CD133$^+$ cells in pulmonary arterial hypertension

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ABSTRACT Circulating mononuclear cells may play an important role for the vascular remodelling in pulmonary arterial hypertension (PAH), but studies addressing multiple progenitor populations are rare and inconsistent.

We used a comprehensive fluorescence-activated cell sorting analysis of circulating mononuclear cells in 20 PAH patients and 20 age- and sex-matched controls, and additionally analysed CD133$^+$ cells in the lung tissue of five PAH transplant recipients and five healthy controls (donor lungs).

PAH patients were characterised by increased numbers of circulating CD133$^+$ cells and lymphopenia as compared with control. In PAH, CD133$^+$ subpopulations positive for CD117 or CD45 were significantly increased, whereas CD133$^+$CD309$^+$, CD133$^+$CXCR2$^+$ and CD133$^+$CD31$^+$ cells were decreased. In CD133$^+$ cells, SOX2, Nanog, Ki67 and CXCR4 were not detected, but Oct3/4 mRNA was present in both PAH and controls. In the lung tissue, CD133$^+$ cells included three main populations: type 2 pneumocytes, monocytes and undifferentiated cells without significant differences between PAH and controls.

In conclusion, circulating CD133$^+$ progenitor cells are elevated in PAH and consist of phenotypically different subpopulations that may be up- or downregulated. This may explain the inconsistent results in the literature. CD133$^+$ type 2 pneumocytes in the lung tissue are not associated with circulating CD133$^+$ mononuclear cells.

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PAH patients are characterised by lymphopenia and increased numbers of CD133$^+$ cells
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Introduction

Circulating cell populations may play an important role among the different pathologic mechanisms underlying pulmonary hypertension [1, 2]. According to recent studies, circulating mesenchymal precursors of the monocyte/macrophage lineage as well as c-kit$^+$ cells accumulate in the remodelled arteries of patients and contribute to the thickening of the vessel wall [3–5]. In addition, circulating endothelial progenitor cells (EPCs), fibrocytes and mast cells may also contribute to the development of the disease [6–8]. In contrast, it has been shown that circulating cells may be protective against the development of pulmonary hypertension, as regulatory T-cells were able to limit pulmonary vascular injury and endothelial dysfunction [9].

Due to the differences in the number and composition of circulating cells between pulmonary arterial hypertension (PAH) and controls, circulating cells have been discussed as potential biomarkers for the disease [10]. Elevated levels of circulating EPCs have been reported in PAH patients [8, 11]. However, idiopathic PAH (IPAH), Eisenmenger and sickle cell disease-related PAH patients had lower levels of EPCs than controls [12–14]. A potential explanation for the apparently contradictory results is that there is no generally accepted standard for the phenotypic characterisation and definition of these cell populations [15]. Missing standards for cell isolation as well as differences in the pathologic mechanisms of the investigated patients may also have contributed to these discrepancies. Further investigations are necessary to determine whether the number of circulating progenitors is a suitable marker of PAH and what role these cells may play in the pathology of the disease [15].

In this study, we aimed to characterise circulating progenitor cell populations in PAH patients using a multimodal approach, where we employed a combination of surface markers of mononuclear cells and found that the relative number of CD133$^+$ cells was elevated and the relative number of fibrocytes was decreased in PAH as compared with matched healthy controls. Due to their putative importance, CD133$^+$ cells were identified in the lung tissue of PAH patients and healthy donors, and were identified as type 2 pneumocytes, inflammatory cells and undifferentiated cells, but none of them accumulated in the pulmonary vessel wall.

Methods

Patient characteristics

This explorative study was approved by the ethics committee of the Medical University of Graz (23-408 ex 10/11). Written informed consent was obtained from all study participants. Patients with PAH (mean pulmonary arterial pressure $\geqslant$25 mmHg, pulmonary arterial wedge pressure $\leqslant$15 mmHg with no significant heart, lung, liver, kidney and blood diseases) undergoing diagnostic or follow-up right heart catheterisation were included in a prospective manner. Age (±5 years)- and sex-matched healthy volunteers selected on a 1:1 basis served as controls. Blood (3 mL) was taken from n=20 PAH patients within 30 days of right heart catheterisation and from their individual controls (n=20) from an antecubital vein in EDTA-containing Vacutainer® tubes. The samples were processed within 1 h after the blood was drawn. For a subgroup of patients, blood was drawn in parallel from an antecubital vein and a peripheral artery, and was analysed for CD133$^+$ cells. As there were no significant differences (online supplementary figure S4), blood was drawn from the antecubital vein only for the remainder of the study.

The second part of the study focused on the identification of CD133$^+$ cells in the lung tissue. This tissue was obtained from n=5 IPAH patients undergoing lung transplantation in Vienna and from n=5 donor lungs not used for transplantation. The study protocol for tissue donation was approved by the ethical committee of the Medical University of Vienna (976/2010).

Fluorescence-activated cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated *via* Ficoll-Paque PLUS (GE Healthcare Life Sciences, Vienna, Austria) density gradient centrifugation. The obtained mononuclear cells were washed twice in phosphate-buffered saline (PBS) and resuspended in PBS containing 1% bovine serum albumin (BSA). Prior to staining, nonspecific antibody binding was blocked *via* incubation with Fc-receptor-binding inhibitor (eBioscience, Vienna, Austria) for 20 min on ice. Cells were stained simultaneously with fluorescent-conjugated antibodies against the following cell surface markers or their respective isotype controls: CD117 (c-kit), CXCR2, CD309, CD34, CD14, CD31, CD133, CD16 and CD45 (for detailed information, refer to table 1 and online supplementary table S1). The cells were then incubated for 30 min at 4°C. Flow cytometric analysis was performed and analysed on a LSR II with FACSDIVA software version 6.2 (both from Becton Dickinson Biosciences, Oxford, UK). Automatic and manual adjusted compensation was used with single-colour-stained samples. Instrument configuration is given in online supplementary table S2; a compensation matrix is shown in online supplementary table S3. A minimum of 50 000 events was recorded. After exclusion of cell debris and doublets, lymphocytes and monocytic cells were gated. The regions of interest were selected according to the negative and isotype controls. A detailed gating strategy is shown in figure 1.

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TABLE 1 Clusters of differentiation of the investigated antigens

	Antigen description
CD117	c-Kit
CXCR2	Interleukin-8 receptor β
CD309	Vascular endothelial growth factor receptor
CD34	Sialomucin
CD14	Myelomonocytic differentiation antigen
CD31	Platelet endothelial cell adhesion molecule
CD133	Prominin-1
CD16	Aggregated IgG receptor
CD45	Leukocyte common antigen

Cytospin and immunofluorescent staining

PBMCs (from n=5 patients with PAH and n=5 healthy controls) were obtained as previously described. Cells were stained with fluorescence-labelled antibodies against CD133. Briefly, 20 000 cells were diluted in 200 µL PBS containing 1% BSA and put onto microscope slides using the cytospin technique. After fixation with 1.4% paraformaldehyde the slides were mounted with Vectashield® Mounting Medium containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). Negative controls

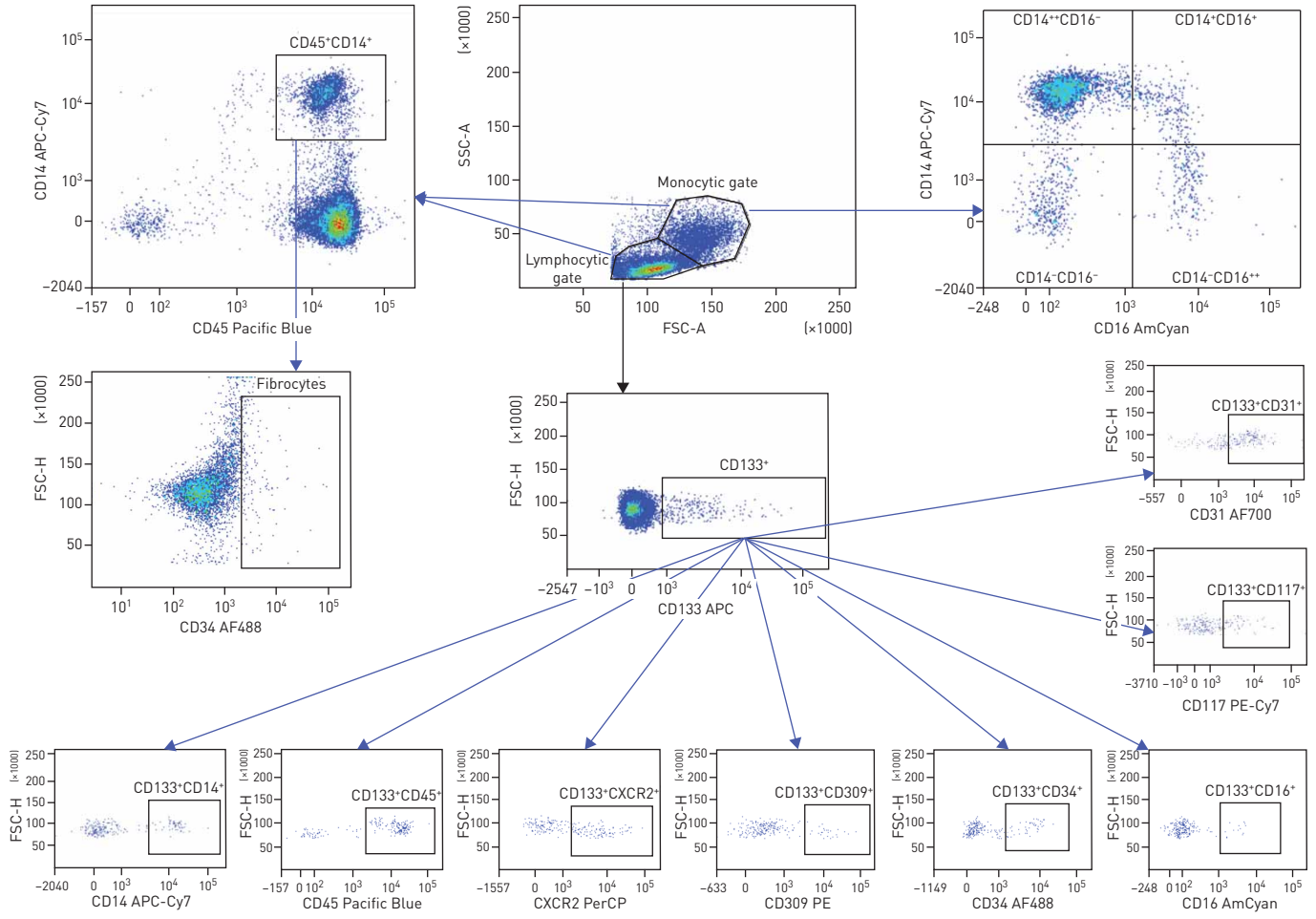

FIGURE 1 Gating strategy for the detection of cell populations from peripheral blood mononuclear cells. A representative example of a pulmonary arterial hypertension patient is shown. Gating for lymphocytic and monocytic cells is demonstrated (*x*-axis: forward scatter (FSC); *y*-axis: side scatter (SSC)). $CD133^+$ cells were identified from the lymphocytic gate. Next, double-positive events were gated from the lymphocytic region. Monocytes were identified according to the expression of CD16 and CD14. Fibrocytes were identified from the $CD45^+$ and $CD14^+$ monocytes defining them as a $CD34^+CD45^+CD14^+$ subpopulation of mononuclear cells. APC: allophycocyanin; PE: phycoerythrin; PerCP: peridinin chlorophyll; AF: Alexa Fluor.

were performed with the isotype-specific antibodies. A Zeiss LSM 510 META (Carl Zeiss, Jena, Germany) scanning laser confocal microscope was used for visualisation.

ELISA

The ELISA for the soluble CD133 (prominin-1) was performed according to the manufacturer's protocol (BIOTREND Chemikalien, Cologne, Germany). Plasma was isolated from blood samples obtained from the patients (n=20) and controls (n=20). Blood samples were kept on ice until centrifugation. Plasma was obtained within 1 h of blood draw, aliquoted and stored at −80°C in the Biobank of the Medical University of Graz.

Immunohistochemistry and immunofluorescence

In order to assess the localisation of $CD133^+$ cells in the lung tissue, we stained n=5 IPAH and n=5 healthy donor lung tissues for this marker using immunohistochemistry and tissue immunofluorescence. The slides were analysed by an expert pathologist in lung cytology (M.T.) and positive cells were counted by means of Visiopharm (Hørsholm, Denmark) software. For a detailed description, see the online supplementary material.

Real-time PCR

Gene expression changes in $CD133^+$ cells were investigated using real-time PCR using a LightCycler 480 (Roche, Mannheim, Germany). Genes which are known to be expressed in stem cells (Oct3/4, SOX2, Nanog, CXCR4 and Ki67) were selected for this set of experiments. Primer sequences are given in online supplementary table S4. The ΔC_T method was used for calculation of mRNA expression, with β_2-macroglobulin as the reference gene. A detailed description is given in the online supplementary material.

Statistical analysis

Data are presented as mean±SD or median (interquartile range (IQR)). Statistical analysis was performed using Prism software version 5.04 (GraphPad, La Jolla, CA, USA). The nonparametric Mann–Whitney U-test was used for calculation of differences between groups.

Results

Fluorescence-activated cell sorting

The characteristics of the 20 PAH patients for this pilot study are given in table 2. The healthy matched controls consisted of 15 females and five males with a mean±SD age of 56.3±13.4 years.

Monocytes (expressed as percentage of lymphomonocytic cells) were elevated in PAH as compared with controls (figure 2a; PAH median (IQR) 23.6% (15.2–31.6) *versus* control 12.4% (7.6–16.7), p<0.001), whereas lymphocytes were decreased in PAH as compared with controls (figure 2b; PAH median (IQR) 73.4% (64.5–80.4) *versus* control 86.9% (81.4–91.3), p<0.001).

Using a novel gating strategy (figure 1) we identified putative progenitors as $CD34^+CD133^+$ double-positive cells from the lymphocytic gate. These cells did not express the chemokine receptor CXCR2, the macrophage/granulocyte marker CD16, vascular endothelial growth factor receptor 2, CD309 (also known as KDR) or CD117 (c-kit), *i.e.* there were no triple-positive events ($CD34^+CD133^+CXCR2^+$, $CD34^+CD133^+CD16^+$, $CD34^+CD133^+CD309^+$ or $CD34^+CD133^+CD117^+$) neither in PAH nor in controls (data not shown). This suggests that cells expressing both $CD34^+$ and $CD133^+$ are undifferentiated in the peripheral blood. One PAH patient had to be excluded *post hoc* from the analysis due to an insufficient number of lymphocytic cells.

The contribution of $CD133^+$ cells to the circulating lymphocytic cells was significantly elevated in PAH as compared with controls (figure 2c; PAH median (IQR) 0.6% (0.4–1) *versus* control 0.3% (0.2–0.5), p<0.003). A $CD133^+$ circulating EPC population is depicted in figure 2 expressed as percentage of the gated lymphocytic cells. Since the $CD133^+$ population was identified from the lymphocytic cells and lymphocytes were lower in PAH as compared with controls, we also compared the arithmetical product of these two fractions. This revealed that the number of $CD133^+$ cells in relation to the number of PBMCs was still significantly higher in PAH as compared with controls (figure 2d; PAH median (IQR) 0.4% (0.3–0.6) *versus* control 0.2% (0.2–0.5), p=0.036). The proportion of circulating $CD133^+$ cells positive for CD45 ($CD133^+CD45^+$), indicating their bone marrow origin, was also significantly increased in PAH as compared with controls (figure 2e; PAH median (IQR) 66.7% (53.3–85.1) *versus* control 57% (40.4–66.6), p=0.047). Another subpopulation of $CD133^+$ cells that was elevated in PAH as compared with controls was $CD133^+CD117^+$ (figure 2f; PAH median (IQR) 31.4% (24.6–56.9) *versus* control 21.9% (10.9–30), p<0.004).

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TABLE 2 Individual characteristics of the 20 pulmonary arterial hypertension (PAH) patients

Sex	Age years	mPAP mmHg	PVR WU	RAP mmHg	PAWP mmHg	CI $L \cdot min^{-1} \cdot m^{-2}$	6MWD m	NYHA class	PAH-specific drug	PAH type
Female	22	81	23.7	6	6	1.9	423	3	None	HPAH
Male	23	71	10.0	8	4	3.8	513	2	Sildenafil+bosentan	CHD-PAH
Female	34	22	2.0	7	9	3.6	494	1	Calcium channel blocker +bosentan	IPAH
Male	48	55	10.6	6	10	2.5	226	3	None	IPAH
Female	50	32	3.7	5	11	3.2	477	2	Calcium channel blocker	IPAH
Male	50	55	14.4	14	10	1.7	426	3	Ambrisentan	SSc-PAH
Female	54	33	4.4	2	2	3.7	533	2	Ambrisentan	IPAH
Male	57	54	12.6	14	8	2.0	375	3	Ambrisentan+iloprost +treprostinil	Po-PAH
Male	58	59	15.3	15	12	3.0	321	3	Bosentan+sildenafil	IPAH
Female	58	66	17.7	6	9	1.8	309	3	Bosentan	SSc-PAH
Female	64	45	7.6	5	5	2.6	275	3	Bosentan+sildenafil	IPAH
Female	64	36	6.3	15	14	2.1	480	2	Ambrisentan	PoPAH
Female	65	47	24.0	12	14	1.4	NA	2	Ambrisentan	IPAH
Female	71	47	9.3	2	6	2.8	429	3	Calcium channel blocker+iloprost	IPAH
Female	71	40	5.2	8	15	2.9	327	3	None	IPAH
Female	74	29	6.9	8	15	2.0	390	3	Sildenafil	IPAH
Female	78	24	3.5	2	9	2.9	311	2	Ambrisentan	IPAH
Female	79	29	5.7	10	14	1.6	NA	3	Bosentan	SSc-PAH
Female	82	49	11.7	4	10	1.9	NA	3	Ambrisentan	SSc-PAH
Female	84	27	3.9	6	8	2.9	120	3	Ambrisentan	SSc-PAH
Mean±SD	59.3±17.9	45±16.4	9.9±6.4	7.7±4.3	9.5±3.7	2.5±0.7	378±111			
Minimum	22	22	2.0	2	2	1.4	120			
Maximum	84	81	24	15	15	3.8	533			

mPAP: mean pulmonary arterial pressure; PVR: pulmonary vascular resistance; WU: Wood Units; RAP: right atrial pressure; PAWP: pulmonary arterial wedge pressure; CI: cardiac index; 6MWD: 6-min walking distance; NYHA: New York Heart Association; HPAH: hereditary PAH; CHD-PAH: congenital heart disease-associated PAH; IPAH: idiopathic PAH; SSc-PAH: systemic sclerosis-associated PAH; Po-PAH: portopulmonary hypertension; NA: not available.

We also identified subpopulations of $CD133^+$ cells that were lower in PAH as compared with controls, *i.e.* $CD133^+CD309^+$ cells (figure 2g; PAH median (IQR) 10.2% (4.4–14.9) *versus* control 23.5% (16.2–32.1), p=0.002), $CD133^+CD31^+$ cells (figure 2h; PAH median (IQR) 44.1 (34–68.7) *versus* control 70.7% (60–78.2), p=0.001) and $CD133^+CXCR2^+$ cells (figure 2i; PAH median (IQR) 34.7% (25.7–40.2) *versus* control 38.7% (34.9–43.7), p=0.047).

The cells derived from the monocytic gate were mostly $CD14^+$. However, the relative contribution of $CD14^+$, $CD14^-$, $CD16^+$ and $CD16^-$ cells was not different between PAH and controls. The contribution of monocytic cells of bone marrow origin ($CD45^+CD14^+$) derived from the lymphomonocytic gate was higher in PAH (figure 2j; PAH median (IQR) 20.2% (16.2–29.6) *versus* control 12.2% (8.6–15.7), p<0.001).

In addition, circulating fibrocytes of bone marrow origin ($CD34^+CD45^+CD14^+$) were decreased in PAH as compared with controls (figure 2k; PAH median (IQR) 0.3% (0.1–0.8) *versus* control 1.2% (0.8–1.5), p<0.001). In an independent set of experiments, peripheral and central blood was taken from patients with PAH and fluorescence-activated cell sorting was performed as described above. There was no difference between the number of circulating progenitors between central and peripheral blood (online supplementary figure S4).

Characterisation of $CD133^+$ cells

Multiple cytospin preparations of mononuclear cells isolated from healthy controls as well as IPAH patients were examined. Positive staining for CD133 was observed on the cell membrane of a small percentage of isolated cells. Phenotypically, the nuclei of these cells were small and condensed. A representative image from an IPAH patient is shown in figure 3. Real-time PCR was performed in order to assess the level of CD133 mRNA in lung homogenates of eight IPAH patients and eight donors. At the mRNA level, CD133 in tissue homogenate did not show any difference between patients and controls (mean±SD IPAH $\Delta C_T = -12.8 \pm 4.6$ *versus* control $\Delta C_T = -12.9 \pm 7.5$, p=0.87). cDNA was obtained from isolated CD133 cells. $CD133^+$ cells did not express SOX2, Nanog, CXCR4 and Ki67 as assessed by

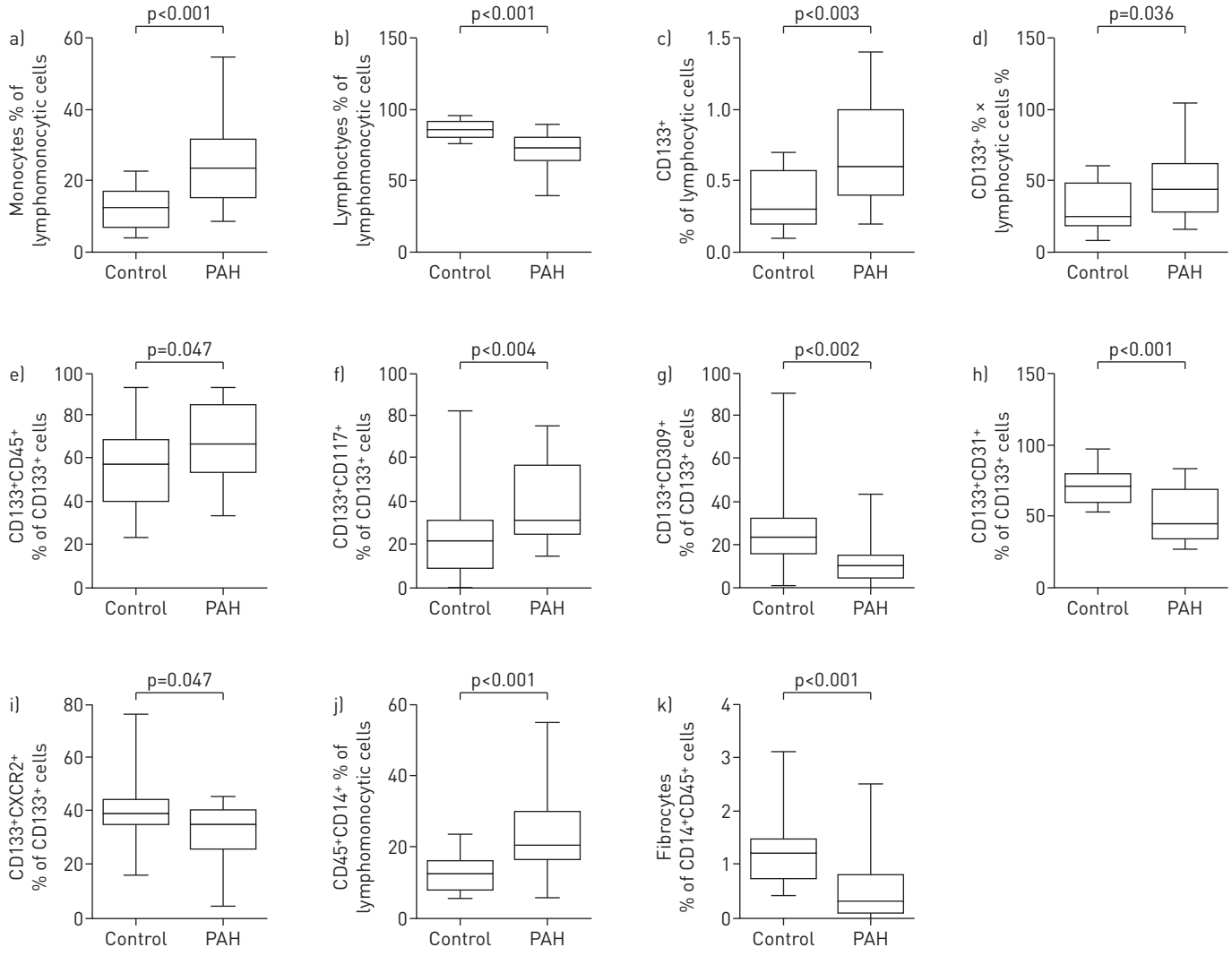

FIGURE 2 Peripheral blood mononuclear cells were stained and analysed using flow cytometry from n=20 pulmonary arterial hypertension (PAH) patients and n=20 age- and sex-matched healthy donors (Control). Data were analysed using the nonparametric Mann–Whitney U-test. Data are presented as median and interquartile range.

real-time PCR. The transcription factor Oct3/4 was expressed an the mRNA level in $CD133^+$ cells derived from PAH and controls with no significant differences (p=0.400; online supplementary figure S1).

ELISA

Plasma samples were analysed for the soluble form of CD133 (prominin-1). As shown in online supplementary figure S2, there were no significant differences between PAH and controls (median (IQR) 0.037 (0.024–0.228) *versus* 0.027 (0.017–0.191) $ng{\cdot}mL^{-1}$, p=0.16).

Immunohistochemistry and immunofluorescence

In IPAH lung tissue the cells that stained positive for CD133 were predominantly type 2 pneumocytes. Other cell types were also occasionally $CD133^+$ and may be described as inflammatory cells or undifferentiated cells (figure 4). There were no apparent differences regarding localisation or number of $CD133^+$ cells between IPAH and donor lung tissues. Figure 5a–e depicts a representative example of serial staining of an IPAH lung. The surface epithelial cells of the alveoli which are larger and cuboidal were identified as type 2 pneumocytes. They are positive for the markers mucin-1 (MUC-1) and surfactant protein C (SPC). $CD133^+$ cells which are located in the alveolar septum mostly represent type 2 pneumocytes. However, not all type 2 pneumocytes express CD133. A representative image is shown in figure 5f. MUC-1 stained most of the $CD133^+$ cells; however, not all type 2 pneumocytes were positive for CD133, which is also shown in the immunohistological staining.

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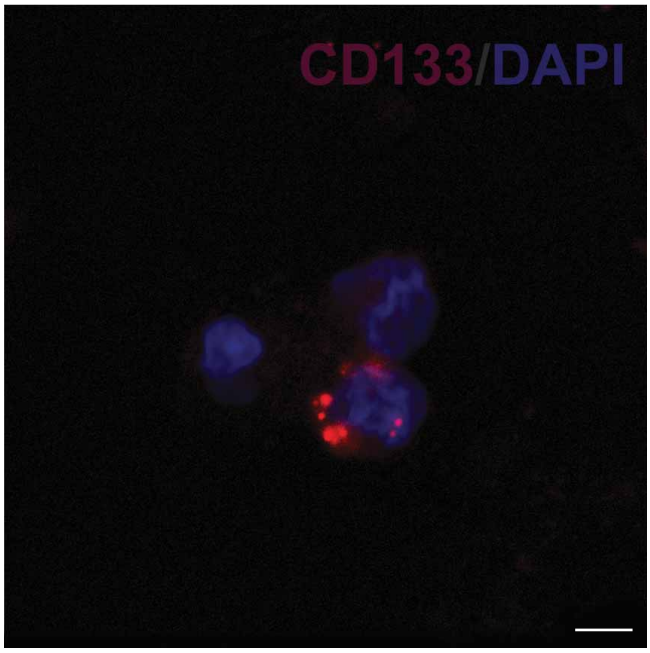

FIGURE 3 Immunofluorescent staining of mononuclear cytospin preparations against CD133. n=5 pulmonary arterial hypertension patients were investigated. Representative image of an idiopathic PAH patient is shown. DAPI: 4′,6-diamidino-2-phenylindole. Scale bar: 5 µm.

Discussion

Technical approach and study population

In contrast to previous studies, we used a more comprehensive gating strategy for quantification of circulating progenitor cells. As the $CD133^+$ population consists of phenotypically diverse subpopulations characterised by the presence of different leukocyte antigens [16], we used a set of stem cell, leukocyte/monocyte and differentiation markers. This applies especially to EPCs, expressing CD133, a member of the prominin family of pentaspan membrane glycoproteins [17]. The leukocyte/monocyte antigens CD45 and CD14 were detectable in the $CD133^+$ population, leading to the conclusion that $CD133^+$ progenitors are mobilised from the bone marrow. During the process of mobilisation and maturation, $CD133^+$ cells may change their phenotype by releasing CD133-containing membrane vesicles [18]. We showed that some subpopulations of $CD133^+$ cells are elevated in PAH, whereas some are decreased as compared with controls. EPCs are essentially hematopoietic cells with variable expression of CD34, CD133 and CD45. The expression of multiple markers for different EPC populations reflects the heterogeneous nature of these cells [19].

Circulating progenitor cells and inflammatory cells as potential biomarkers

Circulating cells are easily accessible (*i.e.* from peripheral blood), and therefore suitable as biomarkers of disease severity and for PAH screening. A recent investigation revealed that the number of progenitor cells in chronic obstructive pulmonary disease (COPD) patients with pulmonary hypertension was higher than in COPD without pulmonary hypertension [20]. In contrast, Diller *et al.* [8] found that the numbers of circulating $CD34^+$, $CD34^+CD133^+$, $CD34^+CD309^+$ and $CD34^+CD133^+CD309^+$ cells were lower in Eisenmenger patients as compared with healthy controls, while IPAH patients showed elevated levels of circulating endothelial cells, but not of EPCs ($CD34^+$, $CD133^+$). Our results demonstrate increased numbers of $CD133^+$ cells and are in line with Toshner *et al.* [8] who reported that $CD133^+CD34^+CD309^+$ cells were elevated in IPAH patients as compared with controls. The discrepancy between the results of Toshner *et al.* [8] and Diller *et al.* [8] may be due to the different patient populations and cell populations that were investigated. Many studies have suggested that inflammation plays an important role in pulmonary hypertension [21–27]. We found that the number of circulating lymphocytes was significantly reduced in PAH patients, whereas circulating monocytes were elevated as compared with controls. We can only speculate on the nature of the missing lymphocytes and it remains an open question if these cells are abundantly recruited in the tissue or if their production is reduced in PAH.

Challenges of progenitor biology in PAH

For many years the characterisation of circulating EPCs has been a matter of debate. Currently, there is no specific marker or combination of markers that reliably defines EPCs both phenotypically and functionally. In our study, phenotypically different subpopulations were detectable and were elevated in PAH patients. This might also indicate that the pulmonary vasculopathy, especially the elevated pulmonary pressure, is a

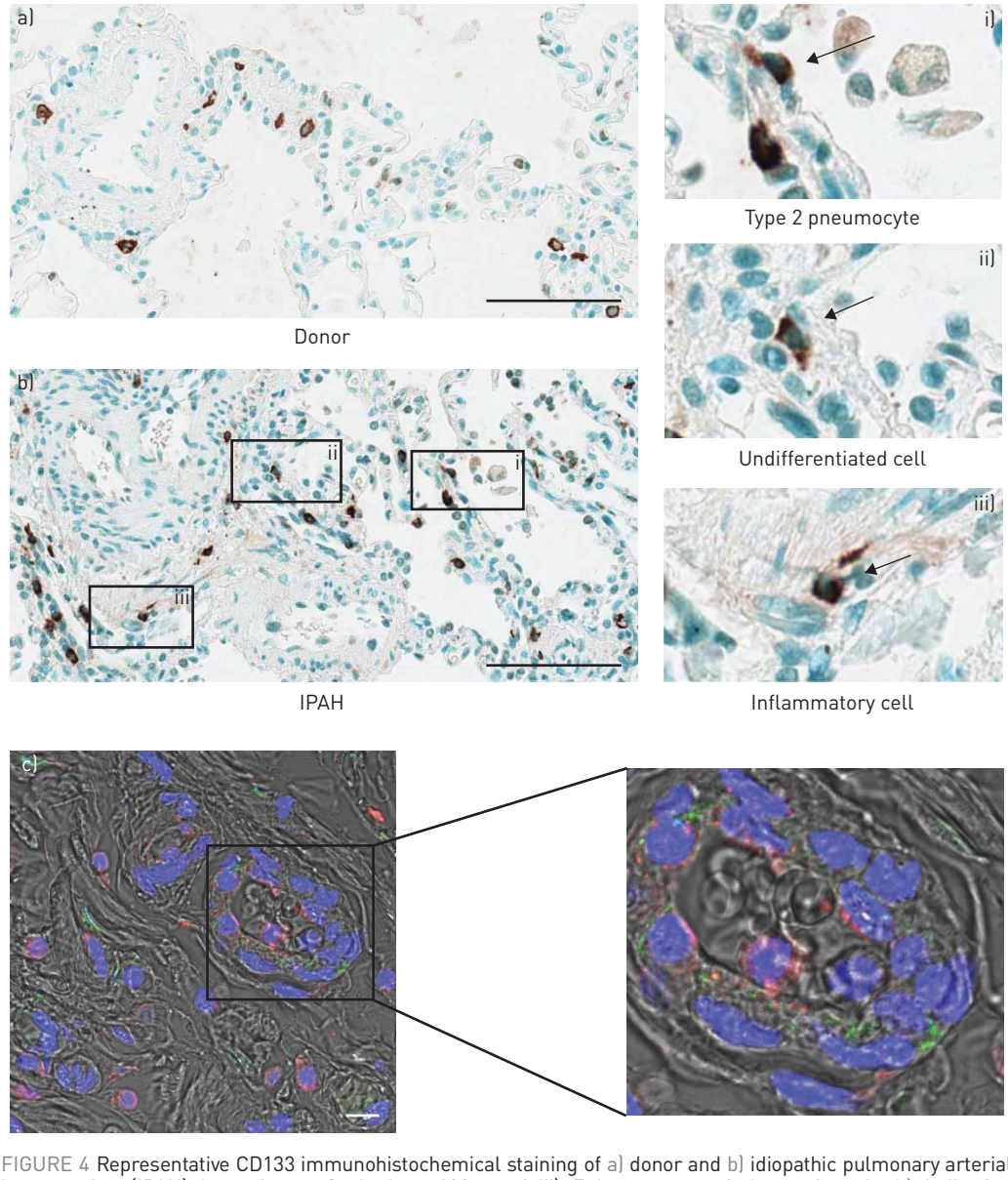

FIGURE 4 Representative CD133 immunohistochemical staining of a) donor and b) idiopathic pulmonary arterial hypertension (IPAH) lung tissue. Scale bar: 100 µm. i–iii) Enlargements of the regions in b) indicating characteristic cells (arrows): i) type 2 pneumocyte, ii) undifferentiated cell and iii) inflammatory cell. c) Representative CD133 immunofluorescent staining of IPAH lung tissue. CD133 is stained in red, von Willebrand Factor is stained in green. Scale bar: 10 µm. The enlargement indicates a $CD133^+$ circulating cell in an arteriole.

driving force for the mobilisation of these cells from the bone marrow. Alternatively, it could indicate that the elevation of the total $CD133^+$ population is pathogenic in PAH. Indeed, it was shown that CD133 cells from PAH patients caused pulmonary hypertension in the nude mouse [28]. Of note, in that study mostly patients with hereditary PAH were enrolled and the responses in the mouse seemed to be patient-specific, suggesting that there may be a complex interaction with other factors.

There is evidence that phosphodiesterase type 5 inhibitors, endothelin receptor antagonists and prostacyclin therapy may affect the number and function of some circulating progenitor cells [29–33]. However, it is not clear if the change in the number of circulating cells affects the severity of PAH. In our study it was not possible to perform subgroup comparisons due to the small sample size.

Molecular characterisation of progenitor cells

We did not find a significant increase in plasma levels of soluble CD133 in our patient cohort; however, we observed a trend towards increased levels in PAH. Compared with that, CD133 in tissue homogenate at the mRNA level did not show any difference between patients and controls, which indicates that the

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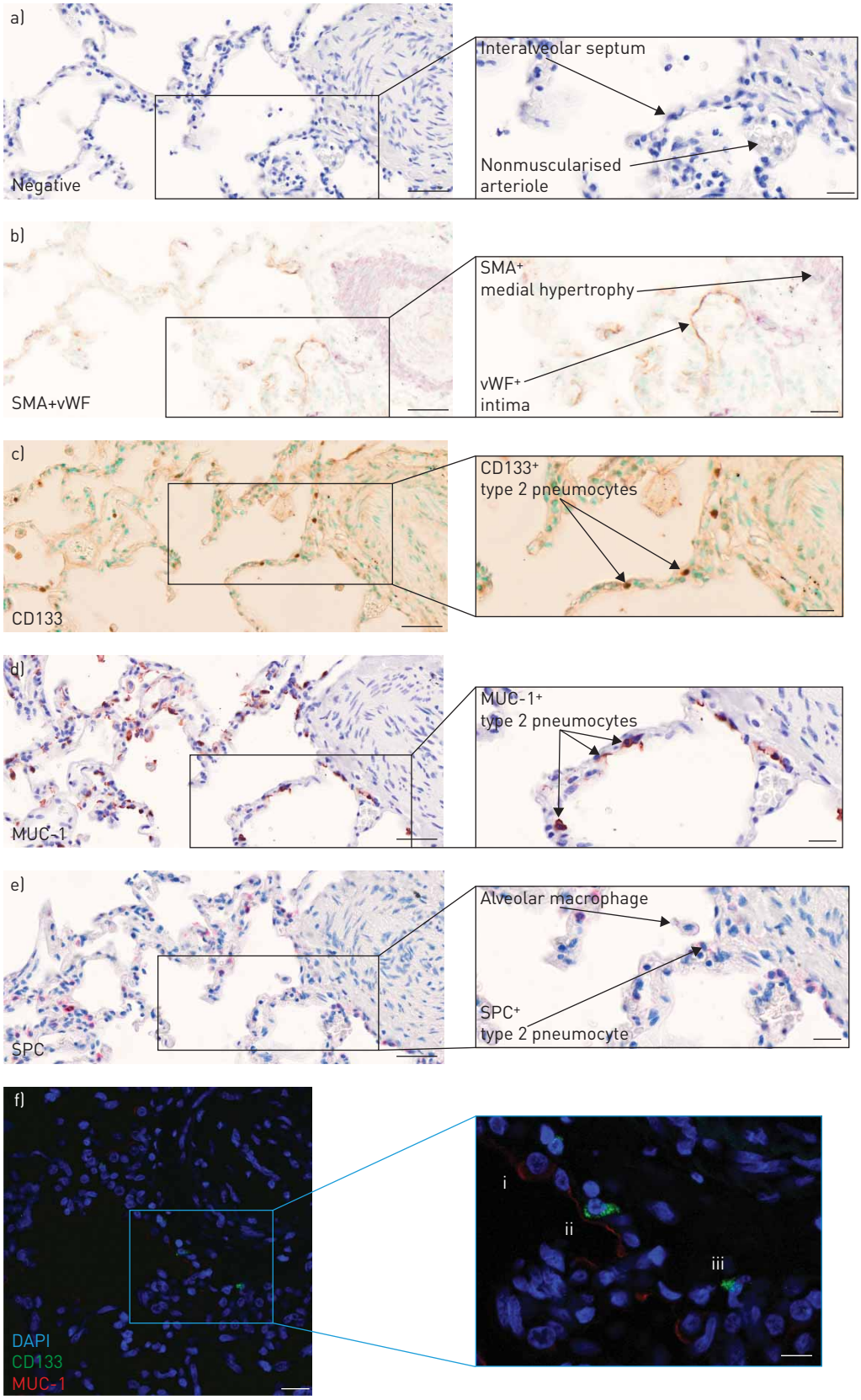

FIGURE 5 Representative immunohistochemical staining of an idiopathic pulmonary arterial hypertension lung tissue. The enlargements show characteristic cells (arrows): a) negative, b) double staining with smooth muscle cell actin (SMA) and von Willebrand Factor (vWF), c) CD133, d) mucin-1 (MUC-1), and e) surfactant protein C (SPC). f) Double immunofluorescent staining with CD133 and MUC-1: i) MUC-1^{+} type 2 pneumocyte, ii) MUC-1^{+} and CD133^{+} type 2 pneumocyte, and iii) CD133^{+} undifferentiated cell. DAPI: 4′,6-diamidino-2-phenylindole. Scale bar: a–d) main, 50 µm; enlargement, 20 µm; f) main, 20 µm; enlargement, 10 µm.

contribution of these cells to the pathogenesis of pulmonary hypertension remains unclear. There may be a paracrine function because the transcription factor Oct3/4, a marker of stem cells, is expressed in the $CD133^+$ cells from both PAH patients and healthy controls. Oct3/4 has often been used as a marker of self-renewal capacity [34]. This could suggest that circulating $CD133^+$ cells may not be lineage committed.

Progenitor cells in the lung tissue

The homing of the circulating progenitor cells remains unclear. It was recently shown that in end-stage lung diseases with pulmonary hypertension there was an increase in the endothelial progenitors in the lung tissue as compared with donor samples [35]. In our study, the main cell population expressing CD133 in the lung tissues was located in the alveolar/septal junction and consisted of type 2 pneumocytes. To the best of our knowledge, this has not been reported previously. Type 2 pneumocytes secrete surfactant and differentiate into type 1 pneumocytes following lung injury. This may indicate that $CD133^+$ type 2 pneumocytes play a role in maintaining the cellularity of the alveolar surface. Our finding is supported by a previous study showing that in the bleomycin-induced fibrosis model, $CD133^+$ epithelial cells were protective against lung injury [36]. In addition to type 2 pneumocytes, $CD133^+$ cells were also found in the adventitia of vessels where they phenotypically resembled monocytes or undifferentiated cells. In agreement with our data, Woo *et al.* [37] found that CD133 was expressed by some of the alveolar epithelial cells in non-neoplastic lung tissue. It remains an open question if the $CD133^+$ type 2 cells, the adventitial cells and the circulating mononuclear cells are related to each other.

Limitations of the study

The low number of PAH patients included (n=20) is an obvious limitation; however, PAH is a rare disease and our patients were haemodynamically well characterised by right heart catheterisation. In addition, our healthy controls were tightly age- and sex-matched to the investigated PAH patients. The lung histology was only performed on end-stage IPAH patients at the time of lung transplantation; however, lung biopsies from patients at an earlier stage are rarely available.

Conclusions

PAH patients are characterised by an elevation of circulating $CD133^+$ progenitor cells and by lymphopenia. Bone marrow-derived circulating CD133 progenitor cells are heterogeneous with respect to their phenotype. In the lung tissue, $CD133^+$ cells consist of phenotypically different populations, mainly consisting of type 2 pneumocytes and some adventitial monocytes and undifferentiated cells.

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