Regenerative Therapy 26 (2024) 299–307

Contents lists available at ScienceDirect

Regenerative Therapy

journal homepage: http://www.elsevier.com/locate/reth

Original Article

Novel cell therapy with *ex vivo* cultured peripheral blood mononuclear cells significantly impacts angiogenesis in the murine ischemic limb model

Satomi Furukawa [a, c], Rie Hirano [a, e], Ai Sugawara [a, d], Satoshi Fujimura [a, d], Rica Tanaka [a, b, c, d, e, *]

[a] Division of Regenerative Therapy, Juntendo University Graduates School of Medicine, Tokyo, Japan
[b] Department of Plastic and Reconstructive Surgery, Juntendo University School of Medicine, Tokyo, Japan
[c] Center for Genomic and Regenerative Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan
[d] Intractable Disease Research Center, Juntendo University Graduates School of Medicine, Tokyo, Japan
[e] ReEir. Inc., Tokyo, Japan

ARTICLE INFO

Article history:
Received 17 April 2024
Received in revised form
6 June 2024
Accepted 12 June 2024

Keywords:
Peripheral blood mononuclear cell
Cell therapy
Vasculogenesis

ABSTRACT

Introduction: Autologous mononuclear cells (MNCs) have been used in vascular regenerative therapy since the identification of endothelial progenitor cells (EPCs). However, the efficacy of autologous EPC therapy for diseases such as diabetes and connective tissue disorders is limited due to deficiencies in the number and function of EPCs. To address this, we developed a novel RE-01 cells that enriches pro-angiogenic cells from peripheral blood MNCs (PBMNCs).

Methods: PBMNCs were collected from healthy volunteers following ethical guidelines. RE-01 cells were cultured in the presence of specific growth factors for 5 days without media change. Flow cytometry was used to analyze cell surface markers. Tube formation assays, EPC culture assays, and mRNA analysis were performed to evaluate angiogenic potential. The efficacy of RE-01 cells upon transplantation into ischemic hind limbs of mice was evaluated.

Results: RE-01 cells exhibited a significant increase in pro-angiogenic cells such as M2 macrophages and angiogenic T cells, in contrast to PBMNCs, while the number of inflammatory cells reduced. *In vitro* assays demonstrated the enhanced angiogenic abilities of RE-01 cells, supported by increased mRNA expression of angiogenesis-related cytokines. *In vivo* studies using mouse ischemic hind limb models have shown that blood flow and angiogenesis improved following RE-01 cell transplantation. Transplantations for 3 consecutive days significantly improved the number of pericyte-recruited vessels in the severely ischemic hind limbs of mice.

Conclusions: RE-01 cells showed promising results in enhancing angiogenesis and arteriogenesis, possibly owing to the presence of M2 macrophages and angiogenic T cells. These cells also demonstrated anti-fibrotic effects. The efficacy of RE-01 cells has been confirmed in mouse models, suggesting their potential for treating ischemic vascular diseases. Clinical trials are planned to validate the safety and efficacy of RE-01 cell therapy in patients with connective tissue disease and unhealed ulcers. We hope that this new RE-01 cell therapy will prevent many patients from undergoing amputation.

Abbreviations: EPCs, endothelial progenitor cells; MNCs, mononuclear cells; G-CSF, granulocyte colony-stimulating factor; MMP, matrix metalloproteinase; VEGF, vascular endothelial growth facto; TPO, thrombopoietin; FLT-3L, Fms-related tyrosine kinase-3 ligand; HUVEC, human umbilical vein endothelial cells; Ac-LDL, acetylated low-density lipoprotein; UEA-I, Ulex europaeus agglutinin I; PFA, paraformaldehyde phosphate buffer solution; ANGPT, angiopoietin; IGF, insulin-like growth factor; HMA, human mitochondrial antibody.

* Corresponding author. Juntendo University Graduate School of Medicine, Division of Regenerative Therapy, 2-1-1 Hongo, Bunkyo-ku, Tokyo, 113-8421, Japan.
E-mail address: rtanaka@juntendo.ac.jp (R. Tanaka).

Peer review under responsibility of the Japanese Society for Regenerative Medicine.

https://doi.org/10.1016/j.reth.2024.06.009

1. Introduction

Since the identification of endothelial progenitor cells (EPCs), autologous mononuclear cells (MNCs) have been used in clinical vascular regenerative therapy [1,2]. Despite confirming its safety in clinical trials, autologous EPC therapy shows limited effectiveness in treating certain diseases including diabetes and connective tissue disorders, primarily due to insufficient numbers and impaired function of EPCs [3–5]. To overcome these problems, various methods have been developed for the *ex vivo* expansion of EPCs. However, these EPC culture methods rely on allogeneic umbilical cord blood, or in the case of peripheral blood MNCs (PBMNCs), mobilization with granulocyte colony-stimulating factor (G-CSF) is required [6]. To achieve effective and minimally invasive autologous cell therapy for vascular regeneration, it is preferred to collect a small amount of peripheral blood without using G-CSF as G-CSF administration could potentially cause adverse effects, such as bone pain, headache, nausea, and fever [7].

Among MNCs, T cells and macrophages are also involved in vascular repair. Angiogenic T cells were first identified by Hur et al., in 2007 and reportedly cooperate with EPCs and enhance endothelial repair through the secretion of pro-angiogenic cytokines [8]. M2 macrophages, primarily known for their anti-inflammatory roles, contribute to extracellular matrix remodeling and promote angiogenesis [9–11]. Therefore, we hypothesized that expanding all angiogenesis-related cells is preferred over isolating and expanding only EPCs.

We developed a new cell therapy using RE-01 cells, which are derived from PBMNCs as well as contain a large number of pro-angiogenic cells and a small number of inflammatory cells. Here, we present the characteristics and angiogenic potential of RE-01 cells both *in vitro* and *in vivo*. RE-01 cells represent a promising novel approach in cell therapy for ischemic diseases.

2. Methods

2.1. PBMNC collection

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee and Review Board of Juntendo University (Tokyo, Japan. Approval number: M12-0902). All participants provided informed consent. Peripheral blood samples were obtained from healthy volunteers. The MNCs were isolated by Ficoll density gradient centrifugation. The cells were treated with ammonium-chloride-potassium lysing buffer (Gibco, Thermo Fisher Scientific, USA) to lyse the remaining blood cells. After washing twice with ethylenediamine tetraacetic acid (EDTA)-phosphate buffer saline (PBS), the cells were suspended in PBS.

2.2. Ex vivo expansion culture for RE-01 cells

PBMNCs were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (including GlutaMAX, Gibco) media supplemented with 0.5% fetal bovine serum, 0.5% human serum albumin (Japan Blood Products Organization, Tokyo, Japan), recombinant human vascular endothelial growth factor (rhVEGF; 50 ng/mL), rh thrombopoietin (rhTPO; 20 ng/mL), rh Fms-related tyrosine kinase-3 ligand (rh FLT-3L; 100 ng/mL; all from Pepro Tech, Rocky Hill, New Jersey, USA), and penicillin-streptomycin, and maintained for 5 days at 37 °C in a humidified incubator under a constant supply of 5% CO_2, without media change. After 5 days, RE-01 cells were harvested by pipetting and washing with EDTA-PBS.

2.3. Flow cytometry

PBMNCs and RE-01 cells suspended in FACS buffer (EDTA-PBS with 2% FBS) at 1×10^6 cell/100 μL were treated with 10 μL FcR blocking reagent (Miltenyi Biotec, Auburn, California, USA) and stained with specific antibodies as follows: FITC anti-human CD19 (3 μL/5 × 10^5 cells, clone: HIB19, mouse IgG1 κ, BioLegend, San Diego, California, USA; 302206), PerCP/Cy5.5 anti-human CCR2 (3 μL/5 × 10^5 cells, clone: K036C2, mouse IgG2a κ, BioLegend; 357204), BV421 anti-human CD56 (3 μL/5 × 10^5 cells, clone: HCD56, mouse IgG1 κ, BioLegend; 318328), PE anti-human CD34 (0.5 μL/5 × 10^5 cells, clone: 581, mouse IgG1 κ, BioLegend; 343506), PE/Cy7 anti-human CD206 (MMR, 2 μL/5 × 10^5 cells, clone: 15–2, mouse IgG1 κ, BioLegend; 321124), APC anti-human CXCR4 (CD184, 2 μL/5 × 10^5 cells, clone: 12G5, mouse IgG2a κ, BioLegend; 306510), AlexaFluor-700 anti-human CD3 (2 μL/5 × 10^5 cells, clone: UCHT1, mouse IgG1 κ, BioLegend; 300424), APC/Cy7 anti-human CD14 (2 μL/5 × 10^5 cells, clone: HCD14, mouse IgG1 κ, BioLegend; 325620), FITC anti-human CD4 (3 μL/5 × 10^5 cells, clone: RPA-T4, mouse IgG1 κ, BioLegend; 300506), PerCP/Cy5.5 anti-human CD25 (2 μL/5 × 10^5 cells, clone: M-A251, mouse IgG1 κ, BioLegend; 356112), BV421 anti-human CD127 (IL-7Ra, 2 μL/5 × 10^5 cells, clone: A019D5, mouse IgG1 κ, BioLegend; 351310), PE anti-human CD133/1 (2 μL/5 × 10^5 cells, clone: AC133, mouse IgG1 κ, Miltenyi Biotec, Bergisch Gladbach, Germany; 130-113-108), PE/Cy7 anti-human CD31 (2 μL/5 × 10^5 cells, clone: WM59, mouse IgG1 κ, BioLegend; 303118), APC/Cy7 anti-human CD8a (2 μL/5 × 10^5 cells, clone: HIT8a, mouse IgG1 κ, BioLegend; 300926) and isotype control antibodies: FITC mouse IgG1 κ (3 μL/5 × 10^5 cells, BD; 555748), PerCP/Cyanine5.5 mouse IgG2a κ (3 μL/5 × 10^5 cells, BioLegend; 400252), PE mouse IgG1 κ (2 μL/5 × 10^5 cells, BioLegend; 400112), PE/Cy7 mouse IgG1 κ (2 μL/5 × 10^5 cells, BioLegend; 400126), Alexa Fluor-700 mouse IgG1 κ (2 μL/5 × 10^5 cells, BioLegend; 400144), APC/Cy7 mouse IgG1 κ (2 μL/5 × 10^5 cells, BioLegend; 400128), BV421 mouse IgG1 κ (3 μL/5 × 10^5 cells, BioLegend; 400158) and APC mouse IgG2a (2 μL/5 × 10^5 cells, Beckman Coulter, California, USA; A12693). After incubation for 30 min at 4 °C, the cells were washed with FACS buffer and analyzed using BD LSRFortessa Flow Cytometer (BD) and FlowJo version 10.8.1 (Tree Star, Inc., Ashland, OR, USA).

2.4. Tube formation assay

Human umbilical vein endothelial cells (HUVECs, Lonza, Basel, Switzerland) were cultured at 2.5–3 × 10^5 cells/dish in EGM-2 MV medium (Lonza) according to the manufacturer's protocol. For the tube formation assay, the medium was replaced with the starvation medium EBM-2 (Lonza) for 1 h. PBMNCs and RE-01 cells were labeled with Alexa Fluor-488 acetylated low-density lipoprotein (Ac-LDL, 1:1000, Invitrogen, Thermo Fisher Scientific; L23380) at 37 °C for 1 h. HUVECs (2×10^4) and PBMNCs or RE-01 cells (4×10^3) were mixed in 200 μL PBS(−) and seeded in 24-well plates pre-coated with 200 μL/well Matrigel Matrix (Corning Inc.). The plates were incubated at 37 °C in 5% CO_2 and photographed under the IX83 time lapse microscope (Olympus, Tokyo, Japan). The number of closed circles of Alexa Fluor-488 Ac-LDL-positive cells incorporated into the tubes was counted and normalized to the controls containing only HUVECs.

2.5. EPC culture assay

The EPC culture assay was performed as described previously [11]. Briefly, 1×10^5 cells/well each of PBMNCs and RE-01 cells were cultured in EGM-2 MV medium (Lonza), in human fibronectin-coated 96-well plates and maintained at 37 °C under 5% CO_2 for 7

days. After 7 days, the attached cells were stained with Alexa Fluor-488 Ac-LDL (Invitrogen) and rhodamine-labeled *Ulex europaeus* agglutinin I (UEA-I, Vector Lab., Burlingame, California, USA) for 4 h in a CO_2 incubator. After fixation with 4% paraformaldehyde phosphate buffer solution (PFA, Wako, Osaka, Japan) at 4 °C for 30 min, the cells were mounted by Vectorshield (Vector Lab.) with DAPI. Overall, 3–5 fields in each well were imaged using a fluorescence microscope (BZ-X710, Keyence, Osaka, Japan). Alexa Fluor-488 Ac-LDL and rhodamine UEA-I double-positive cells were counted as early EPCs.

2.6. RNA analysis

Total RNA was extracted from PBMNCs and RE-01 cells using a RNeasy Micro Kit (Qiagen, Germany) according to the manufacturer's protocol. cDNA was synthesized using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Thermo Fisher Scientific). Real-time PCR was performed using the TaqMan Fast Advanced Master Mix and StepOne Plus (Applied Biosystems, Thermo Fisher Scientific). The Taqman probe and primer mixtures used were *18S-rRNA* (Hs03928990_g1), angiopoietin (*ANGPT*)-1 (Hs00181613_m1), *ANGPT-2* (Hs00169867_m1), vascular endothelial growth factor (*VEGF*)-A (Hs00900055m1), *VEGF-B* (Hs00173634_m1), insulin-like growth factor (*IGF*)-1 (Hs00153126_m1), *FGF-2* (Hs00266645_m1), leptin (Hs00174877_m1), *IL-8* (Hs00174103_m1), *IL-10* (Hs_m1), IL-1β (Hs01555410_m1), TGFB1 (Hs99999918_m1), TNF (Hs00174128_m1), MMP-2 (Hs01548727_m1), and MMP-9 (Hs00234579_m1) (all obtained from Thermo Fisher Scientific).

2.7. Ischemic hind limb model mice and single cell transplantation

Eight-to ten-week-old male BALB/c nude mice (20–25 g; CLEA Japan, Tokyo, Japan) were used to evaluate the effects of RE-01 cells in an ischemic hind limb model. This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Juntendo Animal Care and Ethical Committee (Approval number: 1052). The mice were randomly divided into two groups: control group (n = 9) and RE-01 group (n = 9). The proximal portion of the left femoral artery was suture ligated, and the proximal portions of the saphenous artery were occluded. The overlying skin was closed with silk sutures. Human RE-01 cells were prepared as described above and suspended in various electrolyte solutions. RE-01 cells (5×10^3 cells/10 μL/site) or electrolyte solutions alone for controls were transplanted to the two sites of ischemic hind limbs using 1-mL syringes with 29-gauge needles (Terumo, Tokyo, Japan) immediately after ischemic surgery. Blood flow was assessed on days 1, 3, 7, 10, 14, and 21. After 21 days of transplantation, the mice were euthanized by medetomidine-midazolam-butorphanol overdosage, and tissue samples were harvested for histological assessment.

2.8. Severe ischemic hind limb model mice and three-times cell transplantation

Eight-to ten-week-old male BALB/c nude mice (20–25 g; CLEA Japan, Tokyo, Japan) were used to evaluate the effects of RE-01 cells in an ischemic hind limb model. The mice were randomly divided into three groups: control group (n = 9), RE-01-single group (n = 9), and RE-01-three times group (n = 9). The proximal portion of the left femoral artery was suture ligated, and the proximal portions of the saphenous artery were occluded. The overlying skin was closed with silk sutures. Human RE-01 cells were prepared as described above and suspended in various electrolyte solutions. RE-01 cells (2.5×10^3/10 μL/site) or electrolyte solution as controls were transplanted to the four sites of ischemic hind limbs using 1-mL syringes with 29-gauge needles (Terumo, Tokyo, Japan) on day 1, 2 and 3. Blood flow was assessed on days 1, 3, 7, 10, 14, and 21. After 21 days of transplantation, the mice were euthanized by medetomidine-midazolam-butorphanol overdose, and ischemic hind limb tissue samples were obtained for histological assessment.

2.9. Assessment of blood flow

The blood flow was measured using an OMEGAZONE OZ-3 2D laser blood flow imager and LIA analysis software OMEGAWAVEInc. Tokyo, Japan). Blood flow in identical toe regions of interest between the ischemic and contralateral hind limbs of each mouse was measured, and the blood flow ratio of the ischemic versus contralateral hind limb was calculated.

2.10. Histology and immunofluorescence

After 21 days of surgery, the mice were euthanized under 200 μL medetomidine-midazolam-butorphanol anesthesia. Immediately after euthanization, mice were perfused with 20 mL of 1% heparin/PBS, followed by 15 mL of 4% PFA by cardiac puncture. Resected hind limbs were incubated in 4% PFA at 4 °C overnight. The gastrocnemius was then cut in half; the upper parts were embedded in paraffin for Azan staining, and the inferior parts were frozen with Tissue-Tek O·C.T compound (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) to detect vascular regeneration. To detect fibrosis, high-resolution images of stained sections were processed using a microscope (BZ-X710, Keyence) and BZ-II analyzer (Keyence). The area consisting of collagen fibers divided by the total area of the lateral head of the gastrocnemius muscle was calculated. To detect vascular regeneration, frozen sections were stained with isolectin GS-IB_4-AlexaFluor 594 (Invitrogen, I21413), an endothelial marker. IB4 positive cells in five HPF were counted and averaged. The cells were co-stained with anti-mouse αSMA-FITC antibody (Sigma-Aldrich, F3777) and IB4 antibody to detect arteriogenesis. Double-positive cells in the gastrocnemius muscle were counted.

To detect the transplanted RE-01 cells, frozen sections were co-stained with an anti-human mitochondrial antibody (HMA, Sigma-Aldrich, MAB1273) and isolectin GS-IB_4-AlexaFluor 594 with a Zenon Alexa488 mouse IgG labeling kit (Invitrogen). Tissue sections were mounted using Vectorshield (Vector Lab.) with DAPI. Slides were examined and imaged using a confocal microscope (Olympus FV1000D IX81; Olympus, Tokyo, Japan).

2.11. Statistics

Statistical analysis was performed using GraphPad Prism 9 (version 9.5.1[733]) (GraphPad, LLC, Boston, MA, USA). The mean values were compared using Wilcoxon matched pairs signed-rank test (a paired nonparametric test) to compare the PBMNC and RE-01 groups *in vitro*, and Mann–Whitney test (a nonpaired nonparametric test) to compare the control and RE-01 groups *in vivo*. Two tailed p-values for comparison of two independent means are reported with a significance level of 0.05. All data in graphs are represented as mean ± standard deviation.

3. Results

3.1. Significant increases of pro-angiogenic cell subset in RE-01 cells compared with PBMNCs

Following RE-01 culture, the number of PBMNCs decreased to 49.04% ± 17.69%. Flow cytometry analysis revealed an increase in

large cell populations compared with PBMNCs (14.58 ± 5.35 vs 1.12 ± 0.67; $p < 0.05$) (Fig. 1A and B). The production efficacy of RE-01 cells did not differ significantly between healthy volunteers and patients with diabetes or connective tissue diseases (Fig. 1C). In RE-01 cells, compared with PBMNCs, the proportion of CD206-positive cells significantly increased (11.88 ± 7.61 vs 0.33 ± 0.25; $p < 0.05$) and that of CCR2-positive cells decreased (4.96 ± 5.09 vs 32.77 ± 3.18; $p < 0.05$). Among the immune cells, the proportion of CD19-positive B cells (2.95 ± 0.91 vs 12.09 ± 6.71; $p < 0.05$) and CD56-positive NK cells (15.55 ± 5.40 vs 21.06 ± 6.21; $p < 0.05$) decreased in RE-01 cells. In T-cell subsets, the proportion of $CD3^+/CD4^+/CD25^+/CD127^{low}$ regulatory T cells (2.30 ± 0.55 vs 0.96 ± 0.26; $p < 0.05$) and $CXCR4^+/CD31^+/CD3^+$ angiogenic T cells (35.36 ± 7.75 vs 26.71 ± 5.14; $p < 0.05$) significantly increased in RE-01 cells compared with that of PBMNCs. The expression of the cell surface marker of hematopoietic stem cells CD34 slightly increased in RE-01 cells (0.21 ± 0.12 vs 0.09 ± 0.09; $p < 0.05$), while there was no difference in the expression of the EPC marker CD133 (0.09 ± 0.06 vs 0.19 ± 0.40; $p = 0.5469$) (Fig. 1D).

3.2. Enhanced angiogenic ability of RE-01 cells in vitro

To evaluate the angiogenic ability of RE-01 cells *in vitro*, we performed EPC culture and HUVEC tube formation assays. Although the number of CD34-and CD133-positive cells showed no differences, EPC culture assay detected more Ac-LDL and lectin double-

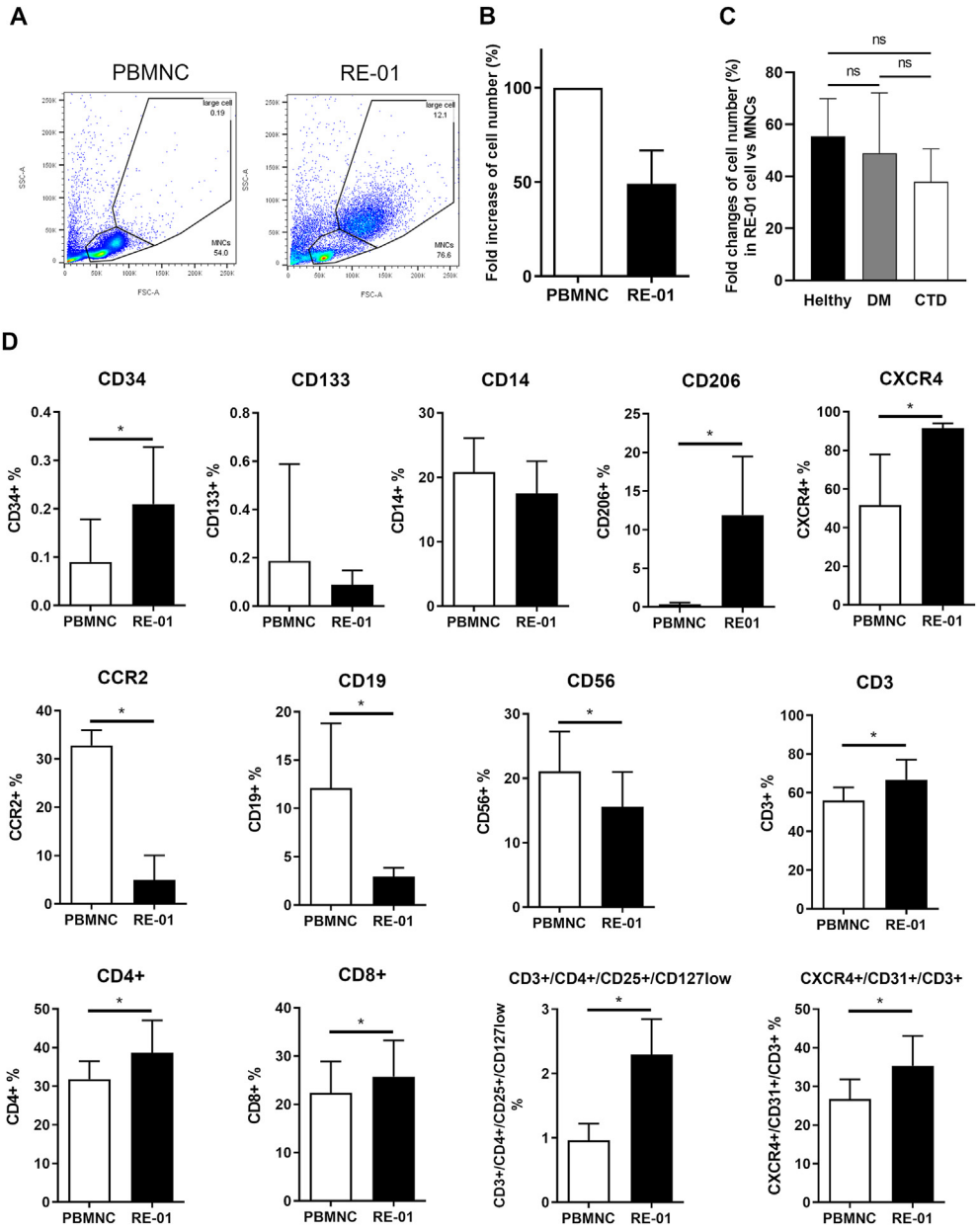

Fig. 1. Characteristics of RE-01 cells. A. The representative image of FACS scattergrams of PBMNC and RE-01 cells B. The cell numbers were counted before (PBMNC) and after RE-01 culture (RE01) in healthy volunteers. (n = 7) C. The changes in cell numbers relative to PBMNCs were compared between healthy people and patients with DM or CTD (healthy, n = 7; DM, n = 6; CTD, n = 8). D. Flow cytometry analysis of PBMNCs and RE-01 cells showing the percentage of cells expressing CD34, CD133, CXCR4, CD206, CCR2, CD14, CD19, CD56, CD3, CD4, CD8, CD3/CD4/CD25/CD127 and CXCR4/CD31/CD3 cell surface marker. Data are presented as mean ± SD. Mean values were compared using the Wilcoxon matched pairs signed-rank test. *p < 0.05. ns, not significant; DM, diabetes mellitus; CTD, connective tissue disease; PBMNCs, peripheral blood mononuclear cells.

positive endothelial cells in RE-01 cells than in PBMNCs (60.4 ± 34.7 vs 33.9 ± 15.6; $p < 0.05$) (Fig. 2A). In the HUVEC tube formation assay, a significantly increased number of closed circles was detected when HUVECs were cultured with RE-01 compared with PBMNCs (5.54 ± 5.10 vs 1.80 ± 0.72; $p < 0.05$). The number of Ac-LDL-positive cells incorporated in tubes significantly increased in RE-01 cells compared to that in PBMNCs (14.68 ± 6.75 vs 1.20 ± 2.07; $p < 0.01$) (Fig. 2B–D). These results demonstrated the increased angiogenic potential of RE-01 cells *in vitro*.

3.3. Significant increases in mRNA expression of angiogenic, anti-inflammatory and anti-fibrotic cytokines in RE-01 cells

To identify the mechanism underlying the angiogenic action of RE-01 cells, we analyzed mRNA expression by qPCR. In RE-01 cells, mRNA expression of the pro-angiogenic cytokines VEGF-B, IGF-1, leptin, and ANG-2 in RE01 cells was significantly upregulated compared with that in PBMNCs (5.4-fold; $p < 0.01$, 231.4-fold; $p < 0.05$, 22.0-fold; $p < 0.05$, and 2.9-fold; $p = 0.0625$, respectively). The expression of the anti-fibrotic cytokines MMP-2 and MMP-9 also increased in RE-01 cells compared with that in PBMNCs (96.4-fold, $p < 0.01$, and 1653.1-fold, $p < 0.01$, respectively). Moreover, RE-01 cells showed increased the mRNA expression of the anti-inflammatory cytokine IL-10 (61.1-fold; $p < 0.05$) but decreased expression of pro-inflammatory cytokines (IL-1β; 0.59-fold; $p = 0.195$, IL-8; 0.51-fold; $p = 0.383$, TGF-β; 0.76-fold; $p < 0.05$) (Fig. 3). We also measured cytokines secreted by PBMNCs and RE-01 cells using human angiogenesis array. Among 55 angiogenesis-related cytokines, the secretion levels of IL-8 and MMP-9 were significantly increased in RE-01 cells (Supplementary material).

3.4. Transplantation of RE-01 cells salvaged mouse ischemic limbs with increased angiogenesis

Next, we evaluated the effects of RE-01 cell transplantation in an ischemic hind limb mouse model. RE-01 cells were transfected into the ischemic limbs immediately after ischemic surgery. The effect of treatment was evaluated using blood flow measurements. RE-01 cell transplantation improved blood flow in the ischemic limbs 4 days after ischemic surgery, whereas the blood flow ratio in the control group did not change for 21 days. The blood flow ratio on day 21 was significantly higher in the RE-01 cell transplantation group than in the control group (0.927 ± 0.127 vs 0.390 ± 0.254; $p < 0.01$) (Fig. 4A and B). Histological assessments were performed to investigate the angiogenesis and fibrosis in limbs treated with RE-01 cells. The number of microvessels stained with isolectin B4 significantly increased in RE-01 cell-treated limbs compared with in the control group (478.4 ± 67.2 vs 320.8 ± 98.1; $p < 0.05$) (Fig. 4C). The number of pericytes recruited to the microvessels stained with SMA and isolectin B4 also significantly increased in RE-01 cells (125.1 ± 27.1 vs 71.9 ± 22.5; $p < 0.05$) (Fig. 4D). HMA and isolectin B4 were co-localized in RE-01 cells (Fig. 4F). These results indicate that RE-01 cell transplantation increases angiogenesis and arteriogenesis in ischemic hind limbs. Furthermore, transplantation of RE-01 cells resulted in decreased fibrosis compared with the control group (17.78 ± 12.46 vs 33.65 ± 18.38; $p < 0.05$) (Fig. 4E).

3.5. Multiple transplantations of RE-01 cells were more effective than single transplantation in mouse models of severe limb ischemia

Since a single transplantation of RE-01 cells was effective, we investigated the effect of repeated transplantations of RE-01 cells in mice with a severe ischemic hind limb. RE-01 cells were transfected into ischemic limbs on days 1, 2, and 3 after ischemic surgery. The blood flow ratio on day 21 significantly increased in the RE-01-single and RE-01-three times groups compared with that in the control group (0.459 ± 0.114 vs 0.644 ± 0.074; $p < 0.01$, and 0.459 ± 0.114 vs 0.751 ± 0.302; $p < 0.01$, respectively), while there was no difference between the RE-01-single and RE-01-three times groups (Fig. 5A and B). Histological assessment was performed to investigate angiogenesis and arteriogenesis. The number of microvessels stained with isolectin B4 significantly increased in both RE-01-single and RE-01-three times groups compared with

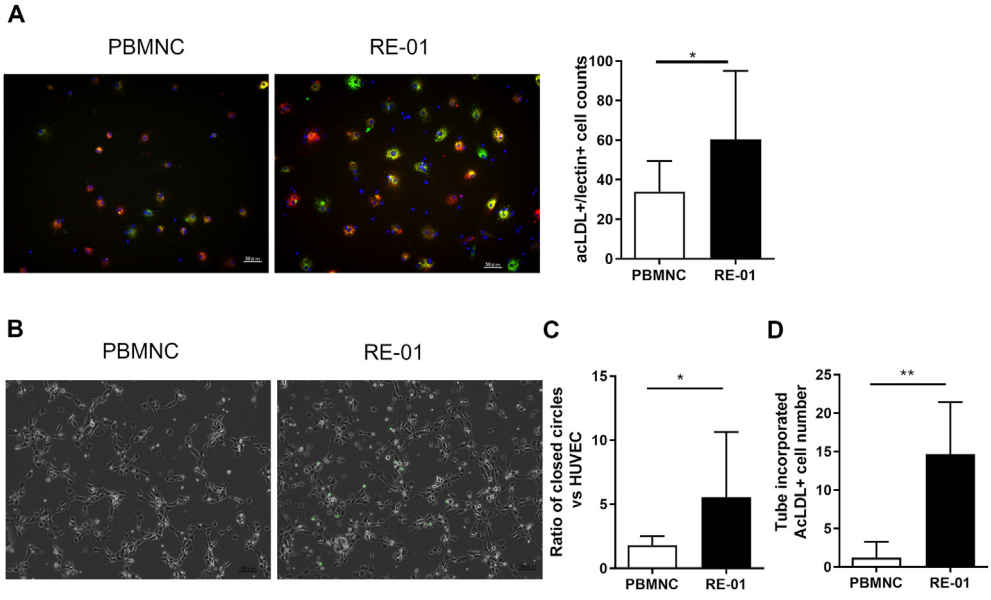

Fig. 2. Angiogenic potential increased in RE-01 cells. A. The EPCs stained with Ac-LDL (green) and lectin (red) in RE-01 cells and PBMNCs were counted after EPC culture ($n = 6$) White bar represents 50 μm. B. The representative image of HUVEC tube formation assay. PBMNC and RE-01 cells were stained with Ac-LDL (green). Black bar represents 100 μm. C. The number of closed circles was counted. The graph shows the ratio of the combination of PBMNCs, RE-01 cells, and HUVECs to the HUVEC-only control group. ($n = 8$). D. The number of Ac-LDL stained (green) cells incorporated into tube formation was counted. ($n = 8$) Data are presented as mean ± SD. Mean values were compared using the Wilcoxon matched pairs signed-rank test. *$p < 0.05$, **$p < 0.01$. PBMNCs, peripheral blood mononuclear cells; EPC, endothelial progenitor cells; Ac-LDL, acetylated low-density lipoprotein.

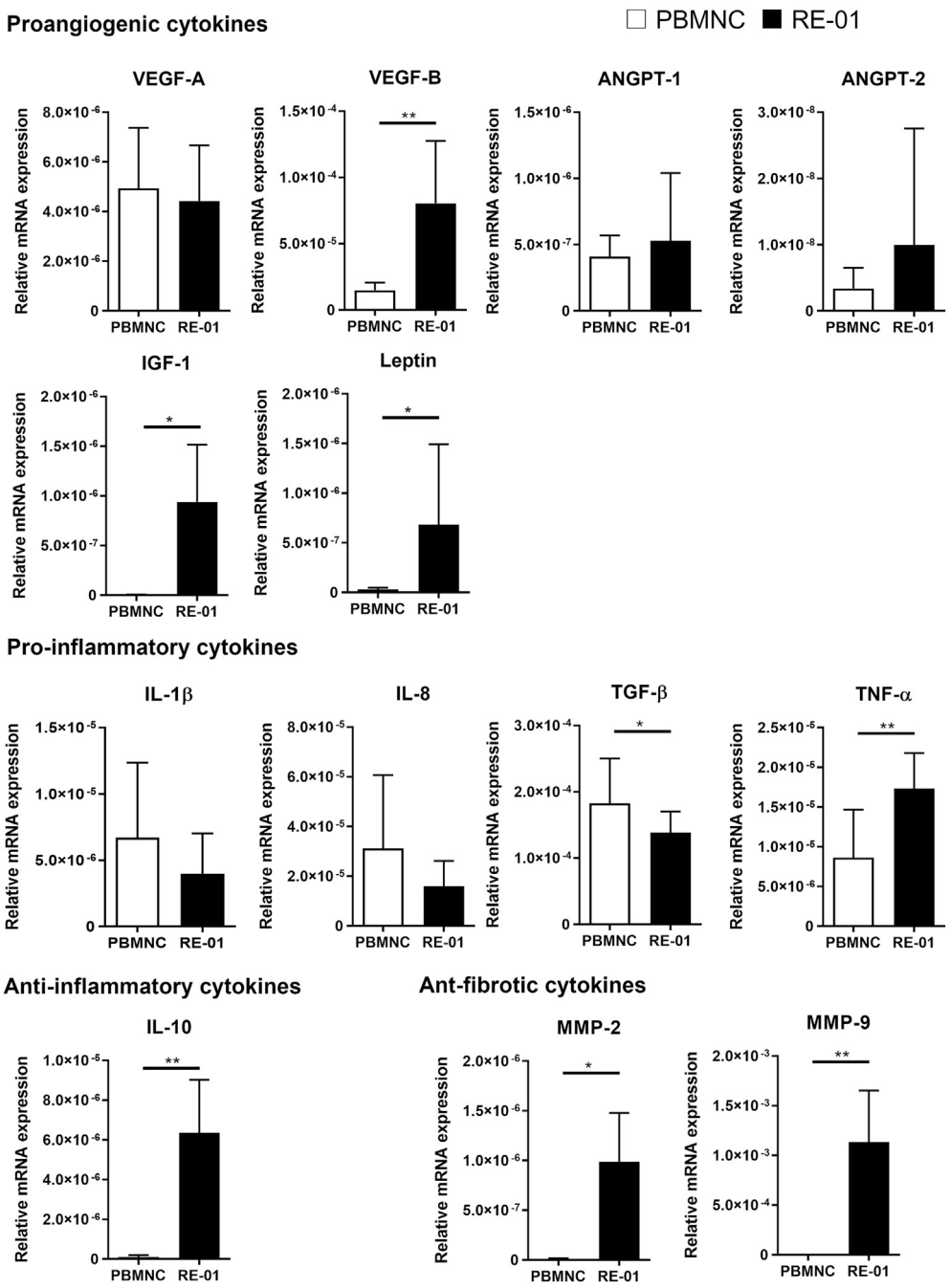

Fig. 3. Cytokines expressed in RE-01 cells. The mRNA expression of pro-angiogenic cytokines, VEGF-A, VEGF-B, ANGPT-1, ANGPT-2, IGF-1, and leptin; pro-inflammatory cytokines, IL-1β, IL-8, TGF-β, and TNF-α; and anti-inflammatory cytokines. Levels of IL-10 anti-fibrotic cytokines, MMP-2, and MMP-9 in PBMNCs and RE-01 cells were analyzed by real-time PCR (n = 8). Data are presented as mean ± SD. Mean values were compared using the Wilcoxon matched pairs signed-rank test. *p < 0.05, **p < 0.01. PBMNCs, peripheral blood mononuclear cells.

that in the control group (339.1 ± 69.1 vs 447.5 ± 104.9; p < 0.01, and 339.1 ± 69.1 vs 468.8 ± 101.8; p < 0.05, respectively) (Fig. 5C). Although the number of pericyte-recruited microvessels stained with SMA and isolectin B4 did not increase in the RE-01-single group compared with that in the control group (40.05 ± 19.58 vs 40.38 ± 15.49; p = 0.65), the RE-01-three times group had significantly increased arteriogenesis compared with the control and RE-01-single groups (40.05 ± 19.58 vs 74.76 ± 25.07; p < 0.05, and 40.38 ± 15.49 vs 74.76 ± 25.07; p < 0.01) (Fig. 5D).

4. Discussion

We developed RE-01 cells, which are a non-invasive and effective peripheral blood cell therapy for revascularization. RE-01 cells were generated from peripheral blood MNCs by *ex vivo* culture in IMDM supplemented with FBS, HSA, growth factors, TPO, FLT-3 ligand, and VEGF for 5 days. The angiogenic potential of RE-01 cells was demonstrated both *in vitro* and *in vivo* using a mouse model of ischemic hind limb. Compared with PBMNCs,

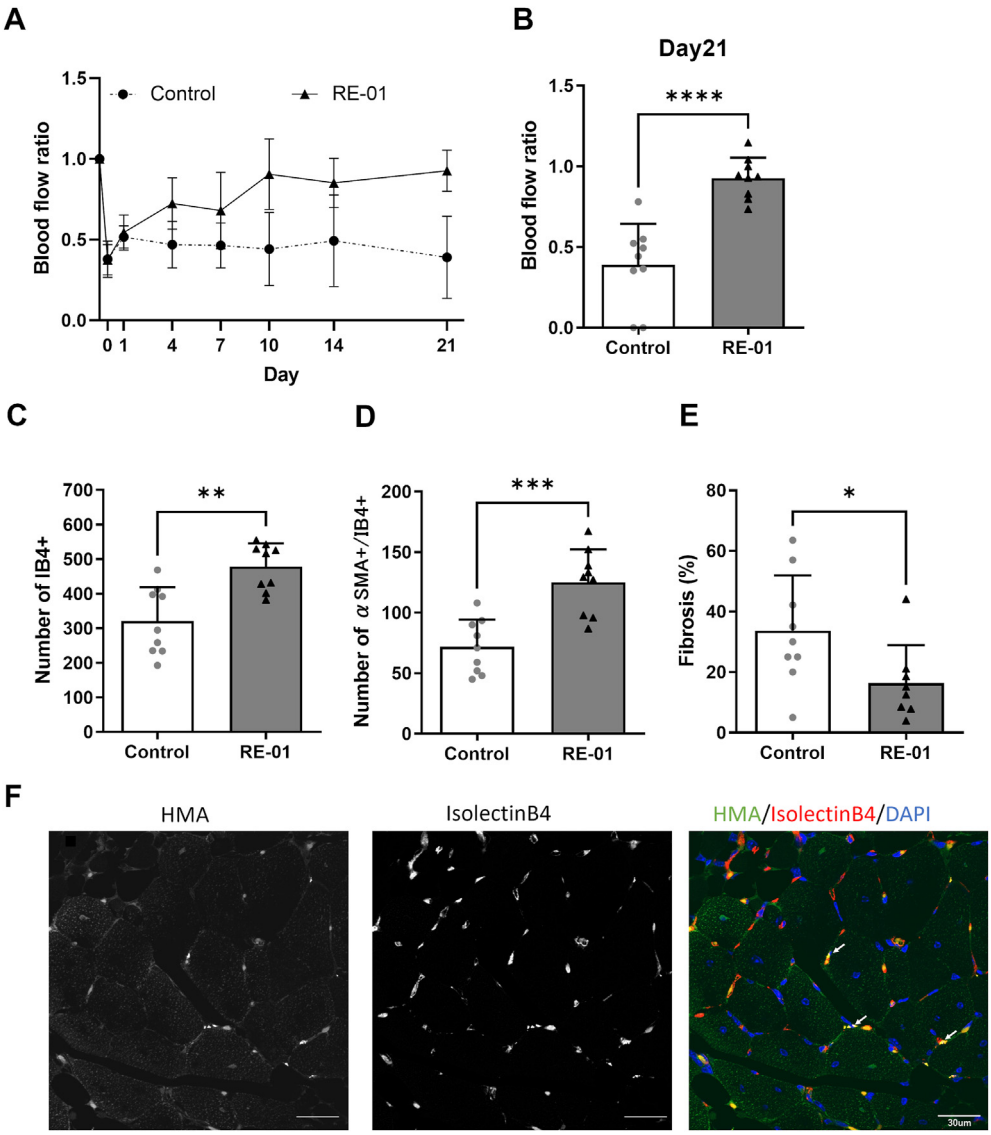

Fig. 4. Efficacy of RE-01 cells in ischemic hind limb mouse model A. RE01 cells were transplanted intramuscularly immediately after ischemic surgery on day 0. Blood flow was measured by laser blood flow imager on days 0, 1, 3, 7, 10, 14 and 21. The graph shows the ratio of blood flow to day 0 (n = 9) B. Blood flow significantly increased on day 21 in the RE-01 group (n = 9) C-E. The quantification of histological examination and immunostaining (n = 9) F. Representative image of co-localization of RE-01 cells stained with HMA (green) and isolectin B4 stained vessels (red). White bar represents 30 μm. Data are presented as mean ± SD. Mean values were compared using the Mann–Whitney test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. IB4, isolectin B4; HMA, human mitochondrial antibody.

RE-01 cells showed an increased number of M2-macrophages, angiogenic T cells, and $CD34^{+}$ stem cells. Increased mRNA expression of angiogenesis-related cytokines, including VEGF-B, IGF-1, Leptin and MMPs, also suggests the angiogenic potential of RE-01 cells. Unlike the PCR results, several cytokines showed no significant difference in the 24-h secretion levels between RE-01 cells and PBMNCs. However, their secretion, although minimal, was confirmed. Histological analysis of RE-01 cells transplanted with ischemic hind limb tissues revealed co-localization of RE-01 cells and mouse endothelial cells. Incorporation of RE-01 cells into the vascular structure was confirmed using an *in vitro* HUVEC tube formation assay. Since RE-01 cells include CD34-positive stem cells, such as EPCs, some RE-01 cells may differentiate into vascular cells. These results suggest that the angiogenic ability of RE-01 cells was not only due to paracrine effects but also due to direct incorporation into the microvascular structure.

As most of the amplified RE-01 cells were CD206-positive macrophages, macrophages seemed to play an important role in the angiogenic potential of RE-01 cells. EPCs, macrophages, and T cells are involved in vascular repair. Phenotypic changes from M1 to M2 macrophages are important during post-ischemic vascular and tissue repair. M2 macrophages contribute to extracellular matrix remodeling by releasing MMPs and promoting angiogenesis by stimulating the proliferation of endothelial cells and recruiting pericytes [9–11]. Angiogenic T cells have been reported to cooperate with EPCs and enhance endothelial repair function through the secretion of pro-angiogenic cytokines [8]. Apart from EPCs, angiogenic T cells also have been reported to decrease in patients with diabetes and rheumatoid arthritis [12,13]. Our results showed an increased number of M2-macrophages and angiogenic T cells in RE-01 cells, which may contribute to the promotion of angiogenesis.

RE-01 cells have also demonstrated anti-fibrotic abilities *in vitro* and *in vivo*. The expression of the anti-fibrotic cytokines MMP-2

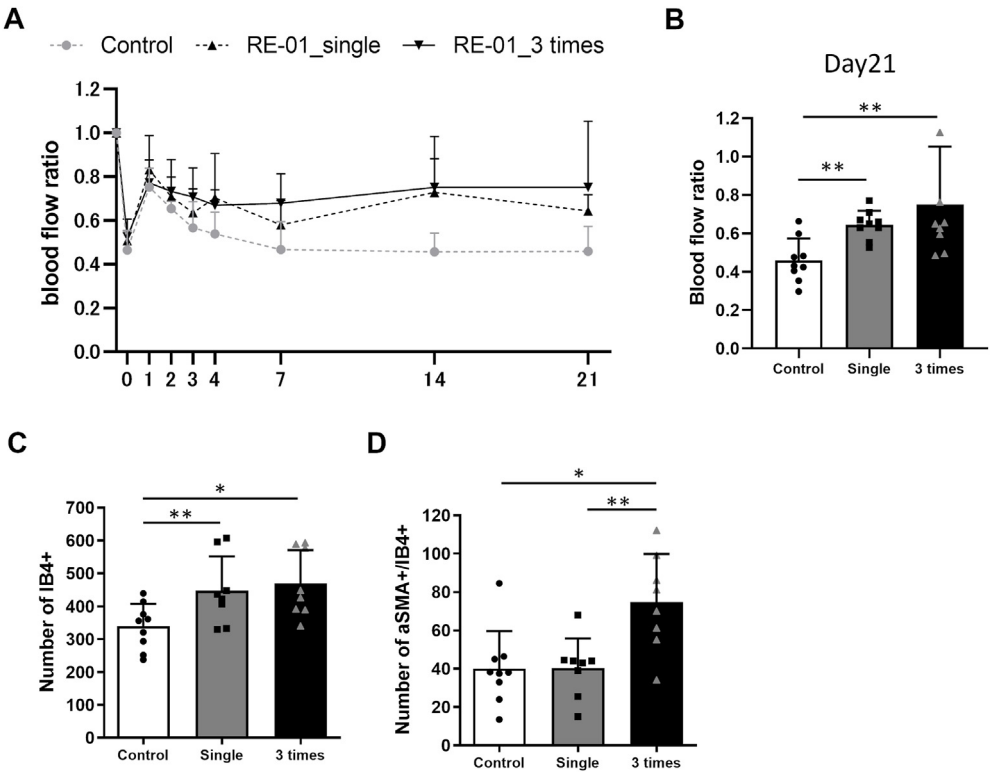

Fig. 5. Multi-administration of RE-01 cells in the ischemic hind limb mouse model A. RE-01 cells were transplanted intramuscularly immediately after ischemic surgery on day 0 (RE-01-single) or on days 0, 1 and 2 (RE-01-three times). Blood flow was measured by laser blood flow imager on days 0, 1, 2, 3, 7, 10, 14, and 21. The graph shows the ratio of blood flow on day 0 (n = 9) B. Blood flow on day 21 significantly increased in both RE-01 groups (n = 9) C-D. The quantification of immunostaining. (n = 9). Data are presented as mean ± SD. Mean values were compared using the Mann–Whitney test. *p < 0.05, **p < 0.01. Single, RE-01-single administration; three times, RE-01-3 administration; IB4, isolectin B4.

and MMP-9 significantly increased in RE-01 cells. Furthermore, MMP-9 secretion from RE-01 cells was significantly higher compared to PBMNCs, consistent with the PCR results. MMP-2 and MMP-9 are gelatinases that digest denatured collagen [14]. Moreover, the level of the anti-inflammatory cytokine IL-10, which is reportedly involved in anti-fibrosis, significantly increased [15]. These results suggest that the decreased fibrosis in RE-01-transplanted mouse ischemic hind limbs was not only due to rapid angiogenesis after ischemia but also due to the secretion of anti-fibrotic cytokines by RE-01 cells.

In the present study, we confirmed the efficacy of RE-01 in a mouse ischemic hind limb model. The number of transplanted cells in mice was 10000 cells/body, which corresponds to 2×10^7 cells/body in humans. This number of cells can be obtained from only 50 mL of peripheral blood. Although the efficacy of RE-01 cell treatment was confirmed in a single transplantation, transplantation for 3 consecutive days significantly improved the number of pericyte-recruited vessels in the severely ischemic hind limb of mice. Thus, we repeated the administration of RE-01 cells to treat patients with chronic limb-threatening ischemia.

In this study, we present new cell products that are non-invasive and highly effective for the treatment of ischemic vascular disease. In addition, RE-01 cells met all criteria for pharmaceutical products in Japan. We will conduct a phase 1 physician-based clinical trial of RE-01 cells in unhealed ulcers of patients with connective tissue diseases to confirm their safety and efficacy. We believe that our new proposed RE-01 cell therapy will save many patients with foot conditions that do not respond to existing treatments.

5. Conclusions

We developed a new vascular regenerative cell therapy called RE-01, which is manufactured from a small amount of peripheral blood. The efficacy of RE-01 cell transplantation was confirmed in an ischemic hind limb mouse model. The minimally invasive and effective RE-01 cell therapy will be a breakthrough treatment for patients with intractable ulcers.

Declaration of competing interest

The authors declare no conflicts of interest associated with this manuscript.

Acknowledgments

We thank the Laboratory of Cell Biology, Biomedical Research Core Facilities, Juntendo University Graduate School of Medicine, for technical assistance. We would like to thank Editage (www.editage.com) for the English language editing. This work was carried out in part at the Intractable Disease Research Center, Juntendo. This research was supported by Japan agency for medica research and development (AMED; Grant Number JP22bk010 4149), the National Institutes of Biomedical Innovation, Health and Nutrition Regenerative Medicine Development Support Program, JST FOREST Program (Grant Number JPMJFR200P, Japan), and JSPS KAKENHI (Grant Number 19H03816 and 23H03067). This work was also supported in part by a Grant-in-Aid for Special Research in Subsidies for ordinary expenses of private schools

from the Promotion and Mutual Aid Corporation for Private Schools of Japan.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.06.009.

References

[1] Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964–7. https://doi.org/10.1126/science.275.5302.964.
[2] Qadura M, Terenzi DC, Verma S, Al-Omran M, Hess DA. Concise review: cell therapy for critical limb ischemia: an integrated review of preclinical and clinical studies. Stem Cell 2018;36:161–71. https://doi.org/10.1002/stem.2751.
[3] Tanaka R, Wada M, Kwon SM, Masuda H, Carr J, Ito R, et al. The effects of flap ischemia on normal and diabetic progenitor cell function. Plast Reconstr Surg 2008;121:1929–42. https://doi.org/10.1097/PRS.0b013e3181715218.
[4] Del Papa N, Pignataro F. The role of endothelial progenitors in the repair of vascular damage in systemic sclerosis. Front Immunol 2018;9:1383. https://doi.org/10.3389/fimmu.2018.01383.
[5] Yoshimi R, Nakajima H. Current state and issues of regenerative medicine for rheumatic diseases. Front Med 2022;9:813952. https://doi.org/10.3389/fmed.2022.813952.
[6] Sukmawati D, Tanaka R. Introduction to next generation of endothelial progenitor cell therapy: a promise in vascular medicine. Am J Transl Res 2015;7:411–21.
[7] D'Souza A, Jaiyesimi I, Trainor L, Venuturumili P. Granulocyte colony–stimulating factor administration: adverse events. Transfus Med Rev 2008;22:280–90. https://doi.org/10.1016/j.tmrv.2008.05.005.
[8] Hur J, Yang HM, Yoon CH, Lee CS, Park KW, Kim JH, et al. Identification of a novel role of T cells in postnatal vasculogenesis: characterization of endothelial progenitor cell colonies. Circulation 2007;116:1671–82. https://doi.org/10.1161/CIRCULATIONAHA.107.694778.
[9] Moore EM, West JL. Harnessing macrophages for vascularization in tissue engineering. Ann Biomed Eng 2019;47:354–65. https://doi.org/10.1007/s10439-018-02170-4.
[10] Hong H, Tian XY. The role of macrophages in vascular repair and regeneration after ischemic injury. Int J Mol Sci 2020;21. https://doi.org/10.3390/ijms21176328.
[11] Wu X, Reboll MR, Korf-Klingebiel M, Wollert KC. Angiogenesis after acute myocardial infarction. Cardiovasc Res 2021;117:1257–73. https://doi.org/10.1093/cvr/cvaa287.
[12] de Boer SA, Reijrink M, Abdulahad WH, Hoekstra ES, Slart RHJA, Heerspink HJL, et al. Angiogenic T cells are decreased in people with type 2 diabetes mellitus and recruited by the dipeptidyl peptidase-4 inhibitor linagliptin: a subanalysis from a randomized, placebo-controlled trial (RELEASE study). Diabetes Obes Metabol 2020;22:1220–5. https://doi.org/10.1111/dom.14024.
[13] Rodríguez-Carrio J, Alperi-López M, López P, Alonso-Castro S, Ballina-García FJ, Suárez A. Angiogenic T cells are decreased in rheumatoid arthritis patients. Ann Rheum Dis 2015;74:921–7. https://doi.org/10.1136/annrheumdis-2013-204250.
[14] Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 2003;92:827–39. https://doi.org/10.1161/01.RES.0000070112.80711.3D.
[15] Steen EH, Wang X, Balaji S, Butte MJ, Bollyky PL, Keswani SG. The role of the anti-inflammatory cytokine interleukin-10 in tissue fibrosis. Adv Wound Care 2020;9:184–98. https://doi.org/10.1089/wound.2019.1032.