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ORIGINAL PAPER

Inhibition of macrophage inflammatory protein-1β improves endothelial progenitor cell function and ischemia-induced angiogenesis in diabetes

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Abstract
Systemic inflammation might contribute to the impairment of neovasculogenesis and endothelial progenitor cell (EPC) function in clinical diabetes mellitus (DM). Macrophage inflammatory protein-1β (MIP-1β) is an inflammatory chemokine that may be up-regulated in clinical DM. Its role in diabetic vasculopathy was not clarified. This study aimed to investigate the role of MIP-1β in human EPCs and in neovasculogenesis in different diabetic animal models with hindlimb ischemia. EPCs chamber assay and in vitro tube formation assay were used to estimate the degree of EPC migration and tube formation abilities. *Lepr*^{db}/JNarl mice, C57BL/6 mice fed a high-fat diet, and streptozotocin-induced diabetic mice were used as different diabetic animal models. Laser Doppler imaging and flow cytometry were used to evaluate the degree of neovasculogenesis and the circulating levels of EPCs, respectively. MIP-1β impaired human EPC function for angiogenesis in vitro. Plasma MIP-1β levels were up-regulated in type 2 DM patients. MIP-1β inhibition enhanced the function and the C-X-C chemokine receptor type 4 expression of EPCs from type 2 diabetic patients, and improved EPC homing for ischemia-induced neovasculogenesis in different types of diabetic animals. MIP-1β directly impaired human EPC function. Inhibition of MIP-1β improved in vitro EPC function, and enhanced in vivo EPC homing and ischemia-induced neovasculogenesis, suggesting the critical role of MIP-1β for vasculopathy in the presence of DM.

Keywords Angiogenesis · Diabetes mellitus · Endothelial progenitor cell · Inflammation · Ischemia · Macrophage inflammatory protein-1β

Introduction

It is indicated that more than half of the diabetic patients may suffer from cardiovascular disease, in particular, peripheral vascular disease. Systemic inflammation has been related to the development of diabetic vasculopathy as well as the progression of hyperglycemia in type 1 and type 2 diabetes mellitus (DM) independently [1, 2]. Clinically, macro- and microvasculopathy may develop with the progression of hyperglycemia [3]. Systemic inflammation might be one of the common contributors to both the deterioration of blood sugar and the development of diabetic vasculopathy [4]. It is then interesting to investigate if anti-inflammatory strategy could improve diabetic vasculopathy with or without the stabilization of blood sugar.

Angiogenesis could be impaired with micro- and macrovasculopathy for peripheral vascular complications in patients with type 1 or type 2 DM [5]. Upon vascular injury and tissue ischemia, endothelial progenitor cells (EPCs) can be mobilized from bone marrow for vascular repair and neovasculogenesis [6]. In type 2 DM patients, the number and function of EPCs are impaired with the presence of atherosclerosis vascular complications [7–9]. Both the activation of stromal cell-derived factor 1α (SDF-1α) and its receptor C-X-C chemokine receptor type 4 (CXCR4) modulate EPC

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mobilization. In experimental DM models, CXCR4 blockade prevents EPC recruitment to the ischemic sites [10–12]. However, the specific inflammatory mechanisms for diabetic vasculopathy were not well defined.

Macrophage inflammatory protein-1β (MIP-1β) is a member of the CC chemokine family [13], which could be enhanced in type 1 DM and clinical ischemic diseases [14–16]. MIP-1β expression may be also increased in patients with vulnerable atherosclerosis plaques and stroke lesions [17, 18]. MIP-1β could directly induce reactive oxygen species production and the adhesion of THP-1 cells to endothelial cells [15], enhance endothelial expression of adhesion molecule E-selectin [19], and inhibit SDF-1-induced chemotaxis on B cells [20]. While independently related to diabetic and atherosclerosis disease [21], one may speculate that MIP-1β might contribute to the development of diabetic vasculopathy with impaired angiogenesis/neovasculogenesis.

This study aimed to investigate the potential role of MIP-1β in diabetic vasculopathy in different experimental models. The specific aims of this study were to determine if direct MIP-1β inhibition could improve EPC functions from type 2 diabetic patients, and could increase EPC homing for ischemia-induced neovasculogenesis in both type 1 and type 2 diabetic animals. Our findings may provide a rationale to the novel role of MIP-1β as a potential therapeutic target for diabetic vasculopathy.

Materials and methods

Cell culture

Study subjects, study protocol, and the culture for human EPCs have been detailed in our earlier publication [22]. In brief, blood samples were obtained from the peripheral veins of the healthy volunteers or patients with type 2 DM in the morning hours after an overnight fasting. Only stable type 2 DM patients without insulin treatment were enrolled. Patients with other significant systemic diseases, receiving major operation in the past 6 months, or currently under medical treatment for other diseases were excluded. The human study was approved by the institute research committee and conformed with the Declaration of Helsinki. Because of the recruitment criteria of this study, only 9 individuals in the control group, 23 individuals in the IGT group, and 40 individuals in the DM group were enrolled at that period. The different number of subjects in each group did not result in statistical analysis errors.

After blood sampling, the total mononuclear cells were isolated by density gradient centrifugation with Histopaque-1077 (1.077 g/mL, Sigma, St. Louis, MO, USA). In brief, mononuclear cells were plated in endothelial growth medium (EGM-2 MV Cambrex, East Rutherford, NJ, USA), with supplements (hydrocortisone, R3-insulin-like growth factor 1, human endothelial growth factor, VEGF, human fibroblast growth factor, gentamicin, amphotericin B, vitamin C, and 20% fetal bovine serum) on fibronectin-coated 6-well plates. After 4 days of culture, the medium was changed and non-adherent cells were removed. EPCs emerged 2–4 weeks after the start of the culture of mononuclear cells and exhibited cobblestone morphology. EPCs were grown in EBM-2 (Cascade Biologics) supplemented with fetal bovine serum (5% v/v final concentration) in an atmosphere of 95% air and 5% CO_2 at 37 °C.

Influence of MIP-1β on the expression of CXCR4

Previous study had chosen the concentration 100 or 1000 ng/mL to conduct their experiments [20]. Here, we followed their suggested concentrations and revealed that incubation with MIP-1β (1000 ng/mL) for 30 min may get the best effects on EPCs.

EPCs were incubated with MIP-1β (1000 ng/mL; R&D Systems) for 30 min in an atmosphere of 95% air and 5% CO_2 at 37 °C. After incubation, EPCs were immediately placed on ice to prevent resurfacing of the receptor and then stained with an anti-CXCR4 antibody (R&D Systems). The level of CXCR4 expression was analyzed by flow cytometry with a Cytomic FC 500 (Beckman Coulter, Miami, FL). For the analyses, 5×10^4 EPCs were scored using CXP software (Beckman Coulter).

Evaluation of MIP-1β concentrations in mononuclear cells and EPC supernatants

Supernatant concentrations of MIP-1β proteins released from EPCs were determined by ELISA (R&D Systems) according to the manufacturer's instructions.

EPC proliferation assay

EPCs were incubated with MIP-1β (1000 ng/mL; R&D Systems) or with MIP-1β (1000 ng/mL; R&D Systems) plus an MIP-1β antibody (30 μg/mL; R&D Systems) for 30 min in an atmosphere of 95% air and 5% CO_2 at 37 °C. For the MTT assay, 5 mg/mL of MTT (Sigma, St. Louis, MO, USA) was added and incubated at 37 °C for 4 h. The supernatant was removed, and 100 μL of DMSO was added to each well. Each sample was mixed using a pipette, and the absorbance at 570 nm was determined. In the BrdU cell proliferation assay (Millipore, Darmstadt, Germany), the proliferation ability of pretreated EPCs was evaluated according to the manufacturer's instructions.

EPC migration assay

The migration of EPCs was evaluated using a chamber assay. EPCs (1×10^4 cells) were resuspended in EBM-2 with 5% FBS after pretreatment with MIP-1β (1000 ng/mL; R&D Systems) or with MIP-1β (1000 ng/mL; R&D Systems) plus an MIP-1β antibody (30 μg/mL; R&D Systems) for 30 min. The cells were added to the upper chambers of 24-well transwell plates with polycarbonate membranes. The EBM-2 was supplemented with recombinant SDF-1α (10 ng/mL; R&D Systems) into the lower chamber. The chambers were incubated for 14 h. After incubation, the membranes were fixed with 4% paraformaldehyde and stained using a hematoxylin solution. The numbers of migrated cells were counted in six random high-power (×100) microscopic fields.

EPC tube formation assay

An in vitro tube formation assay was performed with an angiogenesis assay kit (Invitrogen, Carlsbad, CA, USA). ECMatrix gel solution was mixed with ECMatrix diluent buffer and placed in a 96-well plate. Then, 1×10^4 pretreated EPCs were placed in the matrix solution with EGM-2 and incubated for 14 h. Tubule formation was inspected under an inverted light microscope (×40). Four representative fields were imaged, and the average of the total area of complete tubes formed by cells was compared using Image-Pro Plus software.

Western blotting of EPCs

Equal amounts of protein were subjected to SDS-PAGE using 4–12% gradient gels under reducing conditions (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to nitrocellulose membranes (GE Healthcare). Membranes were incubated with antibodies against VEGF (Santa Cruz Biotechnology, Dallas, TX, USA), SDF-1 (Cell Signalling, Boston, MA, USA), and p-ERK1/2 (Cell Signalling, Boston, MA, USA). The immunoblotting expression of VEGF and SDF-1 was normalized to the α-actin expression determined using a mouse monoclonal anti-α-actin antibody.

Preparation of human monocyte supernatants

Peripheral blood mononuclear cells were isolated by Histopaque 1077 density gradient centrifugation. Monocytes were cultured in fresh RPMI 1640 medium containing 5.5 (control) or 25 (H4D) mmol/L glucose and supplemented with 10% FBS for 4 days. After 4 days in culture, the media were collected and defined as H4D medium.

Animal procedures and the mouse hindlimb ischemic model

Six-week-old male BKS.Cg-*Dock7*^{m}+/+ *Lepr*^{db}/JNarl mice, C57BL/6 mice, and FVB/NJNarl mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan). eGFP transgenic mice were kindly provided by Dr. Feng-Yen Lin (Department of Internal Medicine, Taipei Medical University). Mice were all acclimated for 2 weeks before experiments. LabDiet Rodent 5001 [23.9% protein, 5% fat (ether extract), 5% fat (acid hydrolysis)] was used as a standard diet. C57BL/6 mice were fed a high-fat diet (23.1% protein, 34.9% fat, 25.9% carbohydrates, 58Y1) for 14 weeks, and hindlimb ischemia surgery was then conducted. Animals were raised according to the regulations of the Animal Care Committee of National Yang-Ming University.

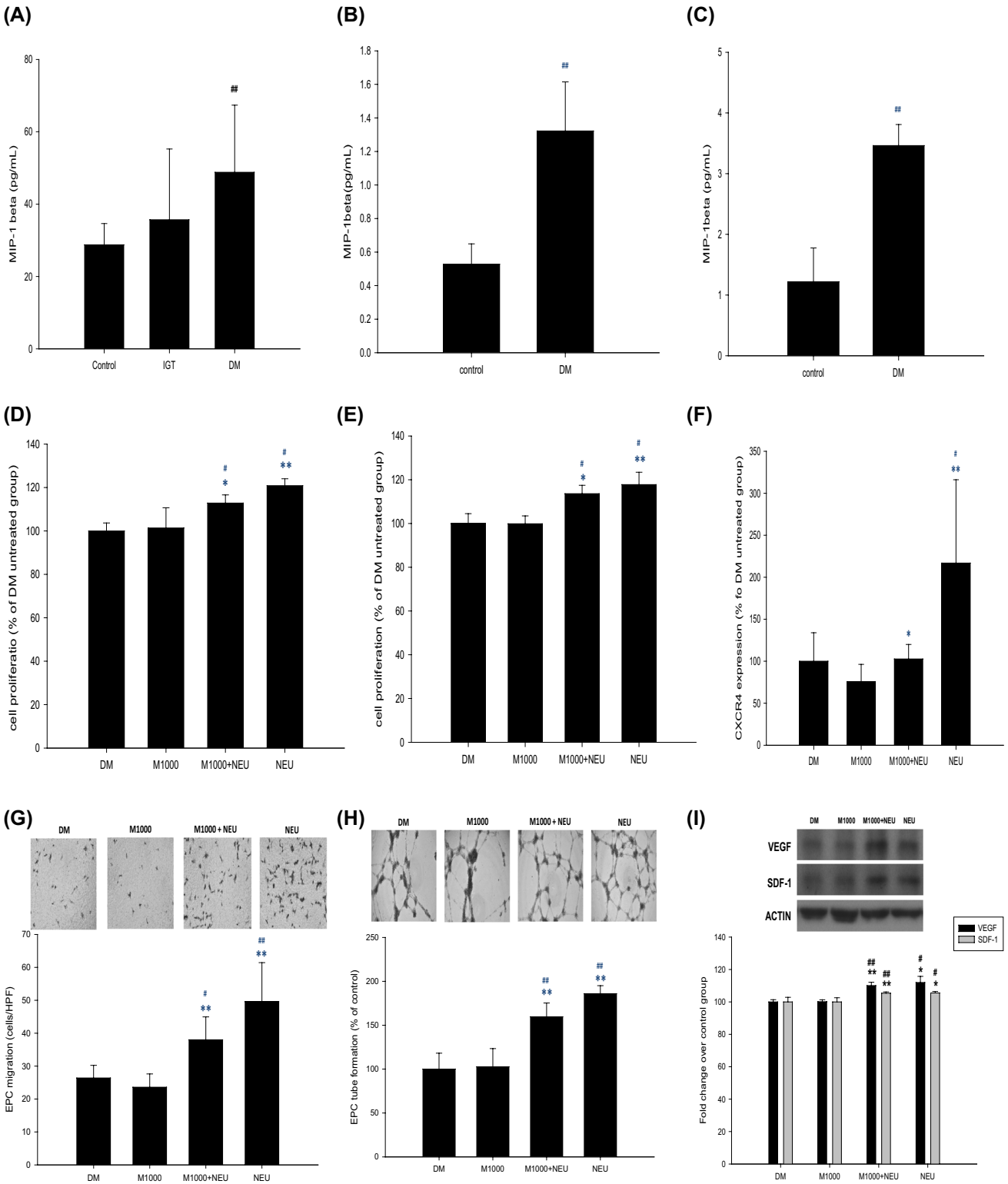
Generation of hyperglycemia in the FVB/NJNarl mice and unilateral hindlimb ischemia surgery have been detailed in our earlier publication [23].

Some diabetic mice received an intraperitoneal injection of an anti-MIP-1β neutralizing monoclonal antibody (mAb, 100 μg; R&D Systems) immediately after the hindlimb ischemia surgery and were injected 3 times per week for 2 or 4 weeks. Mouse IgG_{2A} isotype was administered as a control. Hindlimb blood perfusion was measured with a laser Doppler perfusion imaging system (Moor Instruments Limited, Devon, UK). The results are expressed as the ratio of perfusion in the ischemic limb versus that in the non-ischemic limb. Body weights and blood sugar concentrations were measured.

Bone marrow transplantation model

Recipient male FVB/NJNarl mice at 8 weeks of age were lethally irradiated with a total dose of 9.0 Gy. eGFP transgenic mice that ubiquitously expressed enhanced GFP were used as the donors. After being irradiated, a recipient mouse received unfractionated bone marrow cells (5×10^6) from an eGFP mouse by a tail vein injection.

Six weeks after the bone marrow transplantation, hyperglycemia was generated using streptozotocin (STZ). These diabetic mice underwent unilateral hindlimb ischemic surgery and were injected with an anti-MIP-1β neutralizing monoclonal antibody (mAb, 100 μg; R&D Systems) 3 times per week for 2 or 4 weeks. Repopulation by eGFP positive bone marrow cells was 95%, as measured by flow cytometry. Four weeks after the induction of hindlimb ischemia, ischemic thigh muscles were harvested for histological analysis. eGFP positive cells represented bone marrow-derived cells. Capillaries were investigated using



antibodies directed against CD31 (BD Pharmingen). The EPC density was observed by eGFP/CD31 double-positive cells (yellow color) and evaluated using fluorescence microscopy. Signals were detected by a confocal microscope (Olympus FV1000).

Measurement of capillary densities in the ischemic limbs

Histological analyses of capillary densities in the ischemic limb muscles were determined. The tissues were prepared by

◂**Fig. 1** MIP-1β inhibition sensitized CXCR4 expression and recovered functions of EPCs from diabetic patients and healthy subjects. Levels of MIP-1β in plasma (control, $n=9$; IGT, $n=23$; DM, $n=40$; **a**) and in supernatants from EPCs ($n=6$; **b**) and mononuclear cells ($n=8$; **c**). Cell proliferation was measured by MTT and BrdU cell proliferation assays in EPCs from diabetic patients ($n=3$; **d, e**). CXCR4 expression on EPCs from diabetic patients ($n=6$; **f**). The migration and angiogenesis abilities of EPCs from diabetic patients ($n=5$; **g, h**). Western blotting and statistical analyses of VEGF and SDF-1α expression in EPCs from diabetic patients ($n=3$; **i**). Statistical analyses were performed using unpaired Student's *t* test or analysis of variance, followed by Scheffe's multiple-comparison post hoc test. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the control (untreated basal) group. $^{*}P<0.05$, $^{**}P<0.01$ compared with the MIP-1β 1000 ng/mL alone group

incubation in a 30% sucrose solution for 24 h, then embedding in OCT compound (Sakura), and freezing in liquid nitrogen. For capillary density measurements, two sections taken approximately 3 mm apart were used. The sections were fixed with methanol for 10 min, washed briefly with PBS, and then stained with a monoclonal rat anti-murine platelet–endothelial cell adhesion molecule-1 (CD31) antibody (1:200; BD Pharmingen) at 37 °C for 2 h, followed by incubation with a TRITC-conjugated donkey anti-rat antibody (Jackson ImmunoResearch). Capillaries were counted at three cross-sections and analyzed for each animal, and ten different fields at ×400 from each tissue preparation were randomly selected ($n=6$ in each group). The results were calculated as the number of capillaries per myocyte.

Detection of the mobilization of EPC-like cells by flow cytometry

The mononuclear cells were incubated with fluorescein isothiocyanate (FITC) anti-mouse Sca-1 (eBioscience) and phycoerythrin anti-mouse Flk-1 (VEGFR-2, eBioscience) antibodies at 4 °C for 30 min. The expression of Sca-1^+/Flk-1^+ cells (EPC-like cells) in the mononuclear cells was analyzed by flow cytometry with a Cytomic FC 500 (Beckman Coulter, Miami, FL). For analyses, 10^5 circulating EPC-like cells were quantified by enumerating Sca-1^+/Flk-1^+ cells and were scored using CXP software (Beckman Coulter).

Detection of the CXCR4 protein on bone marrow cells by flow cytometry

The mononuclear cells and bone marrow cells were incubated with fluorescein-conjugated rat anti-mouse CXCR4 (R&D Systems) or phycoerythrin-conjugated rat anti-mouse CXCR4 (R&D Systems) antibodies at 4 °C for 30 min. The expression of CXCR4^+ cells in mononuclear cells was analyzed by flow cytometry with a Cytomic FC 500 (Beckman Coulter, Miami, FL). For analyses, 10^5 circulating CXCR4^+ cells were scored using CXP software (Beckman Coulter).

Detection of the anti-MIP-1β monoclonal antibody in bone marrow

Bone marrow samples were harvested from the thighbones. The expression of the anti-MIP-1β monoclonal antibody in bone marrow cells was detected using a TRITC-conjugated donkey anti-rat antibody (Jackson ImmunoResearch) and the Catch and Release® Reversible Immunoprecipitation System (Millipore, Darmstadt, Germany) according to the manufacturer's instructions.

Western blotting of ischemic thigh muscles

Equal amounts of protein were subjected to SDS-PAGE using 4–12% gradient gels under reducing conditions (Bio-Rad Laboratories) and transferred to nitrocellulose membranes (GE Healthcare). Membranes were incubated with antibodies against VEGF (Santa Cruz Biotechnology, Dallas, TX, USA) and SDF-1α (Cell Signalling, Boston, MA, USA). The immunoblotting expressions of VEGF and SDF-1 were normalized to the α-actin expression determined using a mouse monoclonal anti-α-actin antibody.

Evaluation of MIP-1α, MIP-1β, VEGF, SDF-1 concentrations

Plasma concentrations of MIP-1β proteins were determined by ELISA (R&D Systems) according to the manufacturer's instructions. Plasma concentrations of MIP-1α, MIP-1β, VEGF, and SDF-1 were determined using a MILLIPLEX MAP kit (Millipore, Darmstadt, Germany) according to the manufacturer's instructions.

Statistics

Results are given as the means ± standard errors of the mean (SEM). Statistical analyses were performed using unpaired Student's *t* test or analysis of variance, followed by Scheffe's multiple-comparison post hoc test. SPSS software (version 14; SPSS, Chicago, IL, USA) was used to analyze the data. A *P* value of <0.05 was considered statistically significant.

Results

MIP-1β levels were up-regulated in type 2 diabetic patients

Plasma MIP-1β levels were higher in type 2 diabetic patients than that in control subjects (Fig. 1a). The supernatant MIP-1β expression was also higher in mononuclear cells and EPCs from type 2 diabetic patients than in those from control subjects (Fig. 1b, c).

MIP-1β inhibition sensitized CXCR4 expression and reversed the functions of EPCs from type 2 diabetic patients

The direct effects of MIP-1β and MIP-1β inhibition with an MIP-1β antibody were studied on EPCs from type 2 DM patients. EPC proliferation indicated by MTT and BrdU cell proliferation assays was enhanced by MIP-1β antibody treatment (Fig. 1d, e). While MIP-1β alone had no effects, MIP-1β inhibition increased the CXCR4 expression on EPCs (Fig. 1f). EPCs migration and tube formation abilities were also recovered by MIP-1β inhibition (Fig. 1g, h). Additionally, MIP-1β inhibition increased vascular endothelial growth factor (VEGF) and SDF-1α expression on EPCs (Fig. 1i).

Accordingly, MIP-1β inhibition directly improved the function of EPCs from type 2 DM patients.

MIP-1β desensitized CXCR4 expression and impaired functions of EPCs from healthy human subjects

While MIP-1β or MIP-1β inhibition alone treatments had no effects, MIP-1β inhibition with MIP-1β antibody significantly increased the proliferation of EPCs from control projects, indicating that MIP-1β inhibition only acted its function in high MIP-1β environments (Fig. 2a, b). MIP-1β decreased CXCR4 expression (Fig. 2c), abrogated the migration and tube formation (Fig. 2d, e), and reduced VEGF as well as SDF-1α levels (Fig. 2f) of EPCs. All of them were improved by MIP-1β inhibition (Fig. 2c–f). MIP-1β levels were induced in culture media from monocytes after high glucose condition for 4 days compared with control (Fig. 2g). Moreover, MIP-1β treated directly led to p-ERK1/2 suppression and MIP-1β inhibition could reverse the p-ERK1/2 expressions (Fig. 2h).

Accordingly, MIP-1β could be released from monocytes in high glucose condition. And, MIP-1β may directly impair the functions of EPCs from control subjects via VEGF/SDF-1α/p-ERK1/2.

MIP-1β inhibition improved neovasculogenesis by increasing EPC homing and up-regulating VEGF and SDF-1 expression in streptozotocin-induced diabetic mice

Serum MIP-1β levels were up-regulated in STZ-induced diabetic mice (simulating type 1 DM in humans) compared to the non-diabetic control mice (Fig. 3a). In this set of experiments, a bone marrow transplantation model was used to investigate if MIP-1β inhibition could improve bone marrow-derived EPC homing. Blood flow ratios were improved both in the diabetic mice that received an MIP-1β antibody injection for 2 weeks and for 4 weeks (Fig. 3b, c). Capillary densities and homing numbers of bone marrow-derived EPCs in the ischemic limbs were increased in mice with MIP-1β antibody injection compared to those mice without treatment (Fig. 3d, e). The number of EPCs was recovered and was still increased by MIP-1β inhibition for 2 weeks (Fig. 3f). The tissue expressions of VEGF and SDF-1α in ischemic muscle were higher in mice with MIP-1β inhibition compared to untreated mice (Fig. 3g, h). Besides, plasma MIP-1α and MIP-1β levels were higher in diabetic mice than in non-diabetic mice. Treatment with MIP-1β antibody decreased plasma MIP-1β levels without affecting MIP-1α levels, suggesting the specific neutralization ability of the antibody. MIP-1β inhibition also increased plasma VEGF and SDF-1α level in diabetic mice (Fig. 3i). Bone marrow cells were isolated and detected by a TRITC-conjugated donkey anti-rat antibody to show that the MIP-1β antibody could enter the bone marrow of diabetic mice, (Fig. 3k). Finally, MIP-1β inhibition reversed the decreased CXCR4 expression on peripheral mononuclear cells and bone marrow cells in diabetic mice (Fig. 3j, l).

Accordingly, MIP-1β inhibition enhanced VEGF and SDF-1α expression, reversed CXCR4 expression of peripheral mononuclear cells and bone marrow cells, increased EPC homing, and improved ischemia-related neovasculogenesis in type 1 diabetic animals.

MIP-1β inhibition improved neovasculogenesis in diabetic db/db mice

Compared to non-diabetic mice, serum MIP-1β levels were up-regulated in diabetic db/db mice (a model simulating type 2 DM in humans) (Fig. 4a). In each group of mice, blood flow in the ischemic hindlimb was equally reduced by hindlimb ischemia surgery (Fig. 4b). During the postoperative weeks, perfusion recovery was attenuated in db/db mice compared with non-diabetic mice. Treatment with MIP-1β antibody injection for 2 weeks or for 4 weeks significantly improved blood flow ratios in db/db mice (Fig. 4b, c). Compared to that in non-diabetic mice, capillary densities in ischemic limbs were reduced in db/db mice, which were significantly improved by MIP-1β antibody treatment (Fig. 4d, e). Compared with that in non-diabetic mice, the circulating EPC number was much less increased in the db/db mice at 2 days after ischemic surgery. Treatment with MIP-1β antibody significantly increased circulating EPCs in db/db mice (Fig. 4f). Besides, CXCR4 expression on bone marrow cells was decreased in db/db mice, which could be also improved by MIP-1β inhibition (Fig. 4g).

MIP-1β inhibition improved neovasculogenesis in C57BL/6 mice fed a high-fat diet

Serum MIP-1β levels were up-regulated in mice fed a high-fat diet (another model simulating pre- or early

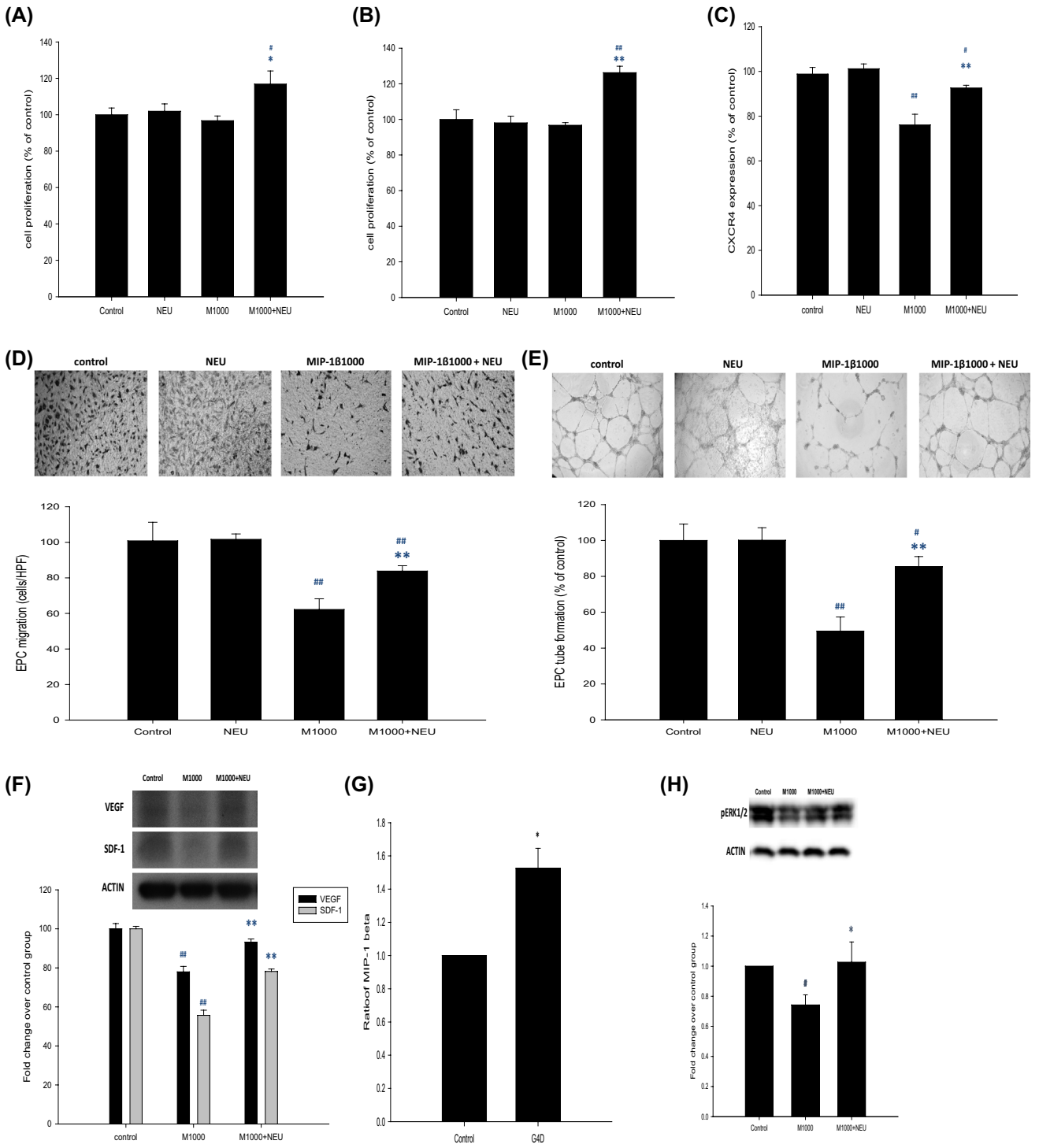
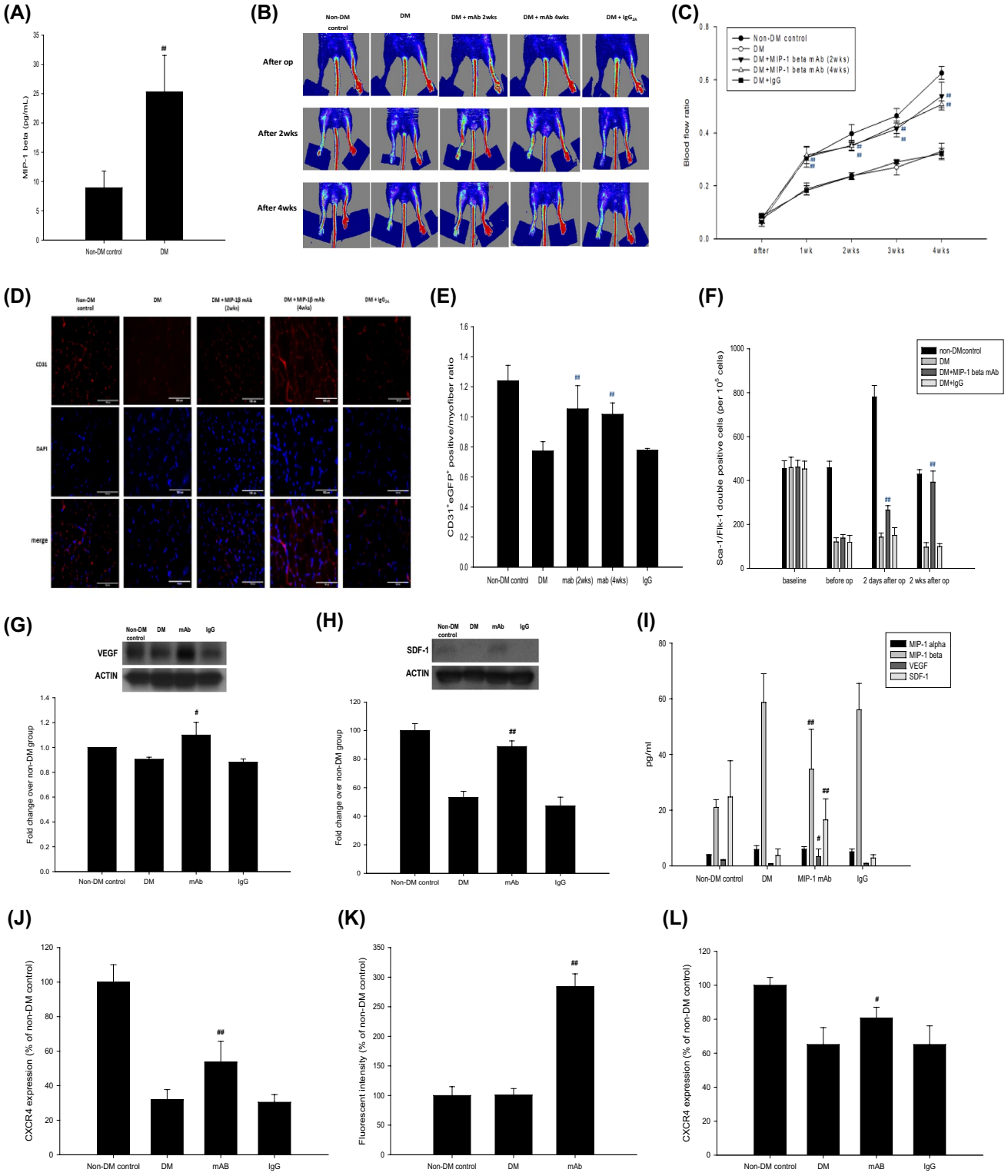


Fig. 2 MIP-1β inhibition sensitized CXCR4 expression and reversed functions of EPCs from healthy subjects. Cell proliferation was measured by MTT and BrdU cell proliferation assays in EPCs from control subjects ($n=3$; **a, b**). CXCR4 expression on EPCs from control subjects ($n=6$; **c**). The migration and angiogenesis abilities of EPCs from control subjects ($n=5$; **d, e**). Western blotting and statistical analyses of VEGF and SDF-1α expression in EPCs from control subjects ($n=3$; **f**). Levels of MIP-1β in supernatants from monocytes were analyzed by ELISA ($n=3$; **g**). Western blotting and statistical analyses of p-ERK1/2 expression in EPCs from control subjects ($n=3$; **h**). Statistical analyses were performed using unpaired Student's *t* test or analysis of variance, followed by Scheffe's multiple-comparison post hoc test. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with the control (untreated basal) group. $^{*}P<0.05$, $^{**}P<0.01$ compared with the MIP-1β 1000 ng/mL alone group



type 2 DM in humans) compared to mice with normal diet (Fig. 4h). Blood flow ratios (Fig. 4i, j) and capillary densities in ischemic hindlimb (Fig. 4k, l) were significantly reduced in high-fat-diet mice compared to normal-diet mice, which was much improved by either MIP-1β antibody injection for 2 weeks or for 4 weeks. Two days after ischemic surgery, the circulating EPC number was much less increased in high-fat-diet mice compared with normal-diet mice. Treatment with MIP-1β antibody significantly improved circulating EPC number (Fig. 4m) and

◂**Fig. 3** Effects of MIP-1β inhibition on bone marrow-derived EPC homing in ischemia-induced neovasculogenesis in STZ-induced diabetic mice. Serum levels of MIP-1β ($n=6$; **a**). Foot blood flow monitored in vivo by LDI in each group of diabetic mice. Representative evaluation of the ischemic (right) and non-ischemic (left) hindlimbs, before, immediately after, and 4 weeks after the surgery. In color-coded images, red indicates normal perfusion and blue indicates a marked reduction in blood flow in the ischemic hindlimb. Color-coded images and blood flow ratios ($n=6$–8; **b, c**). Hindlimb ischemia was created in mice that received eGFP mouse bone marrow cells and then were induced to a diabetic state by STZ injection. MIP-1β inhibition groups showed more GFP/CD31 double-positive cells in ischemic muscle tissues than the untreated diabetic mice group. The histogram shows the eGFP-CD31 double-positive signaling/myofiber ratio. ($n=6$–8; **d, e**). Circulating EPCs ($n=6$; **f**) and Western blotting and statistical analyses of VEGF and SDF-1α after 2 weeks of MIP-1β neutralizing antibody injections in diabetic mice ($n=3$; **g, h**). Plasma MIP-1α, MIP-1β, VEGF, and SDF-1 levels were measured using a MILLIPLEX MAP kit after 2 weeks of MIP-1β neutralizing antibody injections ($n=6$; **i**). Levels of MIP-1β neutralizing antibody in bone marrow ($n=6$; **k**). CXCR4 expression on peripheral mononuclear cells and bone marrow cells ($n=6$–10; **j, l**). Mab represents MIP-1β inhibition group. Statistical analyses were performed using unpaired Student's t test or analysis of variance, followed by Scheffe's multiple-comparison post hoc test. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with control group or untreated DM mice

reversed the decreased CXCR4 expression on bone marrow cells (Fig. 4n) in high-fat-die mice.

The comparable findings of the above two in vivo experiments suggest the universal beneficial effects of MIP-1β inhibition on angiogenesis in different animal models simulating clinical type 2 DM.

MIP-1β impaired neovasculogenesis by desensitizing CXCR4 on mononuclear cells and by decreasing circulating EPCs in non-diabetic mice

To confirm that MIP-1β could directly impair neovasculogenesis through desensitizing CXCR4 expression, MIP-1β was injected into normal mice immediately after the hindlimb ischemic surgery. Perfusion recovery was attenuated in the MIP-1β-injected mice compared with the control group during the postoperative weeks (Fig. 5a, b). The EPC number was also attenuated in the MIP-1β injected mice compared to non-injected mice (Fig. 5c). MIP-1β injections also weakened CXCR4 expression on peripheral mononuclear cells and bone marrow cells in normal mice (Fig. 5d, e). These data imply the direct inhibition of MIP-1β on in vivo neovasculogenesis.

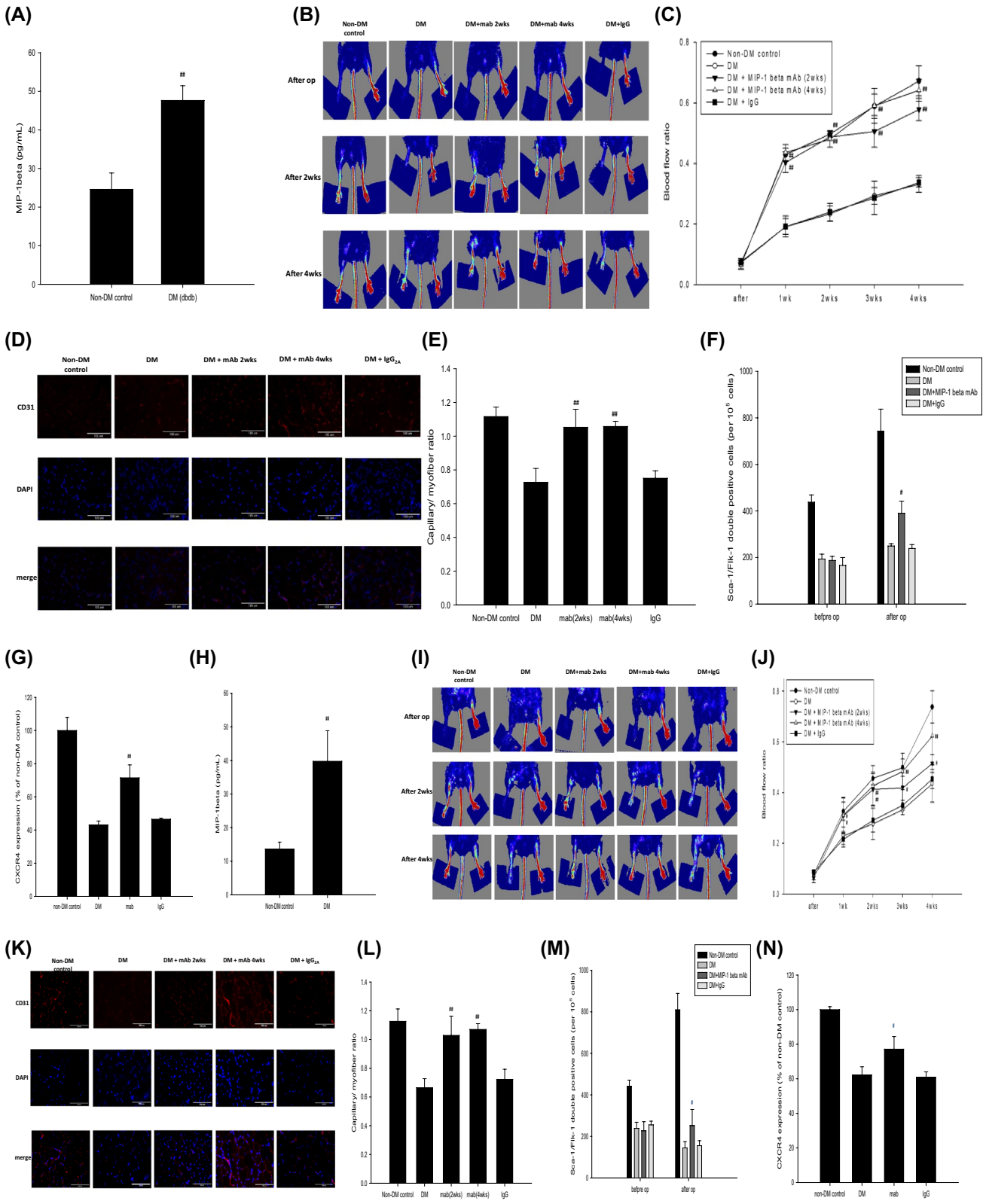
Discussion

There are several fundamental findings of this study. First, mononuclear cells and EPCs from diabetic patients secreted more MIP-1β than that from healthy subjects. Treatment with MIP-1β decreased CXCR4 expression and impaired EPC function. MIP-1β released from monocytes was up-regulated after high glucose treatments. MIP-1β inhibition with a specific MIP-1β antibody reversed the damages of MIP-1β and enhanced VEGF, SDF-1α, and p-ERK1/2 expression on EPCs. Second, MIP-1β inhibition increased the homing abilities of bone marrow-derived EPCs, sensitized CXCR4 expression, enhanced circulating and ischemic muscle VEGF and SDF-1 expression, and improved neovasculogenesis in diabetic mice. Third, MIP-1β attenuated neovasculogenesis by decreasing the number of circulating EPCs and desensitizing CXCR4 expression in non-diabetic mice, indicating the direct effects of MIP-1β on neovasculogenesis.

Our findings are in line with the previous evidence that MIP-1β may play a notorious role in cardiovascular diseases [21]. For example, MIP-1β expression increased in atherosclerosis plaques in stroke patients [17, 18] and could be up-regulated in the infarct mouse myocardium [24, 25]. Further, hypertensive patients with higher serum MIP-1β levels showed a higher risk of future stroke and cardiovascular events [15]. However, angiogenesis is known to be defective in peripheral limbs but enhanced in retinas in diabetes. The VEGF and SDF-1α expressions are decreased in ischemic muscles but elevated in diabetic retinopathy, suggesting that the same angiogenic factors may contribute to opposing results at different sites of a subject [26, 27]. Likewise, MIP-1β could be up-regulated with increased revascularization in hypoxic retinas [28], but might impair neovasculogenesis in ischemic muscles of diabetic animals. VEGF is known to be a pro-angiogenic factor that signals to ERK [29]. In our study, it was first shown that MIP-1β directly attenuated VEGF, SDF-1α, and p-ERK1/2 expressions in vitro. MIP-1β inhibition may reverse the pathway and improve EPC function and neovasculogenesis in different types of diabetic animals. Further study may be conducted to elucidate the potential effects of MIP-1β inhibition on diabetic retinopathy.

MIP-1β levels could be enhanced in type 1 DM and clinical ischemic diseases [14–16]. However, the resources of MIP-1β are not fully known. In this study, we not only revealed that MIP-1β levels were enhanced in type 2 diabetic patients which consisted with previous studies but discovered that supernatant MIP-1β expression was also higher in mononuclear cells and EPCs from type 2 diabetic patients. Moreover, high glucose may act as a direct stimulator for monocytes to release more MIP-1β. These observations may provide some clues to the comprehensive pathological role of MIP-1β in DM.

MIP-1β is one of the ligands of fifth CC chemokine receptor (CCR5). The function of MIP-1β was previously considered to act through CCR5 signaling. However, previous evidence indicated that CCR5 antagonists can inhibit some but not all of the proinflammatory effects of CCR5



ligands [30, 31], suggesting the instability of anti-CCR5 mechanisms. Besides, a naturally truncated MIP-1β, lacking the 2 NH_2-terminal amino acids, could signal through CCR1 and CCR2b [32–34]. The CCRD6 also binds to human MIP-1β [35], implying that the signaling pathways of MIP-1β should not be limited. Since MIP-1β and CCR5

◂**Fig. 4** Effects of MIP-1β inhibition on ischemia-induced neovasculogenesis in db/db mice and in C57BL/6 mice fed a high-fat diet. Serum levels of MIP-1β in db/db mice ($n=6$; **a**) and in C57BL/6 mice fed a high-fat diet ($n=6$; **h**). Color-coded images and blood flow ratios in db/db mice ($n=6$; **b, c**) and in high-fat diet C57BL/6 mice ($n=6$; **i, j**). Anti-CD31 immunostaining showed an increase in capillary formation in the MIP-1β inhibition group in db/db mice ($n=6$; **d, e**) and in the high-fat diet C57BL/6 mice ($n=6$; **k, l**). The number of circulating EPCs was increased in the MIP-1β inhibition group in db/db mice ($n=6$; **f**) and in the high-fat diet C57BL/6 mice ($n=6$; **m**). CXCR4 expression on bone marrow cells in db/db mice ($n=6$; **g**) and in the high-fat diet C57BL/6 mice ($n=6$; **n**). Mab represents MIP-1β inhibition group. Statistical analyses were performed using unpaired Student's *t* test or analysis of variance, followed by Scheffe's multiple-comparison post hoc test. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with untreated DM mice

are not the only ligand and receptor, respectively, for each other, blockade of MIP-1β itself might be a more specific strategy than blocking its receptor(s) to investigate the MIP-1β related effects and mechanisms.

It was shown that non-obese diabetic MIP-1α−/− mice but not non-obese diabetic MIP-1β−/− mice exhibited reduced destructive insulitis and were protected from type 1 DM [36], which suggest that mice born with MIP-1β−/− might not benefit DM. However, no research related to angiogenesis was revealed in MIP-1β−/− mice. In this study, we focused on pathological instead of physiological function of MIP-1β and tried to figure out the direct role of MIP-1β in neovasculogenesis in diabetic animals. While the MIP-1β antibody used in our study was proven specific without interaction with MIP-1α in vivo, a conditional knock-out mice model may be helpful to confirm our findings with MIP-1β inhibition in the future.

Additionally, in this study, the EPCs migration ability toward SDF-1α, which is a ligand of CXCR4, was abrogated afer pretreatment with MIP-1β (1000 ng/mL) for 30 min. Accordingly, our in vitro results indicate the important and direct effect of MIP-1β on CXCR4. However, MIP-1β is usually induced upon assaults and its concentration may be much varied during in vivo disease conditions. Given that the concentration we used in vitro was much higher than the plasma concentration of MIP-1β, further in vivo study may be required to confirm the direct effects of MIP-1β on CXCR4 if indicated.

In conclusion, plasma MIP-1β was increased in type 2 diabetic patients. Both intrinsic and extrinsic MIP-1β impaired human EPC function, which were improved by MIP-1β inhibition. Further, MIP-1β inhibition increased EPC number and function, enhanced bone marrow-derived EPC homing, and improved neovasculogenesis both in type 1 and type 2 DM animals. Accordingly, MIP-1β-related inflammatory mechanisms might contribute to vascular impairment in both clinical and experimental DM. A novel therapeutic strategy targeting on MIP-1β may be validated for potential clinical implication in the future.

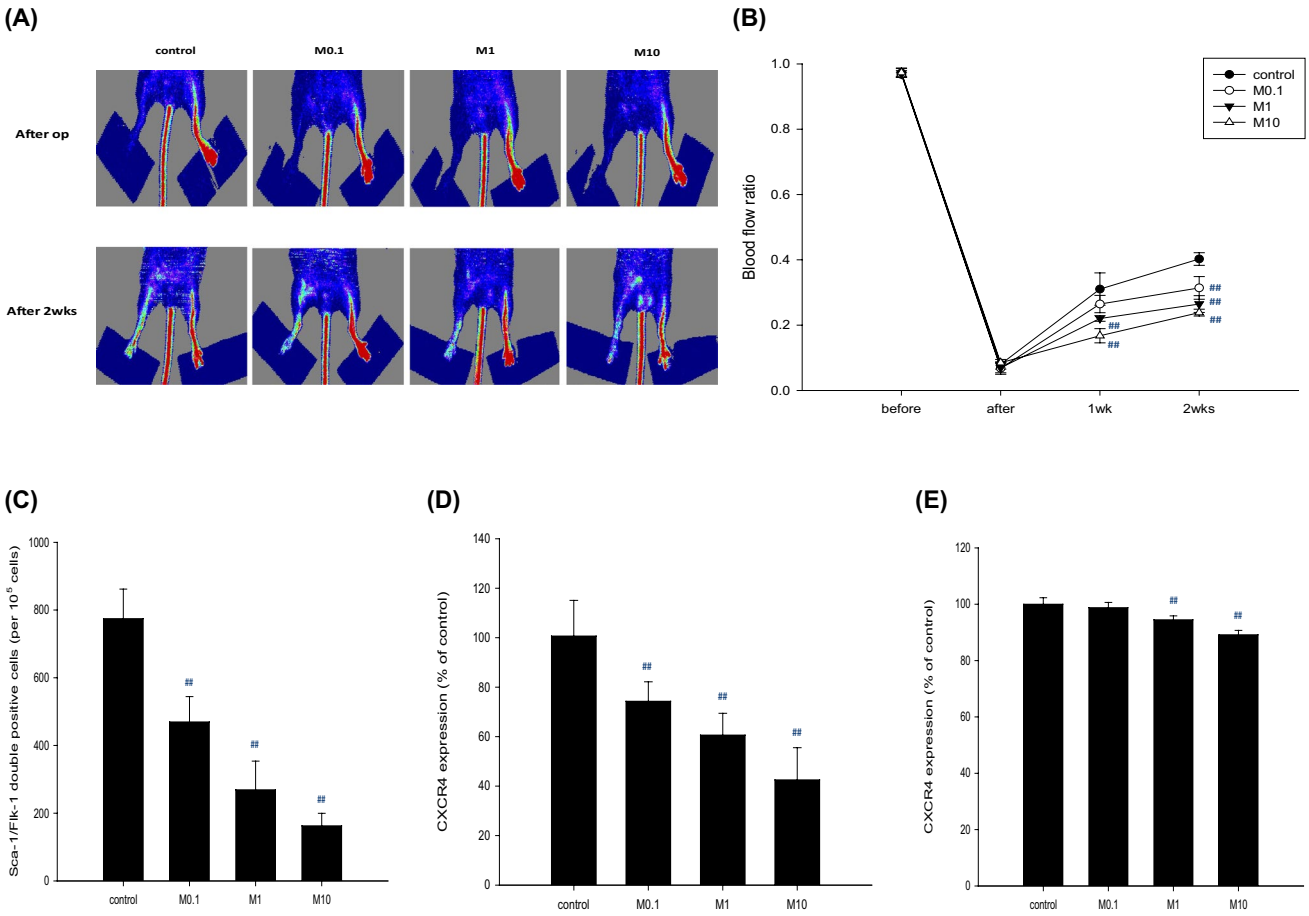


Fig. 5 Effects of MIP-1β injections on neovasculogenesis in non-diabetic mice. Color-coded images and blood flow ratios in non-diabetic mice ($n=6$; **a, b**). The number of circulating EPCs was measured in normal mice 1 day after MIP-1β injection at 0.1, 1, or 10 μg ($n=6$; **c**). CXCR4 expression on peripheral mononuclear cells and bone marrow cells ($n=6$; **d, e**). Mab represents MIP-1β inhibition group. Statistical analyses were performed using unpaired Student's *t* test or analysis of variance, followed by Scheffe's multiple-comparison post hoc test. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with control group or untreated DM mice

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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