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A role for plasma kallikrein-kinin system activation in the synovial recruitment of endothelial progenitor cells in arthritis

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Abstract

Objective—To examine whether the activation of plasma kallikrein-kinin system (KKS) mediates synovial recruitment of endothelial progenitor cells (EPCs) in arthritis.

Methods—EPCs were isolated from Lewis rat bone marrow and characterized by the expression of progenitor cell lineage markers and functional property. EPCs were intravenously injected into Lewis rats bearing arthritis, their recruitment and formation of de novo blood vessels in inflamed synovium were evaluated. The role of plasma KKS was examined using a plasma kallikrein inhibitor EPI-KAL2 and an anti-kallikrein antibody 13G11. Transendothelial migration (TEM) assay was used to determine the role of bradykinin and its receptor in EPC mobilization.

Results—Lewis rat EPCs exhibited strong capacities to form tubes and vacuoles, and expressed higher level of bradykinin type 2 receptor (B2R) and progenitor cell markers CD34 and Sca-1. In Lewis rats bearing arthritis, EPCs were recruited into inflamed synovium at acute phase and formed de novo blood vessels. Inhibition of plasma kallikrein by EPI-KAL2 and 13G11 significantly suppressed synovial recruitment of EPCs and hyperproliferation of synovial cells. Bradykinin concentration-dependently stimulated TEM of EPCs, which was mediated by B2R, as the knockdown of B2R by silencing RNA completely blocked bradykinin-stimulated TEM. Moreover, bradykinin selectively upregulated the expression of homing receptor C-X-C chemokine receptor type 4 (CXCR-4) in EPCs.

Conclusion—These observations demonstrate a novel role for plasma KKS activation in the synovial recruitment of EPCs in arthritis, acting via kallirein activation and B2R-dependent mechanisms. B2R might be involved in the mobilization of EPCs via upregulation of CXCR-4.

Introduction

The pathogenesis of rheumatoid arthritis (RA) is critically dependent on neovascularization of the synovium, occurring before clinical symptoms appear[1]. Synovial neovascularization creates a direct conduit for the entry into the joint of circulating leukocytes that exacerbates inflammation, and provides nutrients to hyperproliferative synovium. Neovascularization in adult tissue was thought to occur exclusively through angiogenesis, it has been recently noted that circulating endothelial progenitor cells (EPCs) are recruited to inflamed synovium

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and participate in synovial neovascularization in arthritis[2–3]. EPCs, which are derived from adult bone marrow, have strong capacity to home to inflamed and ischemic tissues and differentiate into vessel-forming endothelial cells. The mechanism for EPC homing in arthritis, however, remains unknown and the following important issues need to be addressed: How are EPCs regulated under the inflammatory conditions in arthritis ? What are the cellular and molecular mechanisms for EPC homing to inflamed synovium ? How do EPCs differentiate into new blood vessels and to what extent do they contribute to synovial neovascularization ? Understanding the mechanisms that control the process of EPC homing is important, because it will identify key molecules as novel targets for pharmacologic blockade in arthritis by specific inhibitors and receptor antagonists.

The plasma kallikrein-kinin system (KKS) plays an important role in the pathogenesis of arthritis[4–5]. The KKS consists of four plasma proteins, Factor XII, Factor XI, prekallikrein and high molecular weight kininogen (HK)[6]. HK is responsible for the binding of this system complex to cell membrane or the contact surface, which is necessary for the assembly and activation of this system. Upon activation, plasma kallikrein cleaves HK to release a nonapeptide, bradykinin. The inflammatory response in arthritis is composed of an acute phase with edema, pain, and neutrophil migration, all of which are known to be associated with plasma kallikrein activation and the release of bradykinin. In patients with RA, elevated levels of plasma kallikrein and bradykinin are detected in synovial fluid and positively correlated with the degree of joint pain and inflammation[7–8]. The concept that the KKS is important in the pathogenesis of arthritis is supported by the following observations in animal models. Lewis rats have a mutation in HK (S511N) rendering it susceptible to cleavage by plasma kallikrein, the administration of streptococcal cell wall polymers peptidoglycan - polysaccharide (PG-PS) induces systemic inflammatory response including arthritis in Lewis rats, but not in other strains such as Buffalo and Fischer rats[9]. Accompanied with synovitis and joint erosion in Lewis rats, there is a decrease in plasma prekallikrein and HK, likely due to consumption of the precursor proteins following KKS activation. A specific kallikrein inhibitor, by blocking the KKS activation, prevents arthritis and the systemic complications in the PG-PS model[10]. The Brown-Norway-Katholiek (B/N/Ka) rat strain has a severe deficiency of plasma kininogen due to a single point mutation Ala163Thr, which results in defective secretion of kininogen from the liver. Kininogen-deficient rats on a Lewis genetic background exhibit attenuated acute and chronic inflammatory arthritis[11], demonstrating that plasma KKS and HK cleavage product kinins are key mediators of inflammatory diseases. Taken together, in Lewis rat model, the activation of the KKS is critical for the pathogenesis of arthritis. Therefore, this model is appropriate for us to investigate the potential link between the KKS activation and the dynamic recruitment of EPCs into inflamed synovium during the process of arthritis.

The aim of this study was to determine whether plasma KKS activation is associated with the synovial recruitment of EPCs in arthritis. First, we established a method to evaluate the recruitment of EPCs into inflamed synovium in Lewis rat model of arthritis. Second, we investigated the effect of specific plasma kallikrein inhibition on the synovial recruitment of EPCs, and tested whether bradykinin and its receptor B2R are involved in the mobilization of EPCs.

Materials and Methods

Animals

Lewis rats (Charles River Laboratories) at 8 weeks old, with a mean weight of 180–200 g, were used in this study. Green fluorescent protein (GFP) transgenic Lewis rats were provided by Dr. Eiji Kobayashi (Jichi Medical School, Japan). Rats were maintained under climate-controlled conditions in a 12 hours light:12 hours dark cycle. The animals were fed

with standard rodent chow and water *ad libitum*. The health status of the animal colony was monitored in accordance with the guidelines from the Institutional Animal Care and Use Committee.

Isolation of endothelial progenitor cells from Lewis rats

Bone marrow (BM) cells were aspirated from femurs and tibias of 8 week-old female Lewis rats and GFP Lewis rats. Mononuclear cells (MNCs) were isolated by density gradient (Histopaque 1083, Sigma) centrifugation at 400*g* for 30 minutes. Rat $CD34^+$ BM-MNCs were purified using mouse anti-rat CD34 antibody (Santa Cruz Biotechnology) and a CELLection™ Pan Mouse IgG Kit (Invitrogen), they were resuspended in endothelial cell growth culture medium (EGM-2, Cambrex) with 10% fetal bovine serum (FBS), and cultured in tissue cultureplates coated with Type I collagen (Invitrogen) and incubated at 37°C with 5% CO_2. The cells were expanded in large scale. Fluorescent EPCs, isolated from GFP-Lewis rats or labeled with carboxyfluorescein diacetate - succinimidyl ester (CFDA-SE, Molecular Probes), were used in the assays. Rat lung microvessel endothelial cells (rLMEC) purchased from VEC Technologies, Inc., served as a control.

Measurement of mRNA expression by reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real time RT-PCR

Expression of mRNA wasmeasured by RT-PCR as previously described[12]. Briefly, total RNA was prepared using Trizol® reagent (Invitrogen). RNA (a total of 100 ng) was used as template in a one-step RT-PCR reaction (SuperScript One-Step RT-PCR with Platinum® *Taq*, Invitrogen). RT for cDNA synthesis was accomplished in 30 minutes incubation at 50°C, which was followed by PCR cycling as follows: initialization step at 94°C for 2 minutes followed by 30 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute using 0.2 μmol/L primers. The RT-PCR products were identified in 4% agarose gel electrophoresis. The sequence of primers were as follows: B2R, forward 5′-ccatctctccacctgcattg-3′, reverse 5′-cgtctggacctccttgaact-3′; B1R, forward 5′-tcttcctggtggtggctatc-3′, reverse 5′-cgttcaactccaccatcctt-3′; vWF, forward 5′-cacaggtagcacacatcact-3′, reverse 5′-ctcaaagtcttggatgaaga-3′; CD34, forward 5′-gcccagtctgaggttaggcc-3′, reverse 5′-attggcctttccctgagtct-3′; CD31, forward 5′-gccctgtcacgtttcagttt-3′, reverse 5′-ctgcaatgagccctttcttc-3′; Sca-1, forward 5′-cggtcattcagaccacacacag-3′, reverse 5′-tgggttgaagttctcgctcttg-3′; CD14, forward 5′-cttgttgctgttgcctttga-3′, reverse 5′-cgtgtccacacgctttagaa-3′; CD144, forward 5′-aggacgtggtgccagtaaac-3′, reverse 5′-ctgtgatgttggcggtattg-3′; C-X-C chemokine receptor type 4 (CXCR-4), forward 5′-ctgcatcatcatctccaagc-3′, reverse 5′-ggaaaggatcttgaggctgg-3′; α4 integrin, forward 5′-cccaggctacatcgttttgt-3′, reverse 5′-atgggagtgaggatgtctcg-3′; CD11b, forward 5′-ttaccggactgtgtggacaa-3′, reverse 5′-agtctcccaccaccaaagtg-3′; E-selectin, forward 5′-tttttggcacggtatgtgaa-3′, reverse 5′-aggttgctgccacagagagt-3′; Primer pair for β-actin was purchased from Promega (G5740).

Quantitative real-time PCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix kit (Fermentas) according to the manufacturer's instructions. The sequence of primers were as follows: CD31, forward 5′-atcctgtcgggtaacgatgta-3′, reverse 5′-cttcggagactggtcacaatg-3′; CD34, forward 5′-ttaagggagacatcaaatgttca-3′, reverse 5′-gctagattcaaggagcatacacta-3′; Sca-1, forward 5′-agacggcaaaagtcaggttac-3′, reverse 5′-aacctgtatcatttgccctcatc-3′; vWF, forward 5′-caatacggaagcatcaatacca-3′, reverse 5′-ccaaggaggtatccatgatgat-3′; B1R, forward 5′-caggtgaagctgtgagctctt-3′, reverse 5′-aagaagcagatagtgatgacgaa-3′; B2R, forward 5′-aaggacgatcctcactcgtct-3′, reverse 5′-tctgaaaaggtcccgttatga-3′; β-actin, forward 5′-acgttgacatccgtaaagacc-3′, reverse 5′-gccaccaatccacacagagt-3′. In an ABI 7500 system (Applied Biosystems), PCR was conducted over 45 cycles of 95°C for 15 seconds and 60°C for 1 minute, preceded by an

initial step at 95°C for 10 minutes. The results were analyzed using SDS Version 2.1 software.

Three-dimensional (3D) vasculogenesis assay

The vasculogenic capacity of Lewis rat EPCs was evaluated in culture within 3D collagen gels as we recently described[13]. Briefly, EPCs at passages 2 were resuspended in endothelial cell basal medium EBM-2 (2×10^6 cells/mL) and mixed with neutralized collagen type-I (4 mg/mL, BD Bioscience) at a volume ratio of 1:1. One hundred microliters of mixture was transferred to one well of the 96-well culture plate and incubated at 37°C for 20 minutes to allow gel formation. The gel matrices were then overlaid with EGM-2 containing growth factor and 10% FBS and cultured for 48 hours. After fixation with 2% paraformaldehyde (PFA), the gels were stained with 0.1% toluidine blue (Sigma) and visualized with a microscope (Nikon Eclipse TE300).

Transendothelial migration (TEM) of EPCs

The TEM was analyzed using 96-transwell filters (3.0 μm polycarbonate membrane, Corning Costar). After rLMECs were cultured on collagen-coated transwell filters until becoming confluent, fluorescent EPCs (3.0×10^4) in endothelial basal medium (EBM-2) containing 2% FBS were placed on top of the rLMECs monolayer. EBM-2 containing 2% FBS and bradykinin (Sigma) was added to the lower compartment. After incubation at 37°C for 18 hours, the filters were fixed with 2% PFA, and fluorescent EPCs that had migrated into the lower side of the filters were visualized with a fluorescent microscope and counted in random microscopic fields.

Construction of small interfering RNA (siRNA) and transfection of EPCs with siRNA

The sequences selected as the targeting region were as follows: for rat B2R 5′-CCGCACTGGAGAACATCTTTGTCCT-3′, and for CXCR-4 5′-GGATAACTACTCCGAAGAA-3′. A nonsilencing sequence 5′-AACCTGCGGGAAGAAGTGG-3′ was used as a control (control siRNA). The siRNA oligos labeled with fluorescein were synthesized by Invitrogen. Transfection of EPCs was performed with HiPerfect (Qiagen).

Rat models of arthritis and implantation of EPCs

In PG-PS-induced arthritis model, female-specific pathogen-free (SPF) Lewis rats received a single dose of intraperitoneal injection with PG-PS (15 μg of rhamnose/g of average body weight; BD Lee Laboratories)[11]. In collagen-induced arthritis (CIA) model, female SPF Lewis rats received injection of 250 μg bovine type II collagen in complete Freund's adjuvant (Chondrex, Inc.) at the base of tail at one week interval. The severity of arthritis was assessed using ankle joint diameters with digital caliper Ultra-Call Mark III (F.V. Fowler Co., Inc.). The mean of triplicate measurements of each hind paw were recorded every day during the first week and then every other day during the entire course of the protocol. The joint diameter was reported as change in joint diameter in mm from the baseline on day 0. Fluorescent rEPCs (1×10^7) were injected into recipient animals through tail vein in a volume of 300 μl. After a period of time as indicated, the animals were euthanized by CO_2 narcosis. Hind limb joints were harvested, fixed in buffered formalin.

Histopathology and immunohistochemistry

The paws were decalcified in formic acid (Fisher Scientific) or EDTA. The tissue was embedded in paraffin and the sections were stained with hematoxylin and eosin for microscopic examination. Some sections were stained with polyclonal anti-vWF antibody (Abcam) plus 0.2% Triton X-100 (Sigma) and Alexa Fluor 633-labeled goat anti-rabbit

antibody (Molecular Probes). The sections were photographed under a Leica TCS SP5 confocal laser-scanning microscope. The images were processed using Adobe Photoshop 9.0 software.

Quantitative analysis of synovial recruitment of EPCs at acute phase of arthritis

The paraffin-embedded sections at 10-μm thickness of ankle joint were stained with SYTO® Red Fluorescent Nucleic Acid Stain (SYTO® Red, Molecular Probes) for visualizing total synovial cells including the implanted fluorescent EPCs. The sections were examined under a Leica TCS SP5 confocal microscope. Total synovial cells (red) and EPCs (green) in synovial tissues were enumerated.

Data analysis

The data were calculated as average ± SEM from experiments done at least 3 times, and statistically analyzed by Student's *t* test (two groups only) or One Way Analysis of variance (ANOVA) and Student-Newman-Keuls test (multiple groups). Difference with probability values below 0.05 were considered significant.

Results

Isolation and characterization of rat EPCs

In this study, we first isolated EPCs from Lewis rats. Bone marrow (BM) cells were cultured on Type I collagen surfaces, multiple colonies often appeared after 7 days. As shown in the Figure 1A, a typical colony with cobblestone-like morphology was pictured on day 7 and day 12, respectively, which was subsequently collected for large-scale expansion. EPCs are often characterized by the progenitor capacity of tube and vacuole formation and expression of progenitor and endothelial markers[13]. In an in vitro angiogenesis assay we recently established[13], EPCs formed vacuoles and tubes (Figure 1B), suggesting that EPCs possess the endothelial progenitor capacity for differentiation. To characterize whether the collected cells express the markers for endothelial cell and progenitor cell lineage, we measured their mRNA expression by RT-PCR, because the availability of antibodies that recognize rat antigens is limited. Rat lung microvessel endothelial cells (rLMECs) were used as a control for differentiated endothelial lineage. Total RNA was extracted from passage 3 of EPCs and rLMECs. As shown in the Figure 1C, rEPCs and rLMECs expressed similar levels of certain endothelial cell marker mRNA such as CD144 (VE-cadherin), CD31, and vWF. EPCs exclusively expressed hematopoietic progenitor cell markers Sca-1 and CD34, and they did not express monocytic lineage marker CD14. Both of EPCs and rLMECs expressed B2R but not B1R. We also isolated EPCs from bone marrow of GFP-Lewis rats, the formation of original colonies appeared on day 7 (Figure 1D,i). A colony was collected for expansion, and their mRNA expression of endothelial cell and progenitor cell lineage markers was quantitated by real time RT-PCR. As shown in the Figure 1D(ii), compared with rLMECs, EPCs expressed higher level of progenitor cell lineage markers CD34 (1.00±0.01 v.s. 8.13±0.03) and Sca-1 (1.00±0.01 v.s. 2.00±0.01), but expressed lower level of endothelial cell lineage markers CD31 (1.00±0.07 v.s. 0.72±0.05) and vWF (1.00±0.04 v.s. 0.13±0.08). Interestingly, EPCs expressed 4-fold higher B2R than rLMECs (1.00±0.03 v.s. 3.90±0.03), and both EPCs and rLMECs did not express B1R (Figure 1D, ii).

EPCs home to inflamed synovium and formed new blood vessels in Lewis rats bearing arthritis

The KKS activation is tightly associated with the pathogenesis of arthritis in Lewis rat model induced by PG-PS[10]. After receiving a single intraperitoneal injection of PG-PS, the Lewis rats developed an acute arthropathy within 72 hours (Figure 2A). Light

microscopic examination of ankle sections, stained with hematoxylin-eosin, revealed inflamed and hyperplastic synovium with joint destruction, mononuclear cell infiltration and pannus formation (Figure 2B). It has been known that acute inflammation of this model usually resolves within 7 days and is followed by a chronic and secondary phase, which begins ~14 days after injection. In this study, we used this model to investigate the synovial recruitment of EPCs during the acute phase, and the new vessel formation at the chronic phase, respectively. Arthritis was induced by PG-PS in Lewis rats on day 0, followed by venous injection of fluorescent EPCs on day 1. On day 5, although fluorescent EPCs were not detected in the synovial tissue of disease-free ankle joint, they accumulated in the inflamed synovium (Figure 2C). The inflamed synovium from arthritic rat without implantation of EPCs served as a control (Figure 2C). To assess the vessel formation by implanted EPCs in the inflamed synovium of arthritic rats on day 12, the sections from the ankle joints were stained with rabbit anti-vWF antibody and Alexa Fluor 633-labeled goat anti-rabbit antibody. As shown in the Figure 3A, the implanted fluorescent EPCs formed blood vessels, which were positive for anti-vWF staining (arrows in d; bar, 60 μm). The higher magnification of the selected field within the box in panel d is demonstrated in the Figure 3B (bar, 20 μm). The recruitment of implanted EPCs to inflamed joints and formation of new blood vessels suggest that EPCs home to inflamed synovium at the acute phase of arthritis and participate in the formation of new blood vessels.

Inhibition of plasma kallikrein by EPI-KAL2 and 13G11 attenuates synovial recruitment of EPCs at acute phase of arthritis

The differentiation of EPCs into new vessels in synovium at chronic phase suggests that EPCs are recruited during the acute phase. Synovial recruitment of implanted EPCs was quantified six days post-injection of PG-PS. The paraffin-embedded sections of ankle joints were stained with SYTO® Red Fluorescent Nucleic Acid Stain for visualizing total synovial cells. As shown in the Figure 4A, total synovial cells (red) and fluorescent EPCs (green) in the synovial tissues could be viewed and enumerated. This method allows us to quantitatively evaluate synovial hyperplasia and synovial recruitment of EPCs.

In rat model of arthritis, activation of the KKS can be modulated by the inhibition of plasma kallikrein [10]. In this study, we first used a potent plasma kallikrein inhibitor EPI-KAL2 (Ki < 1 nM), which is the Kunitz domain inhibitor specific for plasma kallikrein[14]. As shown in the Figure 4B, joint swelling in the Group I rats (disease, treated with PBS) started after 24 hours post PG-PS injection, the increase in the joint diameter became marked on day 2, and peaked on day 3 (1.78 ± 0.23 mm), followed by a decrease after day 4. In contrast, the Group II rats (disease, treated with EPI-KAL2) did not develop any significant paw swelling during the acute phase (days 2 to 4), although there were mild changes occurring during the chronic phase after days 3 (Figure 4B). These data indicate that EPI-KAL2 prevents the joint swelling (arthritis) throughout the acute phase of the disease and greatly ameliorated joint inflammation at the chronic phase. Concomitantly, the treatment with EPI-KAL2 significantly reduced the total number of synovial cells by about 50 % and the number of recruited EPCs by near 75% (Figure 4C). Additionally, in a CIA model, we reexamined the effect of plasma kallikrein inhibition using an inhibitory anti-kallikrein antibody 13G11[15]. As shown in the Figure 4A, joint swelling of Lewis rats started 14 days after first injection of collagen, and reached at the maximal level on day 20. Treatment with 13G11 significantly inhibited joint swelling over the course of observation (Figure 5A). Moreover, the administration of 13G11 in this CIA model significantly reduced the total number of synovial cells by about 40 % and the number of recruited EPCs by near 55%, compared with PBS (Figure 5B).

Bradykinin interaction with B2R upregulates homing receptor CXCR-4 expression and stimulates transendothelial migration (TEM) of EPCs

Upon activation, plasma kallikrein cleaves HK to release bradykinin. Transendothelial migration (TEM) is an essential step for EPC homing to sites of inflammatory and ischemic tissue[16]. To further investigate whether and how the plasma KKS activation regulates the homing of EPCs, we measured TEM of EPCs mediated by bradykinin. As EPCs expressed bradykinin receptor B2R, but not B1R (Figure 1C and D), we thus used siRNA approach to knockdown B2R. Figure 6A indicates that B2R mRNA expression in EPCs was markedly downregulated by specific siRNA. In a TEM assay using 96-transwell filters, bradykinin concentration-dependently increased the transmigration of EPCs over the differentiated rLMECs (Figure 6B). Before and after the transmigration, the integrity of the mature endothelial cell monolayer was maintained (Supplemental Figure I). The transmigration of EPCs expressing B2R siRNA was significantly decreased (Figure 6B), suggesting that B2R is required for bradykinin-mediated TEM of EPCs. Further, we examined whether bradykinin modulates the expression of major homing receptors, including CXCR-4, α4 integrin, E-selectin, and CD11b. We found that bradykinin selectively upregulated the expression of CXCR-4 (Figure 6C), but not other three receptors (data not shown). Since the knockdown of B2R prevented the upregulation of CXCR-4 (Figure 6C), the effect of bradykinin is likely via B2R. To further examine whether bradykinin mediates TEM through upregulation of CXCR-4, the expression of CXCR-4 was downregulated by siRNA approach (Figure 6D). As shown in the Figure 6E, TEM of EPCs mediated by bradykinin at 100 and 1000 nM was inhibited by the downregulation of CXCR-4.

Discussion

There is a growing body of evidence indicating that bone marrow-derived EPCs contribute to synovial neovasculogenesis, a critical step for the development of arthritis [2–3]. So far, our understanding of the molecular mechanisms by which EPCs are recruited from the circulation to inflamed tissue remains very limited. Experimental and clinical observations have demonstrated that the KKS plays a critical role in the pathogenesis of arthritis, however, the molecular and cellular mechanisms by which the KKS mediates arthritis remains unknown. Upon activation of the KKS, plasma kallikrein cleaves HK to liberate bradykinin, the remained portion is two chain HKa. Recently, we have demonstrated that HKa regulates EPC functions in vitro [13, 17], revealing the connection between the KKS activation and EPC biology. We now show another role of the KKS in the homing function of EPCs. Our results indicate that 1) in Lewis rat model of arthritis, EPCs are recruited to inflamed synovium and form new vessels; 2) Inhibition of plasma kallikrein by EPI-KAL2 and 13G11 attenuates synovial recruitment of EPCs; 3) EPCs isolated from Lewis rats constitutively express B2R, by which bradykinin stimulates transendothelial migration of EPCs; 4) Bradykinin selectively upregulates expression of homing receptor CXCR4 via B2R. Our current study demonstrates a novel link between synovial neovasculogenesis and the activation of plasma KKS in the pathogenesis of arthritis, and reveals a new mechanism for the homing of EPCs to inflamed synovium.

In this study, the successful isolation of EPCs from Lewis rats and establishment of the model for detecting synovial recruitment of implanted EPCs allow us to document a role for KKS in EPC homing in arthritis. Since EPCs express high level of B2R (Figures 1E), we hypothesized that activation of the KKS, via the release of bradykinin, stimulates synovial recruitment of EPCs in arthritis. Indeed, plasma kallikrein inhibitor EPI-KAL2 and anti-kallikrein MoAb 13G11 attenuated the recruitment of EPCs to the inflamed synovium (Figures 4 and 5), demonstrating that activation of the KKS mediates the homing of EPCs to inflamed synovium in arthritis. The effect of these kallikrein inhibitors is, at least in part, through the inhibition of bradykinin production in plasma at the acute phase of arthritis.

Our previous study has indicated that a selective antagonist of B2R attenuates arthritis[18], suggesting that bradykinin is an important mediator for arthritis, and its effect is mainly through B2R. A recent study has demonstrated that B2R mediates the homing process of EPCs[19]. In this study, we defined the role of B2R in transendothelial migration of EPCs, a critical step of EPC homing process. Bradykinin stimulated TEM of EPCs in vitro, which was blocked by downregulation of B2R expression (Figure 5). During the process of joint inflammation, a chemokine gradient is established, directing the homing of BM-derived circulating EPCs. We now provide a novel cue that the interaction between bradykinin and B2R contributes to the homing of EPCs into inflamed synovium with neovascularization potential. Since elevated level of bradykinin exists in synovial fluid of arthritic animals and patients with RA[20], high expression of B2R in EPCs enables them to sense kinin gradients and to facilitate cell migration and invasion of EPCs. CXCR4 is a critical receptor for stem/progenitor homing function. Bradykinin selectively upregulates the homing receptor CXCR-4, via activation of B2R (Figure 5), thereby stimulating the synovial recruitment of EPCs. Thus, EPCs are likely a new target for bradykinin in the setting of acute arthritis.

The continuous recruitment of EPCs to inflamed synovium and the excessive utilization for neovascularization result in the depletion of circulating EPCs in RA patients, which may dampen vascular repair[21]. We have recently reported that HKa, another cleavage product of HK by plasma kallikrein, inhibits vasculogenic differentiation of EPCs and accelerates the onset of their senescence[13, 17]. Therefore, our current observations that the inhibition of plasma kallikrein suppresses synovial recruitment of EPCs reveal likelihood that plasma kallikrein may serve as a target for treatment of arthritis with dual benefits, inhibition of synovial neovasculogenesis and improvement of circulating EPC function. Further investigations into the cellular and molecular mechanisms by which the KKS regulates EPC homing and differentiation should improve in depth our understanding of the pathogenesis of arthritis.

Taken together, this study demonstrates for the first time that EPC homing to inflamed synovium at acute phase of arthritis is associated with the activation of plasma KKS, which underlies a novel role of this system in the pathogenesis of arthritis. As illustrated in the Supplemental Figure II, in the development and progress of arthritis, plasma kallikrein activation results in the cleavage of HK releasing bradykinin. Bradykinin upregulates the expression of CXCR-4, thereby mediating the transendothelial migration of EPCs and their homing to inflamed synovium, in which they participate in synovial neovascularization. This study not only provides additional information for understanding the molecular and cellular mechanisms of synovial neovasculogenesis, but also supports the hypothesis that therapeutic approaches targeting the KKS activation have considerable potential in arthritis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Szekanecz Z, et al. New insights in synovial angiogenesis. Joint Bone Spine. 2010; 77(1):13–19. [PubMed: 20022538]
2. Ruger B, et al. Endothelial precursor cells in the synovial tissue of patients with rheumatoid arthritis and osteoarthritis. Arthritis Rheum. 2004; 50(7):2157–66. [PubMed: 15248213]
3. Silverman MD, et al. The role of vascular cell adhesion molecule 1/very late activation antigen 4 in endothelial progenitor cell recruitment to rheumatoid arthritis synovium. Arthritis Rheum. 2007; 56(6):1817–26. [PubMed: 17530710]
4. Colman RW. Regulation of angiogenesis by the kallikrein-kinin system. Curr Pharm Des. 2006; 12(21):2599–607. [PubMed: 16842160]
5. Isordia-Salas I, et al. The role of plasma high molecular weight kininogen in experimental intestinal and systemic inflammation. Arch Med Res. 2005; 36(1):87–95. [PubMed: 15900628]
6. Colman RW, Schmaier AH. Contact System: A Vascular Biology Modulator With Anticoagulant, Profibrinolytic, Antiadhesive, and Proinflammatory Attributes. Blood. 1997; 90(10):3819–3843. [PubMed: 9354649]
7. Hernandez CC, Donadi EA, Reis ML. Kallikreins and kininogens in saliva and plasma of patients presenting with rheumatoid arthritis. Scand J Rheumatol. 2002; 31(1):38–40. [PubMed: 11924648]
8. Cassim B, et al. Kallikreins, kininogens and kinin receptors on circulating and synovial fluid neutrophils: role in kinin generation in rheumatoid arthritis. Rheumatology. 2009; 48(5):490–496. [PubMed: 19254919]
9. DeLa Cadena RA, et al. Role of kallikrein-kinin system in pathogenesis of bacterial cell wall-induced inflammation. American Journal of Physiology - Gastrointestinal and Liver Physiology. 1991; 260(2):G213–G219.
10. Dela Cadena R, et al. Inhibition of plasma kallikrein prevents peptidoglycan-induced arthritis in the Lewis rat. The FASEB Journal. 1995; 9(5):446–452.
11. Sainz IM, et al. Modulation of inflammation by kininogen deficiency in a rat model of inflammatory arthritis. Arthritis & Rheumatism. 2005; 52(8):2549–2552. [PubMed: 16059911]
12. Wu Y, et al. A role for Mer tyrosine kinase in αvβ5 integrin-mediated phagocytosis of apoptotic cells. Journal of Cell Science. 2005; 118(3):539–553. [PubMed: 15673687]
13. Wu Y, et al. Cleaved high molecular weight kininogen inhibits tube formation of endothelial progenitor cells via suppression of matrix metalloproteinase 2. Journal of Thrombosis and Haemostasis. 2010; 8(1):185–193. [PubMed: 19874467]
14. Markland W, Ley AC, Ladner RC. Iterative optimization of high-affinity protease inhibitors using phage display. 2. Plasma kallikrein and thrombin. Biochemistry. 1996; 35(24):8058–67. [PubMed: 8672510]
15. Veloso D, et al. A monoclonal anti-human plasma prekallikrein antibody that inhibits activation of prekallikrein by factor XIIa on a surface. Blood. 1987; 70(4):1053–1062. [PubMed: 2443201]
16. Nilsson SK, Simmons PJ. Transplantable stem cells: home to specific niches. Current Opinion in Hematology. 2004; 11(2):102–106. [PubMed: 15257026]
17. Dai J, et al. Cleaved High-Molecular-Weight Kininogen Accelerates the Onset of Endothelial Progenitor Cell Senescence by Induction of Reactive Oxygen Species. Arteriosclerosis, Thrombosis, and Vascular Biology. 2011; 31(4):883–889.
18. Sainz IM, et al. Interactions between bradykinin (BK) and cell adhesion molecule (CAM) expression in peptidoglycan-polysaccharide (PG-PS)-induced arthritis. The FASEB Journal. 2004
19. Kränkel N, et al. Role of Kinin B2 Receptor Signaling in the Recruitment of Circulating Progenitor Cells With Neovascularization Potential. Circulation Research. 2008; 103(11):1335–1343. [PubMed: 18927465]
20. Bond AP, et al. Generation of kinins in synovial fluid from patients with arthropathy. Immunopharmacology. 1997; 36(2–3):209–16. [PubMed: 9228548]
21. Grisar J, et al. Depletion of Endothelial Progenitor Cells in the Peripheral Blood of Patients With Rheumatoid Arthritis. Circulation. 2005; 111(2):204–211. [PubMed: 15642766]

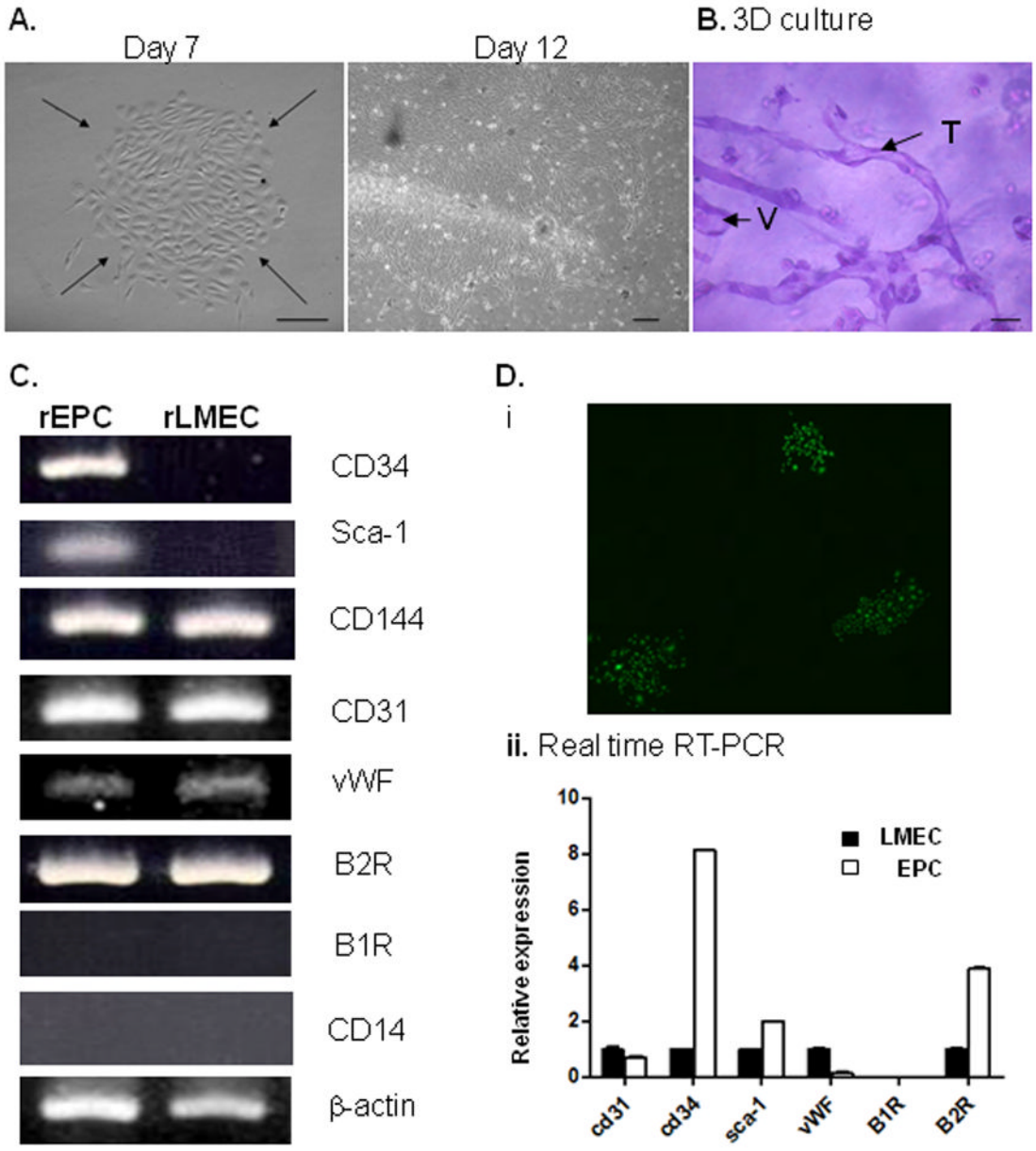

Figure 1. Isolation and characterization of rat EPCs

A. After CD34-positive bone marrow cells isolated from Lewis rats were cultured on collagen surfaces for 7 days, a colony with cobblestone-like morphology appeared and became a large colony after culture for 12 days. Scale bars represent 100 μm. **B.** After the isolated EPCs were cultured in the presence of 25 ng/mL VEGF in the 3D Type I collagen gel for 48 hours, they formed vacuoles (V) and tubes (T). Scale bar represents 50 μm. **C.** Endothelial cell and progenitor cell lineage markers of EPCs were characterized by RT-PCR. Total RNA was extracted from passage 3 of EPCs and rLMECs. **D.** Formation of multiple colonies of GFP-EPCs isolated from GFP Lewis rat bone marrow (**i**). Quantitation of mRNA level of progenitor cell and endothelial cell lineage markers of GFP-EPCs by real time RT-PCR (n=3). Results were normalized against mRNA of rLMECs, and relative quantification (RQ) between rLMECs and EPCs was calculated using the equation: $RQ = 2 - \Delta\Delta Ct$ by SDS Version 2.1 software (**ii**).

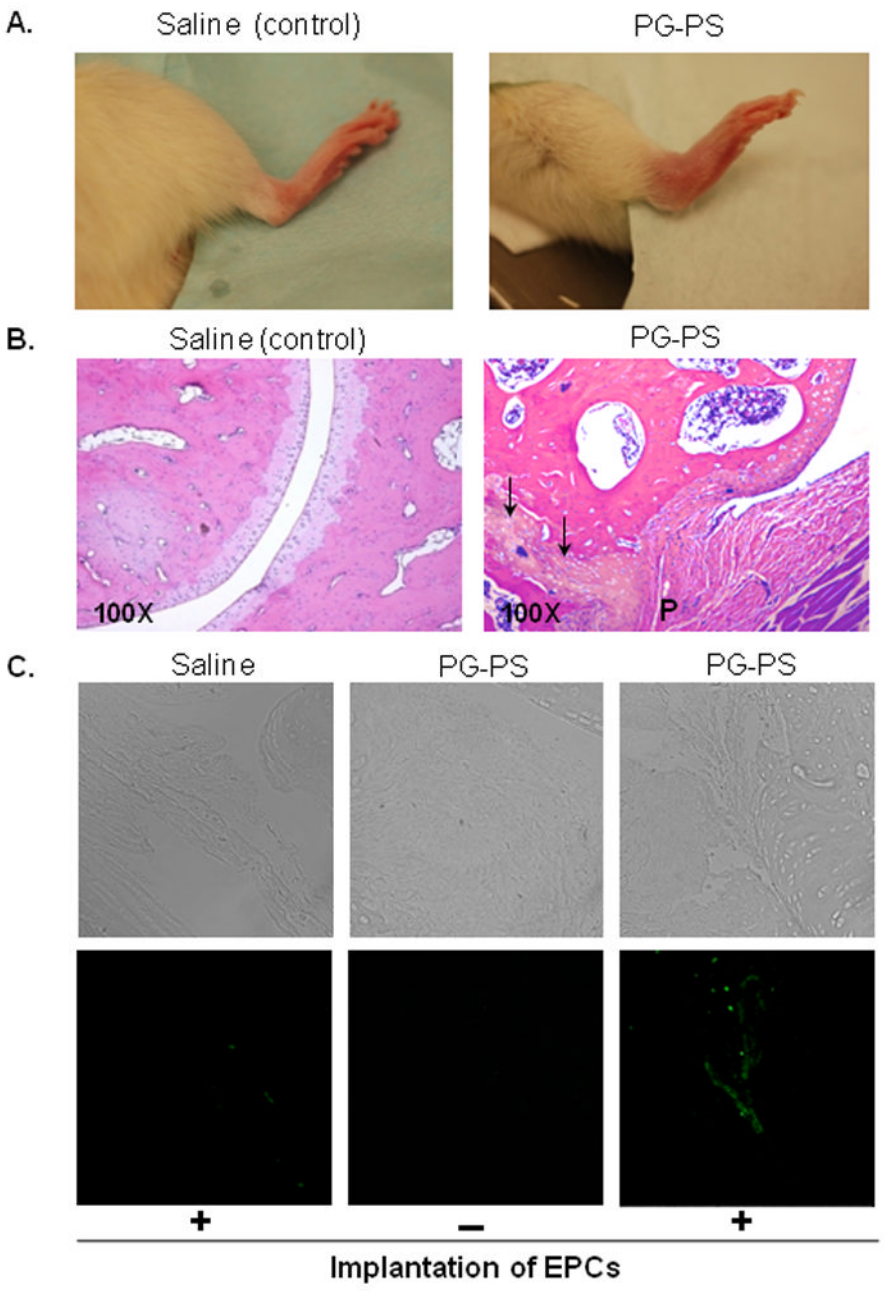

Figure 2. EPCs home to inflamed synovium

Female-specific pathogen-free Lewis rats received a single dose of intraperitoneal injection of saline (control) or PG-PS (15 μg of rhamnose/g of average body weight) on day 0. **A.** Photograph of the hind paw of Lewis rats, 5 days after injection of saline (control) and PG-PS. **B.** Representative section stained with hematoxylin and eosin showing destructive features of arthritis with mononuclear cell infiltration (arrows) and advanced pannus (P). **C.** Female-specific pathogen-free Lewis rats were intraperitoneally injected with saline or a single dose of PG-PS (15 μg of rhamnose/g of average body weight) on day 0, and received an injection through tail vein with or without fluorescent EPCs on day 1. On day 5, rats were euthanized, hind ankles were fixed in 4% PFA and decalcified. The paraffin sections were viewed and photographed under a fluorescent microscope.

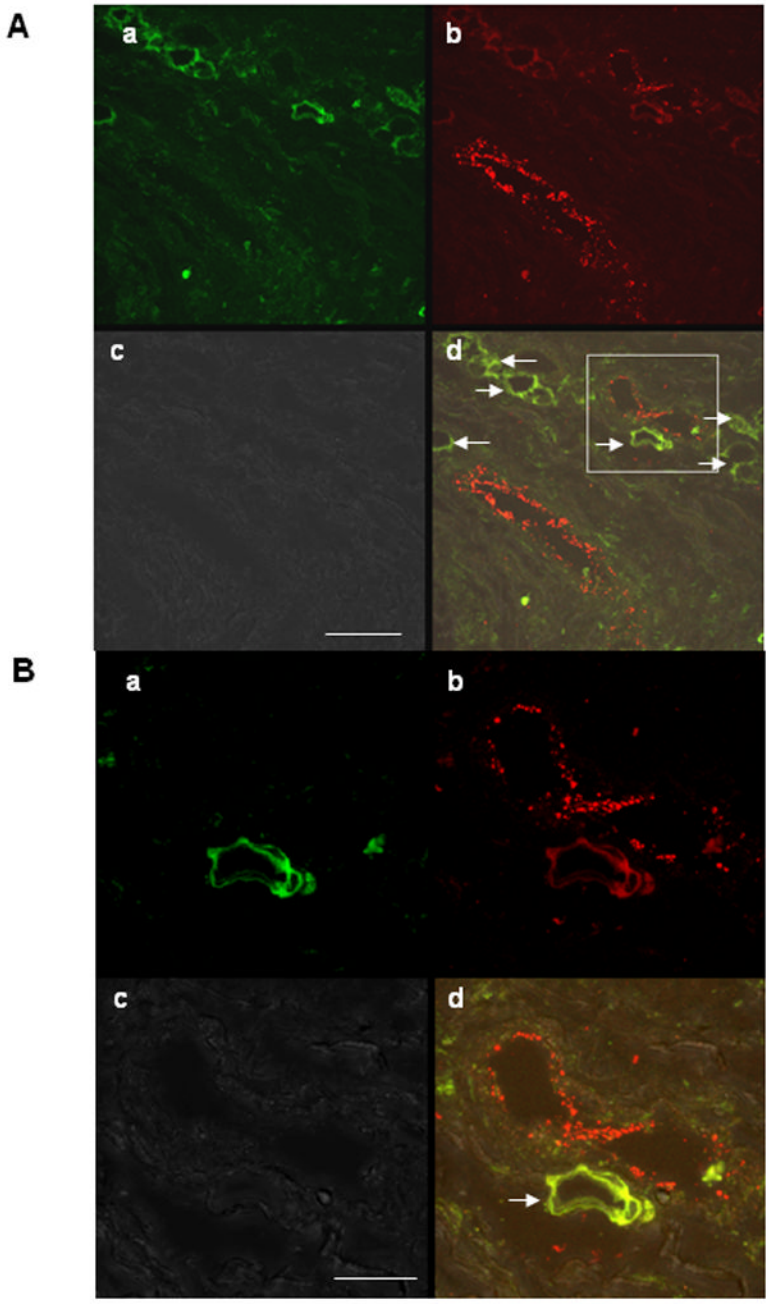

Figure 3. EPCs form blood vessel in inflamed synovium

A. To observe the de novo vessel formation by EPCs in arthritic joint, Lewis rats received a single intraperitoneal injection of PG-PS (15 μg of rhamnose/g of average body weight) on day 0, and received an injection through tail vein of fluorescent EPCs on day 1. On day 12, rats were euthanized, hind limbs were fixed in 4% PFA and decalcified. The paraffin sections were stained with rabbit anti-vWF antibody and Alexa Fluor 633-labeled goat anti-rabbit antibody. The sections were photographed under a confocal laser-scanning microscope. The images were processed using Adobe Photoshop 9.0 software. New blood vessels formed by fluorescent EPCs were positive for anti-vWF staining (arrows in d; bar,

60 μm). The higher magnification of the selected field in the panel **d** of **A** is demonstrated in **B** (bar, 20 μm).

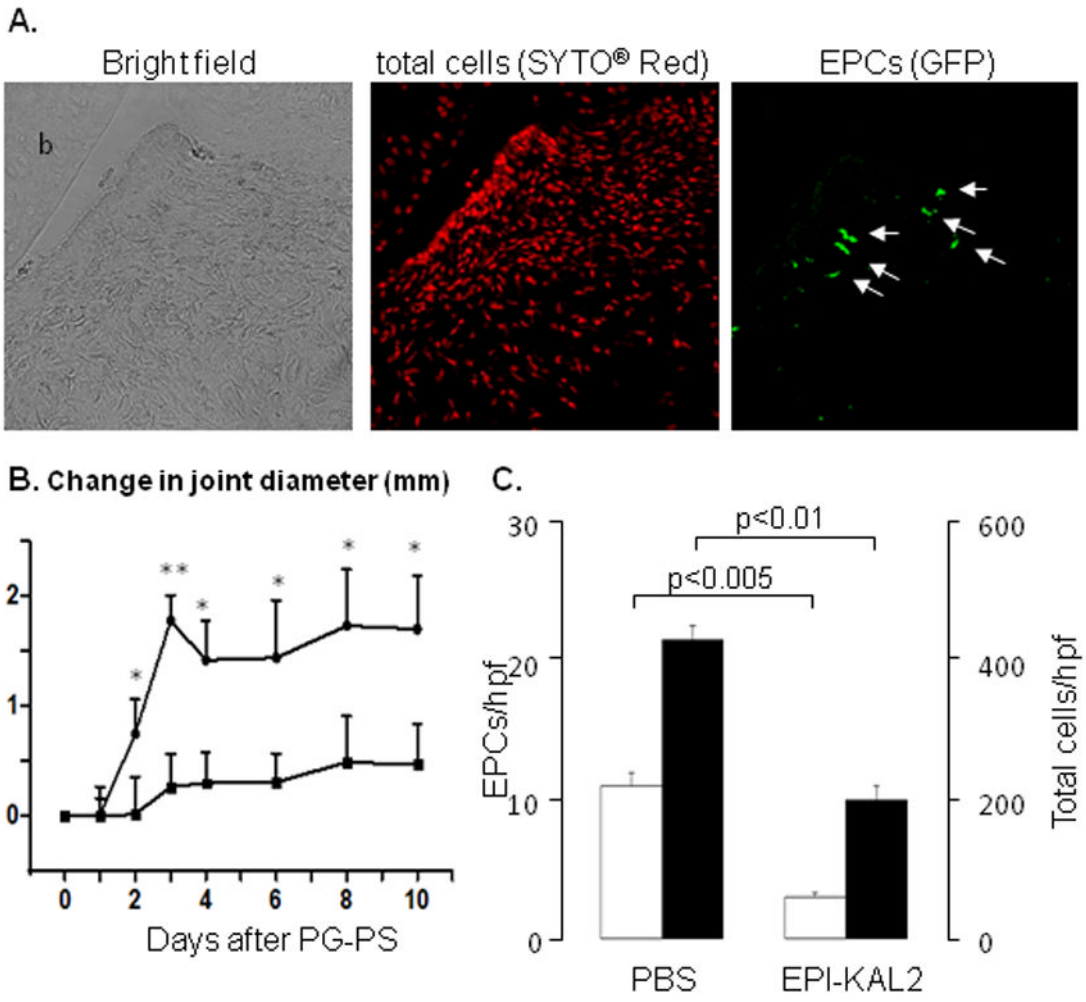

Figure 4. EPI-KAL2 inhibits the recruitment of EPCs into inflamed synovium

A. Quantitative measurement of EPCs homing to inflamed synovium. Lewis rats received an intraperitoneal injection of PG-PS on day 0, and an intravenous injection of fluorescent EPCs on day 1. Rats were euthanized on day 5. Paraffin sections of ankle joints were stained with SYTO® Red. Total synovial cells (red) and EPCs (green, some arrowed) were photographed under a fluorescent microscope (b, bone). **B.** Two groups of female Lewis rats (n=6) received an intraperitoneal injection of PG-PS on day 0 and an intravenous injection of fluorescent EPCs on day 1. From day 0, Group I (control) and Group II (treated) received PBS and EPI-KAL2 (10 mg/Kg body weight, twice a day), respectively. The change in joint diameter was measured (closed circle, PBS; closed box, EPI-KAL2. *, $p<0.01$, **, $p<0.005$). **C.** Two groups of female Lewis rats (n=4) were treated as described in the legend for **B.** Rats were euthanized on day 5. The paraffin-embedded sections of ankle joint were stained with SYTO® Red and photographed under a fluorescent microscope. Ten high-power fields (hpf, 400×) were evaluated per section, and the total synovial cells (red) and fluorescent EPCs (green) were enumerated.

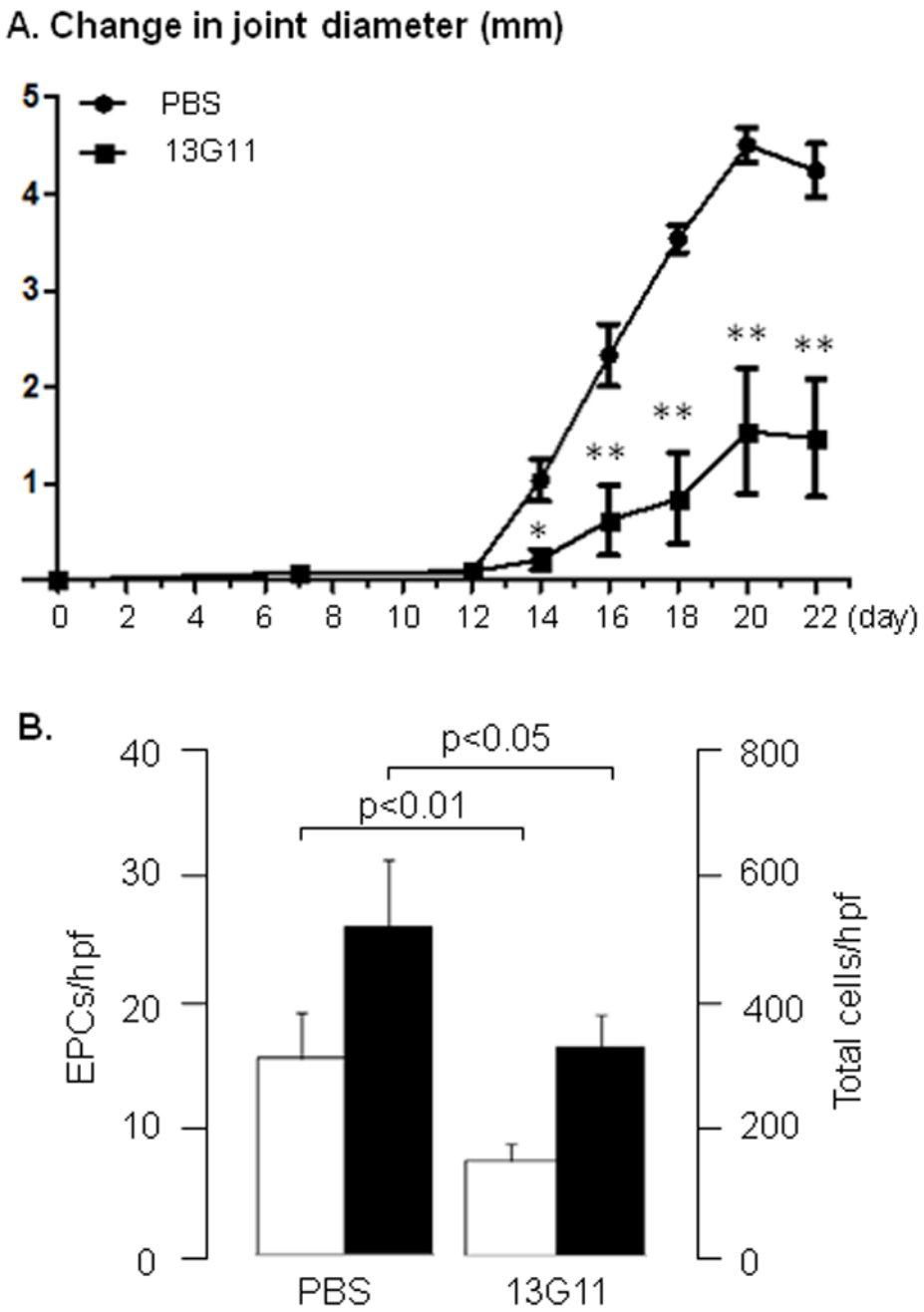

Figure 5. 13G11 inhibits the severity of arthritis and the recruitment of EPCs into inflamed synovium

A. Female Lewis rats were randomly separated into two groups (n=6), and immunized by a mixture of type-II collagen and complete Freund's adjuvant on day 0 and day 7, respectively. From day 7, two groups of rats received intraperitoneal administration of PBS (control) and 13G11 (8 mg/Kg body weight, every other day), respectively. The change in joint diameter was measured since day 0 (*, $p<0.05$, **, $p<0.001$). **B**. Two groups of female Lewis rats (n=4) were treated as described in the legend for **A.** Fluorescent EPCs were injected through tail vein on day 14. Animals were euthanized on day 18 and hind limbs were fixed and decalcified. The paraffin-embedded sections of ankle joint were stained with

SYTO® Red and photographed under a fluorescent microscope. The total synovial cells and fluorescent EPCs in the inflamed synovium were enumerated as described in the legend for Figure 4.

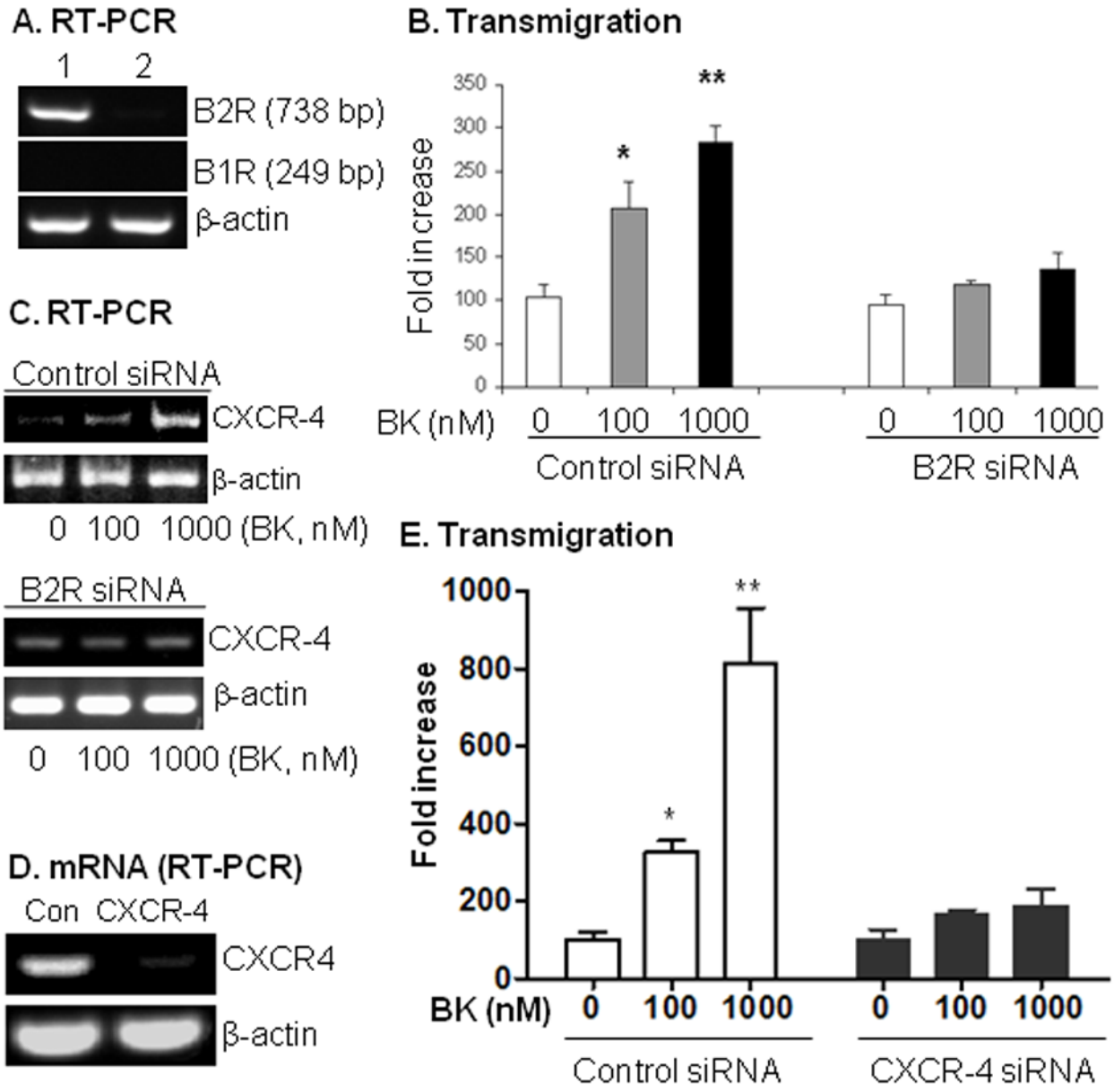

Figure 6. B2R mediates transendothelial migration of EPCs by upregulation of CXCR-4 expression

A. After EPCs were transfected with control siRNA (lane 1) and B2R siRNA (lane 2) for 48 hours, B2R and B1R mRNA expression was examined with RT-PCR. The expression of β-actin mRNA serves as a control. **B.** After transfected with fluorescein-labeled siRNA for 48 hours, EPCs were seeded onto confluent rLMECs on transwell filters. Bradykinin at the indicated concentrations was added to the lower compartment, the migration assay was performed as described in the Materials and Methods. *, $p<0.05$, **, $p<0.01$. **C**. Bradykinin upregulation of CXCR-4 expression is dependent on B2R. After transfected with siRNA for 48 hours, EPCs were starved with 1% serum for 6 hours and then stimulated with 0, 100 and 1000 nM bradykinin for 6 hours, respectively. The expression of homing receptor mRNA was analyzed by RT-PCR. The result is representative of three independent experiments. **D**. After transfection of siRNA for 48 hours, CXCR-4 mRNA expression was examined with

RT-PCR. The expression of β-actin mRNA served as a control. **E**. After transfected with fluorescein-labeled control siRNA or CXCR-4 siRNA for 48 hours, EPCs were seeded onto confluent rLMECs. Bradykinin was added to the lower compartment, and the migration assay was performed as described above (B). *, $p<0.005$, **, $p<0.001$.