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Tumor necrosis factor-α-activated mesenchymal stem cells promote endothelial progenitor cell homing and angiogenesis

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ABSTRACT

Mesenchymal stem cells (MSCs) accelerate regeneration of ischemic or injured tissues by stimulation of angiogenesis through a paracrine mechanism. Tumor necrosis factor-α (TNF-α)-activated MSCs secrete pro-angiogenic cytokines, including IL-6 and IL-8. In the present study, using an ischemic hindlimb animal model, we explored the role of IL-6 and IL-8 in the paracrine stimulation of angiogenesis and tissue regeneration by TNF-α-activated MSCs. Intramuscular injection of conditioned medium derived from TNF-α-treated MSCs (TNF-α CM) into the ischemic hindlimb resulted in attenuated severe limb loss and stimulated blood perfusion and angiogenesis in the ischemic limb. Immunodepletion of IL-6 and IL-8 resulted in attenuated TNF-α CM-stimulated tissue repair, blood perfusion, and angiogenesis. In addition, TNF-α CM induced migration of human cord blood-derived endothelial progenitor cells (EPCs) through IL-6- and IL-8-dependent mechanisms in vitro. Intramuscular injection of TNF-α CM into the ischemic limb led to augmented homing of tail vein-injected EPCs into the ischemic limb in vivo and immunodepletion of IL-6 or IL-8 from TNF-α CM attenuated TNF-α CM-stimulated homing of EPCs. In addition, intramuscular injection of recombinant IL-6 and IL-8 proteins resulted in increased homing of intravenously transplanted EPCs into the ischemic limb and improved blood perfusion in vivo. These results suggest that TNF-α CM stimulates angiogenesis and tissue repair through an increase in homing of EPCs through paracrine mechanisms involving IL-6 and IL-8.

1. Introduction

Ischemic disease is the end stage of peripheral vascular disease, resulting in damage or dysfunction of tissues and tissue loss [1]. Ischemic disease has been reported to affect nearly 80 million members of the population worldwide [2]. Currently, due to angiogenesis-stimulating ability of stem cells, stem cell-based therapies have become a promising strategy in treatment of patients with ischemic disease. [3]. Accumulating evidence suggests that circulating endothelial progenitor cells (EPCs) originate from the bone marrow home to sites of tissue injury, facilitating neovascularization, and promoting regeneration of the injured tissues [4]. According to one report, EPCs can integrate into blood vessels and stimulate neovascularization of the ischemic limbs and hearts in animal models of hindlimb ischemia and cardiac infarction [5]. Therefore, mobilization and recruitment of bone marrow-derived stem/progenitor cells are critical for ischemia-induced neovascularization. Exogenously administered EPCs have been reported to improve left ventricular ejection fraction after acute myocardial infarction [6,7]; However, because patients at the highest cardiovascular risk have the lowest number and poorest migratory and homing capacity of endogenous EPCs, the use of autologous EPCs for neovascularization in the clinical setting may prove less effective [8]. Therefore, the ability to increase the homing of exogenously administered EPCs to specific target sites may potentially lead to the improvement of the therapeutic efficacy of EPCs for ischemic diseases.

Mesenchymal stem cells (MSCs) can be isolated from diverse adult tissues, including bone marrow, adipose tissues, umbilical cord blood, and amniotic membrane [9]. They possess stem cell properties, such as self renewal capacity, long term viability, and potential for differentiation into mesodermal lineages, including adipocytes, osteocytes, chondrocytes, and muscle cells [10–12]. In addition to their capacity to differentiate into multiple cell lineages, MSCs play a key role in tissue regeneration through secretion of trophic factors that regulate the local immune system, fibrosis, apoptosis, and angiogenesis [13–15]. Many studies have demonstrated that transplantation of MSCs improves

Abbreviations: MSCs, mesenchymal stem cells; TNF-α, tumor necrosis factor-α; TNF-α CM, conditioned medium derived from TNF-α-treated MSCs; EPCs, endothelial progenitor cells; hASCs, human adipose tissue-derived MSCs

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tissue regeneration in myocardial infarction, ischemic disease, and other disease models [16,17]. These studies suggest that MSCs increased blood perfusion and reduced tissue necrosis in ischemic models through stimulation of angiogenesis. MSC-derived paracrine factors have been reported to be largely responsible for MSC-stimulated neovascularization and tissue regeneration [18,19]. However, the molecular mechanisms associated with the paracrine action of MSCs in vascular regeneration are still elusive.

Tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, is expressed in ischemic and injured tissues [20]. TNF-α plays a key role not only in inflammatory response but also in repair processes of injured tissues [21]. Knockout of TNF-α receptor p75 has been reported to attenuate neovascularization in the hindlimb ischemia animal model and a reduction in the number of circulating bone marrow-derived EPCs is responsible for poor neovascularization [22]. TNF-α-treated human adipose tissue-derived MSCs (hASCs) have been shown to secrete various protein factors, including cytokines, extracellular matrix, proteases, and protease inhibitors [23]. In addition, conditioned medium derived from TNF-α-treated hASCs (TNF-α CM) accelerated cutaneous wound healing and angiogenesis through IL-6- and IL-8-dependent mechanisms [24]. However, the involvement of EPCs in TNF-α CM-induced angiogenesis and repair of ischemic tissues has not been explored.

To demonstrate the possibility of therapeutic application of TNF-α CM in regeneration of injured tissues, we investigated the effects of TNF-α CM on tissue repair, EPC homing, and angiogenesis in an ischemic hindlimb murine model. In addition, we explored the question of whether IL-6 and IL-8 play a role in TNF-α CM-stimulated tissue repair, EPC homing, and angiogenesis in vivo.

2. Materials and methods

2.1. Materials

α-Minimum essential medium, trypsin, and Hank's balanced salt solution were purchased from Invitrogen (Carlsbad, CA). Recombinant human TNF-α, IL-6, IL-8, and VEGF proteins were purchased from R&D Systems (Minneapolis, MN). Enzyme-linked immunosorbent assay (ELISA) kits and neutralizing monoclonal antibodies for human IL-6, IL-8, and VEGF were purchased from BD Biosciences (Bedford, MA). Normal mouse IgG was purchased from Millipore (Billerica, MA). Antibodies against von Willebrand Factor (vWF) (ab6994) and α-SMA (ab5694) were purchased from Abcam PLC (Cambridge, UK).

2.2. Cell culture

Subcutaneous adipose tissues were obtained from elective surgical procedures with the patient's consent as approved by the institutional review board of Pusan National University Hospital and hASCs were isolated as previously described [25]. In brief, adipose tissues were washed at least three times with sterile PBS and treated with an equal volume of collagenase type I suspension (1 g/L of Hank's balanced salt solution buffer with 1% bovine serum albumin) for 60 min at 37 °C with intermittent shaking. Floating adipocytes were separated from the stromal-vascular fraction by centrifugation at 300 ×g for 5 min. The pelleted stromal-vascular fraction was re-suspended in α-minimum essential medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, and cells were cultured for 4–5 days until they reached confluence and were defined as passage "0." The passage number of hASCs used in these experiments was 3–10. The hASCs were positive for CD29, CD44, CD73, CD90, and CD105, whereas they were negative for CD31, CD34, and CD45 [26].

Human EPCs were isolated from human umbilical cord blood, which was collected in disposable sterile pyrogen-free bags (Green Cross, Yongin, Korea) containing anticoagulant. Written informed consent was obtained from all donors as approved by the institutional review board of Pusan National University Hospital. Mononuclear cells were isolated from the blood using Histopaque-1077 (Sigma-Aldrich, Switzerland), as previously described [27]. Cells were seeded on culture dishes coated with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO) and maintained in an endothelial cell basal medium-2 (EBM-2) (Clonetics, San Diego, CA) supplemented with EGM-2 MV SingleQuots containing 5% fetal bovine serum (FBS), human VEGF-1, human fibroblast growth factor-2, human epidermal growth factor, insulin-like growth factor-1, and ascorbic acid. After four days in culture, non-adherent cells were removed and adherent cells were trypsinized and re-plated at a density of 1×10^6 per well through day 7 [27].

2.3. Preparation of conditioned medium

To obtain control CM and TNF-α CM, hASCs were cultured in 150-mm diameter culture dishes until they reached sub-confluence and were washed twice with Hank's balanced salt solution for removal of the serum component. The cells were incubated in 20 mL serum-free α-minimum essential medium in the absence or presence of carrier-free recombinant human TNF-α (10 ng/mL) for 48 h. Conditioned medium were collected and centrifuged at 3000 rpm for 10 min using a MF 300 centrifuge (Hanil Science Industrial Co., Ltd., Inchon, Korea) for removal of cell debris.

2.4. Immunodepletion of IL-6 and IL-8 from TNF-α CM

For immunodepletion of IL-6 and IL-8 from TNF-α CM, aliquots (30 μL) of a suspension (50% slurry) of protein G-agarose beads (Sigma-Aldrich) in PBS were mixed with 0.5 μg of anti-IL-6 and/or anti-IL-8 antibodies or control mouse antibody (Millipore, Billerica, MA) at 4 °C for 1 h with intermittent shaking. After recovery by centrifugation, beads were washed three times and used for immunodepletion of IL-6 and IL-8. TNF-α CM (1 mL) was incubated with protein G-agarose beads immobilized with anti-IL-6 and/or anti-IL-8 antibodies or control mouse antibody for 1 h at 4 °C. Immune complexes absorbed to protein G-agarose beads were precipitated by centrifugation. Resultant supernatants were collected and used immediately for experiments.

2.5. Hindlimb ischemia, cell transplantation, and blood flow measurement

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee of Pusan National University School of Medicine. Athymic nude mice (age 8–10 wk and weighing 17–22 g) were anesthetized with 50 mg/kg pentobarbital i.p. for operative resection of one femoral artery and laser Doppler perfusion imaging. The femoral artery was excised from its proximal origin as a branch of the external iliac artery to the distal point where it bifurcates into the saphenous and popliteal arteries. TNF-α CM, control CM, and HBSS buffer (60 μL each) were injected intramusculary into three sites (20 μL/each site) of the gracilis muscle in the medial thigh immediately after surgery. Conditioned medium or HBSS buffer was injected three times per week for four weeks. To elucidate the role of IL-6 and IL-8 in TNF-α CM-stimulated in vivo vasculogenesis and tissue repair, the ischemic limbs were injected with IL-6- and IL-8-depleted TNF-α CM or recombinant IL-6 and IL-8 proteins. The extent of necrosis in the ischemic hindlimb was recorded on day 28 after surgery. The scores for necrosis were evaluated as follows: 0, limb salvage; 1, toes amputation; 2, foot amputation; and 3, limb amputation. Blood flow of the ischemic and normal limb was measured using a laser Doppler perfusion imaging (LDPI) analyzer (Moor Instruments Ltd., Devon, UK) on days 0, 7, 14, 21, and 28 after induction of hindlimb ischemia. The perfusion of the ischemic and non-ischemic limb was calculated on the basis of colored histogram pixels. Red and blue colors indicate high and low perfusion, respectively. Blood perfusion is expressed as the LDPI index representing the ratio of ischemic versus non-ischemic limb blood flow. A ratio of 1 before operation indicates equal blood perfusion of both legs.

2.6. Histological and immunofluorescence analyses

For histological and immunostaining of the tissue specimens, hindlimb muscles were removed, formalin-fixed, and paraffin-embedded. Three sections measuring 6 μm in thickness were taken from the paraffin-embedded specimens at 150 μm intervals, stained with hematoxylin and eosin (H&E), observed, and photographed with a microscope (Axioimager M2, Carl Zeiss, Heidenheim, Germany). Endothelial cells and smooth muscle cells were immunostained with rabbit anti-vWF and rabbit anti-α-SMA antibodies. The specimens were incubated with Alexa 488 goat anti-rabbit or Alexa 568 goat anti-rabbit secondary antibodies, followed by washing and mounting in Vectashield medium (Vector Laboratories) with 4′,6-diamidino-2-phenylindole (DAPI) for visualization of nuclei. The stained sections were visualized by laser scanning confocal microscopy (Olympus FluoView FV1000). Capillary density and the number of arterioles/arteries were assessed by counting the number of CD31-positive and α-SMA-positive features per high power field (×400). The numbers of CD31-positive and α-SMA-positive cells were quantified by two independent observers blinded to the experimental conditions in nine randomly chosen microscopic fields from three serial sections in each tissue block.

2.7. Cell migration assay

Migration of EPCs was assayed using a disposable 96 well chemotaxis chamber (Neuro Probe, Inc., Gaithersburg, MD). Briefly, EPCs were harvested with 0.05% trypsin containing 0.02% EDTA, washed once, and suspended in EBM-2 at a concentration of 2×10^5 cells/ml. A membrane filter with 8-μm pores of the chemotaxis chamber was pre-coated overnight with 20 μg/ml rat-tail collagen at 4 °C, an aliquot (100 μL) of EPCs suspension was loaded into the upper chamber, and TNF-α CM or control CM was then placed in the lower chamber. After incubation of the cells for 12 h at 37 °C, the filters were disassembled, and the upper surface of each filter was scraped free of cells by wiping it with a cotton swab. The number of cells that had migrated to the lower surface of each filter was determined by counting the cells in four locations under microscopy at ×100 magnification after staining with hematoxylin and eosin.

2.8. Cell tracking analysis

EPCs were labeled with the long-lasting CellTracker CM-DiI according to the manufacturer's instructions. The CM-DiI-labeled EPCs (1×10^6 cells) were transplanted into the hindlimb ischemia animal model by intravenous tail vein injection immediately after surgery, followed by direct injection of TNF-α CM, recombinant IL-6/IL-8 proteins, or vehicles (60 μL each) into the ischemic limbs. Three days after cell transplantation, the adductor muscle of the ischemic leg was isolated, formalin-fixed, and paraffin-embedded. For quantitative analyses, three sections measuring 6 μm in thickness were taken from the specimens at 150 μm intervals. CM-DiI-labeled cells were counted in three randomly chosen microscopic fields from three serial sections in each tissue block.

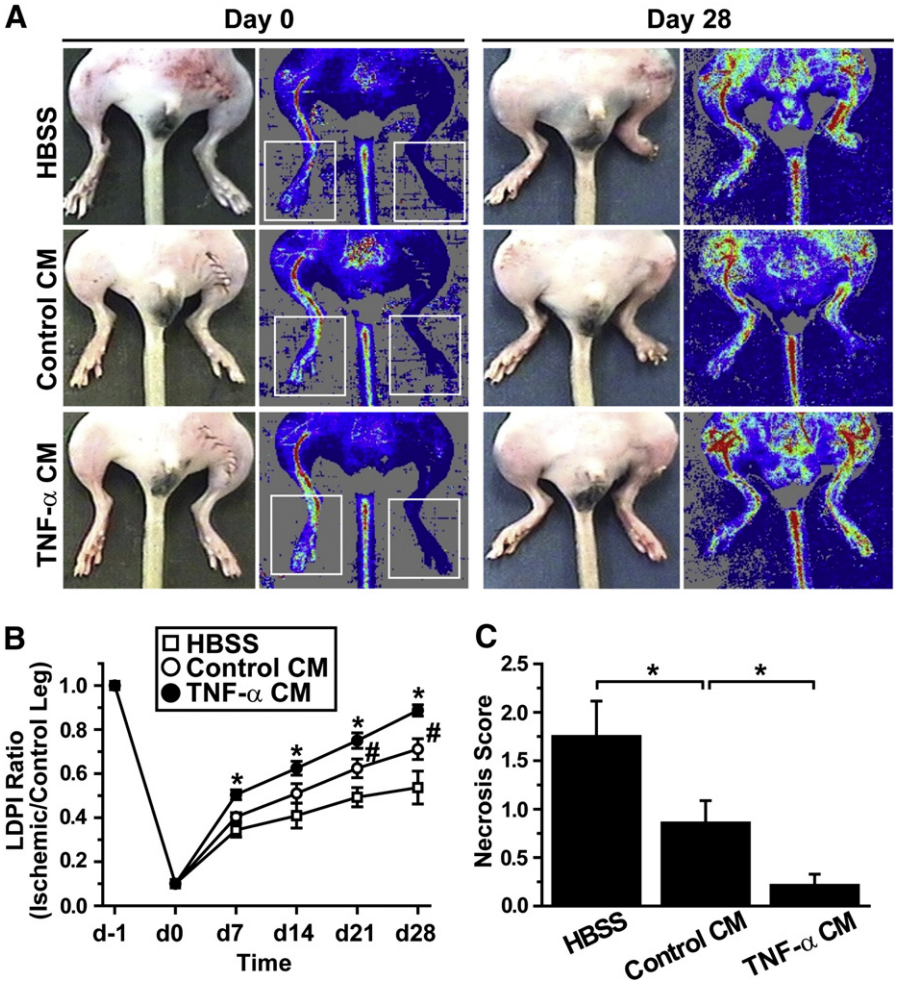

Fig. 1. Effects of TNF-α CM on blood perfusion and tissue necrosis in the hindlimb ischemia murine model. (A) Representative photographs and color-coded laser Doppler perfusion images of mouse hindlimbs on day 0 and 28 after injection of Hank's balanced salt solution (HBBS), control CM, or TNF-α CM. The white box indicates the feet, where blood flow was measured. (B) Quantitative analysis of the blood perfusion recovery measured by LDPI analyzer. The LDPI ratio was calculated as the ratio of ischemic to nonischemic hindlimb blood perfusion. (n = 17 per each experimental group). *, P < 0.05 vs control CM. #, P < 0.05 vs HBSS. (C) Statistical analysis of the necrosis score on day 28. Data indicate mean ± SD (n = 17). *, P < 0.05.

2.9. Statistical analysis

The results of multiple observations are presented as mean ± SD. For multivariate data analysis, group differences were assessed with one-way or two-way ANOVA, followed by Scheffé's post hoc test.

3. Results

3.1. Intramuscular injection of TNF-α CM stimulates blood perfusion of the ischemic hindlimb and inhibits ischemic tissue damage

To explore the therapeutic impact of TNF-α CM on ischemic tissue injury, we determined the effect of TNF-α CM on angiogenesis and tissue repair in an ischemic hindlimb animal model. TNF-α CM was administered by intramuscular injection into the ischemic hindlimb, and blood flow was measured over a period of four weeks using a LDPI analyzer. Intramuscular injection of TNF-α CM into the ischemic hindlimb resulted in significantly improved blood perfusion, as determined by LDPI (Fig. 1A and B). LDPI ratio showed a significant increase in mice injected with TNF-α CM, as compared with the control experimental groups in which control CM or saline were injected (Fig. 1B). Injection of control CM also significantly increased LDPI ratio after day 21; however the stimulatory effect of control CM on blood perfusion was less potent than that of TNF-α CM. TNF-α CM dose-dependently stimulated blood perfusion of ischemic hindlimb with a maximal increase upon intramuscular injection of 60 μL TNF-α CM (Figure I in the online-only Data Supplement). In addition, injection of TNF-α CM resulted in significantly inhibited tissue necrosis and amputation, as demonstrated by reduced necrosis score four weeks after induction of ischemia, as compared with control groups (Fig. 1C). Histological analysis showed necrosis of the ischemic limb in HBSS-injected limbs, and injection of TNF-α CM resulted in reduced muscle necrosis, compared with control groups, which were injected with HBSS or control CM (Figure II in the online-only Data Supplement).

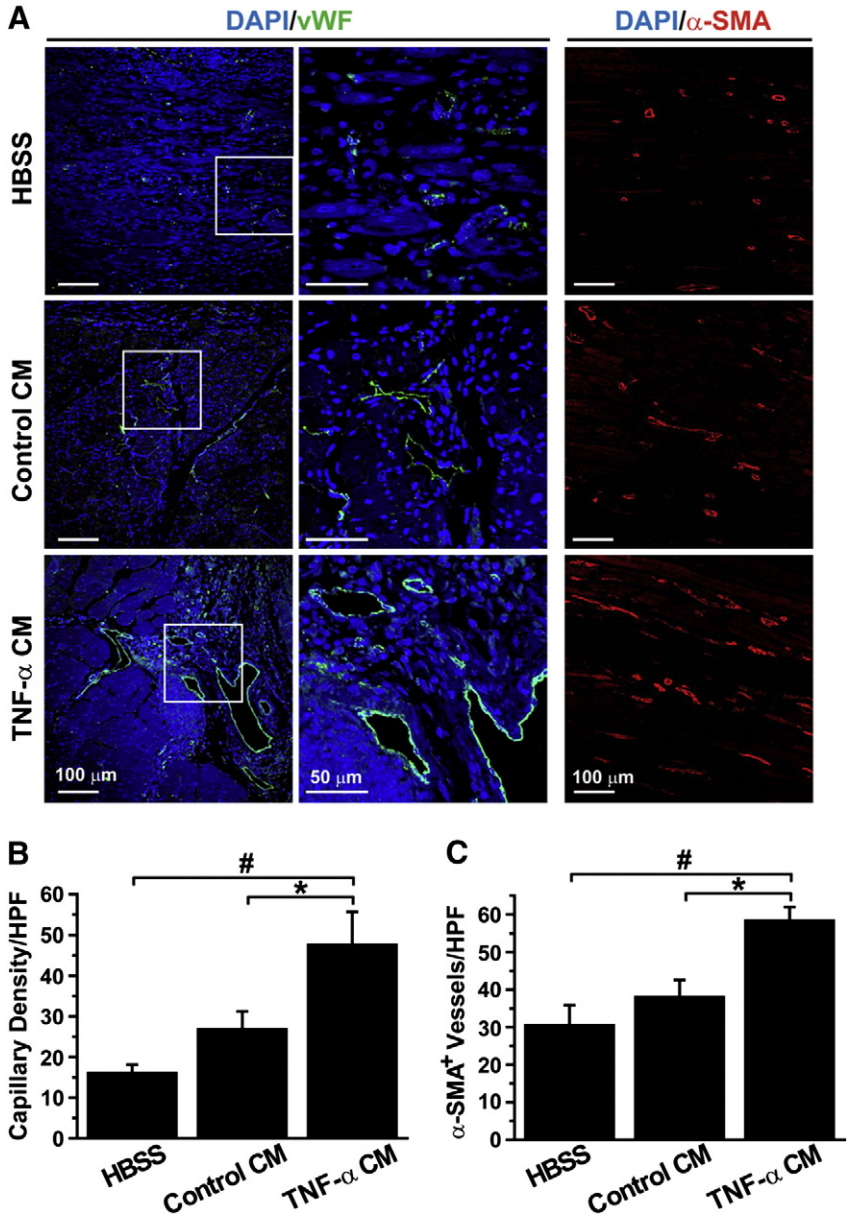

Fig. 2. Effects of TNF-α CM on angiogenesis and vasculogenesis in the mouse hindlimb ischemia model. (A) Immunostaining of vWF-positive capillaries (green color) and α-SMA-positive blood vessels (red color) of the ischemic limbs injected with HBSS, control CM, or TNF-α CM on day 28. Nuclei (blue) were counterstained with DAPI. Magnified images of the areas within the white boxes are shown in the middle panels. (B) Quantification of vWF-positive capillaries in the ischemic limb by immunohistochemistry analysis. (C) Quantification of α-SMA-positive arteries in the ischemic limb by immunohistochemistry analysis. *, $P < 0.05$; #, $P < 0.01$. ($n = 17$ per each group).

To evaluate the question of whether injection of TNF-α CM can stimulate angiogenesis in vivo, we performed immunostaining for determination of capillary density in ischemic muscle. Significantly higher vWF-positive capillary density was observed in the ischemic limb transplanted with TNF-α CM than in the control groups, which were injected with either control CM or saline buffer (Fig. 2A and B). The densities of α-SMA-positive blood vessels were also increased in the ischemic limb injected with TNF-α CM, as compared with the control groups (Fig. 2A and C). These findings of increased densities of vWF-positive capillary and α-SMA-positive arterioles/arteries after TNF-α CM injection are consistent with the increase in blood perfusion and reduction of necrosis in the TNF-α CM-injected ischemic limb.

3.2. TNF-α CM stimulates blood perfusion and inhibits necrosis of the ischemic hindlimb in an IL-6- and IL-8-dependent mechanisms

We have previously reported that immunodepletion of IL-6 or IL-8 from TNF-α CM resulted in abrogated TNF-α CM-stimulated cutaneous wound healing [24]. To determine whether or not these cytokines are responsible for TNF-α CM-inhibited ischemic tissue damage, we next explored the effects of IL-6- or IL-8-depleted TNF-α CM on repair of the ischemic hindlimb. Both IL-6 and IL-8 could be hardly detected in control CM, whereas high concentrations of IL-6 (8.9 ± 1.5) and IL-8 (10.1 ± 0.9) were detected in TNF-α CM. Both IL-6 and IL-8 were depleted from TNF-α CM by immunoprecipitation with antibodies specific for IL-6 and IL-8, respectively (Figure III in the online-only Data Supplement). TNF-α CM-stimulated blood perfusion in the ischemic limb was attenuated by depletion of IL-8 and/or IL-6 from TNF-α CM (Fig. 3A). Intramuscular injection of TNF-α CM resulted in a time-dependent increase of LDPI ratio and immunodepletion of either IL-6 or IL-8 resulted in abrogated TNF-α CM-stimulated increase of LDPI ratio. These results suggest that IL-6 and IL-8 are largely responsible for TNF-α CM-stimulated blood perfusion (Fig. 3B). In addition, immunodepletion of IL-6 or IL-8 resulted in abrogated TNF-α CM-inhibited tissue necrosis and amputation, as shown by reduced necrosis score four weeks after induction of ischemia (Fig. 3A and C).

3.3. Depletion of IL-6 and IL-8 from TNF-α CM blocked TNF-α CM-stimulated angiogenesis in the ischemic limb

To explore the question of whether or not IL-6 and IL-8 are involved in TNF-α CM-stimulated angiogenesis in the ischemic limb, we performed immunostaining in order to examine the effects of IL-6- or IL-8-depleted TNF-α CM on capillary density in ischemic muscle. As shown in Fig. 4A, TNF-α CM-stimulated formation of vWF-positive capillary blood vessels was markedly diminished by depletion of IL-6 or IL-8. Depletion of IL-6 or IL-8 resulted in consistent attenuation of the TNF-α CM-stimulated increase of vWF-positive blood vessels (Fig. 4A and B). In addition, the TNF-α CM-induced increase of α-SMA-positive blood vessel densities was attenuated by depletion of IL-6 or IL-8 from TNF-α CM (Fig. 4A and C), suggesting a principal role for IL-6 and IL-8 in TNF-α CM-stimulated angiogenesis and blood perfusion in the ischemic limb.

3.4. TNF-α CM stimulates homing and engraftment of EPCs into the ischemic limb in an IL-6- and IL-8-dependent mechanisms

To explore whether EPCs are involved in the TNF-α CM-stimulated angiogenesis in vivo, we examined the effect of TNF-α CM on chemotactic migration of EPCs in vitro. As shown in Fig. 5A, TNF-α CM stimulated

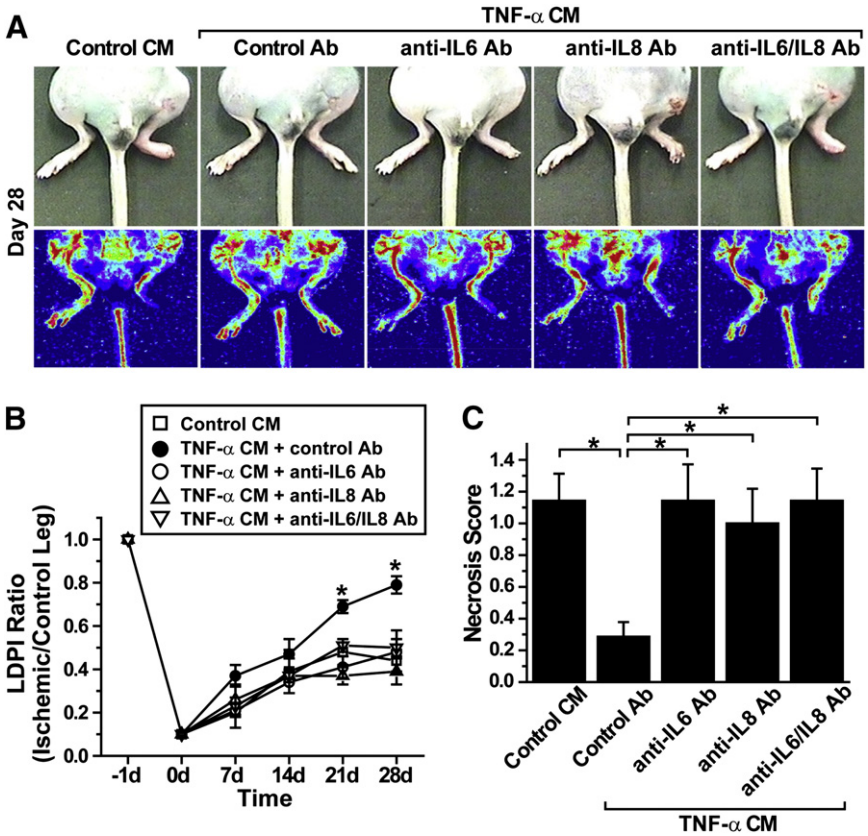

Fig. 3. Role of IL-6 and IL-8 in TNF-α CM-improved blood perfusion and tissue necrosis in the ischemic limb. (A) Representative photographs and LDPI of a mouse hindlimb injected with control CM, TNF-α CM, or IL-6- and/or IL-8-depleted TNF-α CM on day 28. (B) Quantitative analysis of blood perfusion recovery measured by LDPI. The LDPI ratio was calculated as the ratio of ischemic to nonischemic hindlimb blood perfusion. (n = 12 per group). *, $P < 0.05$ vs control CM. (C) Statistical analysis of the necrosis score on day 28. Data indicate mean ± SD (n = 12). *, $P < 0.05$.

dose-dependent chemotactic migration of EPCs and the stimulatory effect of TNF-α CM were more potent than that of control CM. To clarify whether IL-6 and IL-8 are involved in the TNF-α CM-stimulated migration of EPCs, we examined the effects of IL-6- or IL-8-depleted TNF-α CM on migration of EPCs. As shown in Fig. 5B, TNF-α CM-stimulated migration of EPCs was markedly attenuated by immunodepletion of IL-6 or IL-8 from TNF-α CM. In addition, recombinant IL-6 and IL-8 proteins also induced migration of EPCs.

We next examined the question of whether intramuscular injection of TNF-α CM into the ischemic limb can affect homing and engraftment of EPCs into the ischemic hindlimb. To trace engraftment of exogenously transplanted EPCs into the ischemic limb, EPCs were labeled with CM-DiI, a fluorescent tracking dye, followed by i.v. injection of CM-DiI-labeled EPCs and intramuscular injection of TNF-α CM. Sections of the ischemic hindlimb muscle were visualized under confocal microscopy and cell engraftment was quantified. Engraftment of EPCs into the ischemic hindlimb muscle was quantified by counting CM-DiI-positive cells on day 3 after i.v. injection of CM-DiI-labeled EPCs. The number of CM-DiI-positive cells showed an increase in the TNF-α CM-injected group, compared with the HBSS-injected group. Immunodepletion of IL-6 or IL-8 from TNF-α CM resulted in attenuated TNF-α CM-induced engraftment of human EPCs (Fig. 5C and D). In addition, intramuscular injection of recombinant IL-6 or IL-8 proteins stimulated engraftment of CM-DiI-positive cells into the ischemic limb. Furthermore, DM-DiI-positive cells exhibited vWF-positive staining in TNF-α CM-injected tissues on day 28 after transplantation of CM-DiI-labeled EPCs into the ischemic limbs (Fig. 5E). These results suggest that TNF-α CM promotes homing and engraftment of EPCs into newly formed capillaries through IL-6- and IL-8-dependent mechanisms.

To support involvement of IL-6 and IL-8 in TNF-α CM-stimulated neovascularization in the ischemic limbs, we next examined the question of whether intramuscular injection of recombinant IL-6 and IL-8 proteins into ischemic hindlimbs can stimulate neovascularization in vivo. We found that intramuscular injection of IL-6 or IL-8 proteins resulted in a significantly increased blood perfusion in the ischemic limbs four weeks after induction of ischemia (Fig. 6A and B). Injection of recombinant IL-8 protein exhibited higher densities of vWF-positive capillaries and α-SMA-positive vessels than injection of IL-6 protein (Fig. 6C). Co-injection of recombinant IL-6 and IL-8 proteins did not increase further blood perfusion and capillary and vessel densities, compared with the experimental groups injected with either IL-6 or IL-8 protein. Taken together, these results suggest that IL-6 and IL-8 are largely responsible for TNF-α CM-stimulated neovascularization through promotion of homing of EPCs into ischemic tissues, albeit IL-6 and IL-8 do not fully account for TNF-α CM-stimulated neovascularization.

4. Discussion

In the current study, we demonstrate that TNF-α CM stimulated chemotactic migration and in vivo homing of human EPCs through IL-6- and IL-8-dependent mechanisms. In addition, intramuscular injection of TNF-α CM promoted angiogenesis in the ischemic limb through mechanisms involving IL-6 and IL-8. According to one report, significantly increased chronic lesion volumes, worse long-term functional outcome, and impaired angiogenesis were observed in IL-6 knockout mice in a brain stroke animal model [28]. The level of IL-6 was significantly upregulated in the serum and the ischemic adductor muscle of hindlimb ischemic mice and systemic level of IL-6 determined the angiogenic

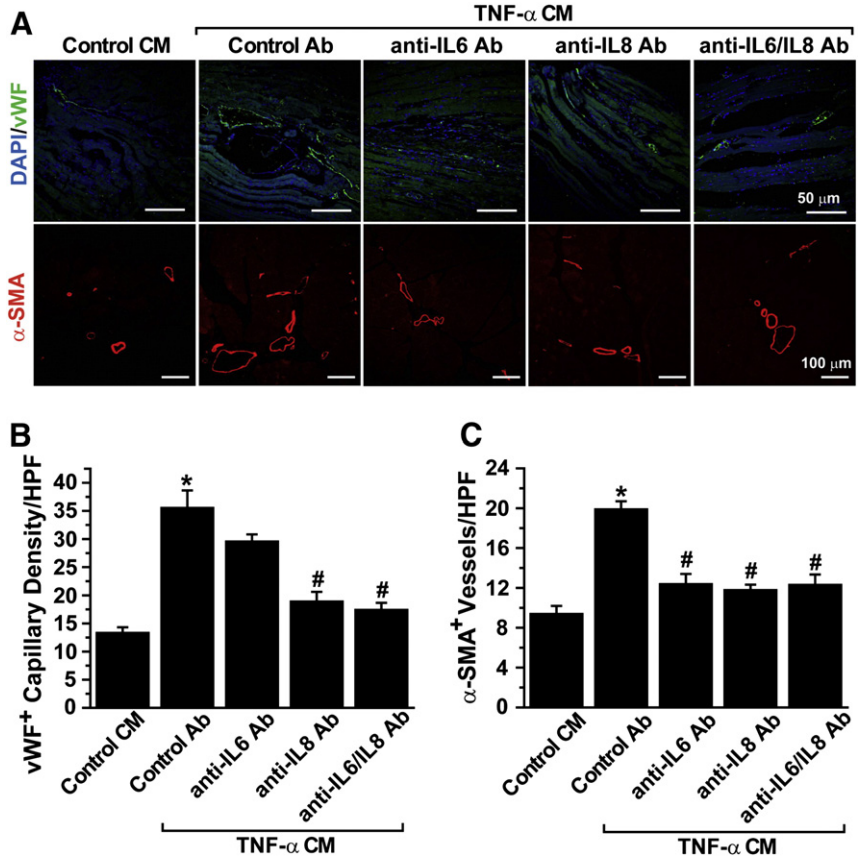

Fig. 4. Role of IL-6 and IL-8 in TNF-α CM-stimulated angiogenesis and vasculogenesis. (A) Immunostaining of vWF-positive capillaries (green color) and α-SMA-positive blood vessels (red color) in the ischemic limbs on day 28 after muscular injection of control CM or TNF-α CM immunodepleted with control, anti-IL-6, and anti-IL-8 antibodies. Nuclei (blue) were counterstained with DAPI, and overlaid images are shown. (B) Quantification of vWF-positive capillaries in the ischemic limb by immunohistochemistry analysis. (C) Quantification of α-SMA-positive arteries in the ischemic limb by immunohistochemistry analysis. *, $P < 0.05$ vs control CM; #, $P < 0.01$ vs control Ab. ($n = 12$ per each group).

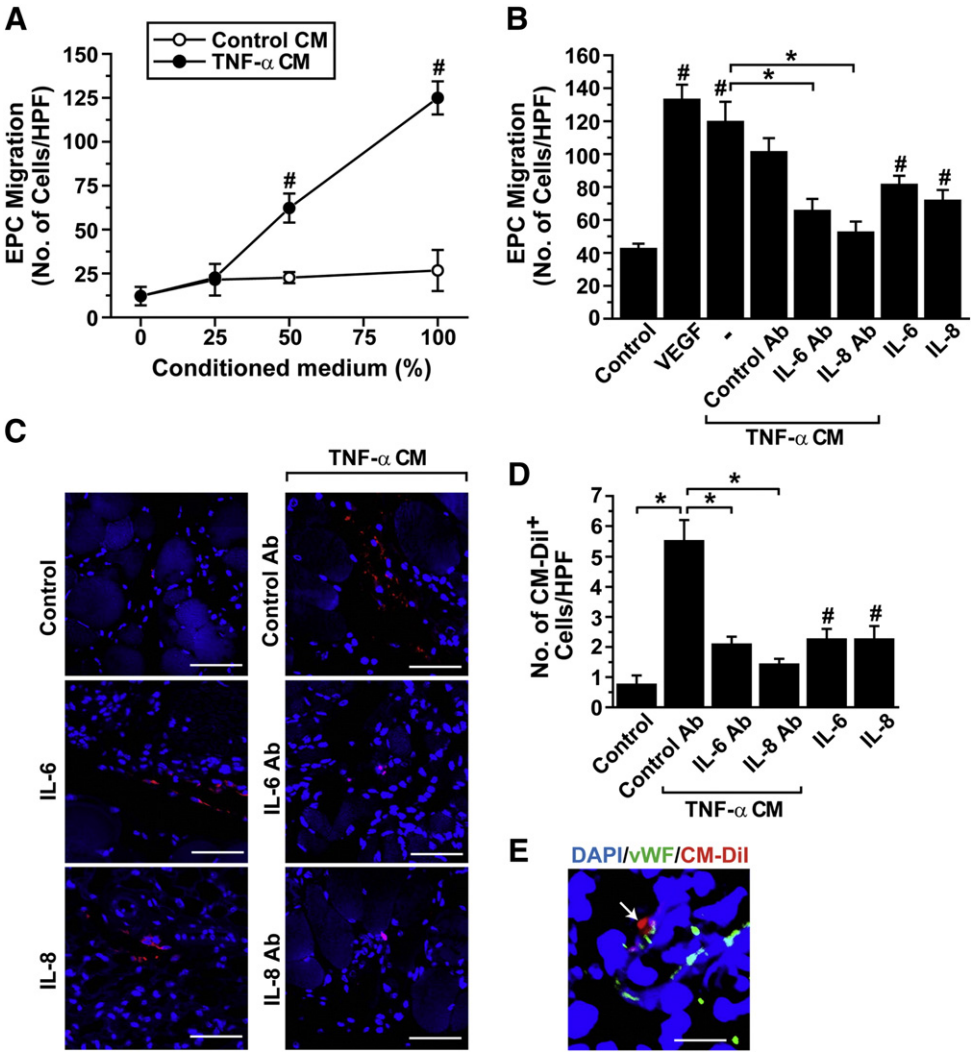

Fig. 5. Role of IL-6 and IL-8 in TNF-α CM-stimulated in vitro migration and in vivo homing of EPCs. (A) Dose-dependent effects of control CM and TNF-α CM on chemotactic migration of EPCs. Data indicate mean ± SD (n = 4). #, P < 0.05. (B) Role of IL-6 and IL-8 in TNF-α CM-stimulated migration of EPCs. Chemotactic migration of EPCs in response to IL-6- or IL-8-depleted TNF-α CM, recombinant IL-6, IL-8, VEGF, and HBSS buffer (control) was examined. Data indicate mean ± SD (n = 4). *, P < 0.05; #, P < 0.05 vs control. (C) Role of IL-6 and IL-8 in TNF-α CM-stimulated in vivo homing of EPCs. The ischemic limbs were injected TNF-α CM immunodepleted with control, anti-IL-6, and anti-IL-8 antibodies, or recombinant IL-6 and IL-8 proteins, and HBSS buffer (control). Homing of EPCs into the ischemic limbs was quantified by confocal microscopic analysis of tissue specimens on day 3 after transplantation of intravenously transplanted CM-DiI-labeled EPCs. Overlaid images of nuclei (DAPI, blue color) and EPCs (red color) are shown. Bar = 50 μm. Representative images from eight different mice are shown (n = 8 per each condition). (D) The number of CM-DiI-positive cells per high power filed was quantified. Data indicate mean ± SD. *, P < 0.05; #, P < 0.05 vs control. (n = 8). (E) Incorporation of EPCs into endothelial vessels. Overlaid image of vWF (endothelial cells), CM-DiI (EPCs), and DAPI (nuclei) is shown and white arrow indicates CM-DiI-positive endothelial cells in TNF-α CM-injected ischemic limbs. Bar = 25 μm.

potential of bone marrow resident monocytes [29]. IL-6 plays an important modulatory role in lung angiogenesis [30]. IL-6 stimulated endothelial cell proliferation and differentiation into capillary-like structures and induced full angiogenic activity in vivo [31]. In addition, IL-6 stimulated angiogenesis of circulating blood-derived EPCs in vitro [32]. In addition to IL-6, up-regulation of IL-8/CXCL8 and related cytokines upon inflammation and ischemia have been reported [33,34]. IL-8 directly enhanced endothelial cell survival, proliferation, and angiogenesis [35,36]. IL-8 was induced in the myocardium after ischemia and reperfusion in vivo [34]. IL-8 stimulates angiogenesis through enhancement of endothelial cell survival, and proliferation [35] and it plays an important role in myocardial neovascularization, protecting against cardiomyocyte apoptosis, and functional cardiac recovery by stimulation of homing of EPCs into ischemic myocardium after acute myocardial infarction [37]. Therefore, these results suggest that both IL-6 and IL-8 promote blood perfusion and repair of ischemic tissues by stimulating the chemotactic migration and angiogenesis of not only mature endothelial cells but also EPCs.

Accumulating evidence suggests that MSCs secrete various angiogenic cytokines such as vascular endothelial growth factor (VEGF), IGF-1, angiopoietin-1, and stromal cell-derived factor-1 (SDF-1) [38–40]. Conditioned medium derived from MSCs stimulated migration of endothelial cell and tube formation [41]. Using liquid chromatography-coupled with tandem mass spectrometry analysis, we reported that TNF-α CM contained high concentrations of various cytokines and chemokines, including IL-6 and IL-8, CXCL2, CXCL5, CXCL6, CXCL10, monocyte chemotactic protein-1, matrix metalloprotease-1, plasminogen activator inhibitor-1, cathepsin L, pentraxin-related protein 3, and complement factors [23]. In addition, treatment with TNF-α has been shown to stimulate secretion of VEGF, hepatocyte growth factor, and insulin-like growth factor I from ASCs [42]. Treatment with TNF-α has been reported to stimulate VEGF secretion up to 1.5 fold in ASCs [42]. However, we could not detect VEGF in control CM and TNF-α treatment did not significantly increase VEGF secretion in hASCs. Furthermore, pre-treatment of TNF-α CM with an anti-VEGF neutralizing antibody did not abrogate TNF-α CM-stimulated migration of EPCs (Figure III in the online-only

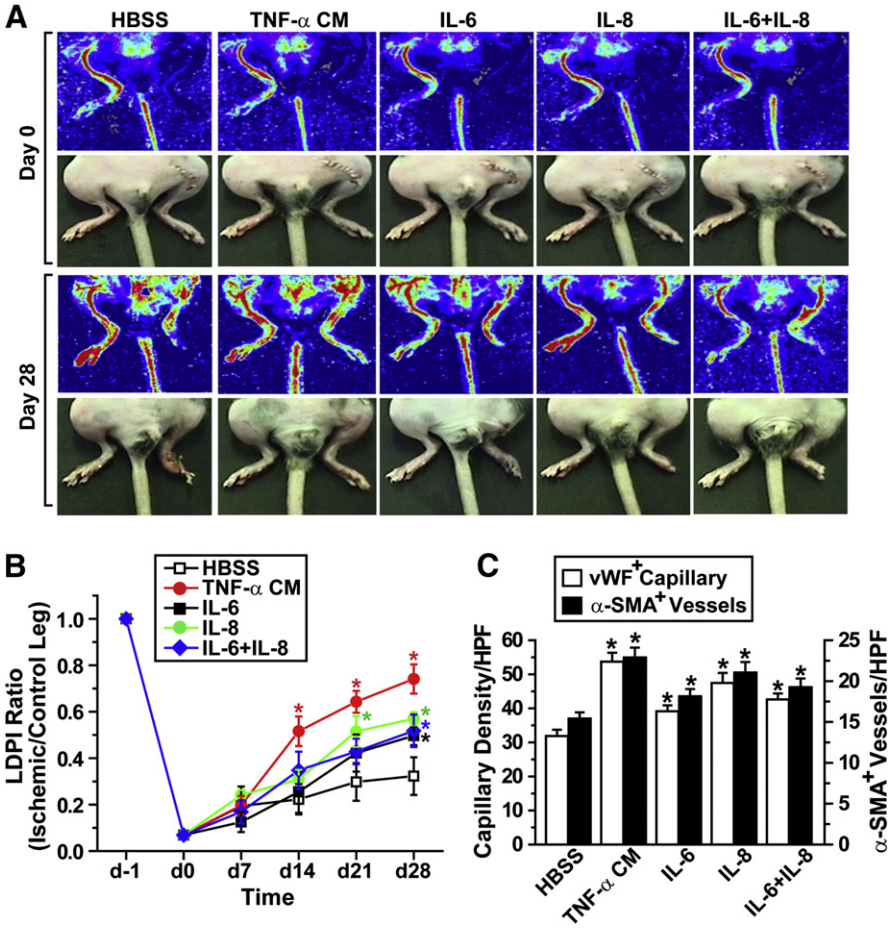

Fig. 6. Effects of recombinant IL-6 and IL-8 proteins in blood perfusion and tissue necrosis in the ischemic hindlimb murine model. (A) Representative photographs and laser Doppler perfusion image (LDPI) of mouse hindlimbs on day 0 and 28 after daily injection of 60 μL Hank's balanced salt solution (HBBS), TNF-α CM, IL-6 (10 ng/mL), IL-8 (10 ng/mL), and IL-6 plus IL-8 (each 10 ng/mL). (B) Quantitative analysis of the blood perfusion recovery measured by LDPI. The LDPI ratio was calculated as the ratio of ischemic to nonischemic hindlimb blood perfusion. (n = 8). *, P < 0.05 vs control (HBSS). (n = 8). (C) Quantification of vWF-positive capillaries and α-SMA-positive arteries in the ischemic limb. *, P < 0.05 vs control (HBSS). (n = 8).

Data Supplement), suggesting that VEGF is not involved in TNF-α CM-stimulated EPC migration and neovascularization. Furthermore, it is possible to hypothesize that exogenously added TNF-α can directly affect blood perfusion and tissue repair. However, this possibility can be excluded because intramuscular injection of recombinant TNF-α did not increase blood perfusion of ischemic hindlimbs (Figure IV in the online-only Data Supplement). In this study, we showed that recombinant IL-6 and IL-8 proteins stimulated chemotactic migration of EPCs, in vivo homing of transplanted EPCs, and blood perfusion in the ischemic limb. However, IL-6 and IL-8 proteins could not fully recapitulate TNF-α CM-stimulated EPC migration, homing, and blood perfusion, whereas immunodepletion of either IL-6 or IL-8 from TNF-α CM abrogated TNF-α CM-stimulated neovascularization, and in vitro migration and in vivo homing of EPCs. These results suggest that both IL-6 and IL-8 are essentially needed for TNF-α CM-stimulated neovascularization as parts of the synergy complex, although IL-6 and IL-8 proteins could not complete the synergy complex. Therefore, it is conceivable that proteases, growth factors, and other paracrine factors secreted from hASCs may be implicated in TNF-α CM-facilitated vasculogenesis and tissue repair in concert with IL-6 and IL-8 as parts of a synergy complex, albeit clarification of the functional role of other paracrine factors in TNF-α CM-stimulated regeneration of ischemic tissues is needed further.

The current study provides evidence that intramuscular injection of TNF-α CM enhanced blood perfusion and inhibited tissue necrosis in the ischemic hindlimb. In addition, the number of proliferating cells was increased in the TNF-α CM-injected ischemic hindlimb. TNF-α CM induced an increase in the number of vWF-positive capillaries and α-SMA-positive arteries/arterioles in the ischemic limb. Consistently, we have shown that topical application of TNF-α CM resulted in accelerated wound healing in a cutaneous wound animal model by stimulating re-epithelialization, proliferation, and angiogenesis [24]. These results suggest that TNF-α CM will be useful for neovascularization and regeneration of not only cutaneous wounds but also peripheral artery disease.

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Disclosures

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.08.002.

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