Assay

Primary human lung microvascular endothelial cells were seeded on 12-well plates at approximately 25,000cells/cm^2 in serum depleted medium (3% FBS minus VEGF) and allowed to settle overnight. The following day, wells were rinsed X2 in the same medium to remove dead cells before addition of treatments. Cells were allowed to proliferate for 24hrs in the CO_2 incubator, trypsinised to remove adhered cells and counted on a Coulter Counter (Beckman, Brea, CA). Each treatment was carried out in duplicate on a plate, and each experiment was carried out n=6 independent times.

Migration Assay

Migration assays were carried out using a modified Boyden chamber (NeuroProbe, Gaithersburg, MD). This multi-well chemotaxis chamber consists of two compartments separated by a polycarbonate membrane, and migration was measured by counting the number of cells crossing the membrane (8µM pores). Prior to experiment start, membranes were incubated with 5µg/ml type I collagen in primary human lung microvascular endothelial cells basal medium (no supplements) for 2hrs at 37°C, followed by overnight air-drying. On the day of the experiment, the lower wells of the chemotaxis chamber were filled with 30µl serum-free medium (minus VEGF) containing recombinant human CXCL12 (Code 350-NS; R&D systems, Minneapolis, MN). The collagen coated membrane was carefully placed on top and the upper chamber secured in place. Primary human lung microvascular endothelial cells (2×10^5 cells in 50µl/well) suspended in serum-free medium (minus VEGF) were added to the upper chambers of the chamber ± inhibitors. The chamber was placed in a CO_2 incubator and cells allowed to migrate for 5hrs. After this, non-migrating cells were washed off the membrane with PBS by drawing the membrane over the wiper blade provided ten times (Neuroprobe, Gaithersburg, MD). The membrane was fixed in methanol, allowed to air-dry and the cells were stained with Podiff 11 stain set according to manufacturer's instructions (Braidwood Laboratories, Sussex, UK). The number of migrating cells was counted using the computer based analysis system (CAST, Visiopharm, Denmark) combined with light microscopy (Leica, Laboratory Instruments, Germany). Random fields of view were acquired (x 40 objective) from the total well area and a point counting grid was digitally superimposed on these images. Results are expressed as average number of migrated cells per mm^2 of membrane. Each treatment was carried out in duplicate on any given day and each experiment was carried out n=6 independent times.

REFERENCES

1. Simonneau, G., N. Galie, L. J. Rubin, D. Langleben, W. Seeger, G. Domenighetti, S. Gibbs, D. Lebrec, R. Speich, M. Beghetti, S. Rich, and A. Fishman. 2004. Clinical classification of pulmonary hypertension. *J Am Coll Cardiol* 43(12 Suppl S):5S-12S.

2. Taraseviciene-Stewart, L., Y. Kasahara, L. Alger, P. Hirth, G. Mc Mahon, J. Waltenberger, N. F. Voelkel, and R. M. Tuder. 2001. Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. *Faseb J* 15(2):427-38.

3. Voelkel, N. F., C. Cool, L. Taraceviene-Stewart, M. W. Geraci, M. Yeager, T. Bull, M. Kasper, and R. M. Tuder. 2002. Janus face of vascular endothelial growth factor: the obligatory survival factor for lung vascular endothelium controls precapillary artery remodeling in severe pulmonary hypertension. *Crit Care Med* 30(5 Suppl):S251-6.

4. Le Cras, T. D., N. E. Markham, R. M. Tuder, N. F. Voelkel, and S. H. Abman. 2002. Treatment of newborn rats with a VEGF receptor inhibitor causes pulmonary hypertension and abnormal lung structure. *Am J Physiol Lung Cell Mol Physiol.* 283(3):L555-62.

5. Rabinovitch, M. 2008. Molecular pathogenesis of pulmonary arterial hypertension. *J Clin Invest* 118(7):2372-9.

6. Humbert, M., D. Montani, F. Perros, P. Dorfmuller, S. Adnot, and S. Eddahibi. 2008. Endothelial cell dysfunction and cross talk between endothelium and smooth muscle cells in pulmonary arterial hypertension. *Vascul Pharmacol* 49(4-6):113-8.

7. Toshner, M., R. Voswinckel, M. Southwood, R. Al-Lamki, L. S. Howard, D. Marchesan, J. Yang, J. Suntharalingam, E. Soon, A. Exley, S. Stewart, M. Hecker, Z. Zhu, U. Gehling, W. Seeger, J. Pepke-Zaba, and N. W. Morrell. 2009. Evidence of dysfunction of endothelial progenitors in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 180(8):780-7.

8. Costello, C. M., K. Howell, E. Cahill, J. McBryan, M. Konigshoff, O. Eickelberg, S. Gaine, F. Martin, and P. McLoughlin. 2008. Lung-selective gene responses to alveolar hypoxia: potential role for the bone morphogenetic antagonist gremlin in pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 295(2):L272-84.

9. Balabanian, K., B. Lagane, S. Infantino, K. Y. Chow, J. Harriague, B. Moepps, F. Arenzana-Seisdedos, M. Thelen, and F. Bachelerie. 2005. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J Biol Chem* 280(42):35760-6.

10. Burns, J. M., B. C. Summers, Y. Wang, A. Melikian, R. Berahovich, Z. Miao, M. E. T. Penfold, M. J. Sunshine, D. R. Littman, C. J. Kuo, K. Wei, B. E. McMaster, K. Wright, M. C. Howard, and T. J. Schall. 2006. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J. Exp. Med.* 203(9):2201-2213.

11. Boldajipour, B., H. Mahabaleshwar, E. Kardash, M. Reichman-Fried, H. Blaser, S. Minina, D. Wilson, Q. Xu, and E. Raz. 2008. Control of chemokine-guided cell migration by ligand sequestration. *Cell* 132(3):463-73.

12. Dambly-Chaudiere, C., N. Cubedo, and A. Ghysen. 2007. Control of cell migration in the development of the posterior lateral line: antagonistic interactions between the chemokine receptors CXCR4 and CXCR7/RDC1. *BMC Dev Biol* 7:23.

13. Naumann, U., E. Cameroni, M. Pruenster, H. Mahabaleshwar, E. Raz, H. G. Zerwes, A. Rot, and M. Thelen. 2010. CXCR7 functions as a scavenger for CXCL12 and CXCL11. *PLoS One* 5(2):e9175.

14. Levoye, A., K. Balabanian, F. Baleux, F. Bachelerie, and B. Lagane. 2009. CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood* 113(24):6085-93.

15. Sierro, F., C. Biben, L. Martinez-Munoz, M. Mellado, R. M. Ransohoff, M. Li, B. Woehl, H. Leung, J. Groom, M. Batten, R. P. Harvey, A. C. Martinez, C. R. Mackay, and F. Mackay. 2007. Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc Natl Acad Sci U S A* 104(37):14759-64.

16. Luker, K. E., M. Gupta, J. M. Steele, B. R. Foerster, and G. D. Luker. 2009. Imaging ligand-dependent activation of CXCR7. *Neoplasia* 11(10):1022-35.

17. Zabel, B. A., Y. Wang, S. Lewen, R. D. Berahovich, M. E. Penfold, P. Zhang, J. Powers, B. C. Summers, Z. Miao, B. Zhao, A. Jalili, A. Janowska-Wieczorek, J. C. Jaen, and T. J. Schall. 2009. Elucidation of CXCR7-mediated signaling events and inhibition of

CXCR4-mediated tumor cell transendothelial migration by CXCR7 ligands. *J Immunol* 183(5):3204-11.

18. Rajagopal, S., J. Kim, S. Ahn, S. Craig, C. M. Lam, N. P. Gerard, C. Gerard, and R. J. Lefkowitz. 2010. Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proc Natl Acad Sci U S A* 107(2):628-32.

19. Gambaryan, N., F. Perros, D. Montani, S. Cohen-Kaminsky, M. Mazmanian, J. F. Renaud, G. Simonneau, A. Lombet, and M. Humbert. 2010. Targeting of c-kit+ hematopoietic progenitor cells prevents hypoxic pulmonary hypertension. *Eur Respir J*.

20. Hoshikawa, Y., P. Nana-Sinkam, M. D. Moore, S. Sotto-Santiago, T. Phang, R. L. Keith, K. G. Morris, T. Kondo, R. M. Tuder, N. F. Voelkel, and M. W. Geraci. 2003. Hypoxia induces different genes in the lungs of rats compared with mice. *Physiol Genomics* 12(3):209-19.

21. Miao, Z., K. E. Luker, B. C. Summers, R. Berahovich, M. S. Bhojani, A. Rehemtulla, C. G. Kleer, J. J. Essner, A. Nasevicius, G. D. Luker, M. C. Howard, and T. J. Schall. 2007. CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. *Proc Natl Acad Sci U S A* 104(40):15735-40.

22. Neusser, M. A., A. K. Kraus, H. Regele, C. D. Cohen, T. Fehr, D. Kerjaschki, R. P. Wuthrich, M. E. Penfold, T. Schall, and S. Segerer. 2010. The chemokine receptor CXCR7 is expressed on lymphatic endothelial cells during renal allograft rejection. *Kidney Int* 77(9):801-8.

23. Balabanian, K., B. Lagane, S. Infantino, K. Y. Chow, J. Harriague, B. Moepps, F. Arenzana-Seisdedos, M. Thelen, and F. Bachelerie. 2005. The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J Biol Chem* 17.

24. Montani, D., F. Perros, N. Gambaryan, B. Girerd, P. Dorfmuller, L. C. Price, A. Huertas, H. Hammad, B. Lambrecht, G. Simonneau, J. M. Launay, S. Cohen-Kaminsky, and M. Humbert. 2011. C-kit-positive cells accumulate in remodeled vessels of idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 184(1):116-23.

25. Mehta, N. N., M. Li, D. William, A. V. Khera, S. DerOhannessian, L. Qu, J. F. Ferguson, C. McLaughlin, L. H. Shaikh, R. Shah, P. N. Patel, J. P. Bradfield, J. He, I. M. Stylianou, H. Hakonarson, D. J. Rader, and M. P. Reilly. 2011. The novel atherosclerosis locus at 10q11 regulates plasma CXCL12 levels. *Eur Heart J* 32(8):963-71.

26. Bleul, C. C., R. C. Fuhlbrigge, J. M. Casasnovas, A. Aiuti, and T. A. Springer. 1996. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med* 184(3):1101-9.

27. Burke, D. L., M. G. Frid, C. L. Kunrath, V. Karoor, A. Anwar, B. D. Wagner, D. Strassheim, and K. R. Stenmark. 2009. Sustained hypoxia promotes the development of a pulmonary artery-specific chronic inflammatory microenvironment. *Am J Physiol Lung Cell Mol Physiol* 297(2):L238-50.

28. Tuder, R. M., B. Groves, D. B. Badesch, and N. F. Voelkel. 1994. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am J Pathol* 144(2):275-85.

29. Young, K. C., E. Torres, K. E. Hatzistergos, D. Hehre, C. Suguihara, and J. M. Hare. 2009. Inhibition of the SDF-1/CXCR4 axis attenuates neonatal hypoxia-induced pulmonary hypertension. *Circ Res* 104(11):1293-301.

30. Satoh, K., Y. Fukumoto, M. Nakano, K. Sugimura, J. Nawata, J. Demachi, A. Karibe, Y. Kagaya, N. Ishii, K. Sugamura, and H. Shimokawa. 2009. Statin ameliorates hypoxia-induced pulmonary hypertension associated with down-regulated stromal cell-derived factor-1. *Cardiovasc Res* 81(1):226-34.

31. Mazzinghi, B., E. Ronconi, E. Lazzeri, C. Sagrinati, L. Ballerini, M. L. Angelotti, E. Parente, R. Mancina, G. S. Netti, F. Becherucci, M. Gacci, M. Carini, L. Gesualdo, M. Rotondi, E. Maggi, L. Lasagni, M. Serio, S. Romagnani, and P. Romagnani. 2008. Essential but differential role for CXCR4 and CXCR7 in the therapeutic homing of human renal progenitor cells. *J Exp Med* 205(2):479-90.

32. Raggo, C., R. Ruhl, S. McAllister, H. Koon, B. J. Dezube, K. Fruh, and A. V. Moses. 2005. Novel cellular genes essential for transformation of endothelial cells by Kaposi's sarcoma-associated herpesvirus. *Cancer Res* 65(12):5084-95.

33. Yamaoka, T., M. R. Frey, R. S. Dise, J. K. Bernard, and D. B. Polk. 2011. Specific epidermal growth factor receptor autophosphorylation sites promote mouse colon epithelial cell chemotaxis and restitution. *Am J Physiol Gastrointest Liver Physiol* 301(2):G368-76.

34. Sands, M., K. Howell, C. M. Costello, and P. McLoughlin. 2011. Placenta growth factor and vascular endothelial growth factor B expression in the hypoxic lung. *Respir Res* 12:17.