CCR2/CCR5-mediated macrophage–smooth muscle cell crosstalk in pulmonary hypertension

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CCR2 and CCR5 are required for collaboration between macrophages and pulmonary artery smooth muscle cells (PASMCs) to initiate and amplify PASMC proliferation. Dual targeting of CCR2 and CCR5 may hold promise for treating pulmonary artery hypertension. http://bit.ly/2L3izU6

Cite this article as: Abid S, Marcos E, Parpaleix A, *et al.* CCR2/CCR5-mediated macrophage–smooth muscle cell crosstalk in pulmonary hypertension. *Eur Respir J* 2019; 54: 1802308 [https://doi.org/10.1183/13993003.02308-2018].

ABSTRACT Macrophages are major players in the pathogenesis of pulmonary arterial hypertension (PAH).

To investigate whether lung macrophages and pulmonary-artery smooth muscle cells (PASMCs) collaborate to stimulate PASMC growth and whether the CCL2-CCR2 and CCL5-CCR5 pathways inhibited macrophage–PASMC interactions and PAH development, we used human CCR5-knock-in mice and PASMCs from patients with PAH and controls.

Conditioned media from murine M1 or M2 macrophages stimulated PASMC growth. This effect was markedly amplified with conditioned media from M2 macrophage/PASMC co-cultures. CCR2, CCR5, CCL2 and CCL5 were upregulated in macrophage/PASMC co-cultures. Compared to inhibiting either receptor, dual CCR2 and CCR5 inhibition more strongly attenuated the growth-promoting effect of conditioned media from M2-macrophage/PASMC co-cultures. Deleting either CCR2 or CCR5 in macrophages or PASMCs attenuated the growth response. In mice with hypoxia- or SUGEN/hypoxia-induced PH, targeting both CCR2 and CCR5 prevented or reversed PH more efficiently than targeting either receptor alone. Patients with PAH exhibited CCR2 and CCR5 upregulation in PASMCs and perivascular macrophages compared to controls. The PASMC growth-promoting effect of conditioned media from M2-macrophage/PASMC co-cultures was greater when PASMCs from PAH patients were used in the co-cultures or as the target cells and was dependent on CCR2 and CCR5. PASMC migration toward M2-macrophages was greater with PASMCs from PAH patients and was attenuated by blocking CCR2 and CCR5.

CCR2 and CCR5 are required for collaboration between macrophages and PASMCs to initiate and amplify PASMC migration and proliferation during PAH development. Dual targeting of CCR2 and CCR5 may hold promise for treating human PAH.

This article has supplementary material available from erj.ersjournals.com

Received: 05 Dec 2018 | Accepted after revision: 27 June 2019

Introduction

Inflammation is a major contributor to the pathogenesis of various types of pulmonary hypertension (PH) including human pulmonary arterial hypertension (PAH) and hypoxia-induced PH in animal models [1, 2]. Macrophages, which are key actors of the innate immune system, are recruited early in experimental PH and are the most abundant inflammatory cells in remodelled pulmonary vessels from patients with PAH [3, 4]. However, the mechanisms underlying interactions between macrophages and pulmonary-artery smooth muscle cells (PASMCs) remain unclear [1]. Deciphering the crosstalk between these cell types is of major importance to better understand how inflammation and PH are connected and which chemokine system pathways could serve as treatment targets to inhibit pulmonary arterial remodelling.

Key chemokine systems involved in macrophage recruitment and pulmonary vascular remodelling include the CCL2-CCR2 [5, 6], CX3CL1-CX3CR1 [7–9] and CCL5-CCR5 pathways [10, 11]. The receptors for each of these systems are present on both PASMCs and macrophages, suggesting that complex interactions may occur between these two cell types. Moreover, both cell types express the specific ligands of the three receptor types. We recently reported that pharmacologically targeting the CCR5 pathway may be useful in PH, notably in patients with PAH complicating HIV infection [11]. Compared to CCL5-CCR5, the CCL2-CCR2 pathway seems to have more complex effects in PH. We previously reported that CCL2 was overexpressed in pulmonary vascular endothelial cells from patients with PAH and contributed to the proliferative PASMC phenotype [6]. In contrast, recent studies in $CCR2^{-/-}$ or $CCL2^{-/-}$ mice showed that CCR2 deficiency was associated with increased severity of hypoxia-induced PH [12, 13]. In these studies, the CX3CL1/CX3CR1 and CCL2/CCR2 systems did not seem to work cooperatively, but instead to have antagonistic effects [13].

Based on these studies, we investigated whether macrophages and PASMCs could communicate *via* the CCL2-CCR2 and CCL5-CCR5 pathways and whether pharmacological targeting of CCR2 and CCR5, individually or in combination, might be useful for treating PH. In these studies, we used the selective CCR5 antagonist maraviroc, as well as newly developed drugs to target either CCR2 alone or both CCR2 and CCR5 with a single molecule [11, 14, 15]. We conducted studies both in mouse models of PH and in lung tissue and cells from patients with PAH compared to controls. Because CCR5 antagonists do not bind to murine CCR5, we used human-CCR5 knock-in mice (h-CCR5ki) for studies in mice or mouse-derived cultured cells.

Methods

Studies in mice

Male mice (C57BL/6J) aged 3 months were used according to institutional guidelines, which complied with national and international regulations (agreement #94-28245). Genetically modified mice with constitutive expression of human CCR5 (h-CCR5ki) or deleted for CCR5 and CCR2 have been described previously [11, 13].

Studies in patients

Human lung tissue was obtained from six patients with idiopathic PAH who underwent lung transplantation at the University Hospital of Leuven (Leuven, Belgium) (supplementary table S1). 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 six patients undergoing lung resection surgery for localised lung tumours at the Montsouris Mutualiste Institute (Paris, France). The controls had a forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) ratio >70%. No patient or control had chronic cardiovascular, hepatic or renal disease or a history of cancer chemotherapy. The study was approved by the institutional review board of the Henri Mondor teaching hospital (Créteil, France). Pulmonary tissue was snap-frozen and stored at −80°C.

Isolation and preparation of human and murine M1-like and M2-like macrophages

Human blood was provided by the French blood transfusion agency (Etablissement Français du Sang). Peripheral blood mononuclear cells were isolated and differentiated to M2 as described in the supplementary methods. Murine macrophages were prepared from bone marrow isolated from the femur and tibia and differentiated into type 0 (M0), type 1 (M1) and type 2 (M2), as described previously [16].

The supplementary material provides details of the animal studies, treatment with CCR2, CCR5 or dual CCR2/CCR5 inhibitors, macrophage isolation and polarisation, PASMC proliferation experiments, analyses of CCL2-CCR2 and CCL5-CCR5 pathways and statistical analysis.

Results

Murine macrophages and PASMCs communicate to promote PASMC proliferation via CCR2 and CCR5

Macrophages from bone marrow of h-CCR5ki mice were polarised to the M1 or M2 phenotypes or left undifferentiated (M0). Exposing murine PASMCs to conditioned media from M1 or M2 stimulated PASMC growth. This effect was greater with conditioned media from M2 than from M1 (figure 1a,b). Exposing murine PASMCs to conditioned media from macrophage/PASMC co-cultures induced an even stronger growth-stimulating effect on PASMCs, and this effect was greatest with conditioned media from M2/PASMC co-cultures (figure 1a,b).

CCR2 and CCR5 expression was upregulated in PASMCs cultured with M1 and, to a greater extent, with M2 (figure 1c,d). CCR2 and CCR5 expression was also increased in macrophages co-cultured with PASMCs: CCR2 upregulation occurred only with M2/PASMC co-cultures, whereas CCR5 upregulation was found with M0/, M1/ and M2/PASMC co-cultures (supplementary figure S1A,B). The concentrations of CCL2 and CCL5 (CCR2 and CCR5 ligands, respectively) were higher in conditioned media from macrophage/PASMC co-cultures than in conditioned media from either macrophages or PASMCs (supplementary figure S1C,D).

To assess whether CCR2 and CCR5 contributed to PASMC proliferation induced by conditioned media from macrophage cultures or macrophage/PASMC co-cultures, we repeated these experiments with the CCR2, CCR5, or dual CCR2/CCR5 antagonist. As shown in figure 1e,f, the growth-stimulating effect of conditioned media from M1 cultures or M1/PASMC co-cultures was reduced by both selective antagonists and the dual antagonist. In response to conditioned media from M2 cultures or M2/PASMC co-cultures, the greatest inhibitory effect was obtained with the dual CCR2-CCR5 antagonist. The antagonist doses were chosen based to their ability to inhibit PASMC growth stimulated by CCL5, CCL2 or both (supplementary figure S2).

To further assess the respective roles played by CCR2 and CCR5 on macrophage/PASMC crosstalk, we co-cultured M2 with PASMCs from either CCR2-knockout (ko) or CCR5-ko mice. The conditioned media thus obtained were then applied to PASMCs from h-CCR5ki mice in the presence of the chemokine receptor antagonists or vehicle. As shown in figure 1g, deletion of CCR2 on M2 markedly inhibited the growth-promoting effect of conditioned media from M2/PASMC co-cultures; deletion of CCR5 on M2 had a smaller effect. In contrast, in PASMCs, deletion of CCR5 was more potent than deletion of CCR2 in attenuating the response (figure 1h). In all cases, treatment with the dual CCR2/CCR5 receptor antagonist produced a stronger inhibitory effect compared to the CCR2 or CCR5 antagonist alone.

Targeting CCR2, CCR5 or both to prevent or reverse PH in mice

To evaluate the functions of CCR2 and CCR5 in the same mouse strain, we performed studies in h-CCR5ki mice, which develop similar PH severity as wild-type mice when exposed to either chronic hypoxia alone or with SUGEN [11].

During normoxic conditions the expression of CCR2/5 was detectable only in perivascular macrophages, as attested by double-labelling with CD68. Exposure to chronic hypoxia induced expression of CCR2/5 in PASMCs of remodelling vessels, co-labelled with α-smooth muscle actin (figure 2a,b). The effects of the CCR2-, CCR5- or dual CCR2-CCR5 antagonist were then assessed *versus* the vehicle in h-CCR5ki mice exposed to 3 weeks' hypoxia. Treatment with the CCR2 or CCR5 antagonist similarly attenuated hypoxia-induced PH, with reductions in pulmonary vessel muscularisation, the percentage of dividing Ki67-positive pulmonary vascular cells and pulmonary vessel wall thickness (figure 3a,b; supplementary figure S3A). Treatment with the dual CCR2-CCR5 antagonist was more potent than blocking either CCR2 or CCR5 alone. The decrease in the number of perivascular macrophages, as assessed using immunohistochemistry, was similar when either CCR2 or CCR5 was blocked, but was more marked with the dual CCR2-CCR5 antagonist (figure 3a,b). Hypoxia exposure was associated with marked elevations in lung levels of mRNAs for the M2-macrophage markers Fizz1 and Mannose RC (MRC1) and as well as for the M1-macrophage markers inducible nitric oxide synthase and tumour necrosis factor-α (supplementary figure S4). These hypoxia-related changes in M1 and M2 macrophages were lessened by inhibitor treatment, with similar effects of blocking CCR2, CCR5 or both.

To assess whether blocking CCR2 and CCR5 could reverse PH, we studied h-CCR5ki mice subjected to SUGEN/hypoxia. After 15 days, the mice had severe PH with marked increases in right ventricular systolic pressure, right ventricle to left ventricle plus septum ratio (Fulton index), pulmonary artery muscularisation and number of $Ki67^+$ cells (figure 4a,b; supplementary figure S3B). These PH parameters increased further between days 15 and 30. Daily administration of the dual CCR2-CCR5 antagonist from day 15 to 30 led to marked decreases in PH compared with SUGEN/hypoxia mice studied on day 15 and with vehicle-treated mice on day 30. In addition, daily administration of either the CCR2 or the CCR5 antagonist from day 15 to day 30 diminished PH severity compared with vehicle-treated mice on day 30,

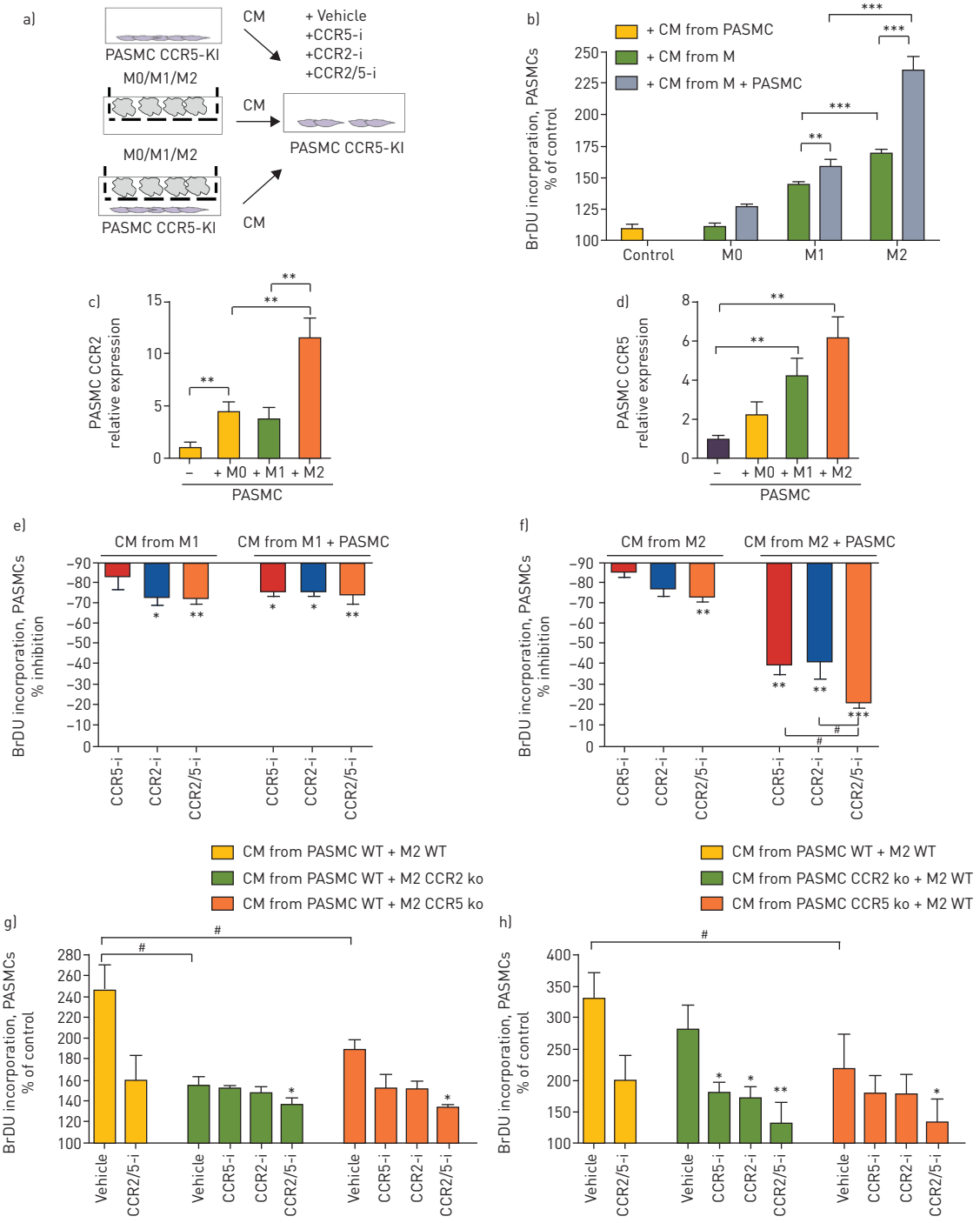

FIGURE 1 Effect of conditioned media (CM) obtained from cultured pulmonary-artery smooth muscle cells (PASMCs), macrophages (M) or co-cultured PASMCs and M on proliferation of PASMCs and induction of CCR2/5 expression. a) Diagram of the protocol for obtaining CM from cultured PASMCs, M or co-cultured PASMCs and M then stimulating the proliferation of PASMCs from human CCR5 knock-in (h-CCR5ki) mice with and without the CCR2, CCR5 or CCR2/CCR5 inhibitors. b) Proliferation of PASMCs from h-CCR5ki mice stimulated with CM from PASMCs, M0-, M1- or M2-macrophages cultured alone or co-cultured with PASMCs. **: p<0.01, ***: p<0.001 for comparisons of the means as indicated. c, d) CCR2 and CCR5 mRNA levels in PASMCs co-cultured with M0-, M1- or M2-macrophages. **: p<0.01 for comparisons of the means as indicated. e, f) Inhibition of proliferation of PASMCs from h-CCR5ki mice stimulated with CM from M1- or M2-macrophages cultured alone or co-cultured with PASMCs and treated with vehicle, the CCR5 inhibitor maraviroc (CCR5-i; 5 μM), the CCR2 inhibitor (CCR2-i; 1 μM) or the dual CCR2/CCR5 inhibitor (CCR2/5-i; 1 μM). *: p<0.05, **: p<0.01, ***: p<0.001 compared with cells treated with vehicle; #: p<0.02 compared with cells treated with the CCR2-i or with cells treated with the CCR5-i. g) Effect of deleting CCR2 or CCR5 in macrophages in the co-culture model. M were from wild-type (WT), CCR2 knock-out (ko) or CCR5 ko mice. Target PASMCs were from h-CCR5ki mice and were treated with CCR5-i (5 μM), CCR2-i (1 μM) or CCR2/5-i (1 μM). h) Effect of deleting CCR2 or CCR5 in PASMCs in the co-culture model. PASMCs co-cultured with M were from WT, CCR2 ko or CCR5 ko mice. Target PASMCs were from h-CCR5ki mice and were treated with CCR5-i (5 μM) maraviroc, CCR2-i (1 μM) or CCR2/5-i (1 μM). *: p<0.05, **: p<0.01 compared with cells treated with vehicle; #: p<0.01 compared with cells treated with CM from co-cultured PASMC and M2 from WT mice. BrDU: bromodeoxyuridine.

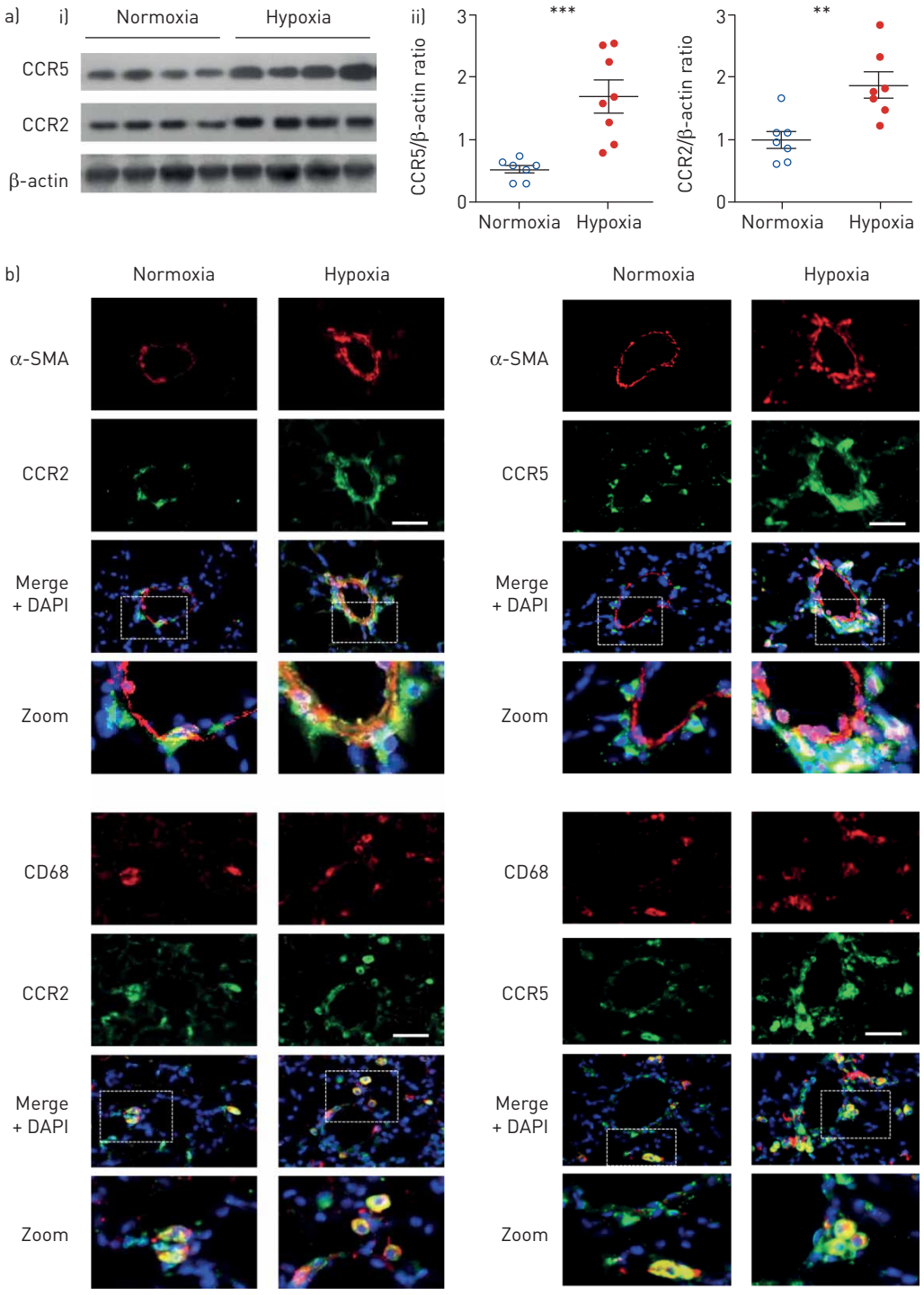

FIGURE 2 CCR2 and CCR5 are overexpressed in pulmonary-artery smooth muscle cells (PASMCs) and perivascular macrophages (M) during exposure to chronic hypoxia. a) i) Typical immunoblot and ii) quantification of CCR2 and CCR5 expression in lungs from normoxic or chronically hypoxic mice. Lines represent the mean±SEM of 7–8 animals. **: p<0.01, ***: p<0.001. b) Representative micrographs of lung tissue from normoxic or chronically hypoxic mice showing CCR5 and CCR2 expression (green) in PASMCs and M. PASMCs were labelled with α-smooth muscle actin (SMA) (red); M were identified by CD68 staining (red); nuclei were labelled with DAPI (blue). Co-localisation of CCR2 or CCR5 with α-SMA or CD68 is in yellow. Scale bars=50 μm.

but not compared with the mice studied on day 15 (figure 4). Moreover, the dual CCR2-CCR5 antagonist reduced the PH parameters and perivascular macrophage count to a greater extent than the selective CCR2-antagonist or the selective CCR5-antagonist on day 30 (figure 4a,b). In some remodelled vessels,

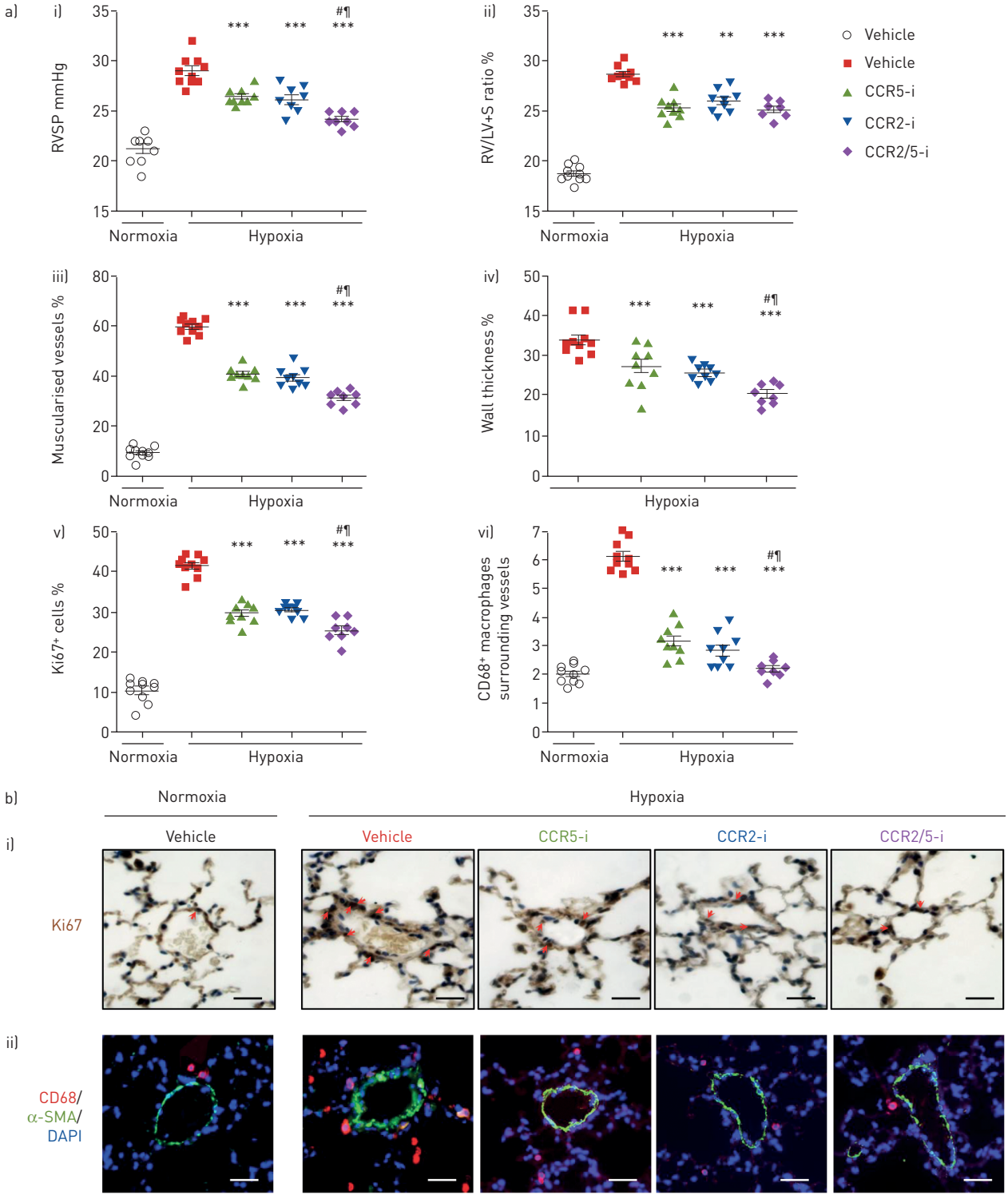

FIGURE 3 Targeting both CCR5 and CCR2 is more protective against hypoxic pulmonary hypertension in mice than targeting CCR5 or CCR2 alone. Human-CCR5 knock-in (h-CCR5ki) mice were exposed to hypoxia (21 days) and treated daily with either the CCR5 inhibitor maraviroc (CCR5-i, 200 mg·kg^{-1} per day, *p.o.*), the CCR2 inhibitor (CCR2-i, 100 mg·kg^{-1} per day, *p.o.*), the dual CCR2/CCR5 inhibitor (CCR2/5-i; 15 mg·kg^{-1} per day, *p.o.*) or vehicle. A control group of mice was kept in normoxic conditions and treated with vehicle. a) Graphs of i) right ventricular systolic pressure (RVSP) and ii) Fulton index (right ventricle to left ventricle plus septum ratio: RV/(LV+S)); iii) pulmonary vessel muscularisation expressed as the percentage of partially or fully muscularised vessels among all pulmonary vessels; iv) wall thickness calculated as the ratio of the difference between the external and internal diameters to the external diameter of the pulmonary artery; v) dividing Ki67$^+$ cells; vi) perivascular CD68$^+$ macrophages. Lines are the mean±SEM of 8–10 animals. **: p<0.01, ***: p<0.001 compared with vehicle-treated mice exposed to hypoxia; $^{\#}$: p<0.01 compared with CCR5-i treated mice; ¶: p<0.01 compared with CCR2-i treated mice. b) Representative micrographs of pulmonary vessels stained for i) Ki67 or ii) perivascular CD68$^+$ macrophages. i) Nuclei are stained with haematoxylin (blue). Proliferating cells are indicated by arrows. ii) Vessels were visualised by α-smooth muscle actin (SMA) staining (green). Nuclei were labelled with DAPI (blue). Scale bars=40 µm.

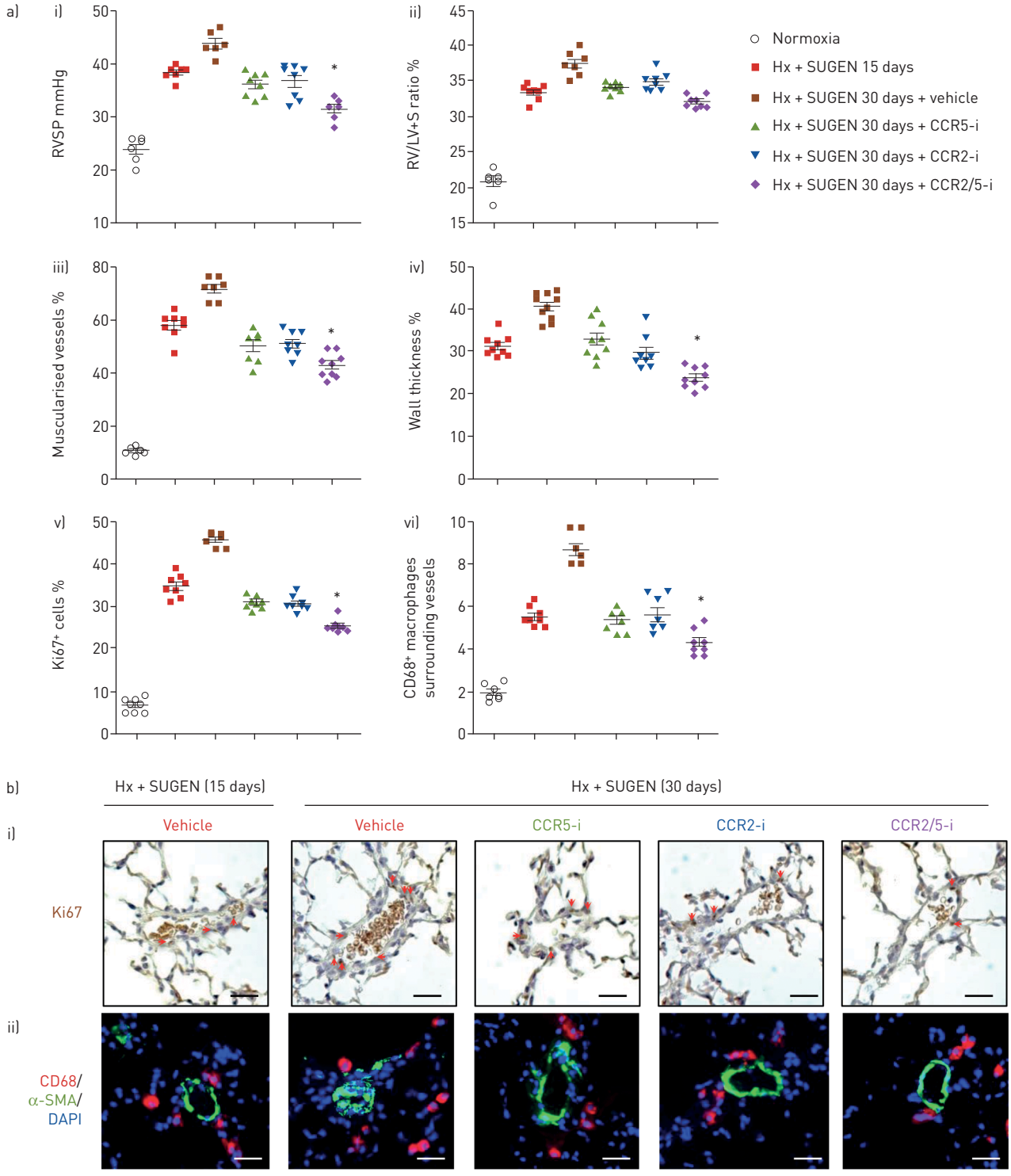

FIGURE 4 Targeting both CCR5 and CCR2 can reverse pulmonary hypertension induced by hypoxia and SUGEN. Human-CCR5 knock-in (h-CCR5ki) mice were exposed to hypoxia (15 days) combined with SUGEN treatment (20 mg·kg^{-1} per week, intraperitoneally) then treated daily with vehicle, the CCR5 inhibitor maraviroc (CCR5-i, 200 mg·kg^{-1} per day, *p.o.*), the CCR2 inhibitor (CCR2-i; 100 mg·kg^{-1} per day, *p.o.*), or the dual CCR2/CCR5 inhibitor (CCR2/5-i; 15 mg·kg^{-1} per day, *p.o.*) for the next 15 days under continuous hypoxia and SUGEN treatment. A control group of mice was kept in normoxic conditions and treated with vehicle. a) Graphs of i) right ventricular systolic pressure (RVSP) and ii) Fulton index (right ventricle to left ventricle plus septum ratio: RV/(LV+S)); iii) pulmonary vessel muscularisation expressed as the percentage of partially or fully muscularised vessels among all pulmonary vessels; iv) wall thickness calculated as the ratio of the difference between the external and internal diameters to the external diameter of the pulmonary artery; v) dividing Ki67$^+$ cells and vi) perivascular CD68$^+$ macrophages. Lines are the mean±SEM of 8–10 animals. *: p<0.05 compared with vehicle-treated mice exposed to hypoxia (15 days) and SUGEN. b) Representative micrographs of pulmonary vessels stained for i) Ki67 or ii) perivascular CD68$^+$ macrophages. i) Nuclei are stained with haematoxylin (blue). Proliferating cells are indicated by arrows. ii) Vessels were visualised using α-smooth muscle actin (SMA) staining (green). Nuclei were labelled with DAPI (blue). Scale bars=40 µm. Hx: hypoxia.

Masson's trichrome staining revealed thickening of the intima, potentially indicating neointima formation (supplementary figure S3C). However, this finding was extremely rare and occurred only in animals not given the inhibitors.

Crosstalk between human macrophages and PASMCs via *CCR2 and CCR5: impact on PASMC proliferation in human PAH*

To determine whether the macrophage/PASMC interactions were involved in human PAH, we investigated lung tissues and cells from patients with PAH and controls. CCR2 immunofluorescence staining in PASMCs predominated in the remodelled vessels of the patients with PAH, whereas no immunofluorescence was observed in the vessels of the controls (figure 5). In contrast, CCR5 staining was found in PASMCs from both controls and patients with PAH, although total staining was greater in the patients, chiefly due to the higher number of PASMCs in their remodelled vessels. In contrast, macrophages surrounding pulmonary vessels were stained equally for CCR2 and CCR5, with an increased number of macrophages in remodelled vessels from the patients compared to vessels from the controls (figure 5). In accordance with these results, we found that CCR2 protein levels as determined by Western blotting were increased in lungs from patients with PAH compared to controls (supplementary figure S5), in a similar fashion to the CCR5 protein levels that we reported previously [11]. Interestingly, most of the perivascular macrophages in patients with PAH were positive for the M2 polarisation marker mannose receptor (MRC1) (figure 6a). This contrasted with the control lungs, in which macrophages were scarce and very rarely stained for MRC1 (figure 6b).

To assess crosstalk between macrophages and PASMCs in PAH, we used macrophages generated from blood monocytes from control donors and PASMCs cultured from pulmonary arteries from three controls and three patients with PAH. Macrophages were polarised toward the M2 phenotype using human interleukin (IL)-4 for comparison with M0. Exposing PASMCs from controls to conditioned media from M0 or M2 induced mild-to-moderate growth rate alterations compared to conditioned media from PASMCs (figure 7a). However, exposure to conditioned media from M0/PASMC or M2/PASMC co-cultures markedly stimulated growth, and this effect was stronger with conditioned media from co-cultures containing PASMCs from PAH patients as opposed to controls (figure 7a). Exposure of control PASMCs to conditioned media from PAH PASMCs stimulated growth, although to a lesser extent (figure 7a). Exposing PASMCs from patients with PAH to the same procedures produced a quantitatively stronger response, and the potentiation seen with conditioned media from macrophage/PASMC co-cultures compared to conditioned media from macrophages alone was still observed, although to a lesser extent (figure 7b). No difference was seen between conditioned media from co-cultures obtained with PASMCs from controls *versus* patients with PAH (figure 7b). Co-culturing PAH or control PASMCs with macrophages considerably increased staining for CCR2 and CCR5 in both cell types (figure 7c,d). To assess the effect of drugs targeting CCR2 and CCR5 on PASMC proliferation, we used conditioned media from M2/PASMC co-cultures in all experiments. While the inhibitory effects of the drugs occurred under most conditions, they were strongest with the dual CCR2-CCR5 antagonist (figure 7e–g), which inhibited the proliferative effect of CCL2 and CCL5 applied to human PASMCs (supplementary figure S6).

Co-culturing macrophages and PASMCs also led to distinctive clustering of CCR5 staining in PASMCs on the side nearest to the macrophages (figure 8a,b). CCR2 expression was uniform on PAMSCs. Treatment with either the CCR2 or the CCR5 inhibitor suppressed macrophages activation, and the CCR5 inhibitor abolished the distinctive localisation or clustering of CCR5 staining. To investigate whether PASMC migratory capacity was also influenced by macrophages *via* CCR2 and CCR5, PASMCs from patients with PAH or controls were placed in Boyden chambers with macrophages in the lower compartment. As shown in figure 8c, the migratory response of PASMCs from both patients with PAH and controls was markedly inhibited by the drugs, the effect being strongest with the dual CCR2-CCR5 antagonist and PASMCs from patients with PAH.

Discussion

That macrophages can contribute to the proliferative phenotype of PASMCs during PH is now well established. While M1 macrophages display a cytotoxic and proinflammatory phenotype, M2 macrophages promote tissue remodelling and tumour progression *via* the release of mitogenic factors [17, 18]. Here, we showed that the proliferating effect of M2 was amplified by PASMCs and that this amplification did not require direct contact between the two cell types. Thus, communication between M2 and PASMCs *via* paracrine mechanisms results in enhanced release of mitogenic stimuli active on PASMCs. Since both macrophages and PASMCs express common receptors for a variety of chemokines, we assessed whether two major molecular pathways, CCL2/CCR2 and CCL5/CCR5, were involved in this process. Exploring these two pathways was further justified by the availability of new drugs targeting each receptor alone or both in combination [11, 14, 15].

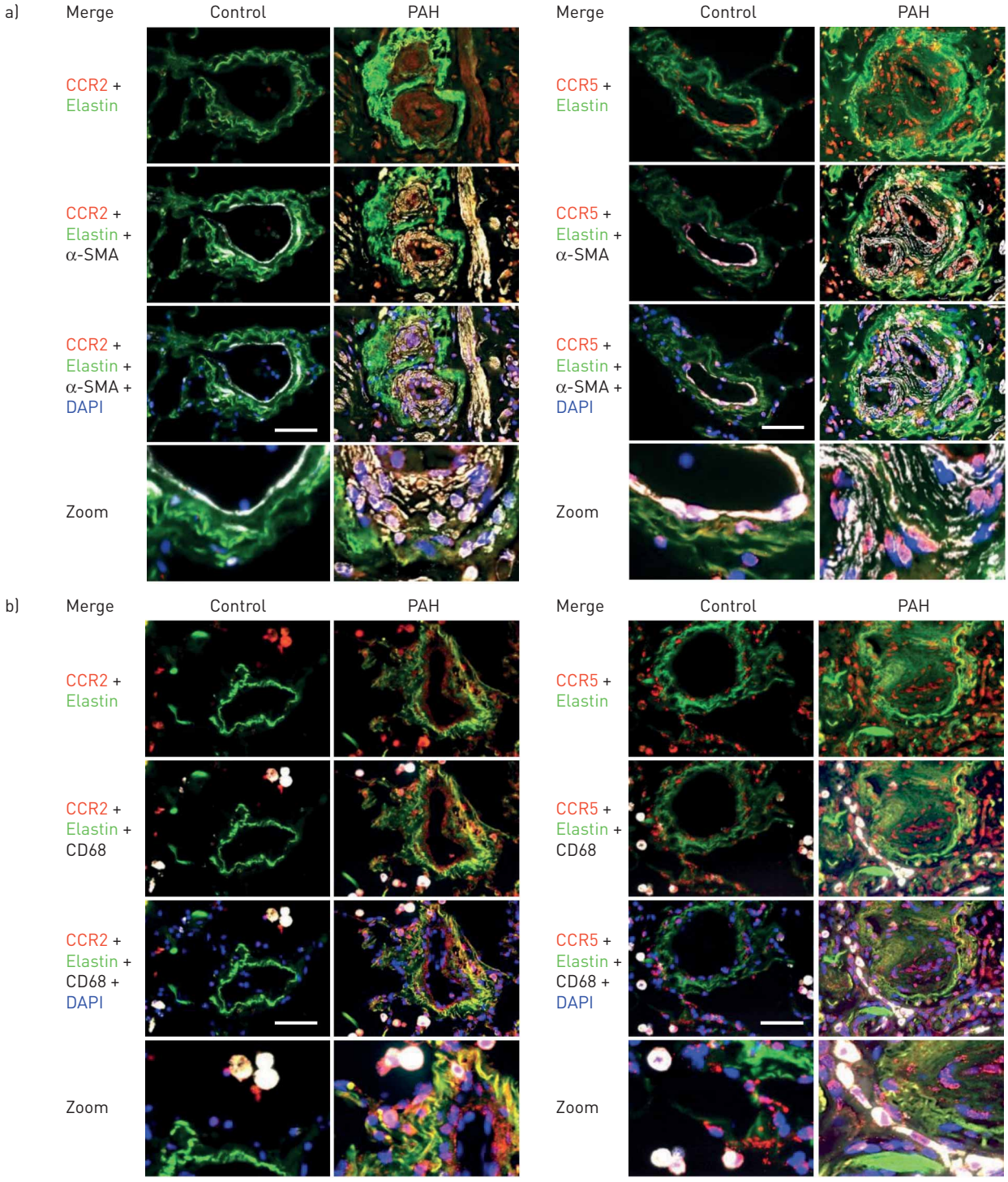

FIGURE 5 CCR2 and CCR5 staining in lung specimens from patients with pulmonary arterial hypertension (PAH) and controls. Representative micrographs of lung tissue from patients with PAH and controls showing CCR2 or CCR5 expression (red) in a) PASMCs from small pulmonary vessels, identified by α-SMA immunofluorescence (white) and b) perivascular macrophages identified by CD68 immunofluorescence (white). Green: elastin autofluorescence. Nuclei were labelled with DAPI (blue). Scale bars=50 μm.

We found that co-culturing macrophages and PASMCs resulted in increased CCR2 and CCR5 expression on both cell types and in greater release of their respective ligands CCL2 and CCL5. Selective inhibition of CCR2 or CCR5 attenuated the growth-promoting effect of conditioned media from M2/PASMC co-cultures, and this effect was enhanced when both CCR2 and CCR5 were inhibited. To determine whether this interaction was linked to CCR2 or CCR5 specifically expressed by macrophages or PASMCs,

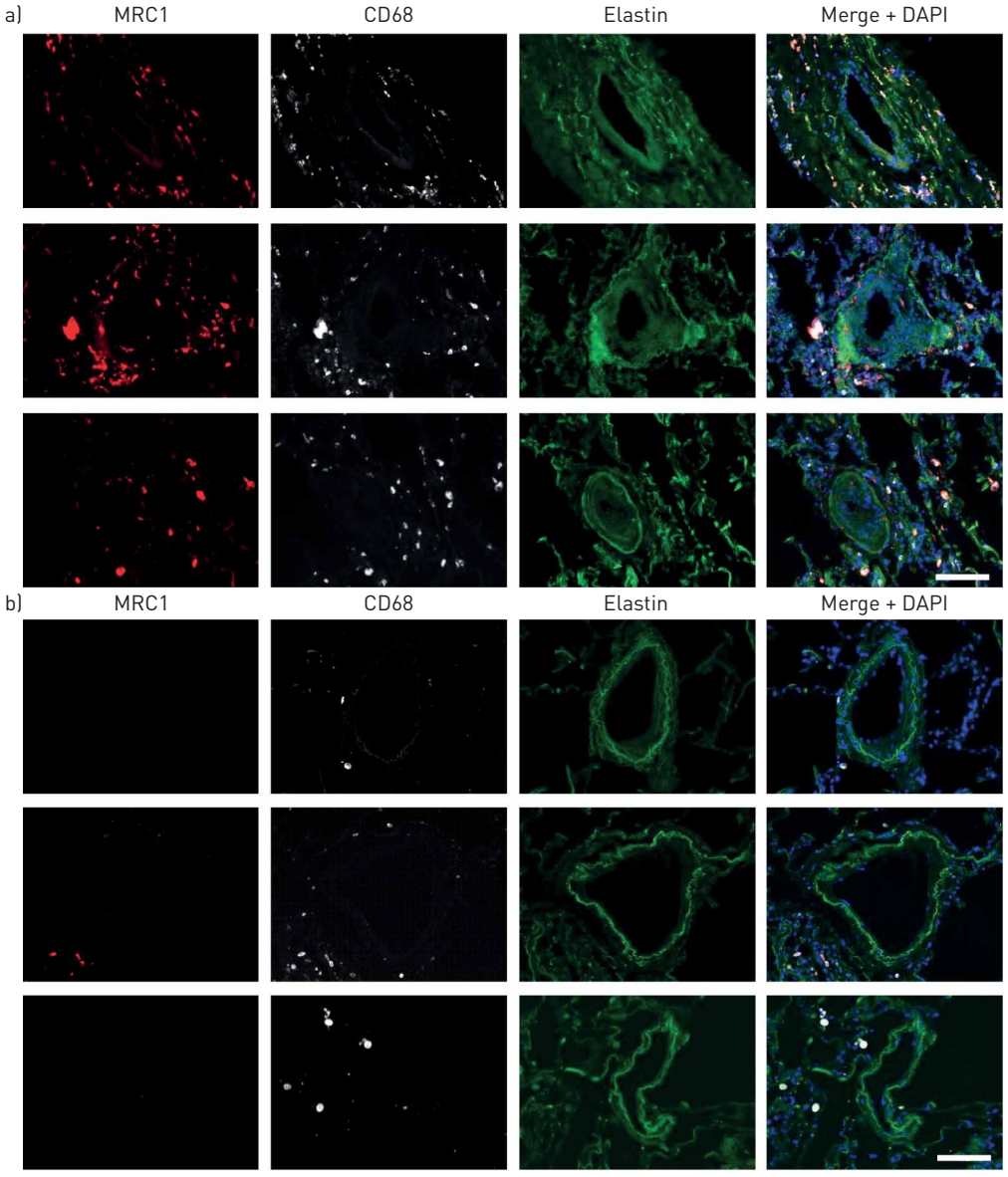

FIGURE 6 Representative micrographs of macrophages stained for CD68 and for the M2 polarisation marker mannose receptor, also known as MRC1 in a) three patients with pulmonary arterial hypertension (PAH) and b) three controls. Green: elastin autofluorescence. Nuclei were labelled with DAPI (blue). Scale bars=100 μm.

we performed experiments in which the CCR2 or CCR5 genes were deleted from macrophages or PASMCs used for the co-cultures. The results showed that both receptors were needed for a complete effect but that CCR5 had a predominant role in PASMCs and CCR2 in macrophages. In all conditions, adding the dual receptor antagonist produced a supplementary effect, indicating that both receptors were required for the complete response. Moreover, since an inhibitory effect was consistently obtained by deleting CCR2 or CCR5, whether in macrophages or in PASMCs, this result demonstrates the two-way nature of the macrophage/PASMC crosstalk mediated by the CCL2/CCR2 and the CCL5/CCR5 systems.

These results suggest that macrophage activation into the M2 phenotype in close proximity to PASMCs may trigger a vicious circle in which both PASMCs and macrophages become activated and interact with each other, ultimately exerting a strong stimulating effect on PASMC proliferation. This may occur in response to many conditions, since most lung cells are able to release both IL-4 and IL-13, the two major M2 phenotype stimuli [19]. Therefore, macrophages may constitute potential triggers of various types of PH. This possibility is further supported by previous studies showing that lung inflammation precedes the development of hypoxia-induced PH and the recruitment of macrophages, which become activated and acquire the M2 phenotype [3]. Moreover, alveolar macrophage depletion has been shown to attenuate

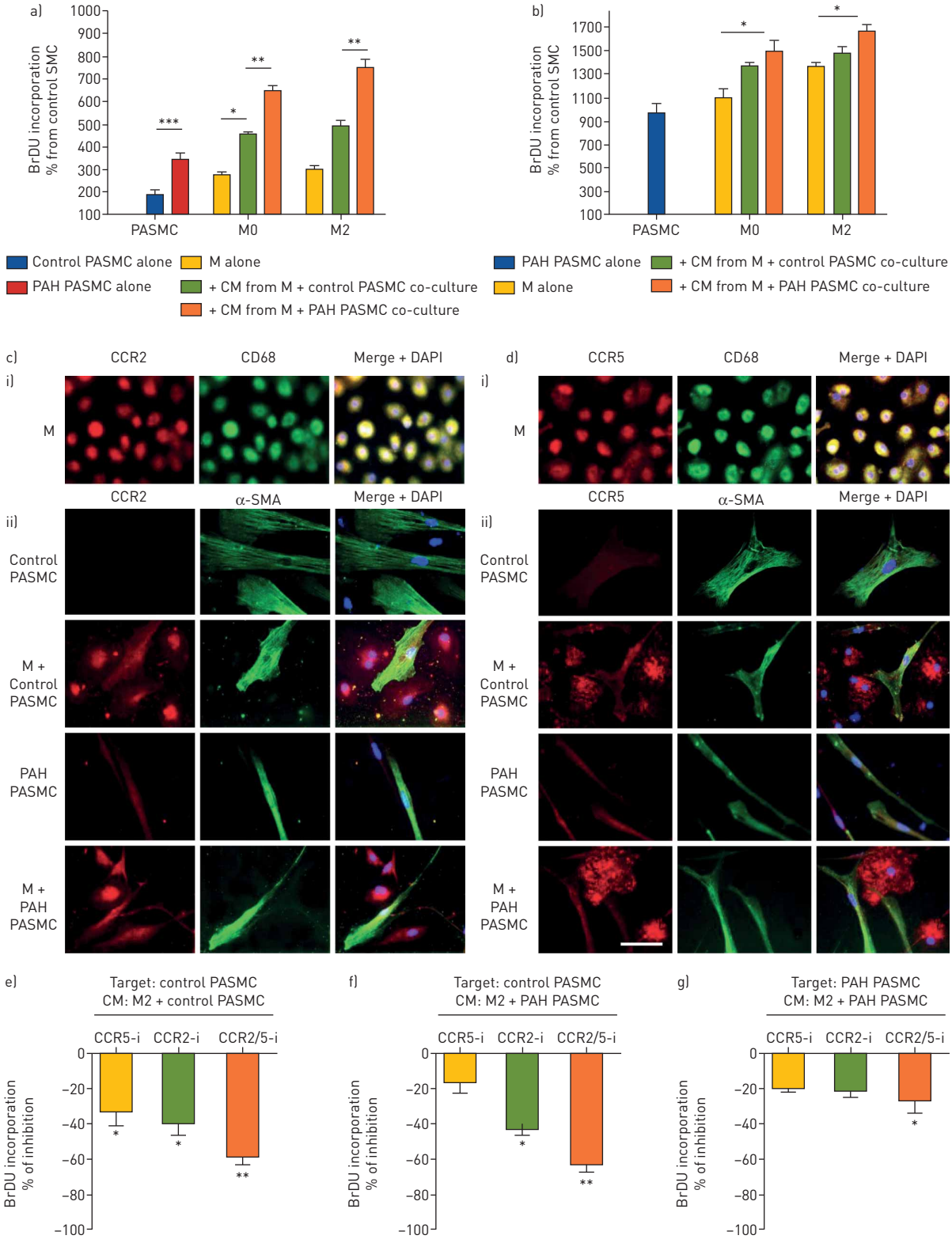

FIGURE 7 Effect of conditioned media (CM) obtained from macrophages alone or co-cultured with pulmonary-artery smooth muscle cells (PASMCs) from patients with PAH or controls on PASMCs from either patients with PAH or controls. a) Proliferation of PASMCs from controls stimulated with CM obtained from PASMCs from controls, PASMCs from patients with PAH, undifferentiated macrophages (M0) or type 2 macrophages (M2) alone or co-cultured with PASMCs from controls or patients with PAH. *: p<0.05, **: p<0.01, ***: p<0.001 for comparisons of the means as indicated. b) Proliferation of PASMCs from PAH patients stimulated with CM obtained from corresponding PASMCs, M0 or M2 alone or co-cultured with PASMCs from patients with PAH. *: p<0.05 for comparisons of the means as indicated.

FIGURE 7 continued. c) CCR2 and d) CCR5 immunofluorescence staining in and PASMCs from controls and patients with PAH. i) CCR2 (red) or CCR5 (red) in cultured macrophages in c) and d), respectively; macrophage preparation purity was verified by anti-CD68 immunofluorescence (green). ii) Expression of CCR2 (red) or CCR5 (red) in PASMCs cultured alone or co-cultured with macrophages, as indicated in c) and d), respectively. PASMCs were identified by anti-α-smooth muscle actin (SMA) immunofluorescence (green); nuclei were stained with DAPI (blue). When co-cultured with PASMCs, macrophages demonstrated increased size and clustered CCR5 receptors. Scale bar=50 μm. e–g) Effect of blocking CCR2, CCR5 or both on CM-induced proliferation of PASMCs from e,f) controls or g) patients with PAH, as indicated. PASMCs were treated either with vehicle or with the CCR5 inhibitor (CCR5-i; 5 μM), the CCR2 inhibitor (CCR2-i; 1 μM) or the dual CCR2/CCR5 inhibitor (CCR2/5-i; 1 μM). *: p<0.05, **: p<0.01, compared to cells treated with vehicle. BrDU: bromodeoxyuridine.

hypoxia-induced PH [20, 21]. Consistent with this, we previously showed that hypoxia exposure was associated with early increases in the lung expression of CCR2 and CCR5 [11, 13]. In the present study, CCR2 and CCR5 were overexpressed by both macrophages and PASMCs in mice exposed to chronic hypoxia and developing PH. Moreover, we found that targeting both CCR2 and CCR5 was more potent in preventing hypoxia-induced PH in mice compared to blocking either CCR2 or CCR5. To evaluate whether targeting CCR2 or CCR5 may not only prevent but also reverse PH, we used the SUGEN-hypoxia model and treated mice only after PH had been established. Treatment with either the CCR2 or the CCR5 antagonist stabilised the disease, but failed to reverse it. In contrast, treatment with the dual receptor antagonist decreased the severity of PH compared to the levels at treatment initiation. The finding that dual CCR2/CCR5 inhibition not only prevented, but also reversed PH may be taken as evidence that CCR2/CCR5-mediated macrophage/PASMC crosstalk is involved in the development and the progression of PH.

In lung cancer, CCR2 was shown to cooperate with CX3CR1 to induce crosstalk between macrophages and cancer cells, resulting in increased cancer-cell proliferation [22]. Contrasting with these results, we showed previously that inactivating both the CX3CL1/CX3CR1 and the CCL2/CCR2 systems in mice led, not to additive, but to opposite effects [13]. Therefore, it is likely that several pathways involved in PH contribute to macrophage/PASMC crosstalk and that targeting two of the key effectors, CCR2 and CCR5, impacts other chemokine systems and limits their effects. Thus, identification of CCR5 and CCR2 as complementary targets provides a rationale for new therapies directed against the inflammatory process in PH.

To investigate the potential relevance of these findings to human PAH, we studied the *in situ* distribution of CCR2 and CCR5 in lungs from patients with PAH and controls. Interestingly, CCR2 in controls was detectable only in macrophages and not in PASMCs, whereas in lungs from patients with PAH, CCR2 was detected in PASMCs in addition. In contrast, CCR5 was found in both macrophages and PASMCs and was more abundant in remodelled vessels from patients with PAH compared to controls. Moreover, most of the perivascular macrophages in patients with PAH were positive for the M2 macrophage marker MRC1. This finding is consistent with previous studies showing a predominance of M2 macrophage markers in the lungs of patients with idiopathic PAH, contrasting with a predominance of lung M1 macrophage subtypes in healthy individuals [23].

To further investigate crosstalk between human macrophages and PASMCs *in vitro*, we used PASMCs from controls or patients with PAH in the co-culture model. We found that co-culturing human macrophages with PASMCs from controls led to marked stimulation, which was stronger when the co-culture used PAH instead of control PASMCs and M2 instead of M0. This last stimulation potency was greater than that obtained with conditioned media from PAH PASMCs. A greater stimulatory effect of M2 compared to M0 was also observed when using PASMCs from patients with PAH as target cells. However, due to the strong proliferative activity of PASMCs from patients with PAH, the potentiated effects of co-culturing macrophages and PASMCs was less apparent than when using PASMCs from controls as target cells. A likely explanation for these different responses involves the higher expression of both CCR2 and CCR5 in PASMCs from patients with PAH, as shown previously and in the present study. Indeed, simultaneous CCR2 and CCR5 blockade inhibited PASMCs growth induced by conditioned media from M2/PASMC co-cultures, whether the target PASMCs were from controls or from patients with PAH. When examining the PASMCs migratory response to macrophages, we found that the strongest inhibitory effect occurred when PASMCs from patients with PAH were exposed to the dual CCR2/CCR5 receptor antagonist. Thus, collaboration between macrophages and PASMCs *via* CCR2 and CCR5 contributes not only to the increased proliferative phenotype of PASMCs but also to the migratory capacity of PASMCs from patients with PAH. Importantly, the CCR2 and CCR5 antagonists more potently inhibited the proliferation of control target cells than of PAH target cells. However, they also more potently inhibited migration of PAH PASMCs than of control PASMCs. We can only speculate about the relevance of these *in vitro* responses to the *in vivo* effectiveness of the antagonists. Indeed, giving these drugs chronically may alter their effects by affecting macrophage/PASMC interactions, reducing M2 macrophage counts, and altering CCR2 and CCR5 expression. Also, we cannot exclude the possibility that therapeutic manipulation of CCR2, CCR5, or both affected PASMC transdifferentiation. It has been shown that capillary endothelial cells can undergo mesenchymal transition in response to chronic inflammatory stimuli [24].

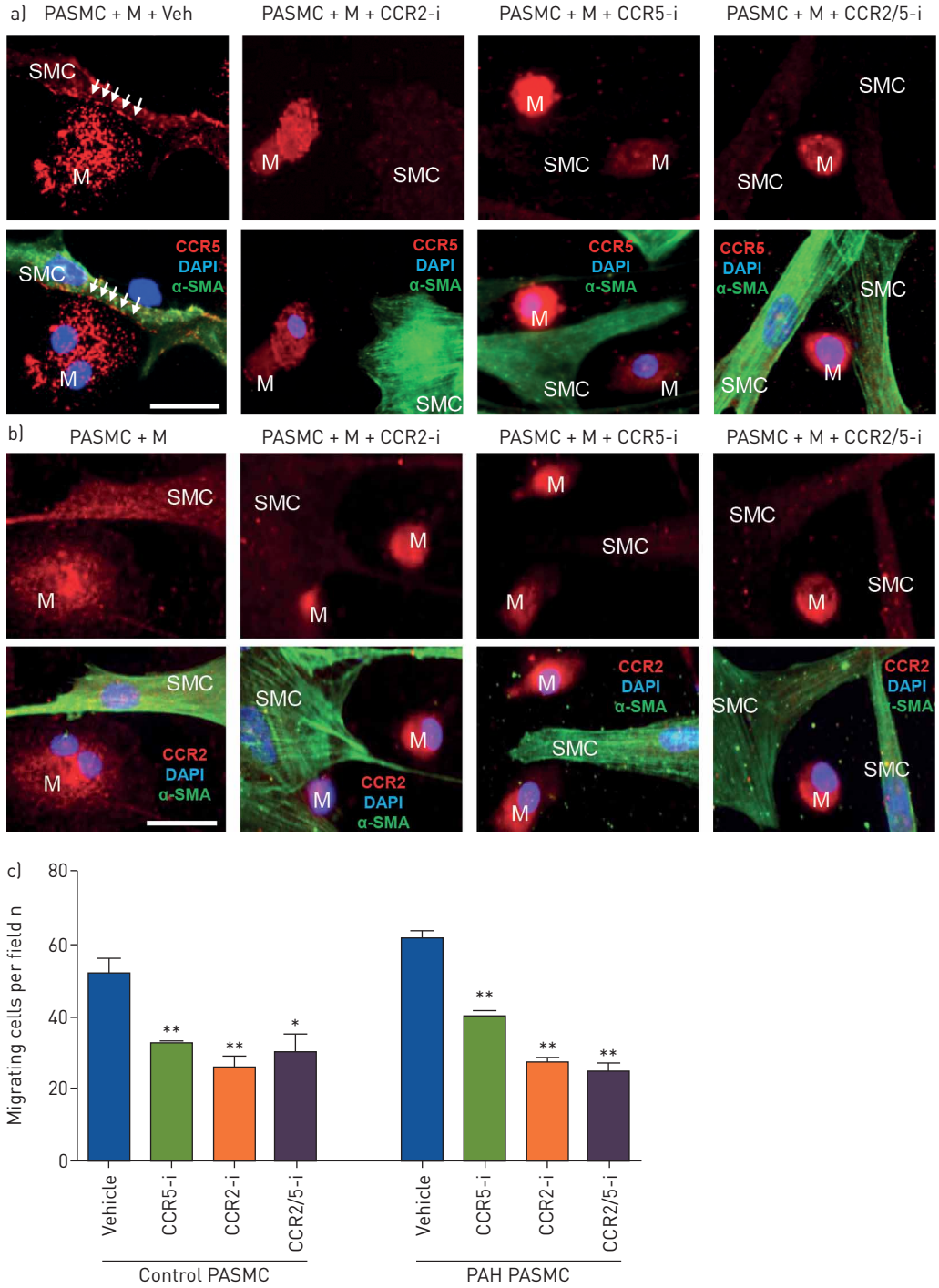

FIGURE 8 Migration of human pulmonary-artery smooth muscle cells (hPASMCs) involves CCR2/5 signalling pathways. Representative micrographs showing expression of a) CCR5 (red) and b) CCR2 (red) on co-cultured type-2 macrophages (M2) and PASMCs. PASMCs were identified by anti-α-smooth muscle actin (SMA) immunofluorescence (green); nuclei were stained with DAPI (blue). When co-cultured with PASMCs, macrophages (M) demonstrated increased size and clustered CCR5 receptors. In PASMCs located near macrophages, CCR5 receptors were clustered on the side nearest the macrophages (arrows), whereas CCR2 expression was more uniform. In presence of the CCR5 inhibitor, the CCR2 inhibitor or the dual CCR2/CCR5 inhibitor, the macrophages appeared inactivated with a reduction in CCR2 and CCR5 staining in both macrophages and PASMCs. Scale bar=10 μm. c) PASMCs from controls or patients with PAH (placed at the upper chamber) in the presence of vehicle, the CCR5 inhibitor (CCR5-i; 5 μM), the CCR2 inhibitor (CCR2-i;1 μM), or the dual CCR2/CCR5 inhibitor (CCR2/5-i; 1 μM). Activated M2 were loaded in the lower chamber. Bar graphs represents the mean count of PASMCs migrating towards the bottom of membrane. Experiments were performed in triplicate with three different patients. *: p<0.05, **: p<0.01, compared to cells treated with vehicle.

Therefore, these results demonstrate that collaboration between macrophages and PASMCs is an important mechanism affecting both cell types and resulting in PASMCs migration and proliferation during both the development and the progression of PH. Macrophage activation into the M2 phenotype appears to be a major trigger for initiating two-way crosstalk between macrophages and PASMCs and may therefore occur as an early critical event in the process of pulmonary vascular remodelling. We show here that both CCR2 and CCR5 expressed by PASMCs and macrophages underlie this synergistic mechanism. Pharmacokinetic and safety data previously obtained with the drugs used in this study are encouraging [25]. Dual targeting of CCR2 and CCR5 may therefore hold promise as a therapeutic strategy for human PH.

Acknowledgements: We are grateful to Rachid Souktani and Philippe Caramelle from the animal facility and to Xavier Decrouy and Christelle Micheli from the imaging platform facility at IMRB Inserm (Créteil, France).

Conflict of interest: S. Abid has nothing to disclose. E. Marcos has nothing to disclose. A. Parpaleix has nothing to disclose. V. Amsellem has nothing to disclose. M. Breau has nothing to disclose. A. Houssaini has nothing to disclose. N. Vienney has nothing to disclose. M. Lefevre has nothing to disclose. G. Derumeaux has nothing to disclose. S. Evans is an employee of Pfizer Inc. C. Hubeau was an employee of Pfizer Inc., during the conduct of the study. M. Delcroix has nothing to disclose. R. Quarck has nothing to disclose. S. Adnot has nothing to disclose. L. Lipskaia has nothing to disclose.

Support statement: This study was supported by grants from the INSERM, Délégation à la Recherche Clinique de l'AP-HP, Fondation pour la Recherche Médicale (FRM), Legs Poix and Agence Nationale pour la Recherche ANR. Funding information for this article has been deposited with the Crossref Funder Registry.

References

1 Hassoun PM, Mouthon L, Barberà JA, *et al.* Inflammation, growth factors, and pulmonary vascular remodeling. *J Am Coll Cardiol* 2009; 54: Suppl. 1, S10–S19.
2 Rabinovitch M, Guignabert C, Humbert M, *et al.* Inflammation and immunity in the pathogenesis of pulmonary arterial hypertension. *Circ Res* 2014; 115: 165–175.
3 Vergadi E, Chang MS, Lee C, *et al.* Early macrophage recruitment and alternative activation are critical for the later development of hypoxia-induced pulmonary hypertension. *Circulation* 2011; 123: 1986–1995.
4 Savai R, Pullamsetti SS, Kolbe J, *et al.* Immune and inflammatory cell involvement in the pathology of idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2012; 186: 897–908.
5 O'Connor T, Borsig L, Heikenwalder M. CCL2-CCR2 signaling in disease pathogenesis. *Endocr Metab Immune Disord Drug Targets* 2015; 15: 105–118.
6 Sanchez O, Marcos E, Perros F, *et al.* Role of endothelium-derived CC chemokine ligand 2 in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2007; 176: 1041–1047.
7 Balabanian K, Foussat A, Dorfmuller P, *et al.* CX(3)C chemokine fractalkine in pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2002; 165: 1419–1425.
8 Perros F, Dorfmüller P, Souza R, *et al.* Fractalkine-induced smooth muscle cell proliferation in pulmonary hypertension. *Eur Respir J* 2007; 29: 937–943.
9 Zhang J, Hu H, Palma NL, *et al.* Hypoxia-induced endothelial CX3CL1 triggers lung smooth muscle cell phenotypic switching and proliferative expansion. *Am J Physiol Lung Cell Mol Physiol* 2012; 303: L912–L922.
10 Dorfmüller P, Zarka V, Durand-Gasselin I, *et al.* Chemokine RANTES in severe pulmonary arterial hypertension. *Am J Respir Crit Care Med* 2002; 165: 534–539.
11 Amsellem V, Lipskaia L, Abid S, *et al.* CCR5 as a treatment target in pulmonary arterial hypertension. *Circulation* 2014; 130: 880–891.
12 Yu YR, Mao L, Piantadosi CA, *et al.* CCR2 deficiency, dysregulation of Notch signaling, and spontaneous pulmonary arterial hypertension. *Am J Respir Cell Mol Biol* 2013; 48: 647–654.
13 Amsellem V, Abid S, Poupel L, *et al.* Roles for the CX3CL1/CX3CR1 and CCL2/CCR2 chemokine systems in hypoxic pulmonary hypertension. *Am J Respir Cell Mol Biol* 2017; 56: 597–608.
14 Xue CB, Wang A, Han Q, *et al.* Discovery of INCB8761/PF-4136309, a potent, selective, and orally bioavailable CCR2 antagonist. *ACS Med Chem Lett* 2011; 2: 913–918.
15 Gale JD, Berger B, Gilbert S, *et al.* A CCR2/5 inhibitor, PF-04634817, is inferior to monthly ranibizumab in the treatment of diabetic macular edema. *Invest Ophthalmol Vis Sci* 2018; 59: 2659–2669.
16 Inaba K, Inaba M, Romani N, *et al.* Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 1992; 176: 1693–1702.
17 Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005; 5: 953–964.
18 Nahrendorf M, Pittet MJ, Swirski FK. Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. *Circulation* 2010; 121: 2437–2445.
19 Munitz A, Brandt EB, Mingler M, *et al.* Distinct roles for IL-13 and IL-4 *via* IL-13 receptor α1 and the type II IL-4 receptor in asthma pathogenesis. *Proc Natl Acad Sci USA* 2008; 105: 7240–7245.
20 Frid MG, Brunetti JA, Burke DL, *et al.* Hypoxia-induced pulmonary vascular remodeling requires recruitment of circulating mesenchymal precursors of a monocyte/macrophage lineage. *Am J Pathol* 2006; 168: 659–669.
21 Žaloudíková M, Vytášek R, Vajnerová O, *et al.* Depletion of alveolar macrophages attenuates hypoxic pulmonary hypertension but not hypoxia-induced increase in serum concentration of MCP-1. *Physiol Res* 2016; 65: 763–768.
22 Schmall A, Al-Tamari HM, Herold S, *et al.* Macrophage and cancer cell cross-talk *via* CCR2 and CX3CR1 is a fundamental mechanism driving lung cancer. *Am J Respir Crit Care Med* 2015; 191: 437–447.
23 Hashimoto-Kataoka T, Hosen N, Sonobe T, *et al.* Interleukin-6/interleukin-21 signaling axis is critical in the pathogenesis of pulmonary arterial hypertension. *Proc Natl Acad Sci USA* 2015; 112: E2677–E2686.
24 Coll-Bonfill N, Musri MM, Ivo V, *et al.* Transdifferentiation of endothelial cells to smooth muscle cells play an important role in vascular remodelling. *Am J Stem Cells* 2015; 4: 13–21.
25 Lefebvre E, Gottwald M, Lasseter K, *et al.* Pharmacokinetics, safety, and CCR2/CCR5 antagonist activity of cenicriviroc in participants with mild or moderate hepatic impairment. *Clin Transl Sci* 2016; 9: 139–148.