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RESEARCH ARTICLE

A novel potential therapy for vascular diseases: blood-derived stem/progenitor cells specifically activated by dendritic cells

Yael Porat[1,2]*
Efrat Assa-Kunik[1,2]
Michael Belkin[3]
Michael Krakovsky[4]
Itschak Lamensdorf[4]
Revital Duvdevani[4]
Galit Sivak[2]
Mark J. Niven[2]
Shlomo Bulvik[2]

[1]*BioGenCell Ltd, Sanz Medical Center, Laniado Hospital, Netanya, Israel*

[2]*Sanz Medical Center, Laniado Hospital, Netanya, Israel*

[3]*Sheba Medical center, Tel-Aviv University, Tel-Aviv, Israel*

[4]*Pharmaseed Ltd, Nes-Ziona, Israel*

*Correspondence to: Yael Porat, BioGenCell Ltd, Laniado Hospital, Netanya 42150, Israel.
E-mail: Yael.porat@biogencell.net

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Abstract

Background Vascular diseases are a major cause of morbidity and mortality, particularly in diabetic patients. Stem/progenitor cell treatments with bone marrow-derived cells show safety and promising outcomes, albeit not without some preprocedural adverse events related to cell collection and mobilization. We describe a novel technology for generating a therapeutic population (BGC101) of enriched endothelial progenitor cells (EPCs) from non-mobilized blood, using dendritic cells to specifically direct stem/progenitor cell activity *in vitro*.

Methods and results Selected immature plasmacytoid and myeloid dendritic cells from 24 healthy and two diabetic donors were activated with anti-inflammatory and pro-angiogenic molecules to induce specific activation signals. Co-culturing of activated dendritic cells with stem/progenitor cells for 12–66 h generated $83.7 \pm 7.4 \times 10^6$ BGC101 cells with 97% viability from 250 mL of blood. BGC101, comprising $52.4 \pm 2.5\%$ EPCs (expressing Ulex-lectin, AcLDL uptake, Tie2, vascular endothelial growth factor receptor 1 and 2, and CD31), $16.1 \pm 1.9\%$ stem/progenitor cells (expressing CD34 and CD184) and residual B and T helper cells, demonstrated angiogenic and stemness potential and secretion of interleukin-8, interleukin-10, vascular endothelial growth factor and osteopontin. When administered to immunodeficient mice with limb ischemia ($n = 40$), BGC101 yielded a high safety profile and significantly increased blood perfusion, capillary density and leg function after 21 days. Cell tracking and biodistribution showed that engraftment was restricted to the ischemic leg.

Conclusions These observations provide preliminary evidence that alternatively activated dendritic cells can promote the generation of EPC-enriched stem/progenitor cells within a 1-day culture. The resulting product BGC101 has the potential for treatment of various vascular conditions such as coronary heart disease, stroke and peripheral ischemia. Copyright © 2014 John Wiley & Sons, Ltd.

Keywords diabetes complications; dendritic cells; cardiovascular diseases; stem/progenitor cell therapy; ischemia; endothelial progenitor cells

Introduction

Vascular diseases, including cardiovascular diseases and peripheral vascular diseases, are major causes of morbidity and mortality; particularly in patients

with diabetes. Atherosclerosis causes diminished tissue perfusion [1–4], resulting in claudication, with progression to rest pain, the inability to heal wounds and eventually gangrene, amputations and even death [5]. In diabetic patients, the medium and small vessels are affected disproportionately, resulting in critical limb ischemia (CLI). Despite aggressive attempts at revascularization with endovascular and open surgical procedures, a significant number of these patients progress to amputation [6,7]. There is a pressing need for new therapeutic modalities, such as the use of stem cell-based products that promote angiogenesis. Treatments, mostly performed using intramuscular injections of bone marrow (BM)-derived cells, have shown high safety profiles and promising therapeutic results. However, adverse events related to BM harvest and mobilization have been reported [6,8,9].

It is well documented that dendritic cells (DCs), originally identified by Steinman *et al*. in 1973 [10], regulate both innate and adaptive immunological responses through their participation in the maintenance of peripheral tolerance and in the triggering of antigen-specific T-cell responses [10–17]. There are no published reports, however, of the use of DCs to direct activation and differentiation of stem/progenitor cell populations *in vitro*, outside of the immune system niche.

Following *in vivo* or *in vitro* activation, immature DCs (iDCs) mature into myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) [18], which stimulate effector T-cell and B-cell responses based both on cell cell interactions and on the pro-inflammatory and anti-inflammatory paracrine effects of cytokines [12–15,17]. In the presence of anti-inflammatory molecules such as calcitriol, prostaglandin E2, transforming growth factor beta, basic fibroblast growth factor and interleukin (IL)-10, iDCs are alternatively activated in an antigen-independent manner to induce regulatory T cells and secrete potent angiogenic factors such as vascular endothelial growth factor (VEGF) and nitric oxide, resulting in tolerance and pro-angiogenic effects [19–24].

The pivotal role of cross-talk between DCs and endothelial cells in angiogenesis was discussed by Sozzani *et al*. [25]. In the presence of pro-angiogenic factors such as ischemia and VEGF, DCs demonstrate the ability to transdifferentiate into endothelial-like cells, thereby contributing to vasculogenesis and angiogenesis [26,27]. Furthermore, by producing and releasing pro-angiogenic mediators leading to neovascularization, DCs influence pathophysiological conditions including inflammation, wound healing, tumor growth and atherosclerosis [25,28,29].

Dendritic cells can be directly isolated from peripheral blood mononuclear cells (PBMCs) [30,31] or generated from hematopoietic stem/progenitor cells (HSPCs) and monocytes during culture in the presence of cytokines such as IL-4, Flt3 (tyrosine kinase) and granulocyte-monocyte colony-stimulating factor [32–34]. Various activation protocols have resulted in functional DCs that can secrete cytokines and activate effector cells *in vitro* and *in vivo* [34]. Studies assessing the kinetics of DC activation demonstrate that brief activation of 3–8 h results in partially matured DCs with migratory abilities that are highly effective in generating an *in vivo* immune response [35]. Bellone *et al*. showed that DC produces IL-12 and marginally upregulates cell-surface molecules as early as 15 min after activation [36].

Here, we describe a novel method for the generation of a therapeutic SPC population of enriched EPC (EnEPCs), termed BGC101 in which alternatively activated non-mobilized blood-derived DCs are used to specifically direct *in vitro* the activity of SPC, which were isolated from the same blood sample.

Methods

Donors

All procedures using human samples were approved by the hospital's Institutional Review Board, and signed informed consent was obtained. Blood samples, at volumes of 250–450 mL, were obtained from either the Israel Blood Bank or Laniado Hospital. Until processed, blood samples were kept in transfusion bags containing 49 mL sodium citrate as an anticoagulant. Fifty-two individual blood samples were used in this study for DCs and SPC enrichment. Thirty-nine of these samples were used for individual EnEPC preparations, 33 from 24 healthy donors and six from two diabetic donors. The healthy donors were aged 18–65 years and had no history for suspected diabetes. The diabetic donors were diagnosed to have type 2 diabetes mellitus as defined by the World Health Organization criteria [37] and had received conventional treatment with oral anti-diabetic agents with or without insulin for at least 6 months. One patient (female; 78 years old; body mass index, 34.5; glucose, 167 mg/dL; HbA_{1c}, 6.6%; microalbumin/creatinine, 1505 mg/g creatinine; cholesterol, 184 mg/dL; triglycerides, 131 mg/dL; HDL, 63 mg/dL; LDL, 95 mg%) had diabetes complications including cardiovascular and peripheral vascular diseases with no history of suspected cerebrovascular disease. The other patient (male, 55 years old; body mass index, 27.2; glucose, 95 mg/dL; HbA_{1c}, 7.2%; microalbumin/creatinine, 8.5 mg/g creatinine; cholesterol, 151 mg/dL; triglycerides, 130 mg/dL; HDL, 36 mg/dL; LDL, 82 mg%) had no history of suspected other diseases.

Cell processing

As illustrated in the flow chart (Figure 1), PBMCs and plasma were isolated from the blood samples on Ficoll

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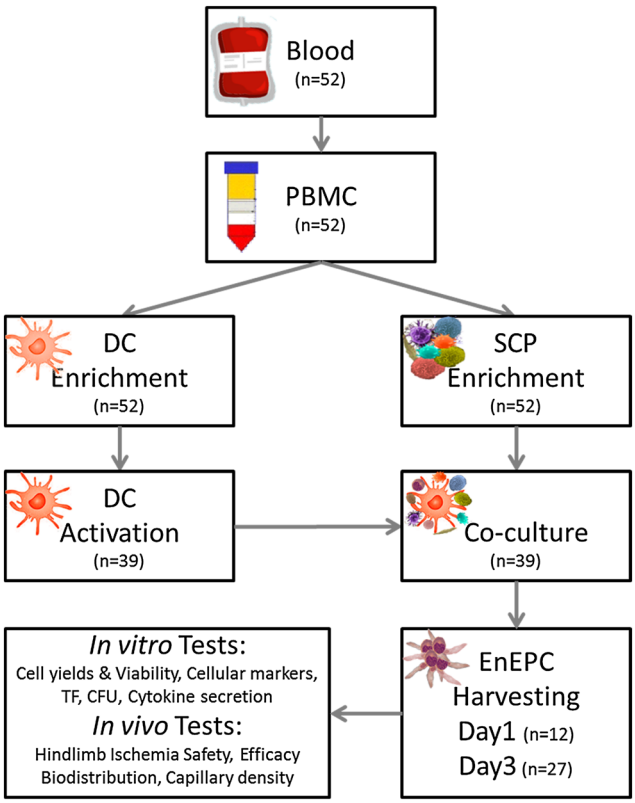

Figure 1. Blood-derived stem/progenitor cells specifically activated by dendritic cells (DCs). Flow chart depicting the generation of a potentially therapeutic enriched endothelial progenitor cells (EnEPCs). Non-mobilized blood-derived plasmacytoid and myeloid dendritic cells activated with toleragenic and pro-angiogenic cytokines (such as IL-10, VEGF) are used *in vitro* to specifically direct the activity of stem/progenitor cells (SPC), which were enriched from the same blood sample and co-cultured for 1 or 3 days. EnEPCs from both healthy and diabetic donors harvested on day 1 or on day 3 were tested for identity and functionality *in vitro* and *in vivo*. *In vitro* tests included cell yield and viability; flow cytometry; angiogenic potential, tube formation (TF) test; stemness, self-renewal potential, colony-forming unit (CFU) assay; and cytokine secretion. *In vivo* tests in the hindlimb ischemia model included (1) safety assessment of high dose-related gross pathology, microscopic pathology, blood chemistry and blood cell count. Additional safety and pharmacokinetic tests examined cells' biodistribution and capillary density in the intact leg. (2) Efficacy tests based on laser Doppler perfusion measurements on days 15 and 21 post injury as well as tracking the human cells and measuring capillary density as mechanisms underlying the observed effect

gradient (Lymphoprep tubes, Novamed, Jerusalem, Israel). Specific cell populations of iDCs and SPCs were enriched on LS isolation columns (Miltenyi Biotec, Bergisch Gladbach, Germany) using biotinylated mouse monoclonal antibodies and mouse monoclonal anti-Biotin MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. First, DCs were enriched by specific markers such as CD141, CD86 and CD1c for mDCs, and CD304 for pDCs (Miltenyi Biotec). SPCs from the same samples were then enriched on the LS separation column by negative selection that excluded CD8 cytotoxic T cells and CD56 natural killer (NK) cells. iDCs at a density of $1–5 \times 10^6$ cells/mL were activated in serum-free medium (X-VIVO15, Lonza, Verviers, Belgium) supplemented with 1–20% autologous serum, 5–25 IU/mL heparin (Bodene, Port Elizabeth, South Africa), 1–50 ng/mL human VEGF and hIL-10 (PeproTech, Rocky Hill, NJ). To generate EnEPCs, activated iDCs were co-cultured with the enriched SPCs from the same blood sample, at ratios of 1 : 5 to 1 : 20, seeded on culture dishes precoated with autologous serum/plasma and subsequently incubated at a density of $1–5 \times 10^6$ cells/mL in growth medium containing X-VIVO15 with or without the added cytokines listed in the preceding text, for 12–18 h (1 day culture) or for 60–66 h (3 day culture) at 37 °C, 5% CO_2. The culture conditions used were based on previous reports [38] and *in vitro* optimization of the aforementioned ranges on blood samples from the healthy donors (specific culture conditions for blood samples for healthy and diabetic donors that were used for animal treatment experiments are provided in the Section on *In Vivo* Assessment of Efficacy and Safety in a Mouse Model of Hindlimb Ischemia). Upon culture termination, nonadherent cells were collected and combined with mechanically detached adherent cells. Harvesting yields are expressed as a percentage of the total number of seeded cells, and this value was used to back-calculate cell yields per 1 and 250 mL of blood (the volume selected for use in clinical applications). To obtain the final product formulation for administration, the EnEPCs were suspended in X-VIVO15 to yield BGC101 doses of 5 and 25×10^6 cells/mL medium (see Supporting information for further details).

In vitro assessment of cell processing efficacy

Cellular markers and functional characteristics were assessed by specific staining detected by flow cytometry; angiogenic potential detected by a tube formation test; stemness, self-renewal potential assessed by a colony-forming unit assay (CFU; CFU-GM, CFU-G, CFU-M, CFU-EPC); and cytokine secretion to the supernatant of cells under starvation conditions was assessed by semi-quantitative FlowCytomix™ human basic kit (eBioScience) (see Supporting information for further details).

In vivo assessment of efficacy and safety in a mouse model of hindlimb ischemia

The efficacy, safety, biodistribution and dosing effect of BGC101 were evaluated in an animal model of stable

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severe hindlimb ischemia mimicking CLI [39,40]. A controlled blinded experiment was performed by Pharmaseed (Rehovot, Israel), a GLP-certified laboratory following the guidelines of OECD Principles of GLP [C (97)186/final]. Animal handling was performed according to guidelines of the National Institutes of Health and the Association for Assessment and Accreditation of Laboratory Animal Care. The study was performed after approval by the Israel Board for Animal Experiments and in compliance with the Israel Animal Welfare Act. The study was performed in 42 CD1 nude mice using blood donations from one healthy donor and one diabetic donor undergoing conservative treatment (metformin 2250 mg/day, glibenclamide 5 mg/day, simvastatin 20 mg/day and aspirin 100 mg/day). On the basis of the planned clinical treatment mode sterile BGC101 samples (cells at concentration of 2.3×10^6 cultured in X-VIVO15 supplemented with 10% autologous serum, IL-10 2.5 ng/mL, VEGF 5 ng/mL and heparin 5 IU/mL, with an average of 97.3% viability, 29.1% EPC and 7.5% HSPC) were delivered as a single administration of two intramuscular injections to the quadriceps muscle of injured, ischemic mice legs. Mice were divided into five groups: vehicle control ($n = 12$); unprocessed cells (PBMC, 2.5×10^6 cells/mouse, $n = 5$, all from diabetic donors); BGC101-1 (cells from 1 day culture, 2.5×10^6 cells/mouse, $n = 10$, four from healthy donors and six from diabetic donors); BGC101-3 (cells from 3 day culture, 2.5×10^6 cells/mouse, $n = 10$, five from healthy donors and five from diabetic donors); and BGC101-31 (cells from 3 day culture, 0.5×10^6 cells/mouse, $n = 5$, all from diabetic donors). The experimental doses used in groups 2–4, 2.5×10^6/mouse and 5, 0.5×10^6/mouse, are equivalent to doses of 700 and 140×10^6 in humans. The higher dose complied with Food and Drug Administration guidelines for estimation of the maximum recommended starting dose for first-time-in-human clinical trials, with the lower dose (0.5×10^6 cells/mouse) representing the range of intended clinical dose [41].

Blood perfusion was measured by laser-doppler perfusion imaging [comparing the ratio of the ischemic (right) to the intact (left) leg blood flow expressed as percent perfusion] before, immediately after and on days 14 and 21 after ischemia induction. Body weight, leg function, macroscopic and microscopic evaluation of safety and mode of action were scored (Patho-Lab Diagnostics, Ness-Ziona, Israel, Pathovet Rehovot, Israel, Pathology Department, Rambam Medical Center, Haifa, Israel, and Veterinary Department of American Medical Laboratories, Herzliya, Israel). Blood samples and tissue from femur at the site of damage (femur I), femur at the implantation site (femur II) and diaphysis of the tibia in both ischemic and intact legs as well as kidney, spleen, lung, liver and lymph nodes of each animal were tested at study termination.

Biodistribution of human cells in the transplanted mice was examined in the tissues collected on day 21 by semi-quantitative polymerase chain reaction (PCR) for human Alu DNA repeats [42–44] (see Supporting information for further details).

Statistical analysis

Results are expressed as mean ± SE unless stated otherwise. Data of all measurable parameters were analyzed by analysis of variance or student's *t*-test. A probability of 5% ($p \leq 0.05$) was regarded as significant.

Results

Enrichment, activation and culturing of specific cell populations

The first enrichment step used magnetic bead cell markers-based specific enrichment of DCs from PBMCs. Enrichment of DCs from 52 individual PBMC samples resulted in a significant increase in the percentage of mDCs and pDCs. The average percentages of immature mDCs and pDCs in the PBMCs were 3.8 ± 0.4% and 2.9 ± 0.4%, respectively, compared with 18.5 ± 1.6% and 19.9 ± 1.7% in the enriched DC population ($n = 52$; $p < 0.005$; representative result Figure 2, A1 and A2).

We further tested in 35 samples the changes in the enriched DCs following their activation in the presence of anti-inflammatory and pro-angiogenic factors and found that the percentage of mDCs expressing CD141 further increased from 15.1 ± 1.7% to 37.5 ± 2.7 ($n = 35$; $p < 0.005$). Total mean fluorescence, determined on the basis of both the number of cells expressing the CD141 marker and the marker's intensity, was also significantly increased (total mean fluorescence = 6.6 ± 0.9; $p < 0005$). Furthermore, the activated cells synthesized and secreted IL-8 and IL-10, which probably exerted their effects on SPCs during the co-culture stage of the process (data not shown).

At the next enrichment step, cytotoxic T and NK cells were removed from the culture in order to enrich the SPC population and promote culturing efficiency by improving the induction of pro-angiogenic conditions and reducing immunogenicity. Depletion on the basis of magnetic beads in 52 individual samples resulted in a significant decrease in the percentage of CD8 cytotoxic T cells and CD56 NK cells. The average percentages of CD8 T cells and CD56 NK cells in the PBMCs were 23.6 ± 1.1% and 21.5 ± 1.4%, respectively, whereas the corresponding average percentages expressed by the enriched SPC

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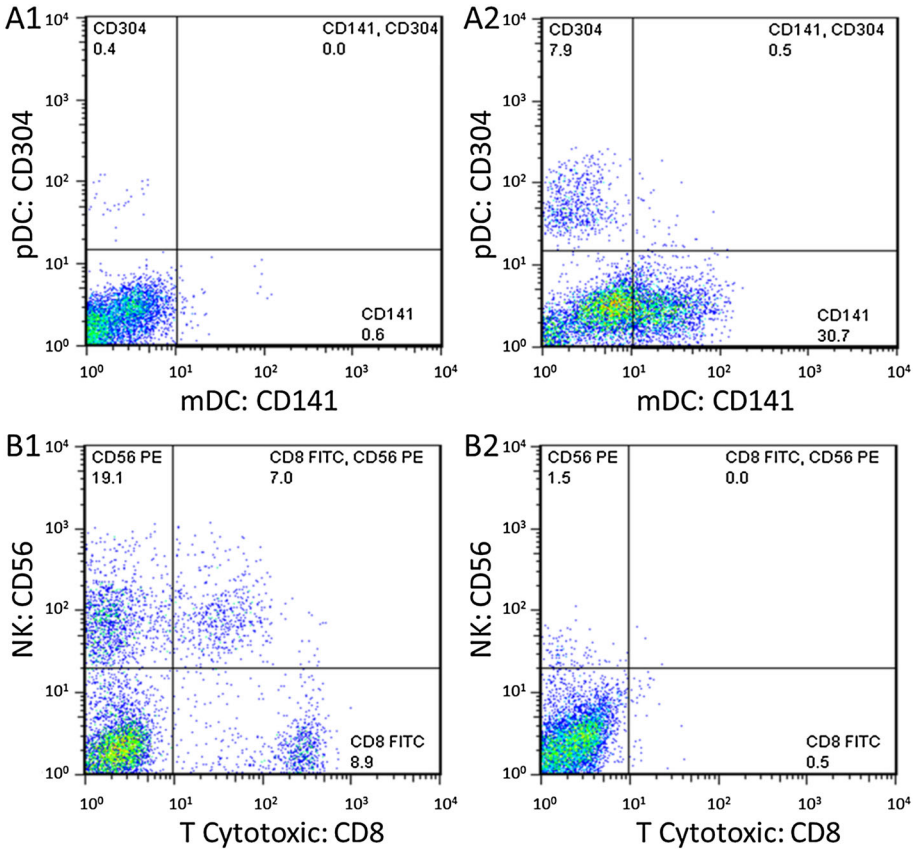

Figure 2. Enrichment of specific cell populations. (A) Representative illustration of flow cytometric analysis of expression of the myeloid dendritic cell (mDC) marker CD141 (*x*-axis) and plasmacytoid dendritic cell (pDC) marker CD303/CD304 (*y*-axis) on peripheral blood mononuclear cells (PBMCs) (A1) and on enriched DCs (A2). (B) Representative illustration of flow cytometric analysis of expression of the cytotoxic T-cell marker CD8 (*x*-axis) and the natural killer (NK) cell marker CD56 (*y*-axis) on PBMCs (B1) and on the enriched SPC (B2)

population were $2.6 \pm 0.4\%$ and $7.5 \pm 1.2\%$ ($n = 52$; $p < 0.005$; representative result Figure 2, B1 and B2).

In vitro cellular characterization of BGC101

To generate the BGC101 population, activated DCs were co-cultured with the enriched SPCs for either 12–18 h (day 1; $n = 12$) or 60–66 h (day 3; $n = 27$). Morphologically, on day 0, the co-cultured cells included mostly small rounded cells typical of resting lymphocyte-like cells (Figure 3A), whereas on day 1 and day 3, harvested cells contained elongated, spindle-shaped cells typical of EPCs as well as large, rounded, dark-nucleated HSPC-like cells (Figure 3B). The initial study was performed on cell preparations from healthy donors (33 samples from 24 donors). To test whether BGC101 cells might be derived from blood from diabetic patients for potential autologous therapy, preliminary studies were performed using six cell preparations from two diabetic donors. Quantitative culture parameters examined on days 1 and 3 were in the same range, except for the percentage of mDCs, which was significantly higher in cells harvested on day 1 than on day 3 (11.7% compared with 0.1%; $n = 9$; $p < 0.005$). The average harvesting yield (tested in samples from healthy and diabetic patients and cultured for 1 and 3 days; $n = 39$) was $57.7 \pm 2.9\%$, and viability was $96.8 \pm 0.3\%$, corresponding to $83.7 \pm 7.4 \times 10^6$ cells per 250 mL of blood.

Cells in samples from both healthy donors and diabetic patients, expressed typical EPC markers such as CD309 (KDR/VEGF-R2), CD202b (Tie2), CD31 and VEGF-R1, and exhibited concomitant binding of Ulex-lectin and uptake of Ac-LDL (Figure 3C), as well as the CD34 and CD184 (CXCR4) markers typical of HSPCs (Figure 3E). Overall, the EPCs accounted for $52.4 \pm 2.5\%$ of the harvested population ($51.5 \pm 2.7\%$ from healthy donors and $56.9 \pm 6.6\%$ from diabetic donors), and HSPCs accounted for $16.1 \pm 1.9\%$ ($16.1 \pm 2.0\%$ from healthy donors and $15.6 \pm 5.5\%$ from diabetic donors).

On the basis of the visualization of tube-like lumen structures and the sprouting of new capillaries in the

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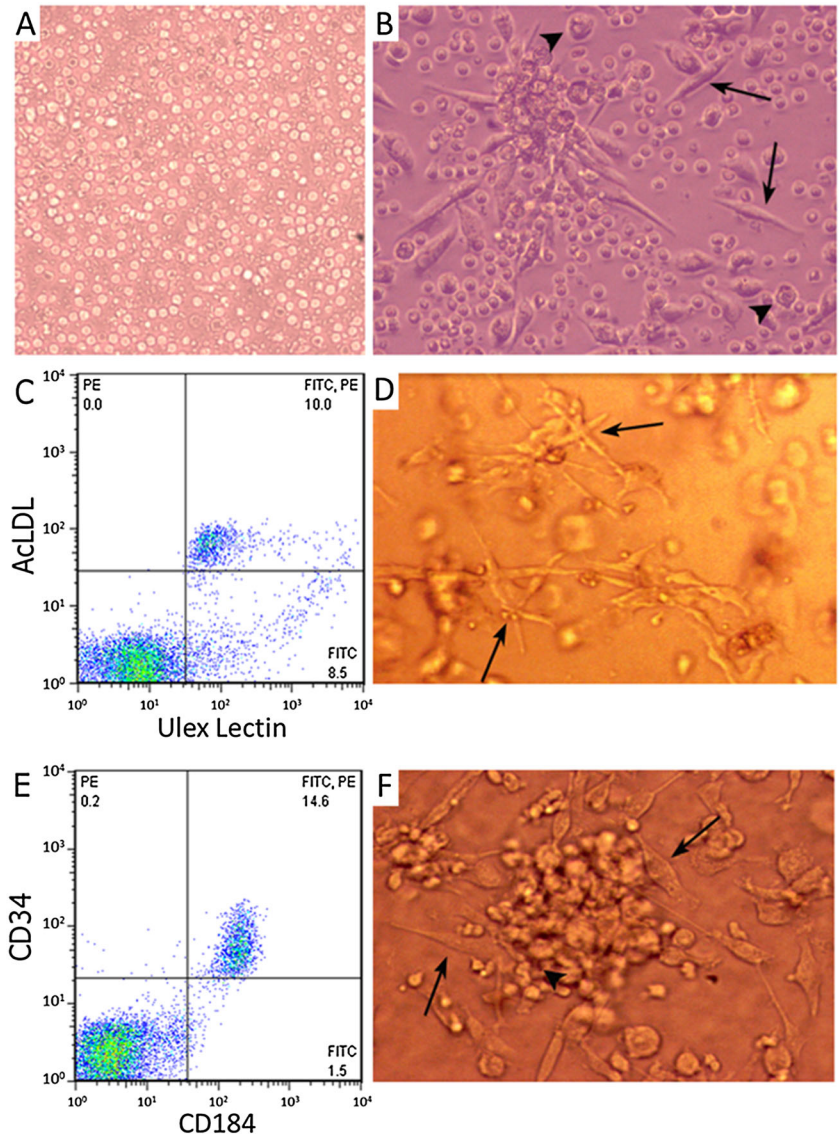

Figure 3. ***In vitro*** **characterization of enriched endothelial progenitor cells (EnEPC). EnEPC were characterized on the basis of morphology, immunostaining and functional examination. Microscopic morphology illustrates (A) co-cultured activated dendritic cells and hematopoietic stem/progenitor cells (HSPCs) on day 0, including mainly small rounded cells typical of resting lymphocyte-like cells. (B) Harvested cells, including elongated, spindle-shaped cells typical of endothelial progenitor cells (arrows) and the large rounded dark-nucleated cells typical of activated HSPCs (arrow heads). (C) Flow cytometric analysis showing concomitant expression of both acetylated low-density lipoprotein (Ac-LDL) uptake and Ulex-Lectin-FITC binding typical of endothelial progenitor cells. (D) Tube formation assay: arrows indicate cell organization into tube-like structure, grade 4. (E) Flow cytometric analysis showing concomitant expression of CD34-PE and CD184-FITC (CXCR4), both typical of HSPCs. (F) Colony-forming unit assay showing typical hematopoietic colonies (arrow head) and EPCs sprouting around them (arrows) 14 days after cell seeding**

angiogenesis test in samples from both day 1 and day 3, the EnEPC average angiogenic potential was scored as grade 3 ($n = 28$; Figure 3D) [45]. The potency of EnEPCs for renewal of HSPCs and EPCs was quantified in the CFU test, which yielded an average of 64.9 ± 10.6 colonies per 10^5 seeded cells. Colonies typical of CFU-GM, CFU-G and CFU-M were observed in addition to the elongated, spindle-shaped colonies typical of CFU-EPC ($n = 22$; Figure 3F). These results show that the BGC101 population contained a significant portion of HSPCs and EPCs with angiogenic potential.

Qualitative screening for cytokine secretion showed *de novo* secretion mainly of IL-10 and IL-8 but also of VEGF and osteopontin (data not shown), thus disclosing a paracrine cytokine profile that supports tolerance, cell survival, migration and vasculogenesis.

In summary, the *in vitro* tests demonstrated that in both day 1 and day 3 cultures, the BGC101 population originating

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from 250 mL of blood contained satisfactory amounts of competent EPCs and HSPCs.

In vivo safety and efficacy assessment in the hindlimb ischemia model

To test whether BGC101 grafting can improve blood flow regeneration, we examined the effect of these cells on recovery from ischemia in the mouse hindlimb ischemia model. The ischemia resulted in a decrease in blood-flow ratio (laser-doppler perfusion imaging ischemic/contralateral intact leg, expressed as percent perfusion) of up to 75 80% from the baseline (with a mean ratio of 23.5 ± 0.7% across all groups). In the vehicle control treated group, the per cent perfusion had increased spontaneously from a baseline of 23.3 ± 1.0% on day 0 to 35.1 ± 1.2% on day 21 and in the PBMCs from 25.7 ± 1.2% on day 0 to 40 ± 4.4% on day 21, an increase that did not reach statistical significance in blood flow. However, in the BGC101 treated groups, BGC101-1, BGC101-3 and BGC101-31, percent perfusion increased markedly between day 0 and day 21, from 24.1 ± 1.09% to 53.7 ± 2.7%, from 21.9 ± 1.0% to 47.7 ± 2.4% and from 23.6 ± 2.5% to 51.8 ± 4.0%, respectively ($p < 0.0002$; Figure 4A and B; Table 1). Calculated perfusion improvement at each time point in each group *versus* that of day 0 demonstrated perfusion improvement of 156 ± 11% and 155 ± 13%

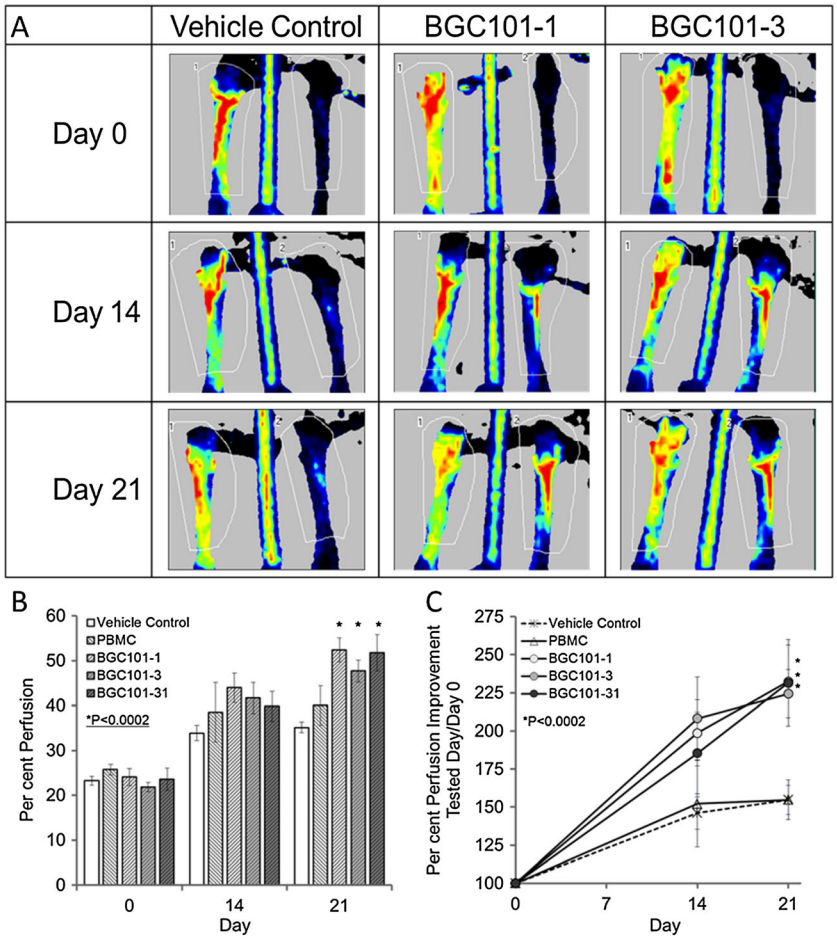

Figure 4. ***In vivo*** **efficacy of BGC101 in a mouse model of hindlimb ischemia, showing significant improvement in perfusion. Ligation and dissection of a femoral artery section led to ischemic damage, which was followed by intramuscular cell injections. (A) Representative laser Doppler scans following infliction of damage (day 0), when blood flow to the right leg was obstructed, and repeated scans 14 and 21 days after treatment. (B) During the experiment, the percent perfusion was tested in the vehicle control treated group ($n = 12$), the peripheral blood mononuclear cell treated group (PBMC, 2.5×10^6 cells/mouse, $n = 5$, all from diabetic donor) and in the BGC101 treated groups BGC101-1 (cells from 1-day culture, 2.5×10^6 cells/mouse, $n = 10$, four from healthy donors and six from diabetic donors), BCGC101-3 (cells from 3-day culture, 2.5×10^6 cells/mouse, $n = 10$, five from healthy donors and five from diabetic donors) and BGC101-31 (cells from 3-day culture, 0.5×10^6 cells/mouse, $n = 5$, all from diabetic donors) ($p < 0.0002$). (C) Percent perfusion improvement at each time point in each group *versus* that of day 0 compared with that of the vehicle control group at the same time point ($p < 0.0002$)**

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Table 1. Percent perfusion following treatment with EnEPCs originated from healthy and diabetic donors

Group	Sub-group	%Perfusion day 21	*p*-value tested group *versus* vehicle control
Vehicle control	All ($n = 12$)	35.1 ± 1.2	
PBMC	All ($n = 5$)	40.0 ± 4.4	$p = 0.167$
	Healthy donor (—)	—	
	Diabetic donor ($n = 5$)	40.0 ± 4.4	
BGC101-1	All ($n = 10$)	53.7 ± 2.7	$p < 0.0002$
	Healthy donor ($n = 4$)	56.8 ± 5.8	
	Diabetic donor ($n = 6$)	49.5 ± 2.1	
BGC101-3	All ($n = 10$)	47.7 ± 2.4	$p < 0.0002$
	Healthy donor ($n = 5$)	48.9 ± 4.1	
	Diabetic Donor ($n = 5$)	46.4 ± 3.0	
BGC101-31	All ($n = 5$)	51.8 ± 4.0	$p < 0.0002$
	Healthy donor (—)	—	
	Diabetic donor ($n = 5$)	51.8 ± 4.0	

Percent perfusion on day 21 is presented in the vehicle control treated group ($n = 12$), the PBMC group (2.5×10^6 cells/mouse, $n = 5$, all from diabetic donor) and in the BGC101 treated groups BGC101-1 (cells from 1-day culture, 2.5×10^6 cells/mouse, $n = 10$, four from healthy donor and six from diabetic donor), BCGC101-3 (cells from 3-day culture, 2.5×10^6 cells/mouse, $n = 10$, five from healthy donor and five from diabetic donor), and BGC101-31 (cells from 3-day culture, 0.5×10^6 cells/mouse, $n = 5$, all from diabetic donor) groups ($p < 0.0002$). Percent perfusion was calculated for all mice (all), as well as separately for mice that received cells from healthy (healthy donor) or diabetic (diabetic donor) donors.

that did not reach statistical significance in the vehicle control and PBMC-treated groups, respectively, in contrast to statistically significant perfusion improvements of 230 ± 24%, 222 ± 16% and 228 ± 28% for groups BGC101-1, BGC101-3 and BGC101-31, respectively ($p < 0.0002$; Figure 4C). Leg function on day 21 assessed semi-quantitatively revealed an improvement in all the treated groups compared with the control group (Table S1).

Histological evaluation of legs

BGC101 cells tracked by hCD45 immunostaining were observed in tissue sections from the injured leg adjacent to blood vessels in the muscle tissue (Figure 5). At 21 days post-administration, such cells were detectable even in the diaphysis of the tibia (far from the injected site), demonstrating homing and engraftment of the human cells along the ischemic areas.

Generation of CD31-expressing new capillaries induced by the injected cells was also observed in different areas along the injured, ischemic leg on day 21. In all examined sections, from femur at the site of damage (femur I), femur at the implantation site (femur II) and at the diaphysis of the tibia (tibia), capillary density was significantly higher in the BGC101-treated mice than in the vehicle control treated group (105.2 ± 5.0 compared with 56.7 ± 2.7 capillaries/field; Figure 6A–G). Notably, the capillary density observed in the intact legs of the treated mice was similar to that in the vehicle control treated group, demonstrating that the BGC101-enhanced vascularization was restricted to the ischemic leg (Figure 6H).

Toxicity and safety

A high treatment safety profile was demonstrated by absence of death significant differences in body weight

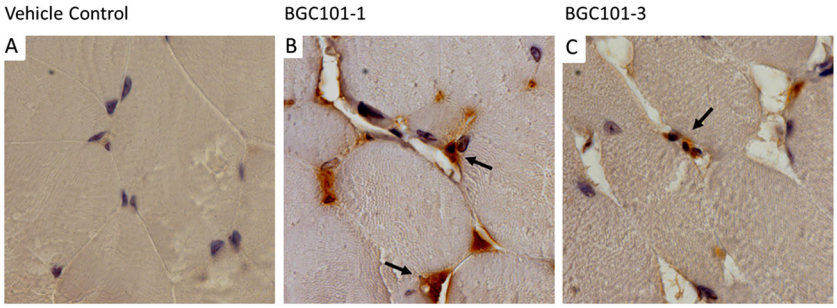

Figure 5. Human cells in mice treated with BGC101 are found adjacent to blood vessels. Representative photomicrographs (×630) of tissue sections from ischemic legs on day 21 show human cells stained for hCD45 (common leukocyte antigen) and counterstained with hematoxylin and eosin. hCD45 staining in tissue sections from (A) a vehicle control treated mouse, (B) BGC101-1 treated mouse and (C) BGC101-3 treated mouse. Stained cells can be seen near blood vessels (arrows)

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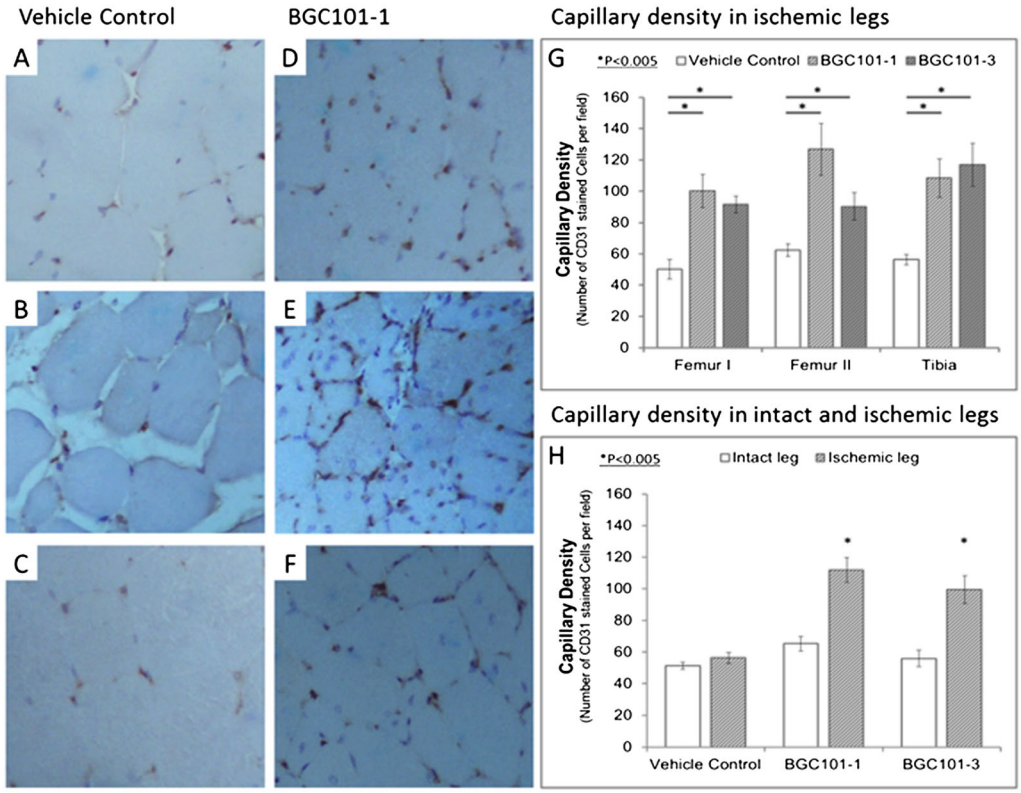

Figure 6. Increased capillary density in BGC101 treated mice is restricted to the injured, ischemic leg. A–F are representative photomicrographs (×630) of tissue sections from ischemic and intact legs on day 21. New capillaries in tissue sections from femur at the site of damage (femur I), femur at the implantation site (femur II) and distally at the diaphysis of the tibia (tibia), were stained with hCD31 (mouse cross-reactive) and hematoxylin and eosin. CD31 staining of (A) femur I, (B) femur II and (C) tibia tissue sections from a vehicle control treated mouse. (D–F) Analogous tissue sections from a BGC101-1 treated mouse. (G) Capillary density (average capillary count per field) in ischemic legs of mice treated with vehicle control, BGC101-1 and BGC101-3 ($p < 0.005$). (H) Capillary density in tissue sections from treated ischemic legs and from intact legs ($p < 0.005$)

and by macroscopic and microscopic evaluations of tissue sections of legs, lungs, spleens, livers, kidneys and lymphatic nodes for tissue damage or cancerous events (data not shown). Tissue damage in ischemic legs occurred in all animal groups, as expected after the injury, followed by spontaneous muscle regeneration. All intact legs showed a normal morphology (Figure 7 shows examples from tibial sections). Normal blood biochemical profile and blood cell counts were observed on day 21 in all study groups other than a minor transient decrease in neutrophils and increase in lymphocytes in group BGC101-3 (Tables S2 and S3).

BGC101 biodistribution

Biodistribution, assessed through detection of human DNA sequences (hAlu) and mouse positive control sequences (mMyo), showed no human cells in either the BM or the

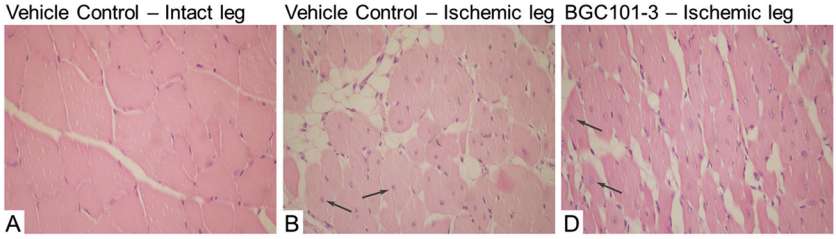

Figure 7. Histopathologic evaluation. Tissue damage was assessed on tissue samples from ischemic and intact mouse legs as well as from samples of mouse kidney, spleen, lung, liver and lymph nodes. Representative photographs high magnification (×400) of hematoxylin and eosin stained histological sections of muscle surrounding tibia and fibula illustrate tissue damage and muscle regeneration, which is indicated by centralization of nuclei (arrows). (A) Intact leg and (B) ischemic leg from vehicle control treated mouse and (C) ischemic leg from mouse treated with BGC101-3

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soft tissues of any of the tested animals (Figure S1). In conjunction with the CD31 immunohistochemistry results, these findings indicate that injected BGC101cells homed only to the ischemic leg and engrafted along it and were not distributed among other body organs.

Altogether, the aforementioned results provide preliminary evidence that BGC101 can safely be used for the regeneration of functional vasculature in ischemic tissues and may lead to safety and efficacy trials in humans.

Discussion

This article describes a novel method for generating EnEPCs with therapeutic potential, termed BGC101, from non-mobilized blood, using DCs to specifically direct SPC activity *in vitro*.

Previous reports have described the *in vivo* anti-inflammatory and pro-angiogenic effects of DCs, as well as methods to obtain and activate them [18,21,24,33]. In the presence of pro-angiogenic factors such as ischemia and the presence of VEGF, DCs were shown to contribute to vasculogenesis and angiogenesis [25–29].

On the basis of those reports, and on the fact that DCs and SPCs are residents of vascular niches in bone and other tissues, we hypothesized that DCs play a role in controlling SPC activation and differentiation towards the endothelial lineage. To test this hypothesis, we activated iDCs, not in the classical, antigen-dependent way, but by an alternative method in which the cells were exposed to anti-inflammatory cytokines in the presence of pro-angiogenic factors. Because the intention was to create potentially therapeutic cells for blood vessel regeneration, it was necessary to augment the pro-angiogenic effects of the DCs as well as to minimize the immune toxicity. To achieve that goal, we developed and tested a method that included depletion of NK and cytotoxic T cells, in addition to positive selection and activation of DCs in the presence of tolerance-inducing cytokines such as IL-10 and transforming growth factor beta and pro-angiogenic cytokines such as VEGF and basic fibroblast growth factor. This activation led to a rapid change in cellular molecules in the DC fraction. IL-8 and IL-10 were secreted, and both the percentage of cells expressing the mDC marker CD141 and the intensity of the marker were increased. These results are in agreement with the reported finding of Riboldi *et al*. showing that alternatively (but not classically) activated mDCs secrete pro-angiogenic factors such as VEGF [22]. Once suitably primed, the DCs directed the activity of the enriched population of blood-derived SPCs towards a rapid and efficient generation of EnEPCs.

Stem/progenitor cells addressing CLI, performed mostly using BM-derived cells, show high safety profile with promising therapeutic results [46–48]. However, pre-procedural adverse events were reported [6,8,9]. Procedures for directly aspirating BM cells require the use of anesthesia and entail pain and discomfort. An alternative method of obtaining large amounts of BM cells is by extraction of mobilized BM cells from peripheral blood by apheresis. In the mobilization method, an inflammatory situation is mimicked by the pretreatment of patients for 4–5 days with high doses of granulocyte CSF (G-CSF; 1400 μg daily). G-CSF administration has been reported to result in fever and chills, headache, muscular pain and bone pain, as well as increased blood viscosity and platelet counts, which are problematic especially in patients suffering from vascular diseases [6,8]. We developed a method based simply on a specimen of the patient's blood obtained by routine venipuncture. However, because the number of EPCs and SPCs in the blood is relatively low, an *ex vivo* method for the enrichment and augmentation of specific cells was needed. To that end, blood-derived immature mDCs and pDCs primed to induce specific pro-angiogenic activation signals were co-cultured with the SPCs. A 1-day culturing process generated a cell mixture composed of EPCs ($52.4 \pm 2.5\%$), HSPCs ($16.1 \pm 1.9\%$) and residual B-cell and T-helper cells that demonstrated angiogenic and stemness potential and secreted IL-8, IL-10, VEGF and osteopontin, a paracrine cytokine profile that supports tolerance (which is also typical of mesenchymal stem cells), cell survival, migration and vasculogenesis. Furthermore, normalization to a blood sample volume of 250 mL showed that the harvested yields, corresponding to $83.7 \pm 7.4 \times 10^6$, gave rise to 13.4×10^6 HSPCs and 43.8×10^6 EPCs. These numbers are comparable with the amount of CD34 HSPC, i.e. within a range of 10 to 40×10^6, obtained from BM specimens [7]. It is particularly noteworthy that the blood of diabetic patients yielded the same range of numbers and specific cells as that of healthy donors although only two patients were sampled. This is an important finding, as a blood volume of 250 mL can be safely and easily acquired even from patients with anemia. Thus, in addition to its scientific merit, the novel technology described here can be expected to facilitate the development of a standardized product with potential clinical applications.

BGC101 produced by this method were assessed for safety, efficacy, dose levels, biodistribution and possible mode of action. We performed a controlled blinded experiment in a mouse model of hindlimb ischemia mimicking CLI [40,49], using blood specimens from a 21-year-old healthy donor and a 53-year-old type 2 diabetes mellitus patient (undergoing conservative treatment) that were administrated intramuscularly to the injured leg. BGC101 administrations significantly increased the blood flow ratio from $23.1 \pm 1.0\%$ to $50.4 \pm 1.7\%$ by day 21 compared with vehicle control treatment ($p < 0.0002$), whereas legs treated with PBMCs showed improvement that did not reach statistical significance in blood flow over time. As reported by Burchfield and Dimmeler [50] and others, accumulating evidence suggests that the mechanisms of action by which SPCs and EPCs provide ischemic tissue

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repair involve cell–cell interactions as well as secretion of paracrine factors. The latter have the potential to directly modify the healing process by the generation of neovascularization, angiogenesis and endogenous repair [50,51]. Likewise, we showed that BGC101 homed to and engrafted in ischemic regions along the damaged leg, close to blood vessels, as demonstrated by hCD45 staining. Moreover, immunohistochemical examination of new capillaries stained with CD31 revealed significantly increased capillary density in BGC101-treated mice compared with controls. Engraftment of human cells and generation of new capillaries were not limited to the injection site but also extended to the site of injury, as well as to secondarily damaged areas such as ischemic areas in the tibia. This regenerative effect, however, was restricted to the injured, ischemic leg; the similarity of the capillary density in intact legs from the BCG101 treated mice to that in the vehicle control treated group, as well as biodistribution testing by human Alu repeats, which has been reported to be a highly specific and sensitive method for tracking of human cells in rodents [42–44], showed that BGC101 did not spread to other body organs.

The safety profile of BGC101 was demonstrated by the absence of clinical signs, as well as through a pathological evaluation and toxicity tests performed on blood samples and tissue sections. Even though these safety results support systemic administration of BGC101, intramuscular administration route was selected on the basis of previous clinical experience with BM-derived cells that have shown high safety profiles and promising therapeutic results [6]. Furthermore, intravenous cell injection is considered less effective in targeting damaged tissues because administrated cells first enter the systemic circulation and are trapped in the lungs before they move gradually into injured sites or eliminated by the kidneys [52–54].

Taken together, these findings provide preliminary evidence that DCs can safely and efficiently direct SPC activity separate from their immunological role. A 1-day culturing process was sufficient for activated DCs to promote the generation of potentially therapeutic functional cells from non-mobilized blood. This process yielded cells comparable in number and characteristics to therapeutic BM products. In spite of their preliminary nature and the incomplete characterization of the cells, these results suggest that following further optimization and standardization, this novel method will potentially be relevant clinically and may consequently support the production of this and similar autologous therapeutic products. Further larger preclinical and clinical studies are needed to assess the safety and efficacy of BGC101 in the treatment of vascular diseases.

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Conflict of interest

This research was performed by BioGenCell Ltd in collaboration with the physicians of the Laniado Hospital diabetes and vascular surgery departments.

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