

SUPPLEMENTAL MATERIALS

A novel CXCR4 antagonist derived from human SDF-1 β enhances angiogenesis in ischemic mice

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1. Supplement of the detail Materials and Methods

1.1 Materials

pET-30a(+) and BL21(DE3) were purchased from Novagen (San Diego, CA, USA), *KpnI/SacI* and Isopropyl beta-D-thiogalactoside (IPTG) were purchased from TAKARA (Dalian, China). ProBondTM Resin and EKMaxTM Enterokinase were purchased from Invitrogen (Carlsbad, CA, USA). Goat anti-human SDF-1 antibody and phycoerythrin (PE) conjugated mouse anti-human CXCR4 (fusin) monoclonal antibody (Clone: 12G5) were purchased from R&D (Minneapolis, MN, USA). MOLT-4 and H9C2 (a line was originally derived from embryonic rat heart tissue and exhibits many of the properties of skeletal muscle) cells were purchase from American Type Culture Collection (Manassas, VA, USA). Transwell trays were purchased from KURABO (Osaka, Japan). Fluo-3/AM was purchased from Molecular Probes (Eugene, OR, USA). AMD3100, monoclonal anti- β -actin antibody and Naphthol AS-D Chloroacetate Esterase staining kit were purchased from Sigma (St Louis, MO, USA) and rat anti-mouse CD31 (1:50) antibody was purchase from BD Bioscience (San Jose, CA, USA). Phospho-Akt (Ser473) (p-Akt) rabbit monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). VEGF rabbit polyclonal antibody, fusin (G-19) goat polyclonal antibody, SDF-1 rabbit polyclonal antibody, phospho-ERK1/2 (p-ERK1/2) rabbit monoclonal antibody, ERK1 rabbit monoclonal antibody and RIPA lysis buffer were purchased from Santa Cruz Biotechnology, INC (Santa Cruz, CA, USA). Proliferating cell nuclear antigen (PCNA, 1:50 dilution) was purchased from DakoCytomation (Carpinteria, CA, USA), and ApopTag In Situ kit was purchased from Chemicon (Temecula, CA, USA).

1.2 Preparation of recombinant human SDF-1 β and SDF-1 β P2G

The primers for cloning human SDF-1 β and its mutant SDF-1 β P2G were designed and synthesized according to cDNA sequences of native SDF-1 β . The followings are the sequences

of these primers: sense primer for SDF-1 β including enterokinase recognizing site: 5'-ggGGTACCGacgacgacgacaagaag**CCC**gtcagcctgagctacagat-3', sense primer for SDF-1 β P2G including enterokinase recognizing site: 5'-ggGGTACCGacgacgacgacaagaag**GGC**gtcagcctgagctacagat-3', and antisense primer for SDF-1 β and SDF-1 β P2G: 5'-acgGAGCTCacatcttgaacctctgtt-3'. SDF-1 β and SDF-1 β P2G cDNA were amplified by reverse transcriptional polymerase chain reaction (RT-PCR) from human bone marrow total mRNA. The PCR products of SDF-1 β and SDF-1 β P2G were digested by restriction enzymes *KpnI/SacI* and inserted into a bacterial expression plasmid pET-30a(+). The recombinants pET-30a(+)/SDF-1 β and pET-30a(+)/SDF-1 β P2G were confirmed and transformed into BL21(DE3), amplified in lactose broth medium and induced with Isopropyl beta-D-thiogalactoside (IPTG).¹ The recombinant proteins were mainly expressed in inclusion bodies. The inclusion bodies were dissolved in inclusion body dissolving buffer (Tris-HCl (pH9.0) 50mmol/L, Urea 8mol/L, β -Mercaptoethanol 0.5%) and purified under denaturing conditions according to the instruction of the user manual of ProBondTM Resin. The purified SDF-1 β and SDF-1 β P2G were refolded and examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and further confirmed by goat anti-human SDF-1 antibody. The purified and verified SDF-1 β and SDF-1 β P2G were digested by EKMaxTM Enterokinase to delete 6xHis-tags and separated by reverse phase-high pressure liquid chromatography (RP-HPLC) and the N-terminal amino acids were sequenced by ABI Procise 492 cLC protein sequencer.^{1, 2}

1.3 Characterization of recombinant human SDF-1 β P2G and SDF-1 β

SDF-1 β P2G and SDF-1 β were characterized as our previous report.^{1, 2} MOLT-4 cells, a T lymphocyte leukemia cell line, is well known to constitutively express CXCR4 on the cell surface.^{1, 3} Therefore, to systemically characterize the antagonistic activity of SDF-1 β P2G,

MOLT-4 cells were used to test receptor internalization, cell migration and calcium influx in response to SDF-1 β and SDF-1 β P2G.

For CXCR4 internalization assay, MOLT-4 cells were plated into each well of a 24-wells tissue culture plate and treated with SDF-1 β or SDF-1 β P2G for 2 h. The cells were harvested and incubated with phycoerythrin (PE) conjugated mouse anti-human CXCR4 (fusin) monoclonal antibody (Clone: 12G5) at 37 °C for 30 min. After two washes with PBS, the cells were analyzed using flow cytometry. The control was the cells incubated with isotype antibody.

Migration of MOLT-4 cells was assessed in disposable transwell trays with 6-mm-diameter chambers and membrane pore size of 5 μ m. SDF-1 β and SDF-1 β P2G were diluted to specified concentration in RPMI 1640 media containing 1 mg/ml of bovine serum albumin (BSA). 700 μ L of each diluted samples was added to the lower wells. To the upper wells, 200 μ L of the suspension of MOLT-4 cells at a concentration of 1×10^7 /ml were added.

For SDF-1 β P2G chemotaxis inhibition assay, various concentrations of SDF-1 β P2G were pre-incubated with MOLT-4 cells at 37 °C for 2 h. 200 μ L of the pre-incubated MOLT-4 cells at a concentration of 1×10^7 /ml was also added to the upper wells. After incubation at 37 °C, 5% CO₂ for 2 h, cells that migrated to the lower chambers were counted. The control was the migrating cells without any treatment.

For intracellular calcium measurement, MOLT-4 cells (1×10^7 /ml) were loaded with the calcium indicator dye Fluo-3/AM at a final concentration of 4 μ mol/L for 45 min in Ca²⁺ flux assay buffer (Hank's balanced salt solution containing 20 mM HEPES and 0.2% BSA, pH 7.4) at room temperature in the dark. Cells were then washed three times with the same buffer and maintained in darkness until use. For each sample, a 40-second baseline monitoring was performed by flow cytometry (BD, Bioscience). Then sample aspiration was briefly paused

and SDF-1 β and SDF-1 β P2G diluted in Ca²⁺ flux assay buffer were quickly added. The Ca²⁺ response was measured against the change in green fluorescence intensity of the cells as a function of time. Analysis was performed with the flow cytometry with an air-cooled 488-nm argon-ion laser and CellQuest software (BD, Bioscience).⁴

For the effects of SDF-1 β or SDF-1 β P2G on skeletal muscle cell assay, the H9C2 cells, which has been used as an *in vitro* cellular model for both skeletal and cardiac muscle,⁵ were cultured in DMEM-high glucose medium supplemented with 1.5 g/L sodium bicarbonate, 10% fetal bovine serum, 100 U/ml of penicillin and 100 μ g/ml of streptomycin in 100 mm tissue culture dishes at 37 °C in a humidified atmosphere of 5% CO₂. Cells once they reached 70 – 80% confluence were treated with SDF-1 β or SDF-1 β P2G at the indicated concentration after 6 h of starvation. Thirty minutes after treatment, cells were harvested and lysized in RIPA buffer. The proteins were collected for further detection of total Akt, p-Akt, ERK1, p-ERK1/2 and VEGF by Western blotting assays (see below).

1.4 Hind limb ischemic mouse model and drug delivery

Male FVB (8 weeks old) were purchased from Jackson laboratory (Jackson Laboratory, Bar Harbor, ME) and maintained under specific pathogen-free conditions at the University of Louisville Animal Facility (Louisville, Kentucky, USA). All experiments were approved by the Animal Care and Use Committee of the University of Louisville. Under sufficient anesthesia with an intraperitoneal injection of Ketamine (100 mg/kg) and Xylazine (10 mg/kg), the hind limbs were shaved and the entire left superficial femoral artery and vein (from just below of deep femoral arteries to popliteal artery and vein) were ligated with 6-0 silk sutures, cut, and excised with an electrical coagulator (Fine Science Tools Inc., Foster City, CA).⁵⁻⁷ The overlying skin was closed with 4-0 silk sutures. Consistency of limb prognosis of these models was confirmed by four repeated pilot experiments using more than 8 mice per model at once

and by the same operator. SDF-1 β P2G at the concentration of 5 mg/kg (body weight) was intravenously injected daily from one day before surgery (subgroup I, n=7-8) or the 3 h after surgery (subgroup II, n=7), until day 14 after surgery. Experimental results were not different for the blood flow restoration between subgroup I and e subgroup II, therefore, these results were pooled together to be present in the result section. AMD3100 5 mg/Kg (subcutaneously) and PBS (intravenously) were injected same as SDF-1 β P2G, as the positive and negative control, respectively.

1.5 Laser doppler perfusion images (LDPI)

The mice were anesthetized with Ketamine (100 mg/kg) and Xylazine (10 mg/kg). After the hind limb was shaved, the mice were affixed supine on a cork plate. Limb blood flow was monitored by using a Laser LDPI analyzer (Periscan PIM II Laser Doppler Perfusion Imager, Perimed AB, Sweden). The image size was 56*48 (points) and scanning time was about 3 min per mouse. Quantitative analysis of blood flow on the limb of the LDPI images was performed using LDPIwin 2.5 program. To minimize the variability in perfusion, the LDPI perfusion data was expressed as the ratio of the ischemic (left) to normal (right) limb blood flow.^{6, 7}

1.6 Immunofluorescent staining

Cryostat sections (5 μ m) from ischemic gastrocnemius muscles dissected from mice at 7 days after the surgical procedure were air dried at room temperature for 30 min, fixed in ice-cold acetone for 5 min and then air dried for another 30 min. The slides were washed with three changes of PBS for 5 min each and incubated with 5% BSA in PBS for 20 min to suppress non-specific binding of IgG. Then the primary antibody cocktails: p-Akt rabbit monoclonal antibody, VEGF rabbit polyclonal antibody, fusin (G-19) goat polyclonal antibody, or SDF-1 rabbit polyclonal antibody was mixed with rat anti-mouse CD31 at 1:50 fold in PBS with 3% BSA solution respectively, and then incubated for 60 min. After washed with PBS three times

for 5 min each, the slides were incubated for 45 min with fluorochrome-conjugated secondary antibodies (1:400) in PBS with 3% BSA in a dark chamber, followed by three washes with PBS containing 0.1% TritonX-100 and mounted coverslips with 90% glycerol in PBS. The expression of different proteins was examined using a fluorescence microscope with appropriate filters.

1.7 Determination of p-Akt, Akt, p-ERK1/2, ERK1 and VEGF protein expression

The expression of p-Akt, Akt, p-ERK1/2, ERK1 and VEGF was assayed by western-blotting as our previous reports.⁹ Tissue samples (gastrocnemius muscle from ischemic hind limbs), obtained at days 7, were thawed and homogenized in 500 μ L of buffer containing protease inhibitors. Proteins from tissues or cells were separated in 12% SDS-PAGE and then blotted onto a nitrocellulose sheet (Hybond ECL, Amersham).⁹ Antibodies against VEGF (1:500), p-Akt (1:500), Akt (1:1000), p-ERK1/2 (1:1000), ERK1 (1:1000), and β -actin (1:2000) were used, and the specific bands were then detected with HRP-conjugated secondary antibodies. The expression of VEGF was expressed as the ratio of quantification of the specific VEGF band to that of β -actin.

References

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2. Supplemental figure legend

Figure 1. Expression and purification of SDF-1 β and SDF-1 β P2G. (A) Recombinant SDF-1 β and SDF-1 β P2G were expressed in inclusion body in BL21(DE3) by Isopropyl beta-D-thiogalactoside (IPTG) induction, the inclusion bodies were washed and identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): lane 1-3, SDF-1 β /His-tag, lane 4-7, SDF-1 β P2G/His-tag, M, marker. (B) The inclusion bodies were dissolved and purified by ProBondTM Resin affinity chromatography, and the recombinant proteins were refolded under optimized conditions and the purities were examined by SDS-PAGE: lane 1, SDF-1 β /His-tag, lane 2, SDF-1 β P2G/His-tag, M, marker. (C) The his-tags of SDF-1 β /His-tag were digested by enterokinase: lane 1, EKMax, lane 2, SDF-1 β /His-tag, lane 3-7, digest 12, 10, 8, 6 and 5 h, M, marker. (D) SDF-1 β P2G/His-tag was digested under the optimized conditions: lane 1, SDF-1 β P2G/His-tag, lane 2, SDF-1 β P2G, lane 3, SDF-1 β P2G/His-tag was digested by enterokinase, M, marker. (E&F) The digested recombinant proteins were separated by RP-HPLC: I: His-tag; II: SDF-1 β or SDF-1 β P2G, respectively.

Figure 2 Characterization of SDF-1 β P2G. (A) CXCR4 internalization was examined by treatment MOLT-4 cells with SDF-1 β or SDF-1 β P2G at 37°C for 2 h at indicated concentrations. CXCR4 on the cell surface was stained with CXCR4-PE antibody (12G5) and detected by flowcytometer. The level of CXCR4 on the cell surface was shown with the mean fluorescence density. (B) The chemotactic activities of both SDF-1 β and SDF-1 β P2G on MOLT-4 cells were performed with transwell trays with a membrane pore size of 5 μ m, in which the background was the mean percentage of migrating cells without any treatment (** P < 0.01 vs. vehicle

control). (C) Cell migration induced by SDF-1 β (1 nM) was measured in MOLT-4 cells pre-treated with various concentrations of SDF-1 β P2G using transwell trays as described above (* $P < 0.05$, ** $P < 0.01$ vs. control [SDF-1 β P2G=0]). (D) The effects of both SDF-1 β P2G and SDF-1 β on intracellular Ca^{2+} influx in MOLT-4 cells were measured by flow cytometry with Fluo-3/AM staining. These images were typical representatives of duplications for each group, in which arrows showing the addition of SDF-1 β or SDF-1 β P2G and the rapid and transient increase of intracellular calcium concentration.

Figure 3 The effects of SDF-1 β P2G and SDF-1 β on Akt, ERK1/2 and VEGF expressions. H9C2 cells were treated with SDF-1 β or SDF-1 β P2G at the indicated concentrations after starvation for 6 h. Thirty minutes after treatment, cells were collected and the expression of Akt, p-Akt, ERK1, p-ERK1/2 and VEGF were detected by Western blotting. β -Actin was detected as loading control (A). No change for total Akt and VEGF expression was found in the cells exposed either to SDF-1 β or to SDF-1 β P2G. The activation of Akt and ERK were quantitatively analyzed and also presented by the ratios of p-Akt to total Akt (B) and p-ERK1 to ERK1 (C), respectively. We analyzed the ratio of p-ERK1 to ERK1 since we did not detect ERK2. The insert in the panel B represents the dose-effect curve for the ratio of p-Akt/Akt of the cells in response to SDF-1 β P2G, and shows that if there is any, SDF-1 β P2G can only induce very small dose-dependent increasing effects on Akt phosphorylation. If comparing the absolute values of ratios of p-Akt/Akt and p-ERK1/ERK1 between SDF-1 β and SDF-1 β P2G both at the concentration of 100 nM, the former are 16.9 and 12.5 folds of those of the latter, respectively.

3. Supplemental

Figure 1

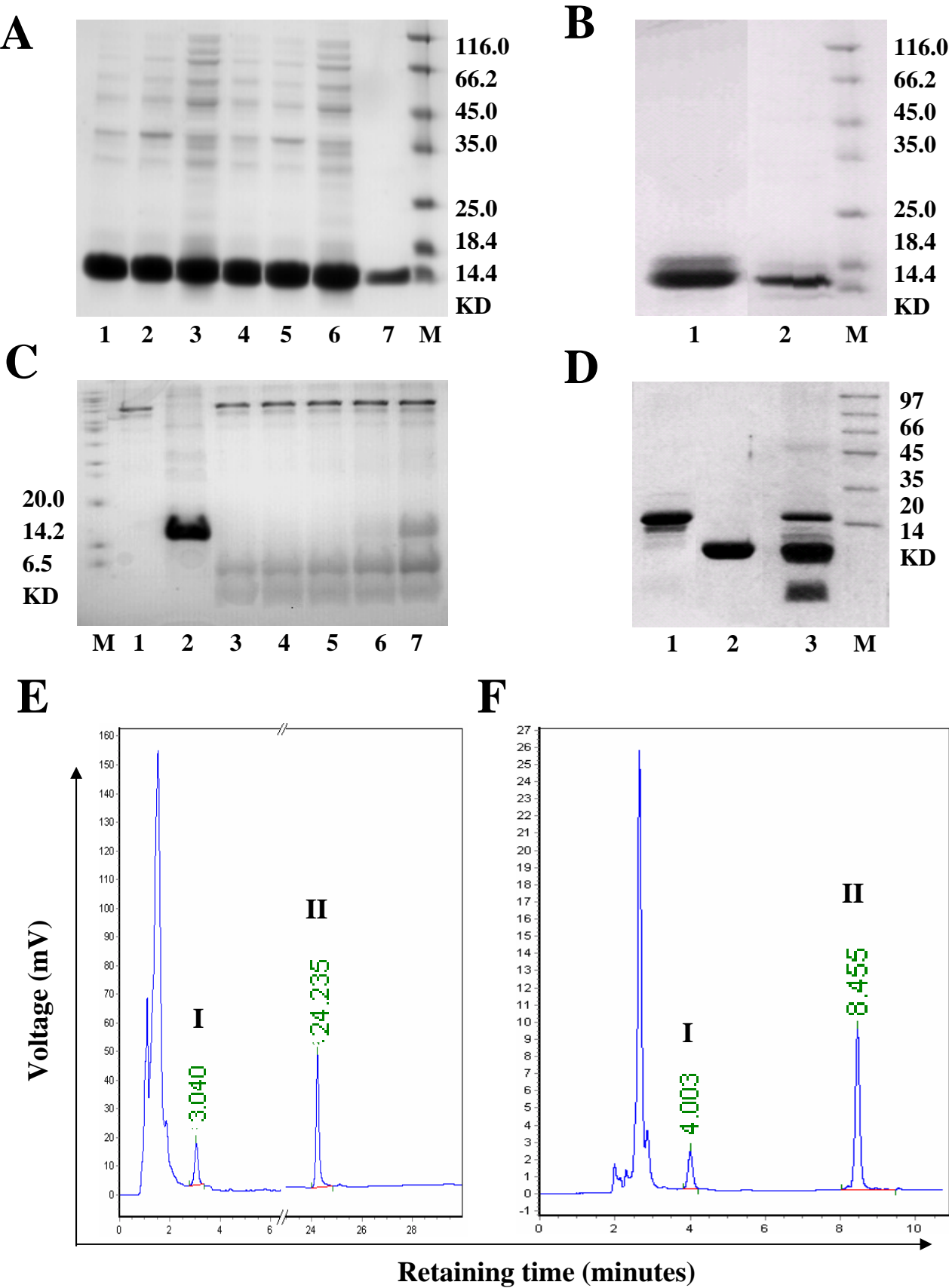


Figure 2

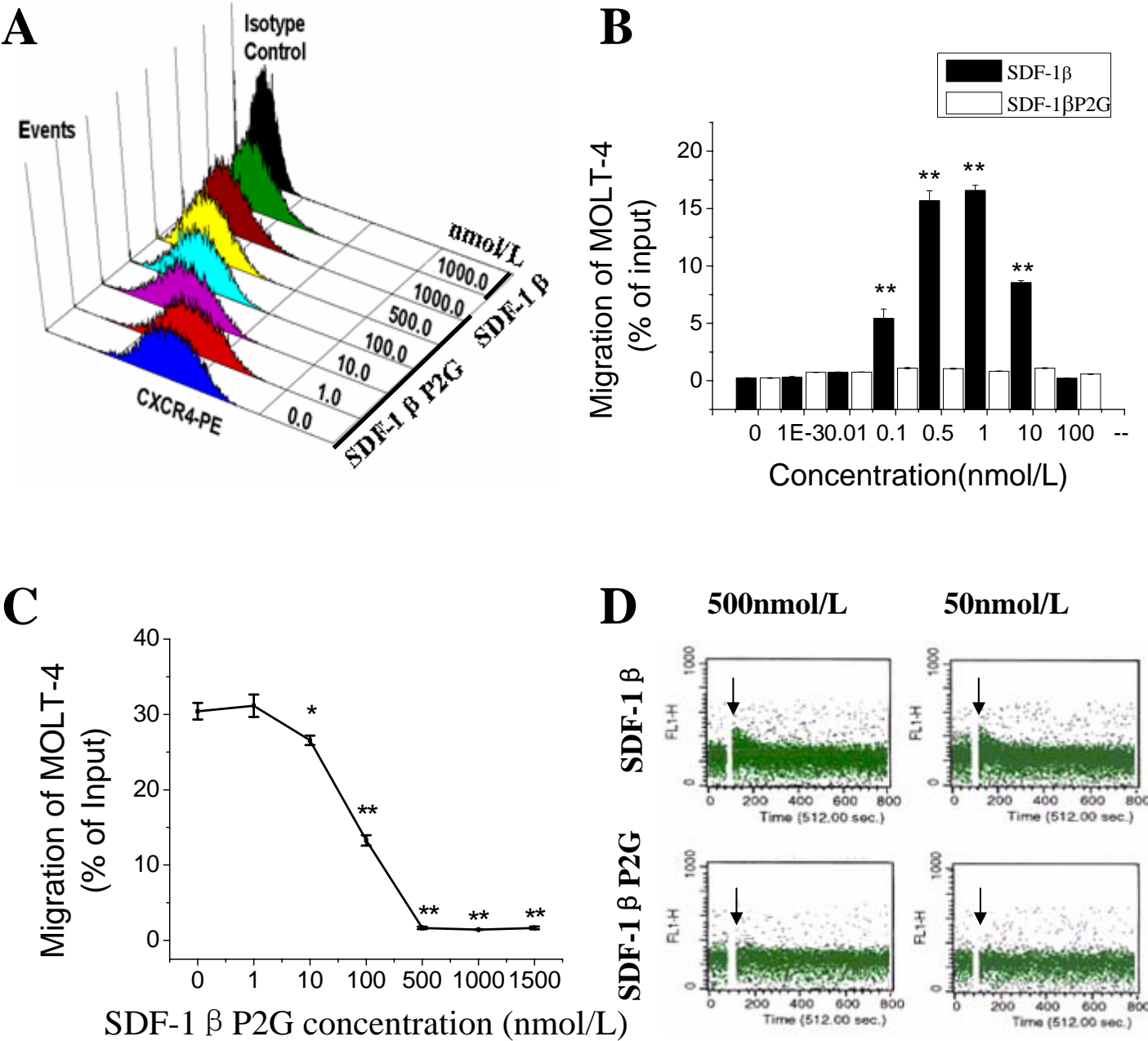


Figure 3

