Biochimica et Biophysica Acta 1853 (2015) 431–444

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Urokinase type plasminogen activator mediates Interleukin-17-induced peripheral blood mesenchymal stem cell motility and transendothelial migration

Jelena Krstić [a,1], Hristina Obradović [a,1], Aleksandra Jauković [a], Ivana Okić-Đorđević [a], Drenka Trivanović [a], Tamara Kukolj [a], Slavko Mojsilović [a], Vesna Ilić [b], Juan F. Santibañez [a,*], Diana Bugarski [a]

[a] Laboratory for Experimental Hematology and Stem Cells Institute for Medical Research, University of Belgrade, Dr Subotića 4, 11129 Belgrade, Serbia
[b] Laboratory for Immunology, Institute for Medical Research, University of Belgrade, Dr Subotića 4, 11129 Belgrade, Serbia

ARTICLE INFO

Article history:
Received 2 July 2014
Received in revised form 18 November 2014
Accepted 19 November 2014
Available online 27 November 2014

Keywords:
Interleukin-17
Peripheral blood mesenchymal stem cells
Urokinase type plasminogen activator
Migration
Transendothelial migration

ABSTRACT

Mesenchymal stem cells (MSCs) have the potential to migrate toward damaged tissues increasing tissue regeneration. Interleukin-17 (IL-17) is a proinflammatory cytokine with pleiotropic effects associated with many inflammatory diseases. Although IL-17 can modulate MSC functions, its capacity to regulate MSC migration is not well elucidated so far. Here, we studied the role of IL-17 on peripheral blood (PB) derived MSC migration and transmigration across endothelial cells. IL-17 increased PB-MSC migration in a wound healing assay as well as cell mobilization from collagen gel. Concomitantly IL-17 induced the expression of urokinase type plasminogen activator (uPA) without affecting matrix metalloproteinase expression. The incremented uPA expression mediated the capacity of IL-17 to enhance PB-MSC migration in a ERK1,2 MAPK dependent way. Also, IL-17 induced PB-MSC migration alongside with changes in cell polarization and uPA localization in cell protrusions. Moreover, IL-17 increased PB-MSC adhesion to endothelial cells and transendothelial migration, as well as increased the capacity of PB-MSC adhesion to fibronectin, in an uPA-dependent fashion. Therefore, our data suggested that IL-17 may act as chemotropic factor for PB-MSCs by incrementing cell motility and uPA expression during inflammation development.

1. Introduction

Cell migration is a complex process involving many both intra- and extracellular components, as well as the signaling events linking them. Due to their ability to preferentially migrate to sites of inflammation and tissue injury, and their immunomodulatory properties, mesenchymal stem cells (MSCs) have great potential for tissue regeneration and cellular therapy [1]. However, the molecular signals that guide MSCs to target tissues remain to be fully elucidated. One of the most remarkable, but least understood findings is the ability of MSCs to migrate from bone marrow or peripheral blood into damaged tissues to promote regeneration [2,3]. Although MSCs can be recruited to damaged or inflamed tissue by inflammatory cytokines [1], the molecular and cellular mechanisms mediating the recruitment of MSCs are not well understood so far.

Various inflammatory and autoimmune diseases, such as rheumatoid arthritis, psoriasis or inflammatory myopathies, include infiltration of Th17 cells into the tissue and high production of Interleukin-17 cytokines (IL-17) [4]. IL-17A, a founding member of the cytokine family has been known to regulate the pro-inflammatory responses by acting on different stromal cells, stimulating them to secrete other soluble and membrane-bound factors, among which are IL-6, G-CSF, GM-CSF, SCF, and NO. In addition, stromal cells, including bone marrow (BM) MSCs, express high levels of IL-17 receptor A (IL-17RA) and IL-17 has been shown to affect the proliferation and differentiation of MSCs [5,6]. Also, IL-17 affects MSC differentiation by inhibiting adipogenesis and myogenesis while promoting osteogenesis [7,8]. Nevertheless, the roles which IL-17 can have in MSC physiology are under research, and little is known about its involvement in MSC migration and homing to inflamed tissue.

In response to tissue damage MSCs are mobilized from bone marrow to the blood circulation and "invade" the target tissue [1,2]. A key requirement for cells to reach distant target sites is their ability to traverse the protein fibers of the extracellular matrix (ECM) which are present between cells of all tissue types [9]. To overcome these matrix barriers, migrating cells require specific proteolytic enzymes. One of the most important enzymes which enables cell migration is urokinase plasminogen activator (uPA). uPA is a proteolytic enzyme which belongs to the group of trypsin-like enzymes. uPA mediates the proteolytic cleavage of

* Corresponding author at: Institute for Medical Research, University of Belgrade, Dr Subotića 4, 11129 Belgrade, Serbia. Tel.: +381 11 2685 788; fax: +381 11 2643 691.
E-mail address: jfsantibanez@imi.bg.ac.rs (J.F. Santibañez).
[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbamcr.2014.11.025
0167-4889/

plasminogen to give plasmin [10,11]. Plasmin, a protease with a broad spectrum of activity, is able to directly break down the components of the ECM, such as fibrin, fibronectin, laminin, collagen IV and the protein backbone of the proteoglycans [11]. Biosynthesis and activity of uPA are associated with the invasive capacity of various cell types such as leukocytes, endothelial cells, and metastasizing tumor cells. In addition to traversing the ECM, during their migration through the body, MSCs need to pass through the endothelium in order to enter the target tissue. Regarding their mobilization to the sites of inflammation, MSCs probably hold properties similar to immune cells. Hence, the well described transendothelial migration of leukocytes can serve as a starting model for MSC transendothelial migration [12]. Certain proinflammatory cytokines, such as IL-1β and TNF-α are known to increase the migratory and adhesion capacity of MSCs in vivo, by up-regulating adhesive proteins and receptors for some chemokines in these cells [1]. Although IL-17 is present in damaged or inflamed tissues and uPA recently has been implicated in migration and tropism to tumor microenvironment [13–15], the capacity of IL-17 to induce peripheral blood MSC (PB-MSC) migration and endothelium transmigration, as well as the involvement of uPA in these processes, was not elucidated so far. Therefore, in this work, we have examined whether IL-17, as a proinflammatory cytokine, is able to modulate the expression of uPA in PB-MSCs, and its importance in PB-MSC motility, endothelial cell adhesion and transendothelial migration.

2. Material and methods

2.1. Cell culture

The PB-MSC isolation was previously described [16]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from the peripheral blood of healthy donors by density gradient centrifugation on lymphocyte separation media (PAA Laboratories, Linz, Austria), and plated in cell culture flasks in growth medium (GM) consisting of 10% fetal bovine serum (FBS) and 100 units/ml Penicillin/Streptomycin (both from PAA Laboratories) in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St Luis, MO, USA) and cultured in a humidified atmosphere at 37 °C with 5% CO_2. The medium was replaced twice a week and nonadherent cells were discarded. Adherent, fibroblast-like cells were detached and seeded in a new flask in GM. Following the first confluence, cells were passaged regularly, and after three passages a homogenous cell culture was obtained. PB-MSCs obtained displayed a multipotent capacity of mesenchymal differentiation, since under appropriate conditions they differentiated to osteogenic, chondrogenic, adipogenic, and myogenic lineages. In addition, they were fully characterized and displayed a normal karyotype [16].

Human microvascular endothelial cells (HMEC cell line) were kindly provided by Dr. C. Bernabeu (CIB, CSIC, Spain). Cells grown in 0.2% gelatin-coated flasks in endothelial cell (EC) medium consisting of 50% DMEM, 50% Ham's F12 (Sigma-Aldrich), 1 μM hydrocortisone (Galenika, Belgrade, Serbia), 20 ng/ml EGF (R&D Systems, Minneapolis, MN, USA) and 5 μl/ml ECGS (Sigma-Aldrich). Myoblast C2C12 cell line was purchased from American Type Culture Collection (ATCC, CRL-1772) and cultured in DMEM supplemented with 10% fetal. The prostatic adenocarcinoma derived PC-3 cell line was obtained from ATCC (CRL-1435) and grown in Hams F12:DMEM (1:1) supplemented with 10% FBS.

2.2. Antibodies and reagents

Anti uPA, IL-17 and IL-17R rabbit antibodies (sc-14019, sc-7927 and sc-30175 respectively) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against MT1-MMP, pERK1,2, ERK1,2, pp38 and p38 were purchased from R&D Systems. For immunofluorescence analysis, anti α-tubulin, anti γ-tubulin, anti-mouse-FITC secondary antibody and DAPI were from Sigma-Aldrich. For F-actin staining, phalloidin coupled to TRITC (Sigma-Aldrich) was used. Recombinant human IL-17 and human recombinant TGF-beta were provided by R&D Systems. Epsilon aminocaproic acid (EACA) and doxycycline were from Sigma-Aldrich. 12-O-tetradecanoylphorbol-13-acetate (TPA), p38 inhibitor SB203580 and MEK1,2 inhibitor PD98059 were obtained from Calbiochem (Darmstadt, Germany), while uPA inhibitor, BC11 hydrobromide was purchased from Tocris Bioscience (Bristol, UK). Anti-uPA mouse Mab, SAM-3, was kindly provided by Dr. F. Castellino (University of Notre Dame, Indiana).

2.3. PBMSC immunophenotyping

To phenotype cell-surface antigens, third-passage cells were stained with fluorescein isothiocyannate (FITC)- or phycoerythrin (PE)-conjugated antibodies specific for the following human antigens CD90-PE, CD44-PE, CD73, CD11b (Biosource), CD45-FITC (R&D System), and CD105-PE (Invitrogen). Stained cells were analyzed using CyFlow CL (Partec, Munster, Germany). For each sample, at least 10,000 events were recorded.

2.4. Wound healing and invasion assays

Cell migration was analyzed by an in vitro wound healing assay. Briefly, 5×10^4 cells/well were seeded in 24 well plates and allowed to grow until confluence, when a scratch wound in the monolayer was made by a 200 μl pipette tip. After the cells were washed three times with PBS, they were allowed to migrate for additional 24 h in GM with the treatments indicated in Results. After the migration period, cells were fixed with ice-cold methanol and stained with 0.1% crystal violet. Cell migration into the scratch area was photographed using an inverted light microscope and quantified by TScratch software (Computational Science and Engineering Laboratory, Swiss Federal Institute of Technology, ETH Zurich, Zurich, Switzerland).

The migration capacity of PB-MSC was also evaluated in a Boyden chamber-based cell migration assay (Costar, Cambridge MA) with 8.0 μm-pore polycarbonate filters (Collaborative Research, Bedford, MA). Briefly, PB-MSCs were labeled with PKH26, according to manufacturer's instructions and seeded in the upper chamber (10^5 cells per transwell) in 100 μl of growth medium. Growth medium (0.5 ml), with or without IL-17, as chemoattractant factor, and the indicated inhibitors, were added in the lower chamber. After 16 h, cells from the upper compartment were cleaned with a cotton swab to remove the nonmigrating cells. Cells attached to the bottom of transwells were fixed by immersing the transwells into 3.7% formaldehyde in PBS. After washing, transwells were turned upside down, mounted with a cover slip and cells were observed using an epifluorescence microscope. Red labeled cells from each sample were counted using ImageJ software in eight random fields per transwell insert.

2.5. Cell mobilization assay

Collagen was prepared as described previously [17]. Briefly, type I collagen was extracted from rat tail tendons in 0.2% acetic acid and 4.4 mg/ml solution was obtained. This solution was subsequently mixed with 0.34 N NaOH and 10 times concentrated DMEM in an 8:1:1 ratio and stored at 4 °C to initiate the gelling process. For cell mobilization assay, 1.2×10^6 PB-MSCs were embedded in 600 μl of collagen I (2.2 mg/ml) and four 25 μl drops were added per well in a 6 well plate. After 1 h of incubation in a humidified atmosphere at 37 °C with 5% CO_2, the cell–collagen drops gelled and 2 ml of GM with 0, 25 or 50 ng/ml IL-17 was added to each well. After a 5-day culture, cell mobilization from the collagen plugs into the surrounding plastic surface was monitored by phase-contrast microscopy. For the experiments using inhibitors, PD98059 (25 μM) or BC11 hydrobromide (50 μM) was added to GM 30 min prior to the addition of rhIL-17.

2.6. Proliferation assay

The proliferation rate of PB-MSCs was analyzed by MTT assay. Briefly, 5×10^3 cells/well were seeded in 96 well plates in GM. The following day rhIL-17 (0, 25 and 50 ng/ml) was added to GM and cells were incubated for additional 3 days. After this period, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MMT, Sigma-Aldrich) was added to each well at 0.5 mg/ml and cells were incubated for additional 2 h. The culture medium was then discarded and the cell-precipitated formazan crystals were dissolved in Isopropanol:DMSO (3:2). The absorbance was read at 540 nm [8].

2.7. Western blot assay

Cells were lysed for 30 min at 4 °C in 200 μl of lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris pH 7.5, with 1 mM Na_3VO_4 and protease inhibitors). Equal amounts of proteins of each extract were separated by SDS-PAGE and transferred to Hybond ECL nitrocellulose membranes (AppliChem, Darmstadt, Germany). The membranes were blocked in 4% non-fat milk in TBS buffer containing 0.05% Tween 20. The membranes were then incubated with primary, and subsequently with horseradish peroxidase-conjugated secondary antibodies. Specific protein bands were visualized using the ECL reagent from Serva, Heidelberg, Germany. Protein bands were quantified by densitometry, using NIH-ImageJ software.

2.8. Zymography assay

Cell culture supernatants were analyzed for the presence of secreted uPA and MMPs by zymography. PB-MSCs were cultured under treatments as described in Results. Briefly, 5×10^4 cells/well were seeded in 24 well plates and cultured overnight, washed three times with PBS and 0.5 ml of serum-free culture medium was added and cells cultured for additional 24 h under treatments indicated in Results. For uPA activity determination, aliquots of protein-normalized conditioned medium (CM) were subjected to 10% SDS-PAGE under non-reducing conditions. After being washed with 2.5% Triton X-100, the gels were placed on 1% agarose gels containing 0.5% casein and 1 μg/ml plasminogen, and incubated at 37 °C for 24 h. uPA-dependent proteolysis was detected as a clear band in the agarose gel. For MMP analyses, aliquots of protein-normalized CM were subjected to 8% SDS-PAGE containing 0.1% gelatin under non-reducing conditions. Gels were washed twice with 2.5% Triton X-100 and rinsed one time with distilled H_2O. The gels were then incubated for 24 h in 100 mM Tris–HCl, pH 8.5 with 10 mM $CaCl_2$. The activity of MMPs was stopped by staining the gels with Coomassie Blue R250 in 50% methanol and 10% acetic acid for 20 min.

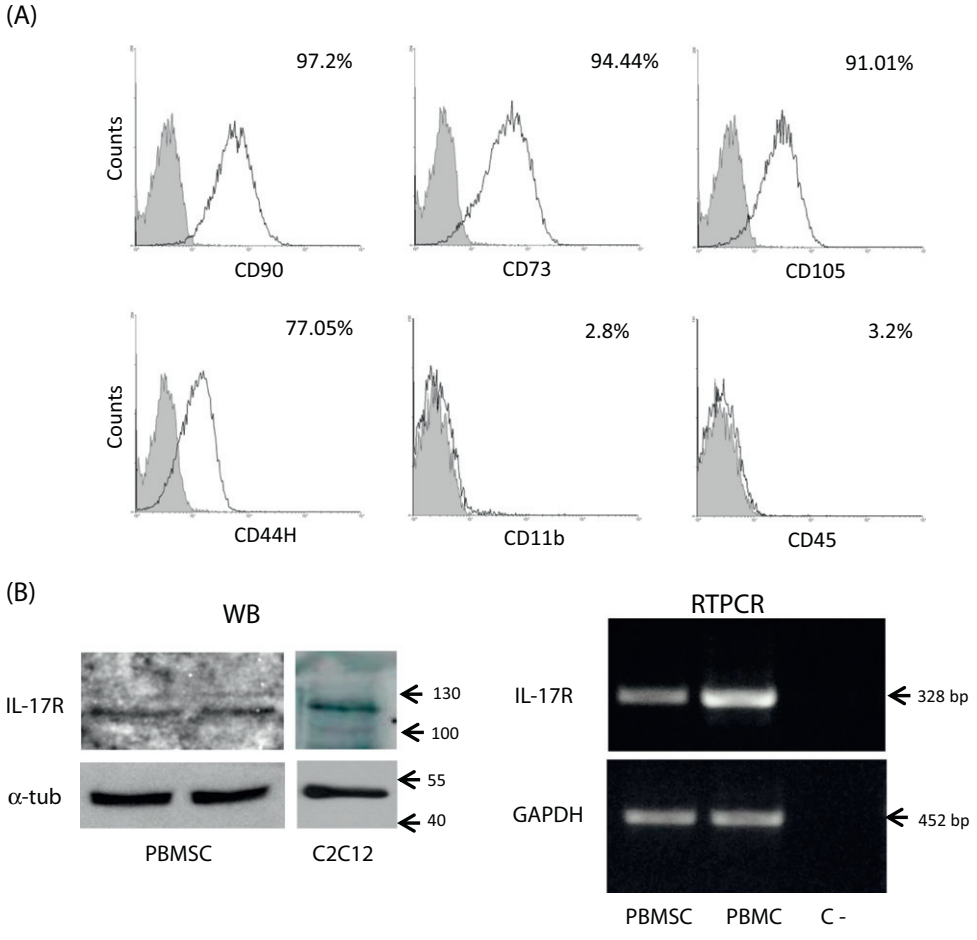

Fig. 1. (A) PB-MSC immunophenotyping. Cells between 3th and 4th passages were subjected to identify the immunophenotypic profile. Flow cytometry histogram of PB-MSCs for mesenchymal stem cell (CD90, CD7, CD105 and CD44) and leukocyte (CD11b and CD45) markers. Gray areas indicate background fluorescence obtained with isotype control, and the white histogram indicates signal for each specific antibody. Results presented are representative from at least 3 experiments performed. (B) IL-17 receptor expression in PB-MSC. IL-17 receptor expression was determined by Western blot (left) in duplicate, C2C12 cells were used as positive control, α-tubulin was used as gel loading control; and by RTPCR assay (right) where cDNA from peripheral mononuclear cells (PBMC) was used as positive control and PCR mix without cDNA as negative control, GAPDH was used as housekeeper gene. Representative results from at least two independent determinations are shown.

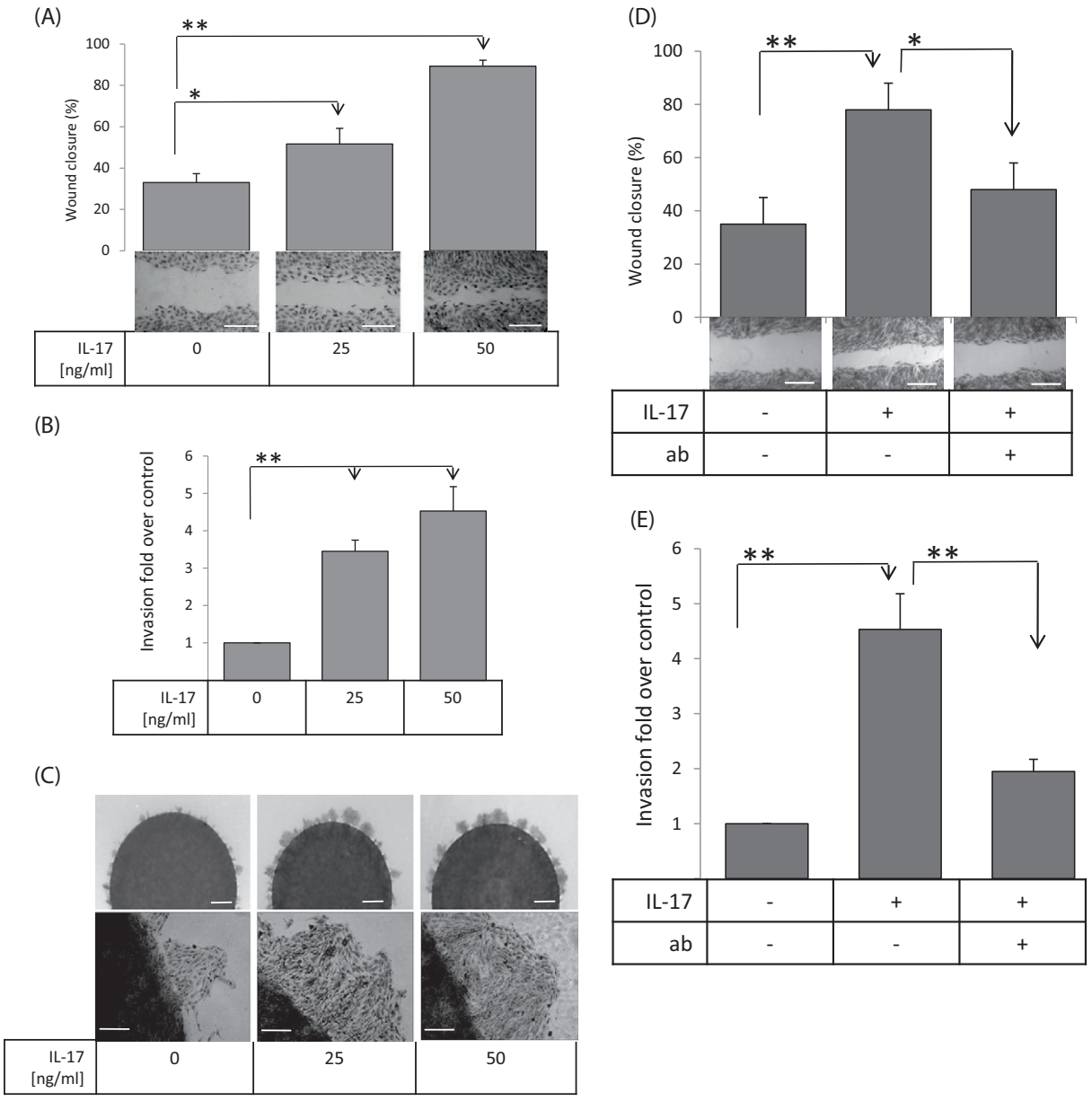

Fig. 2. IL-17 enhances PB-MSC migration and collagen-mobilization. (A) Migration of PB-MSC analyzed by scratch assay: a scratch was made in the confluent monolayer of cells, and cells were cultured in the presence of 0, 25 and 50 ng/ml of IL-17. The recolonization of the scratch by adjoining cells was documented after 24 h; bars represent means ± SEM percentage of the scratch area covered with migrating cells. Magnification ×100 and scale bars 100 μm. Significant difference from the control (untreated cells) by t-test: *$p < 0.05$ and **$p < 0.005$. (B) Cell migration by Boyden chamber-based cell migration assay. PKH26 labeled cells were seeded in the upper chamber and IL-17 (50 ng/ml) was added in lower chamber. After 16 h cells crossing to the bottom face of membrane were fixed and examined by an epi-fluorescence microscope. Ten random fields per sample were photographed and cells counted. Bars represent means ± SEM. Significant difference from the control (untreated cells) by t-test: *$p < 0.05$ and **$p < 0.005$. (C) PB-MSC mobilization from collagen gel. PB-MSCs embedded into collagen drops were treated with or without IL-17 as indicated during 5 days. After fixing and staining the samples were photographed. Drop magnification ×20, scale bars 1 mm, and cell magnification ×100. Results are representative from at least 3 independent experiments performed in triplicate. (D) Migration of PB-MSC analyzed by scratch assay. A scratch was made in the confluent monolayer of cells, and cells were cultured in the presence of or absence of IL-17 (50 ng/ml) with or without anti-IL-17 antibody (ab, 4 μg/ml). The recolonization of the scratch by adjoining cells was documented after 24 h; bars represent means ± SEM percentage of the scratch area covered with migrating cells. Magnification ×100 and scale bars 100 μm. Significant difference from the control (untreated cells) by t-test: *$p < 0.05$ and **$p < 0.005$. (E) Cell migration by Boyden chamber-based cell migration assay. PKH26 labeled cells were seeded in the upper chamber, IL-17 (50 ng/ml), with or without anti-IL-17 antibody (ab, 4 μg/ml), was added in lower chamber. After 16 h cells crossing to the bottom face of membrane were fixed and examined by an epi-fluorescence microscope. Ten random fields per sample were photographed and cells counted. Bars represent means ± SEM. Significant difference from the control (untreated cells) by t-test: **$p < 0.005$.

Zymography was developed by destaining gels with 20% methanol and 5% acetic acid until transparent proteolytic bands were observed. As a positive control for MMP-9 in zymography assay the CM from PC-3 cells treated with 100 ng/ml of TPA for 24 h was used. The uPA and MMP bands were quantified by densitometry, using NIH-ImageJ software [16].

2.9. RT-PCR

After specific cell treatments, indicated in Results, total RNA was obtained using TRIzol (Applichem) and complementary DNA was generated by the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA), using oligo (dT) as a primer.

PCR conditions and the primer set (Invitrogen, Carlsbad, CA, USA) used are described in Supplementary Table. PCR products were obtained after between 25 and 35 cycles of amplification for individual genes with indicated annealing temperature. Amplicons were resolved in 1.5% agarose gel and stained with ethidium bromide. GAPDH was amplified as a control for the amount of cDNA present in each sample. The intensity of the bands was quantified using NIH-ImageJ software.

2.10. PB-MSC polarization during migration

Immunofluorescence assay was used to detect beta-actin; alpha- and gamma-tubulin in migrating PB-MSCs. Briefly, 10^5 cells were seeded per rounded cover slip in GM and allowed to grow until confluence, when a scratch wound in the monolayer was made by a 200 μl pipette tip. After the cells were washed three times with PBS, they were allowed to migrate for additional 6 h in GM with or without IL-17 (50 ng/ml), PD98059 (25 μM), or BC11 (50 μM), as indicated in Results. After being fixed with 4% formaldehyde in PBS, cell monolayers were incubated with specific primary antibodies, followed by incubation with corresponding fluorescently labeled secondary antibodies and 1 μg/ml DAPI. After mounting, the samples were examined and photographed using an epi-fluorescence microscope [18].

2.11. Adhesion of PB-MSCs to endothelial cells

HMECs (10^5 cells/cover slip) were seeded over 0.2% gelatin-coated round glass coverslips and incubated for 48 h in a humidified atmosphere at 37 °C with 5% CO_2, until reaching confluence. Cells were then stained with DAPI (5 μg/ml) for 15 min at room temperature and washed twice with PBS. At the same time when seeding HMECs, 4×10^5 PB-MSCs were seeded per well in a 6 well plate. After they were incubated over night in a humidified atmosphere at 37 °C with 5% CO_2, PB-MSCs were treated with 0 or 50 ng/ml IL-17 for 24 h. Following the treatment, PB-MSCs were detached with 1 mM EDTA and labeled using PKH26 Red Fluorescent Cell Linker Kit for General Cell Membrane Labeling (Sigma-Aldrich), according to manufacturer's instructions. Labeled PB-MSCs were then seeded over HMECs (10^5 cells/cover slip) and incubated in a humidified atmosphere at 37 °C with 5% CO_2 for 30 min. After incubation, cover slips were washed twice with PBS; cells were fixed with 3.7% formaldehyde in PBS and mounted. The samples were examined and photographed using an epi-fluorescence microscope. Eight to ten random fields from each cover slip were photographed and red labeled cells were counted using ImageJ software. To analyze whether IL-17 affects adhesion of PB-MSCs by acting on endothelial cells, the same experiment where HMECs were treated with 0 or 50 ng/ml IL-17 instead of PB-MSCs was conducted.

2.12. Transendothelial migration of PB-MSCs

HMECs were seeded in 24 well plate transwell inserts (Corning, Tewksbury, MA, USA), 6×10^5 cells per transwell, in EC medium and incubated in a humidified atmosphere at 37 °C with 5% CO_2 overnight. PB-MSCs were labeled with PKH26, according to manufacturer's instructions and seeded over HMECs (10^5 cells per transwell) in 100 μl EC medium with or without BC11 (50 μM). Under the transwell inserts, 0.5 ml EC medium with 0 and 50 ng/ml IL-17 in the presence or absence of BC11 (50 μM) was added in the bottom chamber. Cells were then incubated in a humidified atmosphere at 37 °C with 5% CO_2 for 30 h to allow transmigration of PB-MSCs through the HMEC monolayer. The bottom part of transwell inserts was then washed with PBS three times and the top part was cleaned with a cotton swab to remove the nonmigrating cells. Cells attached to the bottom of transwells were fixed by immersing the transwells into 3.7% formaldehyde in PBS. After washing, transwells were turned upside down, mounted with a cover slip and cells were observed using an epi-fluorescence microscope. Red labeled cells in ten random fields from each sample were counted using ImageJ software.

2.13. Fibronectin cell adhesion assay

For the adhesion assay, 24 well plates were coated with 10 μg/ml of fibronectin (Sigma-Aldrich, St Luis, MO, USA) for 24 h at 4 °C and blocked with 1% BSA in PBS at 37 °C for 1 h. PB-MSCs were treated with IL-17 (50 ng/ml) for 24 h, detached with 1 mM EDTA in PBS, resuspended in DMEM containing 0.5% FBS and pretreated with SAM-3 anti-uPA antibody (10 μg/ml) or BC11 (50 μM) in a humidified atmosphere at 37 °C with 5% CO_2 for 1 h. Then, 10^5 cells were seeded in fibronectin coated plates and allowed to adhere for 30 min. The wells blocked with BSA/PBS only were used as a control for basal adhesion. After the incubation time, non-adherent cells were discarded by washing the wells three times with PBS. MTT (0.5 mg/ml) was added to adherent cells which were cultured in GM for additional 2 h. The precipitated formazan was dissolved with Isopropanol:DMSO (3:2) and the absorbance was read at 540 nm.

2.14. Statistical analysis

Data are given as means (±SEM) from at least three independent experiments. Student's t test was performed to evaluate the probability of significant differences among the samples with $p < 0.05$ (*) and $p < 0.005$ (**) considered significant.

3. Results

3.1. Immunophenotyping and IL-17 receptor expression in PB-MSCs

According to criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy [19] the mesenchymal phenotype of PB-MSCs was analyzed by flow cytometry. PB-MSCs isolated by plastic adhesion were subjected to immunophenotyping to MSC specific cell surface antigen expression. More than 90% of cells were positive for CD90, CD73, and CD105; 77% for CD44 and low level for CD45 and CD11b expressions (Fig. 1A). In addition, cells were able to differentiate into three-lineages (osteoblasts, adipocytes and chondrocytes) under standard in vitro condition (data not shown). Next, we elucidated whether PB-MSC express IL-17 receptor by subjecting cells to Western blot analysis. A duplicate determination showed 120 kDa IL-17R protein expression similar to IL-17R expression in C2C12 cells which we previously reported [7] and used here as positive control (Fig. 1B). IL-17R expression also was confirmed by RTPCR assay using peripheral blood mononuclear cells (PBMC) as positive control and PCR reaction in the absence of cDNA as negative control (Fig. 1C).

3.2. IL-17 enhances PB-MSC migration

During injury and inflammation recruitment of MSCs is triggered by specific signals present at the site of damage, and cells are improving their migratory capacity to move toward damaged tissues. IL-17 is known as one of the pro-inflammatory cytokines whose levels are increased during inflammation [20], therefore, we analyzed whether IL-17 modulates PB-MSC migration by wound healing assay. The treatment of PB-MSCs with two different concentrations of IL-17 (25 and 50 ng/ml) for 24 h greatly enhanced the capacity of cells to migrate into the wound in comparison with the cells incubated in the absence of the cytokine (Fig. 2A). IL-17 at higher concentration induced almost a complete scratch closure (about 90%) during the experimental time period. To support the capability of IL-17 to induce cell motility, a chemotaxis Boyden chamber-based cell migration assay was performed. PB-MSCs responded to IL-17 (25 and 50 ng/ml) in the lower chamber, by incrementing their migration through the 8 μm pores to the bottom

side of membrane into the lower compartment, as compared with control cells after 16 h of experimental time period (Fig. 2B). Next, we determined the capacity of PB-MSCs to mobilize from type I collagen hydrogel drops. Therefore, PB-MSCs were embedded within 3D type I collagen drop gels, and mobilization capacities were determined in the absence or presence of IL-17. IL-17 treatment, at both 25 and 50 ng/ml, enhanced cell exit from collagen gels compared with control cells (Fig. 2C). Interestingly, the mobilization of MSCs from collagen showed a collective migration more than individual cell migration. Next, we examined whether IL-17 regulates cell proliferation. PB-MSCs were treated with same concentrations of IL-17 as in the wound healing assay for 3 days and cell proliferation was analyzed by MTT assay. The results showed that IL-17 does not modify cell proliferation (Fig. S1), suggesting that the cytokine acts as a motility factor for MSCs, and that closing of the scratch wound and collagen gel mobilization are mainly due to IL-17 stimulating the cell migration and not by increasing the cell number. In order to determine the direct role of IL-17 on PB-MSCs, additional experiments blocking IL-17 effects were

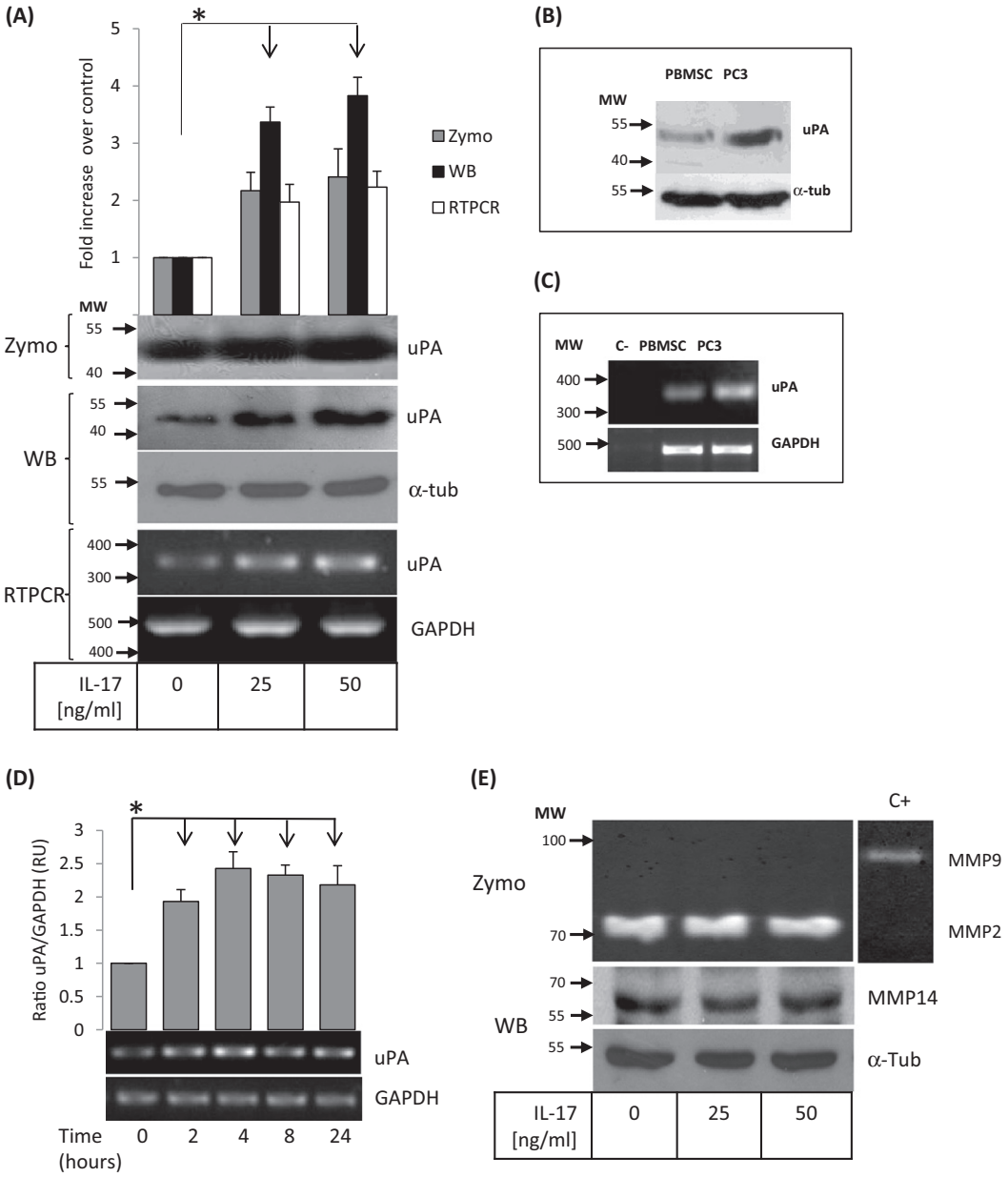

Fig. 3. IL-17 increases uPA production. (A) PB-MSCs were incubated for 24 h in serum-free medium with 0, 25 and 50 ng/ml of IL-17. uPA was analyzed in the serum-free media by zymography (Zymo, gray bars) and Western blot (WB, black bars) assays. Also, uPA mRNA was determined by RTPCR (white bars) using GAPDH as a gel loading control. Bands were analyzed by densitometry. For zymography and WB each band was normalized by determining the correspondent ratio with each α-tubulin band, while for RTPCR each band was normalized by correspondent GAPDH band. All ratios were divided with correspondent control values given value 1. The bars represent uPA expression as fold increase over corresponding control. Molecular weight markers (MW and arrows) are indicated in Kilo Daltons for Zymo and WB, while in base-pair size for RTPCR assay. Representative results from at least three independent determinations are shown. Significant difference from the control (untreated cells): *$p < 0.05$. (B) WB assay using uPA produced by PC3 cells used as positive control for human uPA produced by PB-MSC, α-tubulin was blotted as gel loading control. (C) RTPCR analysis for uPA mRNA expression in PC3 cells as positive control for human uPA mRNA expressed by PB-MSC and GAPDH is used as a gel loading control. (D) Time course of IL-17-induced uPA mRNA expression determined by RT-PCR. Cells were treated with 50 ng/ml of IL-17 for 0, 2, 4, 8 and 24 h. GAPDH was used as a gel loading control. The plot represents uPA mRNA expression relative to GAPDH expression. A value 1 was given to control samples. (E) Effect of IL-17 on metalloproteinase (MMP) expression. PB-MSC cells were treated 24 h with IL-17 (0, 25 and 50 ng/ml) in serum-free medium. MMP2 and MMP9 were determined by zymography assay and TPA-treated PC3 cells-derived conditioned media were used as MMP9 positive control. MMP14 was determined in PB-MSC lysed samples by Western blot assay. α-Tubulin was used as a gel loading control. Representative results from at least three independent determinations are shown. Significant difference from the control (untreated cells): *$p < 0.05$.

performed. By using an antibody against IL-17 (4ug/ml), reduction of PB-MSC responses to IL-17 both in migration and invasion assays were observed (Fig. 2D and E), suggesting that IL-17 ligand is the primary factor that induced the incremented cell motility in our experimental conditions.

3.3. IL-17 induces uPA production in PB-MSCs

One of the key events involved in cell migration is the production of extracellular proteinases which allows cells to modify the ECM or directly enhance the motility of cells [9]. Further experiments aimed to determine whether IL-17 modulates the capacity of PB-MSCs to produce uPA. Cells were incubated in the presence of 0, 25 and 50 ng/ml IL-17 in serum-free culture medium for 24 h and the production of uPA was determined by Western blot and zymography. Western blot analysis allowed the determination of cell-associated uPA, while the amount of uPA secreted into the CM was determined by zymography. IL-17, both at 25 and 50 ng/ml, significantly increased the production of uPA in PB-MSCs. uPA production after IL-17 treatment was also increased at transcriptional level, as determined by RT-PCR analysis (Fig. 3A). uPA produced by PC3 cells was used as a positive control in order to compare and determine the correct MW size of human uPA produced by PB-MSC (Fig. 3B). uPA mRNA from PC3 cells was also used as positive control in order to compare and determine the correct size of corresponding PCR products (Fig. 3C). The IL-17-induced enhancement in uPA transcript expression in PB-MSCs was rapid, with a noticeable increment visible 2 h after treatment, reaching two-fold expression compared to the control in all the following time points tested up to 24 h, which implied that IL-17 directly modulates uPA expression in PB-MSCs (Fig. 3D). Interestingly, IL-17 did not induce the expression of PAI-1, the physiological inhibitor of uPA, as determined by RT-PCR, using PC-3 cells treated with TGF-beta (10 ng/ml) for 24 h as a positive control (Fig. S2).

3.4. Expression of MMPs in PB-MSCs is IL-17 independent

Besides uPA, stem cells are capable of expressing matrix metalloproteinases (MMPs) which are also implicated in MSC migration [21]. Further experiments were performed in order to determine whether IL-17 modulates the expression of MMPs in PB-MSCs. Zymography analysis indicated that PB-MSCs mainly secrete MMP2 and its expression was not modified by IL-17 treatment. However, no activity corresponding to MMP-9 was noticed. Conditioned medium

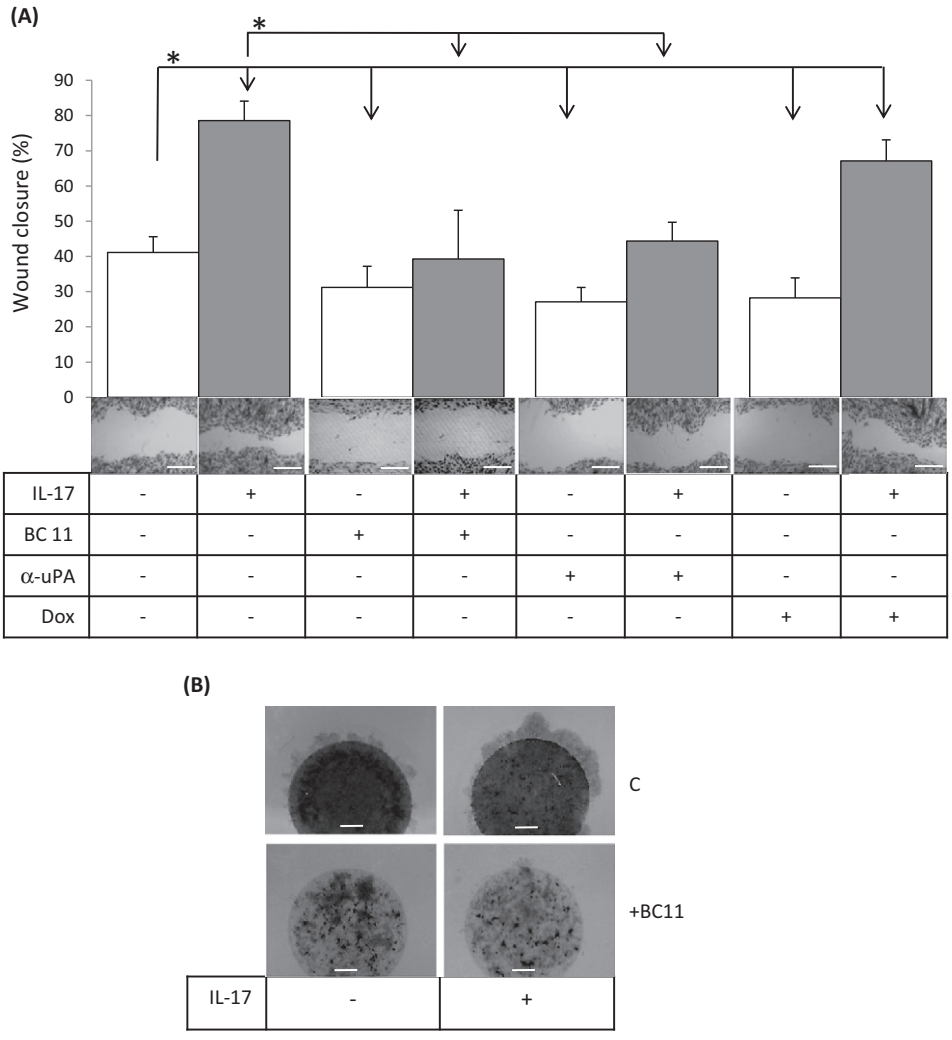

Fig. 4. IL-17-induced PB-MSC migration and collagen-mobilization are uPA dependent. (A) PB-MSCs were subjected to wound healing assay under IL-17 treatment (50 ng/ml) for 24 h in the presence or absence of uPA inhibitor BC11 (50 μM), uPA blocking antibody (5 μg/ml) or doxycycline (1 μg/ml). Bars represent the percentage of the scratch area covered with migrating cells. Significant difference from the control (indicated by arrow): *$p < 0.05$. (B) PB-MSCs embedded into collagen drops were treated with or without IL-17, as indicated, during 5 days in the presence or absence of 50 μM BC11. After fixing and staining the samples were photographed. Collagen drop magnification ×40. Results are representative from at least 3 independent experiments performed in triplicate.

from TPA-treated PC-3 cells was used as positive control (Fig. 3E). Additionally, the expression of MT1-MMP/MMP14, known to be expressed in MSCs [21] was also not modulated by IL-17 treatment, as determined by Western blot assay. The zymography results were confirmed by RT-PCR results, showing that the expression of the MMP2 and MT1-MMP/MMP14 mRNAs were not modified by IL-17, as well as that MMP9 mRNA transcript was not observed in any condition and using PC-3 cells treated with TPA (100 ng/ml) for 24 h as a positive control (Fig. S2B).

3.5. uPA mediates the IL-17- induced PB-MSC migration

The implication of uPA in IL-17-induced cell migration was analyzed by using BC11 hydrobromide, a selective uPA inhibitor, as well as the anti-uPA blocking antibody. As shown in Fig. 4A, BC11 and anti-uPA antibody inhibited both basal and IL-17-induced cell migration. To further support the results suggesting that IL-17 increases PB-MSC migration by enhancing uPA production, we used a broad MMP inhibitor, doxycycline (1 μg/ml). Although doxycycline reduced the basal level of cell migration, its effect on IL-17-induced migration of PB-MSCs was not significant. This result suggested that the enhancement of uPA can overcome the inhibition of MMPs, making this extracellular proteinase responsible for the enhancement of PB-MSC migration by IL-17. Next, we determined the effect of uPA inhibition on collagen gel mobilization (Fig. 4B). The addition of BC11 provoked a strong inhibition of cell mobilization from collagen drops either in basal or in IL-17 treatment conditions. In addition, epsilon-aminocaproic acid (EACA), which blocks the high-

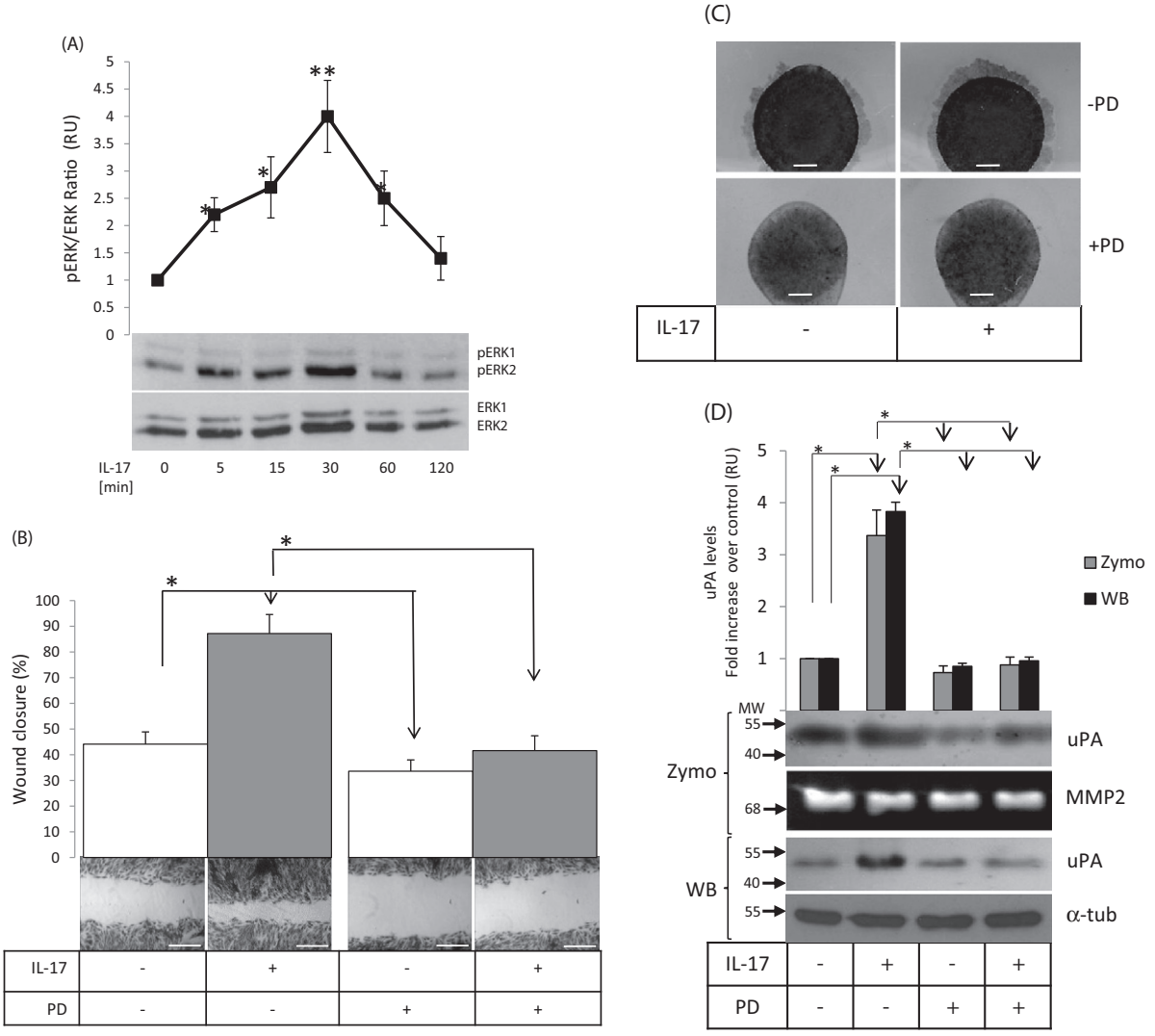

Fig. 5. ERK1,2 signaling mediates IL-17-induced PB-MSC migration and uPA production. (A) IL-17 activates ERK1,2 in PB-MSCs. PB-MSCs were cultured in serum-free medium and treated with 50 ng/ml of IL-17 during the indicated time intervals, phosphorylation status of ERK1,2 was determined by Western blot. Time course changes of ERK1,2 phosphorylation in each time point normalized to the control level are presented as means ± SEM from three independent experiments. Protein status phosphorylations were quantified by densitometry. The ratio of band density for phospho-ERK1,2 to total ERK1,2 for each corresponding time was determined. Each time ratio time was normalized to the zero time level, a value 1 was given to control sample, while the Blot are representative from three independent determinations. (B) ERK1,2 mediates IL-17-induced cell migration. PB-MSC migration in the presence of MAPK inhibitor determined by wound healing assay. Cells were treated for 24 h with 0 or 50 ng/ml of IL-17 in the presence or absence of 25 μM PD98059 and the capacity of cells to close the wounds was documented. Plots represent the average value ± SEM of wound area covered by migrating cells. Results are representative of three independent experiments performed in triplicate. Significant difference from the control (indicated by arrow): *$p < 0.05$. Magnification ×40. (C) ERK1,2 mediates PB-MSC collagen-mobilization. PB-MSCs embedded into collagen drops were treated with or without IL-17, as indicated, during 5 days in the presence or absence of 25 μM PD98059. After fixing and staining the samples were photographed. Collagen drop magnification ×40. (D) ERK1,2 mediates IL-17-induced uPA expression. PB-MSCs were incubated for 24 h in serum-free medium with or without 50 ng/ml of IL-17 and in the presence or absence of 25 μM PD98059. uPA was analyzed in the serum-free media by zymography (Zymo) and Western blot (WB) assays and α-tubulin was used as gel loading control and to normalize uPA activity and expression. MMP2 secretion determined by zymography was used as loading control of media. A quantification plot for uPA enzymatic activity (Zymo, gray bars) and uPA protein expression (WB, Black bars) are presented, above the assays' results. Molecular weight markers (MW and arrows) are indicated in Kilo Daltons for Zymo and WB. Results are representative as means ± SEM of three independent experiments performed in triplicate. Significant difference from the control:*$p < 0.05$.

affinity lysine binding sites on uPA-activated plasmin (the main proteolytic uPA effector) [22], inhibited both IL-17-induced cell migration and collagen mobilization (Fig. S3).

3.6. ERK1,2 mediates IL-17-stimulated cell migration and uPA expression

Subsequent experiments were addressed to determine the involvement of intracellular signaling pathways in IL-17-induced cell migration and uPA expression in PB-MSCs. IL-17 (50 ng/ml) provoked a rapid activation of ERK1,2, which was noticeable 5 min after treatment, reaching maximum at 30 min and subsequently decreasing to basal level (Fig. 5A). To determine the involvement of ERK1,2 on the IL-17-stimulated migration of PB-MSCs, cells were treated with MAPK inhibitors in the absence or presence of IL-17 and both migration capacity and collagen gel mobilization were determined. As shown in Figs. 5B and 5C, PD98059 inhibited the basal level of migration capacity of PB-MSCs and strongly reduced IL-17-stimulated wound closure and collagen gel mobilization. Since ERK1,2 signaling appeared to be involved in the stimulation of cell migration by IL-17, we next aimed to determine the involvement of this signaling pathway in the IL-17-enhanced uPA expression. Cells were treated or not with PD98059 for 30 min prior to adding IL-17 (0 or 50 ng/ml) into the serum-free culture media. After 24 h of incubation, we analyzed the uPA production by Western blot and zymography assays. α-Tubulin was used as loading control in Western blot assay, and MMP2 production was also determined by zymography as a loading control. The presence of PD98059, strongly reduced the IL-17-enhanced uPA expression in PB-MSCs, as well as the amount of uPA secreted into CM, at the same time not affecting MMP2 secretion (Fig. 5D). Conversely, although IL-17 was capable to activate p38 in PB-MSCs, the p38 inhibition by SB203580, neither inhibited the IL-17-induced cell migration nor IL-17-enhanced uPA expression (Fig. S4).

3.7. IL-17 enhances PB-MSC migration by increasing cell polarity

Actin and tubulin cytoskeleton reorganization are among the main mechanisms involved in cell migration [23]. During migration, cells become polarized, and therefore it is possible to distinguish two well-differentiated poles, the leading edge and the trailing edge. The microtubule organizing center (MTOC) consisting of γ-tubulin is oriented toward the "movement path" of the cell. We further investigated whether IL-17 can modulate the cytoskeleton organization and cell polarization during migration in the wound healing assay. Firstly, the "wounded" monolayer of PB-MSCs was treated for 6 h with or without IL-17. Prior to IL-17 addition, cells were treated or not with PD98059 or BC11 for 30 min. When compared to untreated cells, PB-MSCs in the presence of IL-17 reorganized their F-actin cytoskeleton, orientating the fibers into the scratch (Fig. 6A). Also, the cells positioned in the wound edge showed polarization of MTOC, immune-labeled with anti-γ-tubulin, which was mainly localized between the nucleus and the edge of cell protrusions. When cells were pre-treated with the MEK1,2 inhibitor, γ-tubulin showed a random position and F-actin was observed in the position parallel to the wound edge, indicating that PB-MSCs lost their motile phenotype in the presence of the inhibitor. When cells were treated with uPA inhibitor BC11, although cell migration was inhibited and F-actin showed a cortical pattern in wound edge, the effect on IL-17-induced cell polarization was weaker compared with ERK1,2 inhibition. In addition, uPA is localized in PB-MSC cell surface showing a punctuated pattern as was shown by immunofluorescence assay. The IL-17-induced cell movement was accompanied with uPA localization in cell protrusion in the edge of wound (Fig. 6B–b), which was strongly inhibited by either ERK1,2 or uPA inhibition.

3.8. IL-17 enhances adhesion of PB-MSCs to endothelial cells, trans-endothelial migration and fibronectin adhesion

In response to signals from damaged tissues, circulating mesenchymal stem cells have to migrate across endothelium [24]. One of the first steps is the PB-MSC adhesion to endothelium and their subsequent diapedesis across the endothelial monolayer [24]. Therefore to determine whether IL-17 regulates PB-MSC adhesion to endothelial cell monolayer, we pre-treated either MSC or EC monolayer with IL-17 for 24 h and performed cell–cell adhesion assay. As shown in Fig. 7A, the IL-17 treatment of PB-MSCs increased the adhesion capacity to EC monolayer; similarly the treatment of ECs with IL-17 also increased the capacity of PB-MSCs to contact with ECs, suggesting that IL-17 regulates the functionality of both cell types to modulate MSC–EC adhesion.

After MSC cell–cell interaction with ECs, cells are integrated and finally pass and leave endothelial layer barrier [25]. Thus, we next investigated whether IL-17 regulates PB-MSC transendothelial migration, as well as the implication of uPA in this process. PKH-26 labeled PB-MSCs were subjected to migrate across endothelial monolayer in the presence or absence of uPA inhibitor BC11, while IL-17 was added in the bottom chamber as chemoattractant factor. The PB-MSCs underwent an enhanced transmigration capacity in response to IL-17 (Fig. 7B), whereas uPA inhibition disabled IL-17 increasing PB-MSC transendothelial migration and also affected basal capacity of cells to move across EC monolayer. This finding indicates that IL-17 enhances cell–cell adhesion processes concomitantly with increasing PB-MSC transendothelial capacity in uPA activity-dependent way.

To migrate toward damaged or tissue under inflammation MSCs have to interact with sub-endothelial extracellular matrix to move across the basement membrane [26]. Therefore, we next focused on fibronectin, one of the components of sub-endothelial basement membrane implicated in endothelial permeability [27,28]. To determine the capacity of ECs to produce and accumulate fibronectin we performed an immunofluorescence assay. Non-permeabilized fixed EC monolayer showed fibronectin expression which mainly accumulates over cell membrane showing both punctuated and "fibrillar" pattern (Fig. 7C). Subsequently, to determine whether IL-17 modulates PB-MSC adhesion to fibronectin, an adhesion assay using fibronectin coated plates was performed (Fig. 7D). The pre-treatment of PB-MSCs with IL-17 increased the fibronectin adhesion cell capacity compared with control cells; meanwhile the presence of uPA inhibitor BC11 reduced IL-17-increased PB-MSC adhesion to fibronectin. Moreover, using an anti-uPA blocking antibody, which inhibits both uPA binding to cell surface receptor and its activity, dramatically inhibited the cell adhesion to fibronectin under IL-17 stimulus, with value lower than basal cell adhesion capacity. Intriguingly, neither BC11 nor anti-uPA antibody reduced the capacity of cells to interact with fibronectin; suggesting that basal cell-fibronectin adhesion may be independent of uPA.

4. Discussion

During injuries and chronic pathological vascular changes mesenchymal stem cells are mobilized mainly from bone marrow to the blood circulation. MSCs migrate through endothelium and are recruited to the affected tissue, where they can regulate inflammatory responses, and when cued by the appropriate microenvironment may differentiate into tissue specific cells [23,29]. Also during tissue damage, cells from innate and adaptive immune system are recruited and participate in inflammatory response and tissue regeneration. Indeed, by production of cytokines and growth factors immune cells can induce mobilization, recruitment and regenerative capacity of MSCs [30,31]. Recently a subset of CD4 + T cells, the Th17 cells that secrete cytokines IL-17A and IL-17F and express surface IL-23 receptor (IL-23R), have been shown to play significant roles in inflammation and inflammatory diseases [32].

Here, we have investigated the role of IL-17A in peripheral blood mesenchymal stem cell migration and endothelial transmigration. PB-MSCs used in this study exhibited the main characteristic of mesenchymal stem cells, such as plastic adhesion and specific surface antigen expression of the main mesenchymal cluster of differentiation such as CD90, CD105, CD73 and CD44. Low expression of CD11b and CD45 [19] in PB-MCS allows us to exclude leukocyte contamination, including monocytes. Also PB-MSC expressed detectable levels of IL-17 receptor (Fig. 1). Our results demonstrated that IL-17 stimulated in vitro migration and mobilization of PB-MSCs, determined by wound healing and Boyden chamber-based cell migration, as well as that both responses were reverted by using anti-IL-17 antibody, indicating the specificity and main role of IL-17 on PB-MSC motility. In addition, IL-17 stimulated cell mobilization from the three dimensional collagen drops, which indicated that IL-17 enhances the capacity of PB-MSCs to move from extracellular matrix network (Fig. 2). Moreover, this finding also implicates the possibility of extracellular matrix degradation by proteolytic mechanism. In that way, we observed that IL-17 induced the expression of urokinase type plasminogen activator in PB-MSC, mainly by activating ERK1,2 MAPK, as well as that the uPA increased expression was necessary for the IL-17-induced cell migration (Figs. 3 and 4). Among several factors regulating pathophysiological processes required during tissue remodeling after injury, important roles for the multifunctional uPA system have been acknowledged [33]. By binding to its cellular receptor uPA mediates the conversion of plasminogen to plasmin and thereby produces a fine tune regulated cell surface proteolysis in space and time, facilitating cells to migrate across extracellular matrix barrier [34]. Besides, it is known that independent of proteolytic processes uPA–uPAR axis regulates migration, adhesion, proliferation, and differentiation of various cell types in tissue regeneration through activation of an intracellular signaling network [35]. Recently, it has been reported that by binding to its receptor uPA enhances cell migration and mobilization from bone marrow, contributing to hMSC migration into wounded tissue and thus to tissue regeneration. Also, it has been shown that uPA plays roles in tropism of MSCs to solid tumors, as well as that the expression of uPA by MSCs themselves increases MSC migration toward tumors [13,14,28,36,37]. Our data suggested that IL-17, produced within damaged tissue, may increase MSC mobilization and migration by inducing uPA expression. MSCs are also capable to express other types of extracellular matrix proteinases such as matrix metalloproteinase MMP2 and membrane type1 MMP (MMP14) [38], and MMP2, but not MMP9, was shown to play a role in the migration of MSCs [39]. However, although the inhibition of MMPs by doxycycline indicated some role in basal cell migration (Fig. 4A), our results demonstrated that IL-17 does not modify MMP2 and MMP14 expressions in PB-MSCs, pointing that IL-17-mediated uPA expression seems to be critical for the IL-17-induced PB-MSC migration. Additionally, although in several cell types IL-17 was shown to induce MMP9 [20], neither detectable transcript nor activity for MMP9 was demonstrated in IL-17-treated PB-MSC.

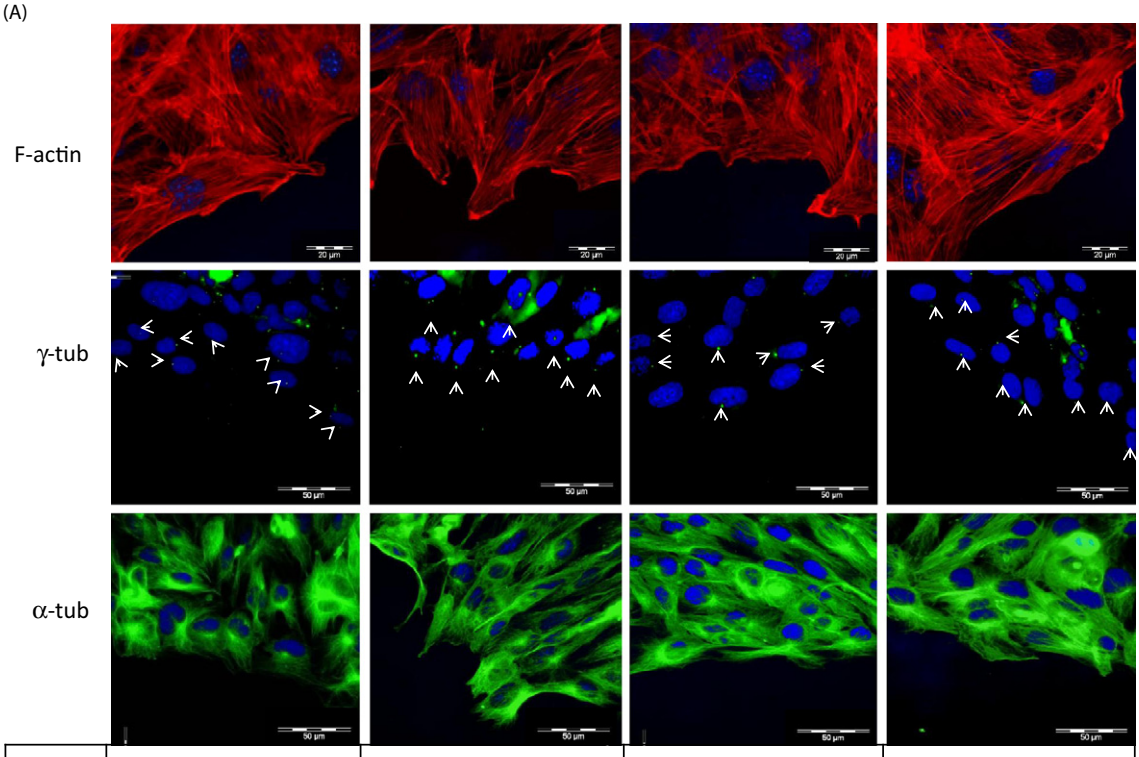

Fig. 6. IL-17 induces cell polarization and uPA cell protrusions localization. (A) IL-17 induces PB-MSC actin cytoskeleton reorganization and cell polarization in wound healing assay in a ERK1,2-dependent way. Cells were subjected to migrate in the wound-healing assay in the presence or absence of 50 ng/ml of IL-17, without or with 50 μM BC11 or 25 μM PD98059 for 6 h. After being fixed, cells were labeled with phalloidin–TRITC for F-actin (red), with gamma-tubulin antibody for MTOC (in green and indicated by arrows) and with DAPI (blue) for the nucleus. Cells in the edge of the "wound" were photographed, in order to detect migratory cells. Scale bar for γ- and α-tubulin: 50 μm, and for F-actin 20 μm. 400× and 630× respectively. (B) PB-MSC treated as above were immune-stained against uPA (green) and with DAPI (blue) for the nucleus. a and b cells without or with IL-17 treatment (5 ng/ml); c and d cell treated with PD98059 (25 μM) without or with IL-17; e and f cells with BC11 treatment (50 μM) without or with IL-17. Zoom indicated uPA localization in cell protrusion under IL-17 treatment (b). Magnification 400× and scale bar: 50 μm. Representative results from at least three independent determinations are shown.

During migration, cells suffer critical changes in the organization of actin cytoskeleton and polarization of tubulin network, mainly by a fine regulated and coordinated localization of MTOC [22,40]. We observed that IL-17 induced actin reorganization with accumulation of cell lamellipodia, and increased MTOC perinuclear location toward the wound edge concomitantly with profound changes in tubulin cytoskeleton (Fig. 6). These effects were mediated by ERK1,2 MAPK since the use of specific inhibitor disabled cells to respond to IL-17 through changes in cell cytoskeleton. Also, urokinase activity inhibition revoked the IL-17-induced actin rearrangement, although it did not significantly affect cell polarization. One possible explanation regarding this observation is that the inhibitor used (BC11) affects uPA activity and does not interfere with its interaction with the receptor, and inhibited uPA activity may trigger intracellular signal necessary for cell polarization. In addition, IL-17 provoked an accumulation of uPA in PB-MCS cell protrusions, which can contribute to the increment of cell migration. The underlying mechanism by which IL-17 induces actin cytoskeleton and MTOC rearrangement to increase cell migration has not been elucidated yet. One plausible mechanism is that IL-17 in MSCs may regulate Rho GTPase family proteins, which are critical regulators of cell migration and polarization, since RhoA and Rac1 are required for stress fibers and cell protrusion of lamellipodia, respectively, and Cdc42 for filopodia and cell polarity [40,41]. This speculation can be supported by the previous studies indicating IL-17 activation of Rac1 to induce cell migration, and of Rho expression and RhoA-kinase activity in endothelial cells [42,43]. Nonetheless, further studies are necessary to elucidate the requirement of Rho proteins for IL-17-induced migration and polarization of PB-MSC.

Both exogenously and endogenous circulating MSCs are known to preferentially engraft at site of inflammation in vivo [44]. MSCs migrate across the endothelial barrier to exit from circulation in response to signals, such as growth factors, cytokines and chemokines, produced by inflamed tissues. These signals may regulate the function of MSC by promoting pathogen clearance, suppression of inflammation and tissue regeneration [1,29]. However, the process and underlying mechanism by which PB-MSC migrate across endothelium toward to the sites of inflammation remained unclear. In addition, although inflammatory cytokines may stimulate MSC transendothelial migration [44], the role of IL-17 is unknown. Here, we demonstrated that IL-17 pre-treatment of either MSC or EC increased cell–cell adhesion, indicating that both MSCs and ECs are IL-17's target cells. Moreover, IL-17 used as chemotactic factor, increased MSC transendothelial migration in an uPA-dependent way (Fig. 7B). uPA has been involved in vivo in paracellular transmigration of neutrophils [45], and is plausible to speculate that uPA may have similar function in MSCs. By incrementing uPA, IL-17 may increase the cleavage of endothelial junctions, as well as by proteolytically degrading the perivenular basement membrane to increase endothelial permeability. Intriguingly, it has been recently reported that MSCs can migrate through endothelial barrier also by transcellular mechanism in association with VCAM-1-enriched "transmigratory cups" [44], suggesting a dynamic cell–cell interaction which may actively participate in acceleration of the MSC transendothelial migration to damaged tissue. In the future studies, it would be worthy to determine whether IL-17 may regulate MSC transcellular migration in the endothelium by regulation of uPA expression. In association with TNF-a, IL-17 has also been shown to affect endothelial cell activation and to increase neutrophil transmigration and the expression of adhesion molecules and chemokines in HUVECs, suggesting that IL-17 induces endothelial inflammation by facilitating cell trans-endothelial migration [46,47].

After crossing endothelial cell barrier MSCs interact with subendothelial extracellular matrix [25] and eventually induce the degradation of protein barrier to move toward inflamed tissue. Endothelial cells are known to highly produce fibronectin, and we determined that the IL-17 treatment of PB-MSCs increased cell adhesion to purified fibronectin in uPA-dependent fashion (Fig. 7D). uPA–uPAR interaction with integrin alpha5-beta1, the main fibronectin receptor, is known to modulate integrin-dependent cell adhesion to extracellular matrix, and these interactions actively participate in proteolytic regulation of invasive cell migration [48,49]. Still, we cannot exclude other mechanisms such as an increment of integrin receptor

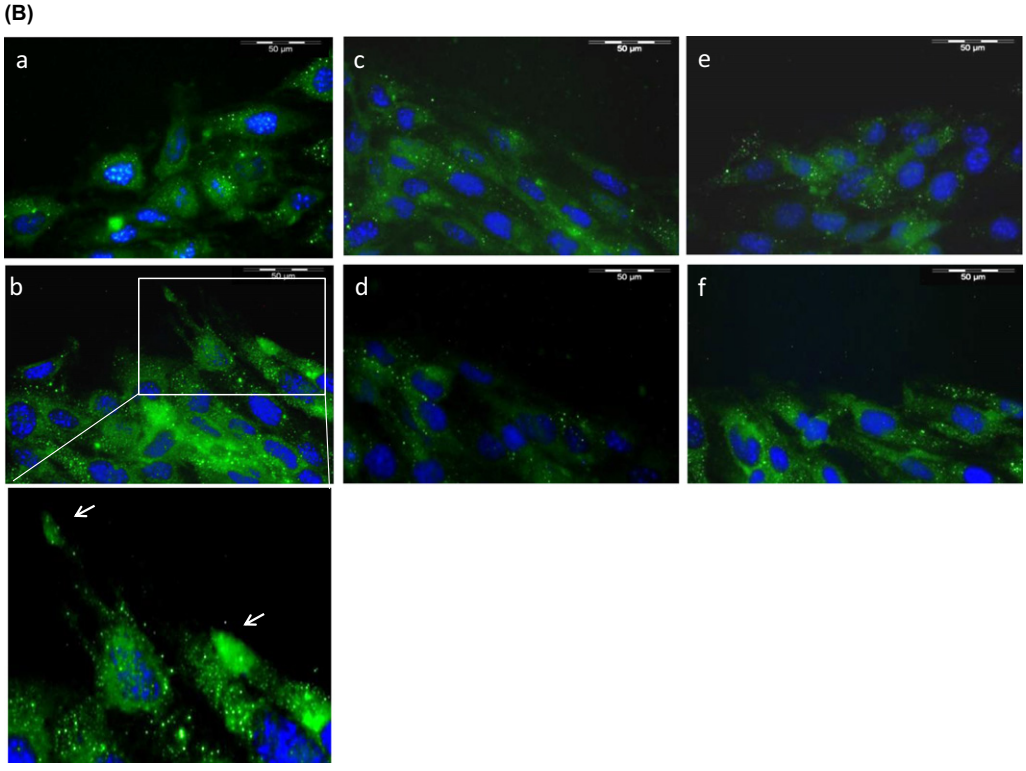

Fig. 6 *(continued).*

expression by IL-17. Our results, obtained by using uPA inhibitor and anti-uPA blocking antibody, suggest that uPA activity and its interaction with cell surface, mediate the capability of IL-17 to increase PB-MSC adhesion to fibronectin.

Abnormal IL-17 expression has been implicated with inflammatory pathology in the setting of autoimmune diseases, and IL-17 depletion has already been adopted as a therapeutic strategy and is currently being tested in clinical trials for several human autoimmune diseases [47]. Our in vitro data suggested that IL-17 can be a recruitment signal for PB-MSCs to migrate and engraft into inflamed tissue. Additionally, as it is believed that MSCs are not spontaneously immunosuppressive, but require 'licensing' or activation to exert their immunosuppressive effects [1], we can speculate that IL-17 may also modulate MSC immunomodulatory capacities. However, MSCs themselves seem to reduce the capacity of Th1 and Th17 cells to produce interferon-γ and IL-17, respectively [50]. Therefore, high IL-17 levels in damaged tissue may serve as a signal for MSC recruitment, which in turn may regulate IL-17 production acting as a negative feedback to control excessive inflammation.

Intriguingly, previous work of our laboratory demonstrated that IL-17 inhibited uPA expression concomitantly with inhibition of myoblast differentiation of C2C12 myoblasts [18]. If we assume that IL-17 could act as PB-MSC tropic signal, engrafted MSCs may in turn regulate the enhanced IL-17 expression in the muscle microenvironment, which has been observed in inflammatory myopathies [15], restoring the level of myoblast expressing uPA and allowing muscle regeneration after injuries. Nonetheless, further studies are necessary to determine if PB-MSCs may regulate IL-17 expression and function in inflammatory myopathies.

4.1. Conclusion

IL-17 increases in vitro PB-MSC migration and endothelial barrier transmigration by inducing uPA expression through ERK1,2 MAPK activation. These data allow us to better understand the effect of IL-17 on PB-MSC migration, and point to IL-17 as one of the signals implicated in MSC recruitment to damaged tissue to enhance regulation of inflammation and tissue regeneration.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.11.025.

Acknowledgements

This work was supported by Grant No 175062 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

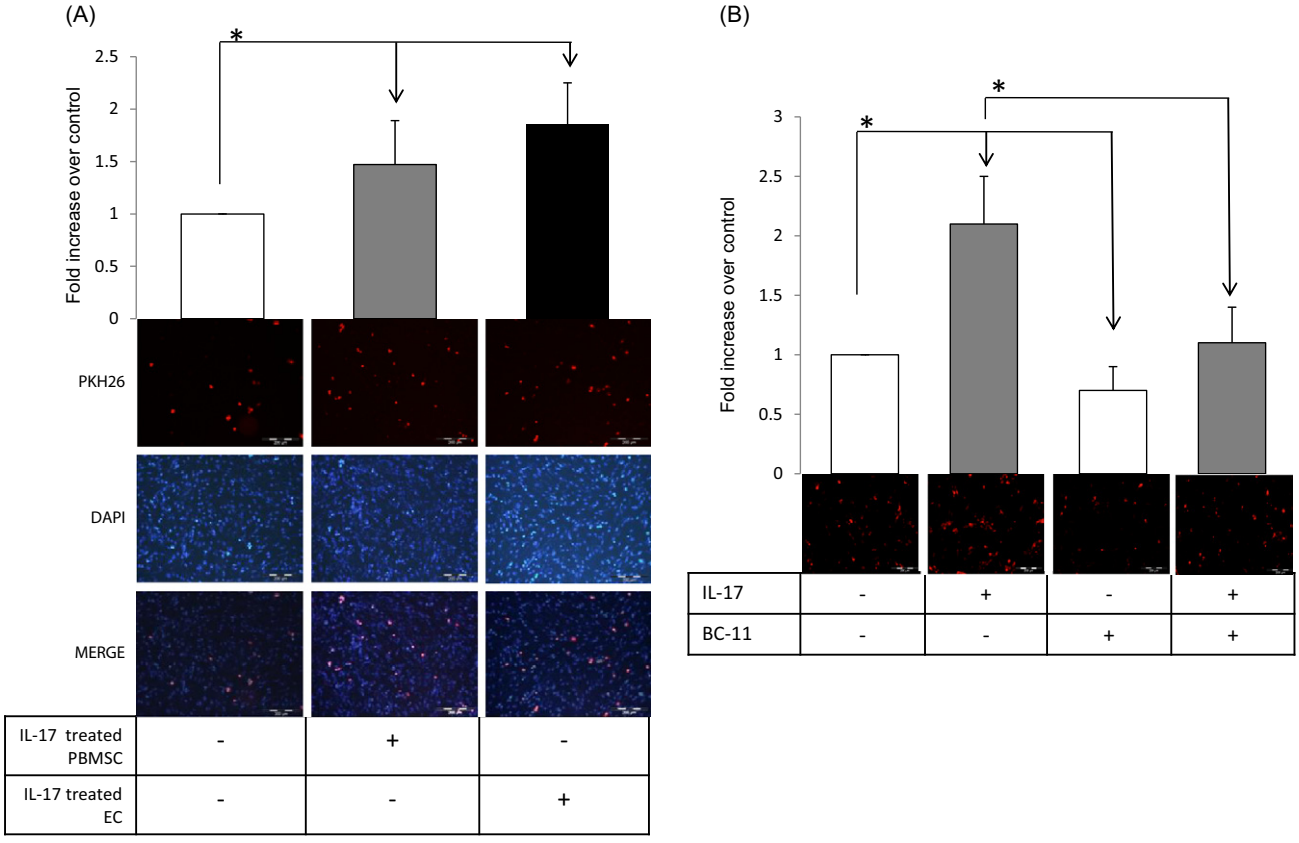

Fig. 7. IL-17 stimulates PB-MSC adhesion and transmigration on endothelial monolayer, and fibronectin adhesion. (A) Adhesion of PB-MSCs on endothelial cell monolayer. PB-MSCs labeled with PKH26 Red Fluorescent reagent were subjected to adhesion assay on endothelial cell monolayer stained with DAPI. Control (white bar) is PB-MSC adhesion on endothelial monolayer without IL-17 treatment; gray bar corresponds to PB-MSCs pretreated with IL-17 (50 ng/ml, 24 h) and subjected to adhesion on endothelial cells; black bar corresponds to PB-MSC adhesion on endothelial cell monolayer pretreated with IL-17 (50 ng/ml, 24 h). Photographs represent PKH26 Red Fluorescent labeled PB-MSC adhesion on endothelial cell monolayer for each treatment. Magnification 200× and scale bar: 200 μm. Representative results as means ± SEM from at least three independent determinations are shown. Significant difference from the control: *$p < 0.05$. (B) PB-MSC transmigration across endothelial cell monolayer. PB-MSCs labeled with PKH26 Red Fluorescent reagent were subjected to transmigrate across endothelial cells seeded in the upper compartment of transwell insert, and IL-17 (50 ng/ml) was added in the bottom compartment as chemoattractant signal. The uPA inhibitor BC11 (50 μM) was added in both compartments. Transmigrating fluorescent labeled cells were fixed and photographed. Magnification 200×, scale bar: 200 μm. Representative results as means ± SEM from at least three independent determinations are shown. Significant difference from the control: *$p < 0.05$. (C) Fibronectin expression in endothelial cell monolayer. Endothelial cell cultures in rounded glasses were subjected to immune-staining to fibronectin (green) and nuclei were stained with DAPI (blue). Magnification 400× and scale bar: 50 μm. (D) PB-MSC fibronectin adhesion. Cells were pretreated with IL-17 (50 ng/ml, 24 h) and subjected to adhesion assay on fibronectin pre-coated 24 well plates, in the presence or absence of uPA inhibitor BC11 or uPA blocking antibody. Representative results as means ± SEM from at least three independent determinations are shown. Significant difference from the control: *$p < 0.05$. Results are representative of three independent experiments.

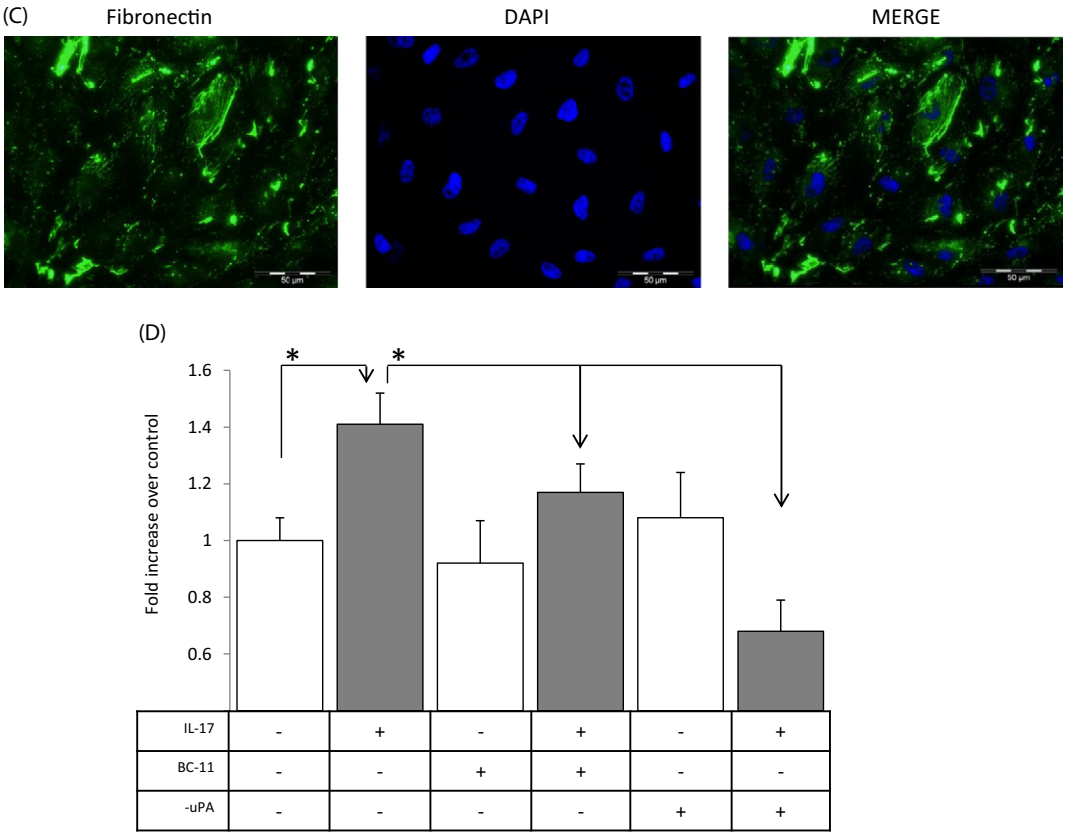

Fig. 7 (*continued*).

References

[1] K. English, Mechanisms of mesenchymal stromal cell immunomodulation, Immunol. Cell Bio. 91 (1) (2013) 19–26, http://dx.doi.org/10.1038/icb.2012.56.

[2] C.A. Roufosse, N.C. Direkze, W.R. Otto, N.A. Wright, Circulating mesenchymal stem cells, Int. J. Biochem. Cell Biol. 36 (4) (2004) 585–597, http://dx.doi.org/10.1016/j.biocel.2003.10.007.

[3] S.L. Tomchuck, K.J. Zwezdaryk, S.B. Coffelt, R.S. Waterman, E.S. Danka, A.B. Scandurro, Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses, Stem Cells 26 (1) (2008) 99–107, http://dx.doi.org/10.1634/stemcells. 2007-0563.

[4] C.M. Wilke, K. Bishop, D. Fox, W. Zou, Deciphering the role of Th17 cells in human disease, Trends Immunol. 32 (12) (2011) 603–611, http://dx.doi.org/10.1016/j.it.2011.08.003.

[5] A. Krstic, S. Mojsilovic, G. Jovcic, D. Bugarski, The potential of interleukin-17 to mediate hematopoietic response, Immunol. Res. 52 (1–2) (2012) 34–41, http://dx.doi.org/10.1007/s12026-012-8276-8.

[6] S. Mojsilović, A. Krstić, V. Ilić, I. Okić-Đorđević, J. Kocić, D. Trivanović, J.F. Santibañez, G. Jovčić, D. Bugarski, IL-17 and FGF signaling involved in mouse mesenchymal stem cell proliferation, Cell Tissue Res. 346 (3) (2011) 305–316, http://dx.doi.org/10.1007/s00441-011-1284-5.

[7] Y. Lee, The role of interleukin-17 in bone metabolism and inflammatory skeletal diseases, BMB Rep. 46 (10) (2013) 479–483.

[8] J. Kocić, J.F. Santibañez, A. Krstić, S. Mojsilović, I.O. Dorđević, D. Trivanović, V. Ilić, D. Bugarski, Interleukin 17 inhibits myogenic and promotes osteogenic differentiation of C2C12 myoblasts by activating ERK1,2, Biochim. Biophys. Acta 1823 (4) (2012) 838–849, http://dx.doi.org/10.1016/j.bbamcr.2012.01.001.

[9] R. Kalluri, Basement membranes: structure, assembly and role in tumour angiogenesis, Nat. Rev. Cancer 3 (6) (2003) 422–433, http://dx.doi.org/10.1038/nrc1094.

[10] J.F. Santibanez, Transforming growth factor-beta and urokinase-type plasminogen activator: dangerous partners in tumorigenesis-implications in skin cancer, ISRN Dermatol. (2013) 597927, http://dx.doi.org/10.1155/2013/597927.

[11] S. Ulisse, E. Baldini, S. Sorrenti, M. D'Armiento, The urokinase plasminogen activator system: a target for anti-cancer therapy, Curr. Cancer Drug Targets 9 (1) (2009) 32–71, http://dx.doi.org/10.2174/156800909787314002.

[12] E. Spaeth, A. Klopp, J. Dembinski, M. Andreeff, F. Marini, Inflammation and tumor microenvironments: defining the migratory itinerary of mesenchymal stem cells, Gene Ther. 15 (10) (2008) 730–738, http://dx.doi.org/10.1038/gt.2008.39.

[13] M. Gutova, J. Najbauer, R.T. Frank, S.E. Kendall, A. Gevorgyan, M.Z. Metz, M. Guevorkian, M. Edmiston, D. Zhao, C.A. Glackin, S.U. Kim, K.S. Aboody, Urokinase plasminogen activator and urokinase plasminogen activator receptor mediate human stem cell tropism to malignant solid tumors, Stem Cells 26 (6) (2008) 1406–1413, http://dx.doi.org/10.1634/stemcells. 2008-0141.

[14] S.M. Pulukuri, B. Gorantla, V.R. Dasari, C.S. Gondi, J.S. Rao, Epigenetic upregulation of urokinase plasminogen activator promotes the tropism of mesenchymal stem cells for tumor cells, Mol. Cancer Res. 8 (8) (2010) 1074–1083, http://dx.doi.org/10.1158/1541-7786.MCR-09-0495.

[15] P. Miossec, J.K. Kolls, Targeting IL-17 and TH17 cells in chronic inflammation, Nat. Rev. Drug Discov. 11 (10) (2012) 763–776, http://dx.doi.org/10.1038/nrd3794.

[16] D. Trivanović, J. Kocić, S. Mojsilović, A. Krstić, V. Ilić, I.O. Djordjević, J.F. Santibanez, G. Jovcić, M. Terzić, D. Bugarski, Mesenchymal stem cells isolated from peripheral blood and umbilical cord Wharton's jelly, Srp. Arh. Celok. Lek. 141 (3–4) (2013) 178–186.

[17] J. Krstić, A. Jauković, S. Mojsilović, I.O. Đorđević, D. Trivanović, V. Ilić, J.F. Santibañez, D. Bugarski, In vitro effects of IL-17 on angiogenic properties of endothelial cells in relation to oxygen levels, Cell Biol. Int. 37 (11) (2013) 1162–1170, http://dx.doi.org/10.1002/cbin.10144.

[18] J. Kocić, J.F. Santibañez, A. Krstić, S. Mojsilović, V. Ilić, D. Bugarski, Interleukin-17 modulates myoblast cell migration by inhibiting urokinase type plasminogen activator expression through p38 mitogen-activated protein kinase, Int. J. Biochem. Cell Biol. 45 (2) (2013) 464–475, http://dx.doi.org/10.1016/j.biocel.2012.11.010.

[19] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, Prockop Dj, E. Horwitz, Minimal criteria for defining multipotent mesenchymal stromal cells, The Int. Soc. Cell. Ther. pos. statement. Cytotherapy 8 (2006) 315–317.

[20] A. Tournadre, P. Miossec, Interleukin-17 in inflammatory myopathies, Curr. Rheumatol. Rep. 14 (3) (2012) 252–256, http://dx.doi.org/10.1007/s11926-012-0242-x.

[21] C. Ries, V. Egea, M. Karow, H. Kolb, M. Jochum, P. Neth, MMP-2, MT1-MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stem cells: differential regulation by inflammatory cytokines, Blood 109 (9) (2007) 4055–4063, http://dx.doi.org/10.1182/blood-2006-10-051060.

[22] Z. Sun, Y.H. Chen, P. Wang, J. Zhang, V. Gurewich, P. Zhang, J.N. Liu, The blockage of the high-affinity lysine binding sites of plasminogen by EACA significantly inhibits prourokinase-induced plasminogen activation, Biochim. Biophys. Acta 1596 (2) (2002) 182–192, http://dx.doi.org/10.1016/S0167-4838(02)00233-9.

[23] C. Le Clainche, M.F. Carlier, Regulation of actin assembly associated with protrusion and adhesion in cell migration, Physiol. Rev. 88 (2) (2008) 489–513, http://dx.doi.org/10.1152/physrev.00021.2007.

[24] A.M. Dimarino, A.I. Caplan, T.L. Bonfield, Mesenchymal stem cells in tissue repair, Front Immunol 4 (2013) 201, http://dx.doi.org/10.3389/fimmu.2013.00201 (eCollection 2013).

[25] A. Schmidt, D. Ladage, C. Steingen, K. Brixius, T. Schinköthe, F.J. Klinz, R.H. Schwinger, U. Mehlhorn, W. Bloch, Mesenchymal stem cells transmigrate over the

endothelial barrier, Eur. J. Cell Biol. 85 (11) (2006) 1179–1188, http://dx.doi.org/10.1016/j.ejcb.2006.05.015.
[26] C. Steingen, F. Brenig, L. Baumgartner, J. Schmidt, A. Schmidt, W. Bloch, Characterization of key mechanisms in transmigration and invasion of mesenchymal stem cells, J. Mol. Cell. Cardiol. 44 (6) (2008) 1072–1084, http://dx.doi.org/10.1016/j.yjmcc.2008.03.010.
[27] A. Mettouchi, The role of extracellular matrix in vascular branching morphogenesis, Cell Adhes. Migr. 6 (6) (2012) 528–534, http://dx.doi.org/10.4161/cam.22862.
[28] A. Chronopoulos, K. Trudeau, S. Roy, H. Huang, S.A. Vinores, S. Roy, High glucose-induced altered basement membrane composition and structure increases trans-endothelial permeability: implications for diabetic retinopathy, Curr. Eye Res. 36 (8) (2011) 747–753, http://dx.doi.org/10.3109/02713683.2011.585735.
[29] K.C. Vallabhaneni, S. Tkachuk, Y. Kiyan, N. Shushakova, H. Haller, I. Dumler, G. Eden, Urokinase receptor mediates mobilization, migration, and differentiation of mesenchymal stem cells, Cardiovasc. Res. 90 (1) (2011) 113–121, http://dx.doi.org/10.1093/cvr/cvq362.
[30] S. Maxson, E.A. Lopez, D. Yoo, A. Danilkovitch-Miagkova, M.A. Leroux, Concise review: role of mesenchymal stem cells in wound repair, Stem Cells Transl. Med. 1 (2) (2012) 142–149, http://dx.doi.org/10.5966/sctm. 2011-0018.
[31] Y. Liu, S. Wang, S. Shi, The role of recipient T cells in mesenchymal stem cell-based tissue regeneration, Int. J. Biochem. Cell Biol. 44 (11) (2012) 2044–2045, http://dx.doi.org/10.1016/j.biocel.2012.08.003.
[32] M.S. Maddur, P. Miossec, S.V. Kaveri, J. Bayry, Th17 cells: biology, pathogenesis of autoimmune and inflammatory diseases, and therapeutic strategies, Am. J. Pathol. 181 (1) (2012) 8–18, http://dx.doi.org/10.1016/j.ajpath.2012.03.044.
[33] B.R. Binder, J. Mihaly, G.W. Prager, uPAR–uPA–PAI-1 interactions and signaling: a vascular biologist's view, Thromb. Haemost. 97 (2007) 336–342, http://dx.doi.org/10.1160/TH06-11-0669.
[34] F. Blasi, N. Behrendt, M.V. Cubellis, V. Ellis, L.R. Lund, M.T. Masucci, L.B. Møller, D.P. Olson, N. Pedersen, N. Behrendt, V. Ellis, L.F. Lund, M. Ploug, E. ERønne, K. Danø, The urokinase receptor and regulation of cell surface plasminogen activation, Cell Differ. Dev. 32 (1990) 247–253.
[35] F. Blasi, P. Carmeliet, uPAR: a versatile signalling orchestrator, Nat. Rev. Mol. Cell Biol. 3 (2002) 932–943, http://dx.doi.org/10.1038/nrm977.
[36] M. Abedin, Y. Tintut, L.L. Demer, Mesenchymal stem cells and the artery wall, Circ. Res. 95 (2004) 671–676, http://dx.doi.org/10.1161/01.RES.0000143421.27684.12.
[37] S. Neuss, R.K. Schneider, L. Tietze, R. Knüchel, W. Jahnen-Dechent, Secretion of fibrinolytic enzymes facilitates human mesenchymal stem cell invasion into fibrin clots, Cells Tissues Organs 191 (1) (2010) 36–46, http://dx.doi.org/10.1159/000215579.
[38] B.R. Son, L.A. Marquez-Curtis, M. Kucia, M. Wysoczynski, A.R. Turner, J. Ratajczak, M.Z. Ratajczak, A. Janowska-Wieczorek, Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases, Stem Cells 24 (5) (2006) 1254–1264, http://dx.doi.org/10.1634/stemcells. 2005-0271.
[39] A. De Becker, P. Van Hummelen, M. Bakkus, I. Vande Broek, J. De Wever, M. De Waele, I. Van Riet, Migration of culture-expanded human mesenchymal stem cells through bone marrow endothelium is regulated by matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-3, Haematologica 92 (4) (2007) 440–449, http://dx.doi.org/10.3324/haematol.10475.
[40] A.J. Ridley, M.A. Schwartz, K. Burridge, R.A. Firtel, M.H. Ginsberg, G. Borisy, J.T. Parsons, A.R. Horwitz, Cell migration: integrating signals from front to back, Science 302 (5651) (2003) 1704–1709, http://dx.doi.org/10.1126/science.1092053.
[41] A. Hall, Rho GTPases and the control of cell behaviour, Biochem. Soc. Trans. 33 (Pt 5) (2005) 891–895, http://dx.doi.org/10.1042/BST20050891.
[42] E.M. Moran, M. Connolly, W. Gao, J. McCormick, U. Fearon, D.J. Veale, Interleukin-17A induction of angiogenesis, cell migration, and cytoskeletal rearrangement, Arthritis Rheum. 63 (11) (2011) 3263–3273, http://dx.doi.org/10.1002/art.30582.
[43] H. Nguyen, V.L. Chiasson, P. Chatterjee, S.E. Kopriva, K.J. Young, B.M. Mitchell, Interleukin-17 causes Rho-kinase-mediated endothelial dysfunction and hypertension, Cardiovasc. Res. 97 (4) (2013) 696–704, http://dx.doi.org/10.1093/cvr/cvs422.
[44] G.S. Teo, J.A. Ankrum, R. Martinelli, S.E. Boetto, K. Simms, T.E. Sciuto, A.M. Dvorak, J.M. Karp, C.V. Carman, Mesenchymal stem cells transmigrate between and directly through tumor necrosis factor-α-activated endothelial cells via both leukocyte-like and novel mechanisms, Stem Cells 30 (11) (2012) 2472–2486, http://dx.doi.org/10.1002/stem.1198.
[45] C.A. Reichel, B. Uhl, M. Lerchenberger, D. Puhr-Westerheide, M. Rehberg, J. Liebl, A. Khandoga, W. Schmalix, S. Zahler, E. Deindl, S. Lorenzl, P.J. Declerck, S. Kanse, F. Krombach, Urokinase-type plasminogen activator promotes paracellular transmigration of neutrophils via Mac-1, but independently of urokinase-type plasminogen activator receptor, Circulation 124 (17) (2011) 1848–1859, http://dx.doi.org/10.1161/CIRCULATIONAHA. 110.017012.
[46] X. Xing, J. Yang, X. Yang, Y. Wei, L. Zhu, D. Gao, M. Li, IL-17A induces endothelial inflammation in systemic sclerosis via the ERK signaling pathway, PLoS One 8 (12) (2013) e85032, http://dx.doi.org/10.1371/journal.pone.0085032.
[47] G.K. Griffin, G. Newton, M.L. Tarrio, D.X. Bu, E. Maganto-Garcia, V. Azcutia, P. Alcaide, N. Grabie, F.W. Luscinskas, K.J. Croce, A.H. Lichtman, IL-17 and TNF-α sustain neutrophil recruitment during inflammation through synergistic effects on endothelial activation, J. Immunol. 188 (12) (2012) 6287–6299, http://dx.doi.org/10.4049/jimmunol.1200385.
[48] R. Bass, V. Ellis, Regulation of urokinase receptor function and pericellular proteolysis by the integrin alpha(5)beta(1), Thromb. Haemost. 101 (5) (2009) 954–962, http://dx.doi.org/10.1160/TH08-08-0558.
[49] J. Yue, K. Zhang, J. Chen, Role of integrins in regulating proteases to mediate extracellular matrix remodeling, Cancer Microenviron. 5 (3) (2012) 275–283, http://dx.doi.org/10.1007/s12307-012-0101-3.
[50] L. Wang, Y. Zhao, S. Shi, Interplay between mesenchymal stem cells and lymphocytes: implications for immunotherapy and tissue regeneration, J. Dent. Res. 91 (11) (2012) 1003–1010, http://dx.doi.org/10.1177/0022034512460404.