



ANGIOGENESIS

Characterization of the Pall Celeris system as a point-of-care device for therapeutic angiogenesis

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Abstract

Background aims. The Pall Celeris system is a filtration-based point-of-care device designed to obtain a high concentrate of peripheral blood total nucleated cells (PB-TNCs). We have characterized the Pall Celeris–derived TNCs for their *in vitro* and *in vivo* angiogenic potency. **Methods.** PB-TNCs isolated from healthy donors were characterized through the use of flow cytometry and functional assays, aiming to assess migratory capacity, ability to form capillary-like structures, endothelial trans-differentiation and paracrine factor secretion. In a hind limb ischemia mouse model, we evaluated perfusion immediately and 7 days after surgery, along with capillary, arteriole and regenerative fiber density and local bio-distribution. **Results.** Human PB-TNCs isolated by use of the Pall Celeris filtration system were shown to secrete a panel of angiogenic factors and migrate in response to vascular endothelial growth factor and stromal-derived factor-1 stimuli. Moreover, after injection in a mouse model of hind limb ischemia, PB-TNCs induced neovascularization by increasing capillary, arteriole and regenerative fiber numbers, with human cells detected in murine tissue up to 7 days after ischemia. **Conclusions.** The Pall Celeris system may represent a novel, effective and reliable point-of-care device to obtain a PB-derived cell product with adequate potency for therapeutic angiogenesis.

Key Words: *angiogenesis, hind limb ischemia, peripheral blood cells, point-of-care*

Introduction

Critical limb ischemia (CLI) is the severest form of peripheral arterial disease, characterized by ischemic rest pain eventually leading to lower-extremity ulceration and amputation. CLI has an incidence of 500 to 1000 per million population in Western countries, representing a heavy social and economic burden as the result of high mortality and morbidity rates [1–3]. The therapeutic frontline of CLI is mechanical revascularization through percutaneous or surgical procedures. Unfortunately, up to 50% of patients with CLI are not suitable for revascularization because of poor anatomy

or high operative risk [1]. Among these patients, 35% to 50% undergo major amputation within 12 months, and 20% die within 6 months [4,5]. Notably, no effective pharmacological therapy is currently available [6].

The development of a vicarious collateralization is the therapeutic and prognostic determinant in CLI [7]. In the past decades, advancements in understanding the biological mechanisms underpinning neovascularization processes in adult tissues have focused on the role of bone marrow (BM)/peripheral blood (PB)-derived cells in contributing to the development of collateral artery growth, that is, arteriogenesis [8]. After

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endothelium activation on ischemia, the main cellular effectors of the complex scenario of neovascularization are circulating leukocytes [9]. In particular, the mononucleated compartment present in the bloodstream (PB-MNCs), constituted by monocyte/macrophage and lymphocyte populations, exerts a crucial role in arterio-arterial collateral growth. Mechanistically, these cells act as secreting “cytokine factories,” promoting vascular growth through paracrine mechanisms [10–14], including extracellular matrix remodeling, endothelial progenitor cell recruitment, trophic support for neo-endothelium and finally, the promotion of *de novo* arteriogenesis [15]. Notably, it has been observed that neo-vascularization is significantly diminished in the absence of monocytes and that capillary density increases in proportion with their accumulation [10,16].

On the basis of a such evidence, different controlled clinical studies have tested the hypothesis that the exogenous inoculation of BM- or PB-derived autologous mononuclear cells may be beneficial for the treatment of patients who are no longer suitable for conventional revascularization therapies [17–25]. Although large confirmative studies are still lacking, available results of phase I–II trials are encouraging, suggesting preliminary efficacy in the absence of major complications [26]. PB-MNCs represent a more accessible cell source for cell therapy in CLI with respect to the more widely adopted BM-MNCs [27]. Importantly, evidence of non-inferiority with respect to conservative therapy has been shown as for PB-MNCs versus BM-MNCs in restoring lower-limb perfusion. Notably, patients treated with PB-derived cells showed a more enriched MNC fraction with respect to the BM counterpart [28].

Interestingly, an increasing number of ongoing trials take advantage of point-of-care (POC) systems for cell selection. These devices offer the advantage of proximity to patients and time-saving throughout the cell harvesting, selection and delivery processes. In addition, these systems are cheaper, user-friendly and have wider applications with respect to standard good manufacturing-grade procedures. Although different POC devices for the isolation of BM cells are currently under investigation, no system that selects cells from PB has been tested to date.

The Pall Celeris system is a simple and effective POC device designed to obtain a high concentration of total nucleated cells (TNCs) from PB by means of whole-blood gravity filtration. With the long-term objective to evaluate the therapeutic efficacy of autologous TNC delivery obtained with the use of the Pall Celeris system in CLI patients, in the present work, we characterized the PB-TNCs obtained with the use of the Pall Celeris system for their identity and for their *in vitro* and *in vivo* angiogenic potency.

Methods

TNC enrichment with the use of the Pall Celeris system

A 20- to 120-mL volume of peripheral blood was collected from 27 healthy donors. Collection was performed after the donor signed a research donation consent form. All blood samples were collected in ACD-A anticoagulant and held at room temperature until use. Experiments were performed within 24 h of blood collection. PB-TNCs were isolated through the use of the Pall Celeris system (Pall Medical Corporation) according to the manufacturer’s instructions (Figure 1A). Briefly, the cell collection bag that receives the TNC concentrate was connected to port A with the use of a Luer lock mechanism. Whole blood was loaded through port B (Figure 1B). Gravity filtration (Figure 1C) was allowed to proceed until the upstream side of the filter had no remaining blood and the fluid no longer flowed into the lower filtrate bag (Figure 1D). During filtration, TNCs were captured in the filter; the majority of plasma, platelets (PLTs) and red blood cells (RBCs) were not retained. The enriched TNCs were recovered by back-flushing the filter with 14 mL of sterile saline through port C (Figure 1E).

Complete blood counts

The volume of whole blood (WB) and TNCs, before and after filtration with the Pall Celeris system, was carefully measured for each donor. A sample (200 μ L) of WB and TNCs was tested with the use of the XS-1000i Analyzer (Sysmex Europe GmbH) to obtain a complete blood count. Data were analyzed to obtain recovery, yield enrichment and cell dose for each cell subpopulation. Table I shows data from five samples with the higher clinical-grade volume of starting material (WB).

Flow cytometry

WB and TNCs were stained immediately after filtration with fluorescent-conjugated antibodies for 15 min at room temperature; red blood cells were lysed with the use of BD Pharm Lyse (BD Biosciences) and analyzed with the use of a FACSCalibur (BD Biosciences) equipped with Cell-Quest Software. The following anti-human antibodies were used: CD3 PE, CD19 PE, CD14 FITC, CD66b FITC, CD34 PE, CD45 APC (BD Biosciences), CD3APC/CD16FITC/CD56PE (Dako North America, Inc) and KDR (R&D Systems).

Migration assay

A total of 5×10^5 WB or TNCs per well were resuspended in 300 μ L of medium M199 (Life Technologies) and placed in the upper chamber of a

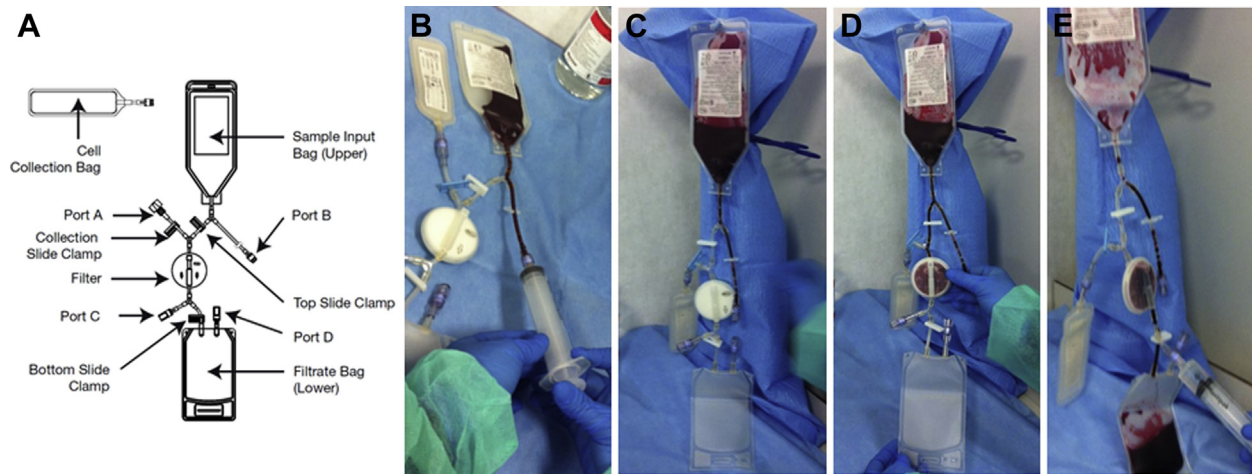


Figure 1. Pall Celeris system. (A) Schematic of the Pall Celeris system; (B–E) representative images for Pall Celeris system use. (B) Blood loading into the sample input bag through port B. (C and D) Filtration by gravity. (E) Filter backflush to recover TNCs with the use of 14 mL of saline solution through port C.

modified Boyden chamber (Corning Corporation; 5- μm pore size). The upper chamber was placed in a 24-well culture dish containing 500 μL of M199 medium supplemented with 100 ng/mL of stromal cell–derived factor 1 (or C-X-C motif chemokine 12 or CXCL12) (SDF1) (R&D Systems), 50 ng/mL of vascular endothelial growth factor (VEGF) (R&D Systems), 10% fetal bovine serum (positive control) or M199 medium alone (negative control).

After 16 h of incubation at 37°C, 5% CO₂, transmigrated cells were counted. Non-migratory cells on the upper side of the membrane were scraped off with the use of wet cotton swabs. Migrated cells in the lower chamber were manually counted. Cells present both in the lower chamber and on the lower side of the filter were counted and considered as migrated cells.

Capillary-like structure-forming assay

To assess the ability of WB and TNCs to form vascular structures *in vitro*, cells were seeded onto Cultrex (Cultrex Reduced Growth Factor Basement Membrane Extract, Trevigen Inc) artificial cell basal

membrane. Cultrex (250 μL) was allowed to polymerize onto 24-well plates at 37°C, 5% CO₂ for 30 min. WB and TNCs were seeded at a concentration of 5×10^4 cells per well in complete EGM-2 medium (Life Technologies). Branching point number was evaluated after 1, 2 and 7 days. Human umbilical vein endothelial cells (HUVEC) (Lonza Group Ltd) were used as a positive control for these experiments and were exposed to the same culture conditions.

Cytokine, chemokine and growth factor assay

A bead-based multiplex immunoassay, Bio-Plex assay (Bio-Rad Laboratories), was used to compare cytokines, chemokines and growth factors released from TNCs immediately after the Pall Celeris system filtration ($n = 8$) versus Ficoll (Ficoll Paque Plus, GE Healthcare Life Sciences) gradient centrifugation according to the manufacturer's instructions ($n = 5$). Samples were centrifuged at 4000g for 20 min. The supernatant was removed and frozen at -80°C until use. Samples were evaluated in duplicate for the presence of the following angiogenic factors:

Table I. Cell concentration after Pall Celeris system filtration.

Population	Concentration WB (before Pall Celeris filtration)	Concentration TNCs (after Pall Celeris filtration)	Yield enrichment	Recovery %	Dose
TNCs	$5.46 \pm 1.85 (\times 10^3/\mu\text{L})$	$16.24 \pm 3.97 (\times 10^3/\mu\text{L})$	2.97	39.85 ± 6.30	$2.08 \pm 0.53 (\times 10^8)$
RBCs	$4.12 \pm 0.41 (\times 10^6/\mu\text{L})$	$1.58 \pm 0.42 (\times 10^6/\mu\text{L})$	0.38	4.99 ± 1.18	$1.99 \pm 0.42 (\times 10^{10})$
PLTs	$226.60 \pm 68.82 (\times 10^3/\mu\text{L})$	$292.60 \pm 35.44 (\times 10^3/\mu\text{L})$	1.29	17.83 ± 4.46	$37.72 \pm 7.34 (\times 10^8)$
Neutrophils	$3.31 \pm 1.45 (\times 10^3/\mu\text{L})$	$7.23 \pm 2.34 (\times 10^3/\mu\text{L})$	2.18	29.81 ± 8.06	$0.93 \pm 0.32 (\times 10^8)$
Lymphocytes	$1.66 \pm 0.34 (\times 10^3/\mu\text{L})$	$7.05 \pm 2.16 (\times 10^3/\mu\text{L})$	4.25	55.05 ± 7.28	$0.90 \pm 0.25 (\times 10^8)$
Monocytes	$0.32 \pm 0.11 (\times 10^3/\mu\text{L})$	$1.25 \pm 0.27 (\times 10^3/\mu\text{L})$	3.94	54.20 ± 12.16	$0.16 \pm 0.04 (\times 10^8)$
MNCs	$1.98 \pm 0.41 (\times 10^3/\mu\text{L})$	$8.30 \pm 2.31 (\times 10^3/\mu\text{L})$	4.20	54.76 ± 7.93	$1.06 \pm 0.28 (\times 10^8)$

Anticoagulated (ACD-A) whole blood obtained from healthy donors. The whole blood volume processed was $98.2 \text{ mL} \pm 8.49$ ($n = 5$) and the volume of the enriched TNC concentrate obtained was $12.84 \pm 1.29 \text{ mL}$. Values are mean \pm standard deviation ($n = 5$).

Angiotensin-1, platelet-derived growth factor-AB/BB (PDGF-AB/BB), hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), regulated on activation, normal T-cell expressed and secreted (or Chemokine [C-C motif] ligand 5 or CCL5) (RANTES), interleukin-8 (IL-8), growth-regulated oncogene- α (or C-X-C motif ligand 1) (GRO- α), IL-10, VEGF-A, tumor necrosis factor- α (TNF- α), IL-1 β , IL-6, soluble VEGF receptor type 2 (sVEGFR-2), platelet endothelial cell adhesion molecule-1 (PECAM), basic fibroblast growth factor (bFGF) and SDF-1, by use of Luminex Technology (Bio-Plex assay, Bio-Rad), according to the instructions for use.

Mouse model of limb ischemia

All experimental procedures were approved by the internal Animal Research Ethical Committee (protocol HH39) according to the Italian Ministry of Health and complied with the National Institutes of Health (USA) *Guide for the Care and Use of Laboratory Animals*. CD1 male mice (Charles River), 2 months old, weighing 25 to 30 g, were used for all experiments. Animals were anesthetized with a mixture of 1 g of tribromoethyl alcohol in 1 mL of tert-amyl alcohol (Avertin, Sigma-Aldrich), diluted 1:50 and intraperitoneally injected, 20 μ L/g body weight. Acute hind limb ischemia in CD1 mice was induced by femoral artery dissection as previously described [29]. Mice ($n = 5$ /group) received five injections of either normal saline or 1×10^5 enriched TNCs into the adductor muscle. Induction of ischemia was confirmed by means of laser Doppler perfusion imaging (Lisca, PeriMed AB) immediately after surgery and at 7 days before euthanasia, as established previously [29]. Briefly, blood flow was measured in ischemic and contralateral hind limbs by use of Lisca at baseline, immediately after induction of ischemia (day 0, to confirm efficient induction of ischemia) and 7 days after ischemia. Before imaging, mice were placed on a heating mat at 37°C. Low or no perfusion was displayed in dark blue, whereas high perfusion was displayed in red. To avoid the influence of ambient light and temperature, results were expressed as the ratio between perfusion in the left (ischemic) versus right (non-ischemic) limb.

Histological analysis

Seven days after surgery, anesthetized mice were killed and perfused with phosphate-buffered saline, followed by 10% buffered formalin (10 min) at 100 mm Hg through the left ventricle. After paraffin embedding, 3- μ m-thick sections were cut from each sample with muscle fibers oriented in the transverse direction.

Hematoxylin and eosin staining was used to identify capillaries and regenerating myofibers. Capillaries were identified by their morphology and adjacent localization to or associated with muscle fibers. The number of capillaries from 10 randomly selected separate fields was counted for each muscle. Capillary density was defined as the mean number of capillaries per field as previously established [29]. Regenerating myofibers are defined as fibers with centrally located nuclei; fibers intersecting the right and top border of the field were not counted. Images were taken with the use of a Zeiss Axioplan microscope. Ten fields ($\times 20$ objective) were analyzed in each mouse by two individuals in a blinded manner.

Arterioles were identified by use of α -smooth muscle actin (α -SMA)-positive (Sigma-Aldrich) fluorescent staining and appropriate morphology and localization (adjacent to or associated with muscle fibers, 4 to 40 μ m diameter) and counted by two independent operators blinded to the treatment regimen. The number of arterioles from 10 randomly selected different fields was counted for each muscle. Arteriole density was defined as the mean number of capillaries per field as previously established [29].

To assess the local bio-distribution of injected cells, human cells were identified by means of anti-human nuclei-positive fluorescent staining (Millipore Corporation). Sections were observed with the use of an inverted Zeiss Axioplan fluorescence microscope. Images were acquired by use of a $\times 40$ objective and a digital camera system and analyzed with the use of IAS Delta System software.

Statistical analysis

Statistical analyses were performed by use of Student's paired or unpaired t -tests, with the use of GraphPad statistical software, except for migration assay. A value of $P < 0.05$ was considered statistically significant.

For the migration assay, to compare means of the two "subsamples" (WB and TNCs), one-way analysis of variance was performed. Outcomes confirmed no significant differences in means among the WB and TNCs ($P = 0.4$). A comparison of the means for four subsamples: CTR-neg, SERUM, VEGF and SDF-1 was performed with the use of one-way analysis of variance. Outcomes confirm a significant difference in means among the four subsamples ($P < 0.01$).

Results

TNC characterization

The mean volume of whole blood processed was 98.2 ± 8.49 mL ($n = 5$) and the mean volume of the enriched TNC concentrate obtained was 12.84 ± 1.29

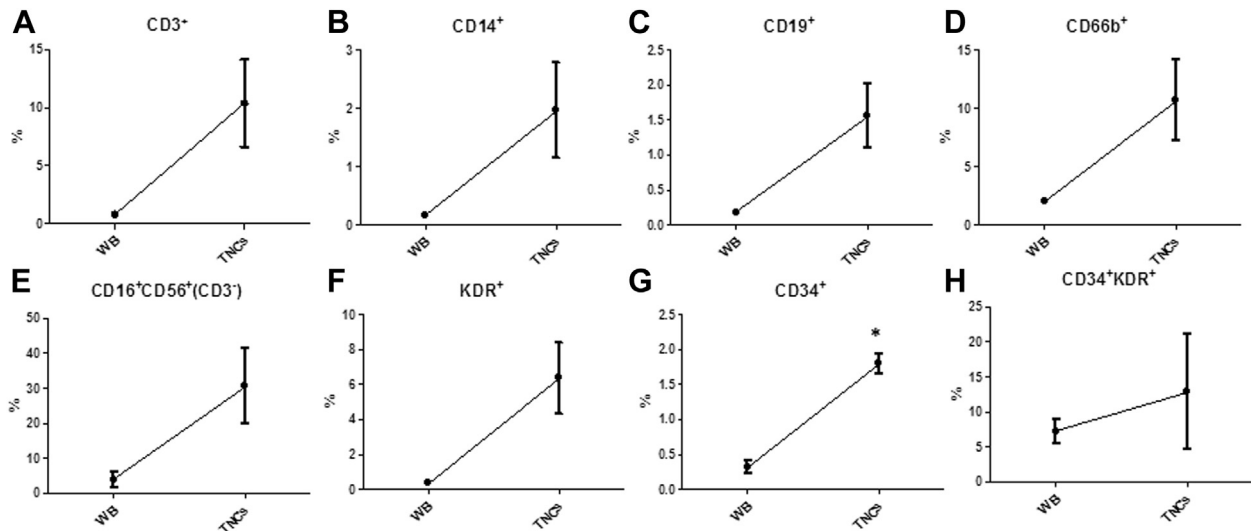


Figure 2. FACS analysis of TNCs: (A) CD45⁺CD3⁺ T lymphocytes, (B) CD45⁺CD14⁺ monocytes, (C) CD45⁺CD19⁺ B lymphocytes, (D) CD45⁺CD66b⁺ neutrophil granulocytes, (E) (CD3⁻)CD16⁺CD56⁺ natural killer, (F) CD45⁺CD34⁺ stem cell component, (G) CD45⁺KDR⁺ and (H) CD34⁺KDR⁺ endothelial progenitor markers. Values are mean \pm standard error of the mean ($n = 4$). Paired *t*-test. * $P < 0.05$.

mL ($n = 5$). TNCs were enriched 2.97-fold (concentration factor averaged) and MNCs were enriched 4.2-fold (Table I); the CD34⁺ progenitor cell subpopulation was enriched by $5.6\% \pm 4.2\%$ versus controls, $P < 0.01$; $n = 4$ (Figure 2). No differences were found for the various surface markers used to identify subpopulations of leukocyte cells including B and T lymphocytes, monocytes and granulocytes.

Cytokine, chemokine and growth factor assay

The pro-angiogenic potential of TNCs has been tested through the use of multiplex analysis by comparing multiple cytokine content in the supernatant obtained after filtration with the Celeris system versus the Ficoll gradient centrifugation counterpart (Table II). As shown in Table II, a variety of angiogenic cytokines have been found in TNC supernatant: bFGF, G-CSF, HGF, PDGF-AB/BB, PECAM-1, sVEGFR-2, IL-8, TNF- α , VEGF-A, IL-1 β , IL-10, RANTES, SDF-1 and IL-6. Notably, we found that the levels of angiogenic cytokines in supernatant of the Pall Celeris filtrate are significantly higher than for Ficoll for 10 of 14 tested molecules ($P < 0.05$) (Table II).

Migration assay

A prerequisite for the therapeutic success of intramuscularly delivered TNCs is their ability to migrate toward chemoattractant factors upregulated in ischemic tissue such as SDF-1 and VEGF. Both WB and the enriched TNCs showed a comparable ($P = 0.4$) and significant migratory ability compared with negative control (CTR neg) ($P < 0.05$), confirming that Celeris filtration does

not impair the ability of the cells to migrate on chemoattractant stimuli ($n = 8$) (Figure 3B).

In vitro endothelial differentiation

Positive control cells (HUVECs) formed capillary-like structures within 24 h on seeding on to synthetic media (Cultrex), whereas native whole blood (WB) cells and Celeris-derived TNCs did not form capillary structures after 7 days of observation (Figure 3). After 7 days in differentiation medium, immunofluorescence analyses were performed to evaluate markers of endothelial differentiation: Von Willebrand Factor (vWF) and the uptake of

Table II. Released cytokines in TNC supernatant after Pall Celeris filtration versus Ficoll centrifugation.

Cytokine	Supernatant TNCs (pg/mL)	Supernatant Ficoll (pg/mL)	<i>P</i> value
	mean \pm SD $n = 8$	mean \pm SD $n = 5$	
bFGF	201.32 \pm 106.51	213.01 \pm 48.52	0.8236
G-CSF	367.03 \pm 206.67	231.41 \pm 82.26	0.1945
HGF	743.32 \pm 540.80	141.81 \pm 59.52	0.0330
PDGF-AB/BB	783.09 \pm 264.62	394.90 \pm 168.87	0.0143
PECAM-1	2830.04 \pm 808.87	4388.69 \pm 1553.17	0.0350
sVEGFR-2	2499.42 \pm 1776.57	54.76 \pm 32.43	0.0116
IL-8	18.09 \pm 7.98	0.41 \pm 0.75	0.0005
TNF- α	54.32 \pm 20.71	0.77 \pm 0.09	0.0001
VEGF-A	144.81 \pm 95.80	19.07 \pm 7.45	0.0149
IL-1b	0.72 \pm 0.20	0.12 \pm 0.07	0.0001
IL-10	2.00 \pm 1.71	0.72 \pm 0.19	0.1287
RANTES	6188.49 \pm 1676.56	5582.95 \pm 4028.66	0.7090
SDF-1	172.55 \pm 54.95	34.60 \pm 25.21	0.0003
IL-6	2.68 \pm 2.03	0.30 \pm 0.19	0.0258

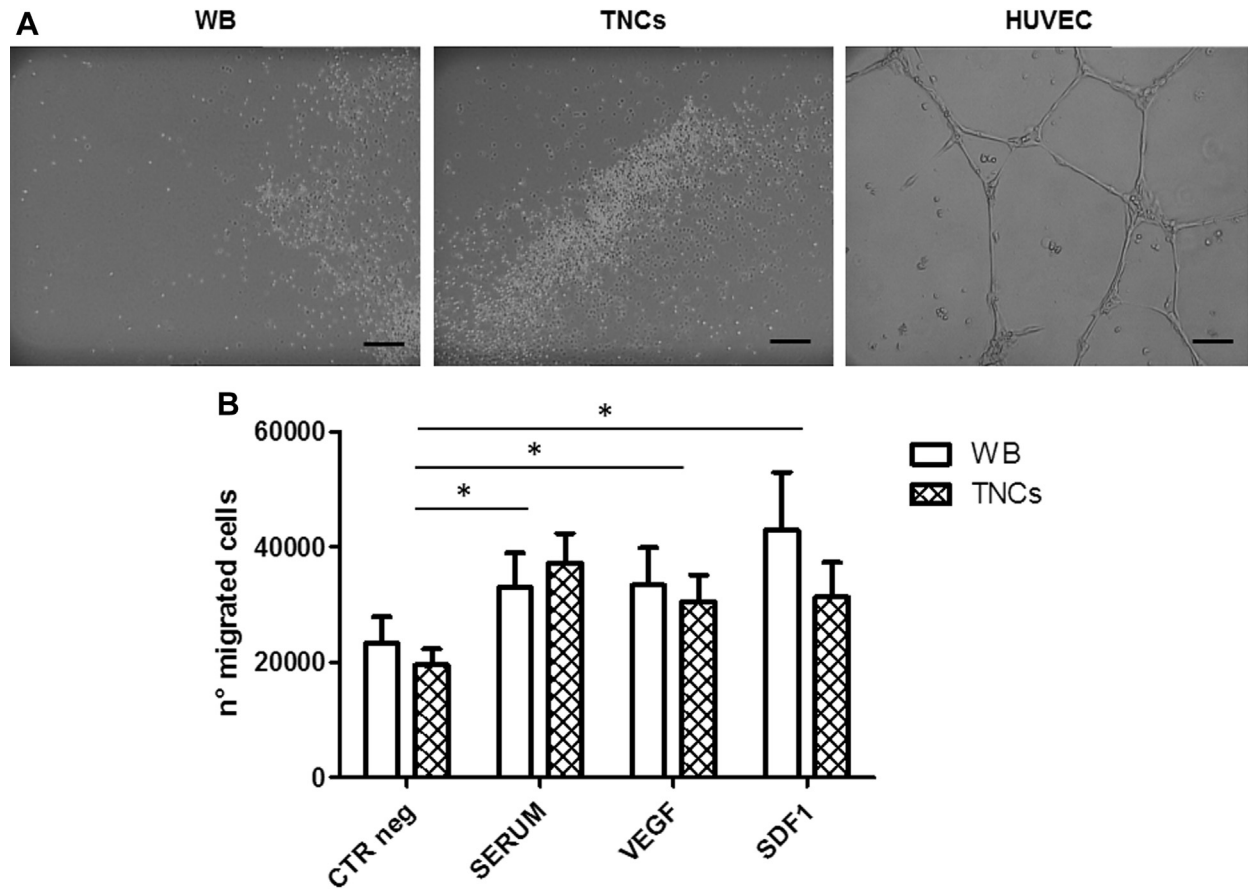


Figure 3. (A) Capillary tube formation assay ($n = 6$) formation of tubular-like structures by WB, TNCs and HUVECs onto Cultrex basement membrane. Capillary formation was found only in the positive control (HUVECs). Representative images $\times 10$. Scale bar = 200 μm . (B) Migration assay. Stimuli were culture medium M199 (CTR neg), culture medium + 10% fetal bovine serum (SERUM), culture medium VEGF + 50 ng/mL (VEGF) and SDF1 culture medium + 100 ng/mL (SDF1). No significant differences are observed between WB and TNCs in any of the conditions considered ($P = 0.4$). Stimuli with SERUM, VEGF and SDF1 cause a significant increase in the number of migrated cells compared with CTR neg ($*P < 0.05$) ($n = 8$).

acetylated low-density lipoprotein (DiI-Ac-LDL). After 7 days of culture, we observed in WB and TNCs a very low number of endothelial-like cells characterized by the expression of vWF and DiI-Ac-LDL, as compared with HUVECs (Supplementary Figure 1S).

In vivo perfusion

Laser color Doppler perfusion evaluation 7 days after ischemia showed a significant improvement of blood flow in the hind limb of mice treated with enriched human TNCs with respect to controls ($P = 0.036$) (Figure 4A,C).

In vivo immunohistochemistry and immunofluorescence

The local bio-distribution of human cells was assessed by anti-human nuclei-positive fluorescent staining (Figure 4D,E). This assay showed that 7

days after transplantation, isolated clusters of human cells in murine muscle tissue can be identified.

Histological analysis of ischemic tissues demonstrated that human TNC delivery was associated with a significant increase in capillary and arteriole formation compared with that in saline-treated animals ($P = 0.0025$ and $P = 0.0001$, respectively) (Figure 5A–C).

Furthermore, to evaluate whether human cells play a role in skeletal muscle regeneration, the number of regenerating fibers were counted on adductor muscle sections at day 7 after ischemia and treatment delivery. At this time point, the number of regenerating fibers increased in the cell-treated group when compared with untreated animals (Figure 5A–C) ($P = 0.047$).

Discussion

In this report, we have provided evidence that human peripheral blood TNCs isolated with the Pall Celeris

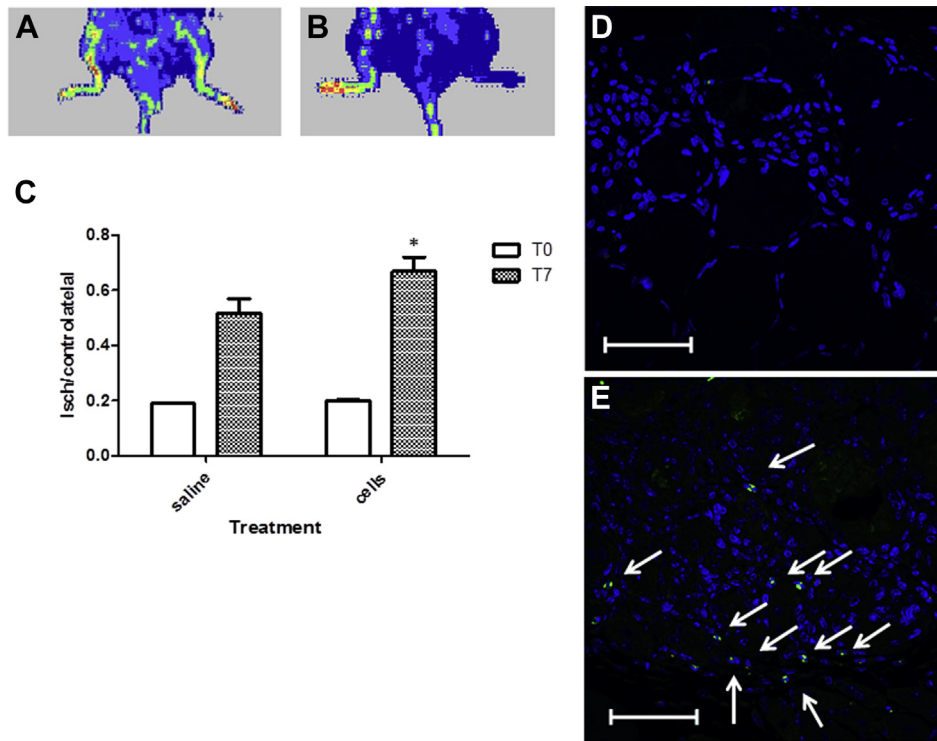


Figure 4. (A–C) Laser Doppler perfusion imaging (LDPI) in a murine model before (A) and after (B) microsurgery to induce hind limb ischemia. (C) Seven days after ischemia, LDPI showed a significant increase in the group of mice treated with 10^5 cells, compared with the group treated with saline. * $P < 0.05$ (T7 cells versus T7 saline) ($n = 5$). (D, E) Local bio-distribution of injected cells after 7 days after transplantation ($n = 5$). Immunofluorescence for anti-human nuclei identifies human cells (white arrows) in murine tissue, with Hoechst 33258 staining. Representative images $\times 40$. (D) Mice treated with saline; (E) mice treated with 1×10^5 TNCs. Scale bar = 50 μm .

filtration system have high angiogenic potency *in vitro* and *in vivo*. In particular, we showed that TNCs secrete a variety of angiogenic factors and can migrate in response to VEGF and SDF-1. Moreover, when injected in a mouse model of hind limb ischemia, TNCs induced neovascularization by increasing capillary and arteriole number.

BM- or PB-derived cell therapy has recently gained interest as a potential treatment option for refractory CLI, with the goal to increase blood circulation in the ischemic limb [26]. Different cell lineages have been delivered to affected limbs through the intramuscular or intra-arterial route to achieve therapeutic angiogenesis. These approaches aimed to boost the physiological active role that leukocytes, and in particular circulating mononuclear and endothelial progenitor cells (EPCs), are exerted into ischemic tissues through initiating a cascade of events leading to collateral artery growth [30–36]. As consistently shown [37,38], BM-derived nucleated cells with monocytic and/or macrophage markers [39] invade ischemic tissues after endothelium-dependent activation and promote vascular growth, not directly incorporating into vessel walls but rather acting as “cytokine factories,” releasing angiogenic factors in a paracrine manner.

Circulating EPCs, first described by Asahara [40] and classified on the basis of CD34^+ expression, are generally defined as a BM-derived circulating cell population with endothelial potential [41]. Despite the lack of a precise classification, these cells have been purported to originate from the monocyte-macrophage lineage, representing approximately 1% of total MNC population [13]. EPCs are responsive to ischemia similarly to BM-derived monocytic cells, acting as cytokine factories in the perivascular collateral artery space [42]. Consequently, BM and PB are possible sources of cells with angiogenic potential.

Different isolation procedures have been used to concentrate the mononuclear fractions or to positively isolate EPCs on the basis of their surface markers from BM or PB. From a regulatory standpoint, the techniques involving cell separation (Table III), such as Ficoll density gradient system centrifugation [30], plasma pheresis systems (ie, COBE Spectra, Gambro; CS 3000-Plus, Baxter Healthcare) [27,43] or immune-magnetic selection (ISOLEX 300i Magnetic Cell Selection System, Baxter Healthcare; Sepax 2 BioSafe SA; MACS, Miltenyi Biotec S.r.l.) [33,36] require complex and stringent Good Clinical Practice—or Good

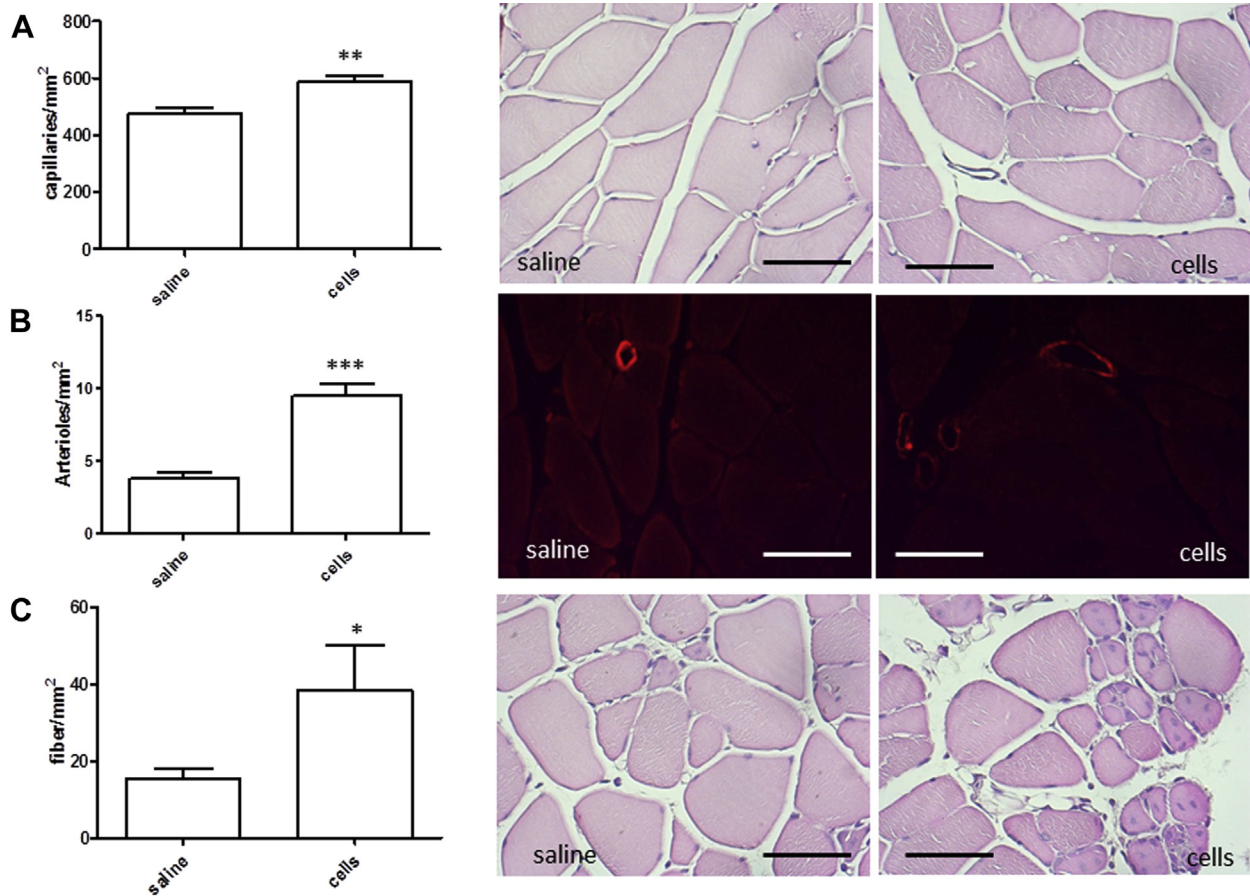


Figure 5. Capillary, arteriole and regenerative fiber counts after 7 days. The number of capillaries (A), regenerative fibers (B) and arterioles (C) per unit of area showed a significant increase in the cell-treated mouse group compared with the saline-treated group. Representative images $\times 20$ ($n = 5$). Scale bar = 200 μm . * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Manufacturing Practice—certified procedures. In the European community, tight regulation is applied for such advanced therapies, which results in high manufacturing costs and complex authorization processes (WHO Technical Report Series, No. 908, 2003 Annex 4 Good Manufacturing Practices for pharmaceutical products: main principles). In an attempt to overcome these issues, POC systems have been recently implemented. POC systems are single-step, bedside, closed isolation systems that allow for a relatively cost effective and quicker cell processing. To date, different centrifugation-based POC systems (SmartPreP, Harvest Technologies Corp Res-Q60 BMC, TotipotentSC; Magellan, Arterioocyte Medical System) have been designed to process BM [9]. Some of them are currently under investigation in controlled clinical trials of BM-derived cell therapy in CLI: MarrowStim (Biomet, Inc), NCT01049919, Magellan, NCT01386216, (Icellator Cell Isolation System, Tissue Genesis, Inc), NCT02234778, BMAC 2 (Harvest Technologies), NCT01232673, NCT01245335, NCT00595257, NCT00498069, Res-Q60 BMC and NCT01472289 (Table III). Of

note, four clinical trials are currently ongoing (www.clinicaltrials.gov) that use the BMAC 2 system, which concentrates, by means of a centrifugation-based principle, 4.20-fold BM-derived nucleated cells. In a phase II study, Prochazka *et al.* [35] reported a 79% limb salvage rate in patients with CLI and foot ulcers. Notably, the efficiency in CD34⁺ hematopoietic stem cell enrichment with the use of BMAC 2 to concentrate BM cells is comparable to the use of the Pall Celeris system with PB cells, [35]. Another phase I trial that used the MarrowStim device delivers a high BM-MNC number (2×10^9) to affect ankle-Brachial Index, TcPO₂ and rest pain. By way of contrast, the present work confirms that a dose of approximately one-tenth of PB cells is enough to provide significant improvement in perfusion and capillary and arteriolar density in the mouse model studied. The ISOLEX 300i Magnetic Cell Selection System [36] is not strictly a POC system. This device is used in a Good Manufacturing Practice environment to concentrate a single stem cell population (CD34⁺) through the use of a magnetic selection approach. Losordo *et al.* [36] report a

Table III. Clinical studies of cell therapy with the use of devices in patients with critical limb ischemia.

Clinical Trials. gov Id	Phase	Patients (n)	Raw material	Quantity	Cell type	Dose	Device	Route of administration	Ref.
NCT01049919	II	152	BM	360–370 mL	BM-MNCs	$2.0 \pm 1.6 \times 10^9$ MNCs	MarrowStim	IM	[31]
NCT01065337	II	30	BM	40–50 mL	BM-MNCs/ BM-MSCs	NA	Sepax/MACS	IM/IA	[33]
NCT01386216	I	20	BM	NA	BM-MNCs	NA	Magellan	IM	-
NCT00523731	I	6	PB	250 mL	EPCs	NA	Vescell	IM	[34]
NCT02234778	I	20	Adipose tissue	NA	Stromal vascular fraction	NA	Icellator Tissue Genesis	IM	-
NCT01232673	II	96	BM	240 mL	BM-MNCs	$6.44 \pm 0.5 \times 10^8$ MNCs $2.85 \pm 0.04 \times 10^9$ TNCs	BMAC 2	IM	[35]
NCT01245335	III	210	BM	300 mL	BM-MNCs	NA	BMAC 2	IM	-
NCT00595257	I/II	60	BM	NA	BM-MNCs	NA	BMAC 2	IM	-
NCT01472289	I/II	15	BM	120 mL	BM-MNCs	NA	Res-Q60	IM	-
NCT00498069	I/II	48	BM	300 mL	BM-MNCs	NA	BMAC 2	IM	-
NCT00616980	I/II	28	Mobilized PB	NA	CD34 ⁺ cells	Low/high/placebo 1:1:1	ISOLEX 300i	IM	[36]

MSC, mesenchymal stromal cell; NA, not available.

trend toward reduced amputation rates in cell-treated subjects versus control subjects. No published data are available for other centrifugation-based devices (Res-Q60 BMC; Magellan) currently under investigation in clinical trials.

Given the current lack of evidence of the superiority of BM versus mobilized PB cells [27], the advantage of PB as a cell source is the avoidance of BM harvesting drawbacks, including local pain, hematomas and anemia [44]. To date, more than 200 patients have been enrolled in phase I–II clinical trials on PB cell therapy for CLI [19–24,34,45–47]. Cells are primarily obtained through apheresis to retrieve the MNC fraction with or without G-CSF–induced cell mobilization. No adverse effects have been reported, along with preliminary observations of enhanced wound healing, perfusion and pain in the ischemic limb [9]. Although G-CSF has proven to be effective for a large mobilization in the bloodstream of BM cells, concerns about rare but possible serious adverse events [46,48,49] have prompted researchers to test cell harvesting from PB in the absence of endogenous stimulation [22,23,34]. In a pilot study on six patients with CLI, Mutirangura *et al.* [34] reported that implanting nucleated cells from PB was safe in terms of systemic and local reactions. More recently, Morija *et al.* [11] intramuscularly injected ischemic limbs of 42 CLI patients with PB-derived MNCs harvested without G-CSF–induced mobilization [11] by means of an apheresis-based appliance (COBE Spectra). Although this was not a case-controlled study, preliminary efficacy data suggest a good safety profile and potential

angiogenic efficacy in this severely compromised patient population.

The Pall Celeris system is the first POC device conceived to concentrate an MNC-enriched population of TNCs with high angiogenic potential from PB without apheresis, by means of a filtration system. As reported by Jin *et al.* [50], ischemia induces elevation in plasma of different cell-derived active cytokines, including sKitL (Soluble Kit-ligand) and thrombopoietin, and, to a lesser extent, progenitor-active cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin. In agreement with these results, we found that Pall Celeris-derived TNCs produced in supernatant a large pool of cytokines with angiogenic properties, including FGF, VEGF, HGF and G-CSF. Importantly, our data show that supernatant cytokine levels of the Pall Celeris system are significantly higher than that in the Ficoll counterpart as for 10 of 14 tested angiogenic molecules. Because Ficoll is a gold standard for MNC isolation [51,52], this finding is supportive for a high angiogenic potency of the Pall Celeris system.

Our finding that Pall Celeris–derived TNCs do not trans-differentiate into endothelium reinforces the concept that their contribution to angiogenesis promotion is indirectly through secreted factors, reminiscent of the previously described role of BM-derived cells in angiogenesis promotion [26]. Interestingly, PLTs have been identified as a co-player in the secretion process [53,54] by releasing SDF-1, a key compound responsible for cell ability to migrate on ischemia [55]. Our data show that the Pall Celeris filtration system can enrich PLT concentration by

approximately 1.3-fold and that SDF-1, along with PDGF, are liberated in the TNC supernatant. Functionally, these findings are paralleled by the preserved capacity of filtered TNCs to migrate on SDF-1 or VEGF stimuli, thus suggesting their well-maintained chemo-attractive response. An additional positive factor possibly related to efficacy after BM-MNC delivery was attributed to the supply of EPCs (including CD34⁺ fraction) [43]. The meta-analysis by Fadini *et al.* [44] reported that in BM, the mean CD34⁺ cell count in published studies was $5.0 \pm 1.48 \times 10^7$, indicating that approximately 1.4% of transplanted cells were CD34⁺. Our data demonstrated a significant 5.6-fold enrichment in CD34⁺ cell population after Pall Celeris filtration (from 0.32% in WB to 1.79% in Pall Celeris TNCs), with a mean CD34⁺ cell count of 1.37×10^6 . In addition, in comparison to previously published data with centrifugation-based POC systems, we first report the contamination rate of red blood cells. The data have not been reported thus far and the presence of red blood cells in ischemic tissue is still controversial. Ozawa *et al.* [56] report that treatment of limb-ischemic mice with whole BM cells improved limb survival and blood flow. However, the implantation of purified erythroid cells did not rescue limbs but appeared not to impair the limb salvage if present in a mixed population.

As for *in vivo* potency, similarly to BM-derived cells [33], we found that the Pall Celeris-derived TNCs induce, under ischemic conditions, a significant collateral growth, by increasing capillary and arteriole number, and improve limb perfusion accompanied by an increase of regenerating fibers, possibly as a consequence of neovascularization. As previously described [55], limited clusters of transplanted cells persisting 1 week after TNC introduction have been observed.

Although this study was not conceived with a dose-finding design, the cell dose we injected in mice (1×10^5 TNCs) was selected for a calculated TNC number corresponding to $2.08 \pm 0.53 \times 10^8$ TNCs and $1.06 \pm 0.28 \times 10^8$ MNC in humans. This cell count can be easily obtained through filtration of 120 mL of PB from patients (see Table I). In published clinical studies that used BM-POC systems, numbers of BM-TNCs ranging between 2.0 and 2.85×10^9 have been obtained, with an approximate 4-fold-enrichment for each subpopulation; however, we show in the present work that approximately a tenth of the PB-derived cell dose is sufficient to promote neo-angiogenesis in a mouse model of hind limb ischemia. Very recently, a large pilot study in CLI patients ($n = 43$) with intramuscular injection of Pall Celeris-derived TNCs has been published [57]. The starting volume of PB (WB) was 120 mL,

corresponding to our Pall Celeris setup. Results are encouraging in terms of procedure safety and preliminary efficacy (limb rescue).

In conclusion, our data indicate that the Pall Celeris system may represent an interesting novel and reliable POC device to obtain a PB-derived cell product with adequate potency for therapeutic angiogenesis in limb ischemia.

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Supplementary data

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