Myeloid angiogenic cells exhibit impaired migration, reduced expression of endothelial markers and increased apoptosis in idiopathic pulmonary arterial hypertension

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Zucco, Liana; Guy's and St Thomas' Hospital NHS Trust  
Toshner, Mark; University of Cambridge School of Clinical Medicine  
Morrell, Nicholas; University of Cambridge School of Clinical Medicine  
Granton, John; University Health Network, Respiratory  
Stewart, Duncan; Ottawa Hospital Research Institute  
Kutryk, Michael; St. Michael's Hospital, Cardiology |
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Myeloid angiogenic cells exhibit impaired migration, reduced expression of endothelial markers and increased apoptosis in idiopathic pulmonary arterial hypertension

Qiuwang Zhang¹†, Liana Zucco²†, Mark Toshner³, Nicholas W. Morrell³, John Granton⁴, Duncan J. Stewart⁵, and Michael J.B. Kutryk¹*

¹Division of Cardiology, Keenan Research Center for Biomedical Science, St. Michael's Hospital, University of Toronto, Toronto, Ontario, Canada. ²Guy’s and St Thomas’ Hospital NHS Trust, London, UK. ³University of Cambridge School of Clinical Medicine, Addenbrooke's and Papworth Hospitals, Cambridge, UK. ⁴University Health Network, University of Toronto, Toronto, Ontario, Canada. ⁵Ottawa Hospital Research Institute, Ottawa, Ontario, Canada

*Corresponding author: Michael J.B. Kutryk, MD, PhD, Room 7084 Bond Wing, 30 Bond Street, Toronto, Ontario, M5B 1W8 Canada

Tel. (416)-864-6060 ext. 6155
Fax (416)-864-5989

Email address: kutrykm@smh.ca

†These authors contributed equally to this work
Abstract

Idiopathic pulmonary arterial hypertension (IPAH) is a rare and devastating condition. There is no known cure for IPAH, and current treatment options are not always effective. Autologous myeloid angiogenic cells (MACs) have been explored as a novel therapy for IPAH but preliminary data from clinical trials show limited beneficial effects. A complete understanding of IPAH MAC function remains elusive. This study was designed to comprehensively compare cell function between IPAH MACs and healthy control MACs. MACs were procured through the culture of peripheral blood mononuclear cells in endothelial selective medium for 7 days. Compared with healthy MACs, IPAH MACs exhibited 1) significantly lower levels of endothelial markers as shown by fluorescence microscopy; 2) a markedly higher rate of apoptosis under both normal culture condition and serum starvation as shown by the TUNEL assay; 3) significantly decreased migration towards VEGF as shown by a modified Boyden chamber migration assay; and 4) similar VEGF and eNOS mRNA levels as shown by RT-qPCR. In conclusion, various aspects of IPAH MAC function are impaired. In order to achieve greater therapeutic benefits, pharmacologic and/or genetic manipulations to improve IPAH MAC function, particularly to promote cell survival and migration, are warranted.

Key Words: Cell therapy; idiopathic pulmonary artery hypertension; myeloid angiogenic cell; cell apoptosis; cell migration; gene expression
Introduction

Pulmonary arterial hypertension (PAH) is a rare disease, with an estimated prevalence of 10-50 per million people (Hoepner and Simon R Gibbs 2014). The disease is characterized by pathological changes in the pulmonary vasculature, with a resultant increase in pulmonary vascular resistance, elevated pulmonary arterial pressures, and eventually right ventricular dysfunction and death (Farber and Loscalzo 2004; Simonneau et al. 2013). PAH can be idiopathic (IPAH), hereditable (HPH), or associated with other conditions such as congenital heart disease, connective tissue disease, portal hypertension, certain viral infections, anorexigen exposure, or schistosomiasis. IPAH occurs sporadically with unknown etiology with an estimated 3-year mortality of 20-30% (Kanwar et al. 2017). There is no cure for IPAH and current treatment options for IPAH are not always effective (Gomberg-Maitland et al. 2013; Kanwar et al. 2017).

Myeloid angiogenic cells (MACs) (Medina et al. 2017), also termed “early endothelial progenitor cells” (Asahara et al. 1998; Takahashi et al. 1999; Asahara et al. 1999) or “circulating angiogenic cells” (Fadini et al. 2012), possess angiogenic and vascular repair capacity. They are commonly generated through the culture of peripheral blood mononuclear cells (PBMNCs) in endothelial cell selective medium containing vascular endothelial growth factor (VEGF) for 4-7 days (Kalka et al. 2000; Vasa et al. 2001; Tepper et al. 2002; Ward et al. 2011). The therapeutic potential of allogeneic MACs to provide an alternative therapy for PAH has been demonstrated in different animal models (Nagaya et al. 2003; Takahashi et al. 2004; Zhao et al. 2005). However, in open-label clinical trials of IPAH, transplantation of autologous MACs has shown only modest benefits (Wang et al. 2007a; Zhu et al. 2008). A potential reason for the limited
benefits may be due to impaired angiogenic activity of MACs in IPAH. Junhui et al. (2008) have reported reduced MAC migration in IPAH patients; and there is accumulating evidence that MAC function is impaired in a variety of diseases (Rodríguez-Carrio et al. 2017). Ward et al. (2011) reported that MACs from subjects with cardiovascular disease exhibited reduced migration towards VEGF compared with cells from healthy controls. Tepper et al. (2002) showed that MACs from type II diabetics had impaired proliferation, adhesion, and incorporation into vascular structures. Vasa et al. (2001) observed an inverse correlation between the number and migratory activity of MACs and risk factors for coronary artery disease. The purpose of this study was to compare MAC function in patients suffering IPAH and those from healthy individuals using a series of surrogate assays for angiogenic activity.

**Materials and Methods**

**Patient recruitment**

Patients clinically diagnosed with IPAH and age- and gender-matched healthy controls were recruited. The diagnosis of PAH was made based on the criteria described by Galiè (2004) and updated in 2015 (Galiè et al. 2015). PAH was defined by a mean pulmonary arterial pressure >25 mmHg at rest or >30 mmHg with exercise, a pulmonary wedge pressure <15 mmHg and pulmonary vascular resistance >3 mmHg/L/min. Only patients with IPAH as defined by the clinical classification of pulmonary hypertension (Simonneau et al. 2013) were included in this analysis. Informed written consent for study participation was obtained from all patients and healthy volunteers. All protocols involving human samples were approved (approval # 02-236) by the Research Ethics
Board of St. Michael’s Hospital, University of Toronto, in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

**MAC generation**

PBMNCs were isolated from 80 ml of venous blood by Ficoll density gradient centrifugation and plated at a density of $0.75 \times 10^6$/ml onto human fibronectin-coated (10 µg/ml) dishes/slides as previously described (Zhang et al. 2011). Cells were cultured in endothelial basal medium EBM-2 (Lonza Inc., Missisauga, Canada) supplemented with 20% human serum and growth factors (VEGF, basic fibroblast growth factor and insulin-like growth factor-1). After 48 hours, medium with suspended cells was removed and fresh medium added. Cells were maintained with the change of medium every other day. At day 7, cells were harvested, characterized and functionally assessed.

**MAC characterization**

Expression of CD14, CD45 and CD31 in MACs was analyzed by flow cytometry as we described previously (Ward et al. 2011). Briefly, cells were detached with phosphate buffered saline (PBS, pH 7.4) containing 1 mM EDTA, washed with PBS and re-suspended in PBS at a density of $10^7$/ml. One hundred µl of cell suspension was then incubated with PE-conjugated antibodies in the dark for 30 min (all antibodies were used with a 1:10 dilution). Subsequently, cells were washed once with PBS, re-suspended in 500 µl PBS and analyzed using a Beckman Coulter flow cytometer (Wilmington, DE, USA). All antibodies used were PE-conjugated. Anti-CD31 was from Immunotech (Vaudreuil-Dorion, Canada), anti-CD14 and anti-CD45 were both from Beckman Coulter. Respective isotypes were used as controls.

**Fluorescence microscopy**
MAC uptake of fluorescent dye Dil-labeled acetylated low-density lipoprotein (Dil-Ac-LDL, Molecular Probes, Burlington, Canada) and binding of FITC-labeled UEA-Lectin (FITC-UEA-Lectin) (Sigma, Oakville, Canada) were performed as described previously (Zhang et al. 2013, Zucco et al. 2014). Dil-Ac-LDL/FITC-UEA-1 double positive cells were visualized and counted in a blinded fashion using confocal microscopy (Leica Microsystems, Wetzlar, Germany). The percentage of positive cells against total cells, for each isolation, was calculated and averaged from at least 6 randomly chosen high power fields. MAC VEGF receptor 2 (VEGFR2) was assessed as follows; cells were fixed with 2% paraformaldehyde in PBS containing 0.5% Triton X-100, washed with PBS and incubated with an anti-VEGFR2 antibody (1:200 dilution, Thermo Fisher Scientific, Burlington, Canada) at room temperature for 1 hour. After 3 washes with PBS, cells were incubated with a FITC-conjugated secondary antibody (1:1000 dilution, Thermo Fisher Scientific) followed by 3 washes with PBS. Cells were mounted with VectaShield mounting medium (Vector Lab, Burlingame, USA) with the use of propidium iodide (PI) for nuclear counterstaining. VEGFR2 positive cells were visualized and counted the same way as Dil-Ac-LDL/FITC-UEA-1 double positive cells.

**MAC apoptosis assay**

Basal cell apoptosis (under normal culture condition with complete medium) and serum starvation induced cell apoptosis (cells under culture with serum-free medium for 24 hours) were analyzed using the DeadEnd Fluorometric TUNEL System (Promega, Madison, USA) according to the manufacturer’s instructions. Briefly, cells were fixed in 2% paraformaldehyde in PBS for 10 minutes, washed 3 times with PBS and permeabilized with 0.2% Triton X-100. After 3 washes with PBS, cells were incubated
with TdT reaction mix for 60 minutes at 37°C in a humidified chamber. Following immersion in 2X SSC for 15 minutes and 3 washes with PBS, cells were mounted using Vectashield mounting medium (Vector Lab) with PI dye used for nuclear counterstaining. The green fluorescence of apoptotic cells was visualized under fluorescence microscopy. The percentage of apoptotic cells against total cells, for each isolation, was calculated and averaged from at least 6 randomly chosen fields in a blinded fashion.

**MAC migration assay**

Directional MAC migration towards human VEGF$_{165}$ (hVEGF$_{165}$, Sigma) was assessed using a modified-Boyden chamber migration assay as described previously (Zucco et al. 2014). Briefly, half million MACs in serum free-EBM-2 medium containing 0.5% BSA (migration medium) were added into the top chamber, hVEGF$_{165}$ (50 ng/ml, R & D Systems, Minneapolis, USA) in migration medium, or migration medium alone as a control, was added to the lower chamber. After incubation for 5 hours at 37°C, cells present on the underside of the insert were fixed, stained using Diff Quik (Thermo Fisher Scientific) following the manufacturer’s instructions, and visualized under light microscopy. The average of migrated cells from 5 randomly selected high-power fields (HPF) were calculated and compared.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

VEGF and eNOS mRNA levels in MACs were measured by RT-qPCR. Total RNA was extracted from MACs using the GenElute Mammalian Total RNA Kit (Sigma) according the manufacturer’s instructions, and quantified by spectrophotometry. RT was performed using the Omniscript RT Kit (Qiagen, Mississauga, Canada) as follows, in a total of 20 µl of reaction volume, 500 ng of total RNA was mixed with 2 µl of 10x RT
Buffer, 2 µl of dNTP Mix (5 mM each dNTP), 1 µl (10 units) of RNase inhibitor, 1 µl (4 units) of Omniscript Reverse Transcriptase and 2 µl (3 µg/µl) of random primer. RNase-free water was used to adjust the reaction volume. RT was completed at 37 °C for 1 hr. The SYBR Green qPCR Kit (Thermo Fisher Scientific) was used for measuring VEGF and β-actin mRNA. A total of 20 µl reaction volume contained 1 µl RT product, 10 µl of 2× SYBR Green PCR Master Mix, 0.2 µM (final concentration) each of the primers, and nuclease-free water to adjust the volume. Quantitative PCR was done on the ABI Prism 7900HT Sequence Detection System (Thermo Fisher Scientific) and the thermo-cycling conditions were as follows, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 Sec and 60 °C for 1 min. A dissociation curve was established to determine the specificity of amplification. VEGF primer sequences are as follow: forward, 5’-AAGGAGGAGGGCAGAATCAT-3’; and reverse, 5’-CCAGGCCCTCGTCATTG-3’. β-actin primer sequences: forward, 5’-AGCCTCGCCTTTGCCGA-3’; and reverse, 5’-CTGGTGCCTGGGGCG-3’. The Human eNOS TaqMan Gene Expression Assay Kit (Cat # Hs00167166_m1, Thermo Fisher Scientific) was employed for the measurement of NOS mRNA levels as follows, 1 µl of RT product was mixed with 1 µl of eNOS primers, 10 µl of 2× TaqMan Master Mix and 8 µl of nuclease-free water. The thermost-cycling conditions were the same as with the SYBR Green reaction. Relative VEGF and eNOS mRNA levels normalized against β-actin were calculated using the 2^(-ΔΔCt) method.

**Statistical analysis**

Data normality was determined with the Shapiro-Wilk test. Parametric data were expressed as Mean ± SEM (the standard error of the mean) while nonparametric data were expressed as median (the first quartile, the third quartile). The Mann-Whitney test
was used for nonparametric data analysis while the Welch test for parametric data. A student’s paired T-test was performed for paired migration assays. $P < 0.05$ was considered statistically significant.

**Results**

**Study subjects**

A total of 35 patients clinically diagnosed with IPAH and 33 healthy volunteers were recruited. Demographics of all subjects are summarized in Table 1.

**MAC generation and characterization**

Semi-confluent MACs were obtained after 7-day culture of PBMNCs with more small round cells seen in IPAH MACs (Figure 1, top panels show healthy and IPAH MACs as indicated). The round cells probably represent apoptotic cells. MAC are positive for CD45, CD14 and CD31 (Medina et al. 2017), and we showed by flow cytometric analysis that around 80% of MACs expressed CD14, while over 90% MACs expressed CD45 and CD31 (shown in bottom panels as indicated in Figure 1).

**Reduced endothelial marker expression in IPAH MACs**

Fluorescence microscopy analysis revealed that 61.9 ± 4.7% of IPAH MACs (n=24) were Dil-Ac-LDL/FITC-UEA-1 double positive, which was significantly lower than 75.0 ± 3.9% of control MACs (n=15; $p < 0.05$). The percent of cells positive for VEGFR2 was 46.1 ± 5.6% for IPAH MACs (n=29), which was significantly lower than 76.9 ± 3.8% of control MACs (n=21, $p < 0.05$) (Figure 2).

**Increased apoptosis in IPAH MACs**

Representative images of TUNEL staining for basal apoptosis (red: nuclear staining, green: positive for apoptosis) for control MACs and IPAH MACs were shown
in Figure 3 as indicated. The basal cell apoptosis rate of IPAH MACs was 5.85 ± 0.79% (n=29), which was significantly higher than 3.00 ± 0.48% of control MACs (n=24, \( p < 0.01 \), the rightmost panel of Figure 3).

Serum starvation induced apoptosis was detected by the TUNEL assay and the representative images of TUNEL staining for healthy and IPAH MACs were shown in Figure 4 as indicated. Under the serum starvation, cell apoptosis elevated significantly in both groups compared to their respective basal levels (both \( p < 0.05 \)). Further statistical analysis revealed that serum starvation induced a significantly higher level of apoptosis for IPAH MACs (25.06 ± 5.35%, n=18) compared to 9.90 ± 1.89% of control MACs (n=16, \( p < 0.05 \)) (the rightmost panel of Figure 4).

**Inhibited cell migration in IPAH MACs**

A modified Boyden chamber migration assay showed that the migrated cell number for healthy MACs was 38.2 ± 6.9 cells/HPF in the presence of hVEGF\(_{165}\), which was significantly higher than 11.7 ± 1.5 cells/HPF in the absence of hVEGF\(_{165}\) (\( p < 0.05 \), n=6, Figure 5). In contrast, hVEGF\(_{165}\) failed to significantly augment the migration of IPAH MACs (n=6, Figure 5).

**eNOS and VEGF mRNA expression in MACs**

RT-qPCR analysis showed similar eNOS mRNA levels in IPAH MACs (n=17) and healthy MACs (n=16, \( p = 0.93 \), Figure 6). The VEGF mRNA level tends to decrease in IPAH MACs (n=7) compared with that of healthy MACs (n=7) but the difference was not statistically significant (\( p = 0.1 \), Figure 6).

**Discussion**
MACs have been extensively investigated for therapeutic applications and as biomarkers for diseases. In this study, we focused on examining MAC therapeutic potential; and did not aim to explore MACs as biomarkers for IPAH nor did we intend to translate IPAH MAC activity into the vasculopathy and pathogenesis of IPAH. The therapeutic efficacy of autologous MACs for the treatment of IPAH has been explored in clinical trials (Wang et al. 2007a; Zhu et al. 2008). Apart from a report showing reduced migratory ability of IPAH MACs (Junhui et al. 2008), few studies have comprehensively examined IPAH MAC function. Using a series of surrogate assays we showed that although VEGF and eNOS mRNA levels were not significantly different in healthy and IPAH MACs, proportion of Dil-Ac-LDL/FITC-UEA-1 double positive as well as VEGFR2 positive cells were significantly lower in IPAH MACs. Additionally, IPAH MACs showed a markedly higher rate of apoptosis under both normal and serum starvation culture conditions, and exhibited significantly decreased migration towards VEGF.

In most experimental studies as well as clinical trials, MACs are obtained through the culture of PBMNCs in endothelial cell selective medium. This method was employed in the present study, and we found that the vast majority of MACs expressed CD31, CD14 and CD45, indicating the myeloid origin of MACs, which aligns well with the MAC definition (Urbich et al. 2003; Lopez-Holgado et al. 2007; Medina et al. 2017). The levels of these markers in IPAH and healthy MACs, however, did not differ significantly (data not shown).

It has been shown that MAC angiogenic activity is positively associated with Dil-Ac-LDL/FITC-UEA-1 double positive cell numbers (Kalka et al. 2000; Dimmeler et al. 2001).
Kalka et al reported that direct intramuscular injection of plasmid encoding VEGF gene in patients with critical limb ischemia augmented Dil-Ac-LDL/FITC-UEA-1 double positive MACs, which was associated with enhanced neovascularization (Kalka et al. 2000). In other studies, HMG-CoA reductase inhibitors are shown to promote angiogenesis by increasing the number of Dil-Ac-LDL/FITC-UEA-1 double positive MACs (Dimmeler et al. 2001; Llevadot et al. 2001). We observed in this study that the number of Dil-Ac-LDL/FITC-UEA-1 double positive cells was significantly lower in IPAH MACs, indicating reduced angiogenic capacity of IPAH MACs. Additionally, the percent of VEGFR2 (+) cells in IPAH MACs is also remarkably lower, which could lead to defective MAC response to VEGF, an important angiogenic factor.

Under the normal culture condition, IPAH MACs demonstrated a higher apoptotic rate compared with healthy MACs. With serum starvation, increased cell apoptosis was observed in both control and IPAH MACs, but serum starvation caused a significantly higher level of cell apoptosis in IPAH MACs than in healthy MACs; suggesting IPAH MACs are not only intrinsically fragile but might also be more susceptible to environmental insults.

A modified Boyden chamber migration assay showed that migration of IPAH MACs in response to VEGF was suppressed. VEGFR2 production on IPAH MACs as shown by immunofluorescence microscopy was decreased, which might be responsible for the suppressed cell migration toward VEGF. Junhui et al. also observed impaired migration toward VEGF for MACs isolated from patients with IPAH (Junhui et al. 2008), which is in agreement with our findings. It is known that directional migration is necessary for the homing and engraftment of MACs to regions of tissue damage for their
reparative action. Our migration data added more evidence indicating IPAH MAC dysfunction.

The above findings suggest that pharmacologic and/or genetic modification to improve survival and migration of MACs from IPAH patients may provide a potential for their therapeutic use. Resveratrol, a non-toxic, naturally occurring product mainly extracted from grapes, has been widely studied for its effect in the augmentation of endothelial and MAC function (see review by Schmitt et al. 2010). A number of studies have shown that the treatment of MACs from healthy donors with resveratrol results in enhanced cell proliferation and migration (Gu et al. 2006, Wang et al. 2007b; Balestrieri et al. 2008; Huang et al. 2010). Priming of IPAH MACs with resveratrol in order to improve cell survival and migration might therefore translate into a potential therapeutic strategy.

MACs do not give rise to new blood vessels but rather promote angiogenesis through the production of paracrine factors such as VEGF and eNOS (Ward et al. 2011; Fadini et al. 2012). We previously reported that MACs from patients with coronary artery disease produced similar levels of eNOS mRNA but significantly lower levels of VEGF mRNA compared with healthy MACs (Ward et al. 2011). In this study, we showed that there was a trend toward reduced VEGF mRNA production ($p = 0.1$) in IPAH MACs, although levels of eNOS mRNA did not differ significantly between IPAH MACs and control MACs. The limitations of this study are that the protein levels of VEGF and eNOS were not measured, and the in vivo regenerative capacity of IPAH MACs was not assessed in an animal ischemia model.

Conclusions
In conclusion, although mRNA levels of VEGF and eNOS appear similar in IPAH MACs and healthy MACs, various aspects of IPAH MAC function are impaired. In order to achieve greater therapeutic benefits, pharmacologic and/or genetic manipulations to improve IPAH MAC function, particularly to promote cell survival and migration, are warranted.

Conflicts of interest: None.

Acknowledgements

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Table 1. Summary of patient demographics.

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IPAH: idiopathic pulmonary arterial hypertension; N/A: not available.
**Figure legends**

**Figure 1. MAC generation and characterization.**

Semi-confluent MACs appeared after 7-day culture of PBMNCs. More small round cells were seen in IPAH MACs. Representative histograms of flow cytometry for CD45, CD14 and CD31 in MACs from a healthy individual are shown as indicated.

**Figure 2. Reduced endothelial markers in IPAH MACs.**

The number of Dil-Ac-LDL/FITC-UEA-1 double positive cells (left panel) as well as the percent of VEGFR2 positivity (right panel) were significantly lower in IPAH MACs.

**Figure 3. Basal cell apoptosis measured by the TUNEL assay.**

Representative images of nuclear staining (red) and TUNEL staining (apoptotic cells, green) for healthy and IPAH MACs are shown as indicated. IPAH MACs had a significantly higher rate of apoptosis compared to healthy MACs under the normal culture condition (the rightmost panel).

**Figure 4. Serum starvation induced cell apoptosis measured by the TUNEL assay.**

Representative images of nuclear staining (red) and TUNEL staining (apoptotic cells, green) for healthy and IPAH MACs under serum starvation are shown as indicated. Serum starvation induced a significantly higher level of apoptosis in IPAH MACs compared to control MACs (the rightmost panel).

**Figure 5. Inhibited migration towards VEGF for IPAH MACs.**

Healthy MAC migration towards human VEGF_{165} increased significantly. In contrast, hVEGF_{165} failed to induce significantly elevated migration for IPAH MACs.

**Figure 6. RT-qPCR analysis of VEGF and eNOS mRNA expression.**
eNOS mRNA levels in IPAH MACs and healthy MACs did not differ significantly while the VEGF mRNA level tends to decrease in IPAH MACs compared with that of healthy MACs ($p = 0.1$).
P < 0.05

N=15

Dil-Ac-LDL/FITC-UEA-1 positive double positive cells (%)

Control

IPAH

N=24

P < 0.05

N=21

VEGFR2 positive cells (%)

Control

IPAH

N=29
Control (nuclear staining)  
Control (apoptotic cell staining)  

IPAH (nuclear staining)  
IPAH (apoptotic cell staining)  

Cell apoptosis under serum starvation (%)  
P < 0.05  

N=18  
N=16  

https://mc06.manuscriptcentral.com/cjpp-pubs
P = 0.93

N = 17

P = 0.10

N = 7