

Human bone marrow mesenchymal stem cells secrete endocannabinoids that stimulate in vitro hematopoietic stem cell migration effectively comparable to beta-adrenergic stimulation

Sevil Köse^a, Fatima Aerts-Kaya^a, Çağla Zübeyde Köprü^b, Emirhan Nemutlu^c, Barış Kuşkonmaz^d, Beren Karaosmanoğlu^a, Ekim Zihni Taşkiran^e, Belgin Altun^f, Duygu Uçkan Çetinkaya^{a,d}, and Petek Korkusuz^g

^aDepartment of Stem Cell Sciences, Institute of Health Sciences, Center for Stem Cell Research and Development (PEDI-STEM), Hacettepe University, Ankara, Turkey; ^bDepartment of Histology and Embryology, Faculty of Medicine, Yüksek İhtisas University, Ankara, Turkey; ^cDepartment of Analytical Chemistry, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey; ^dPediatric Hematology, BMT Unit, Hacettepe University Children's Hospital, Ankara, Turkey; ^eDepartment of Medical Genetics, Faculty of Medicine, Hacettepe University, Ankara, Turkey; ^fDepartment of Microbiology, Faculty of Medicine, Hacettepe University, Ankara, Turkey; ^gDepartment of Histology and Embryology, Faculty of Medicine, Hacettepe University, Ankara, Turkey

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Granulocyte colony-stimulating factor (G-CSF) is a well-known hematopoietic stem cell (HSC)-mobilizing agent used in both allogeneic and autologous transplantation. However, a proportion of patients or healthy donors fail to mobilize a sufficient number of cells. New mobilization agents are therefore needed. Endocannabinoids (eCBs) are endogenous lipid mediators generated in the brain and peripheral tissues and activate the cannabinoid receptors CB1 and CB2. We suggest that eCBs may act as mobilizers of HSCs from the bone marrow (BM) under stress conditions as beta-adrenergic receptors (Adr β). This study demonstrates that BM mesenchymal stem cells (MSCs) secrete anandamide (AEA) and 2-arachidonylglycerol (2-AG) and the peripheral blood (PB) and BM microenvironment contain AEA and 2-AG. 2-AG levels are significantly higher in PB of the G-CSF-treated group compared with BM plasma. BM mononuclear cells (MNCs) and CD34⁺ HSCs express CB1, CB2, and Adr β subtypes. CD34⁺ HSCs had higher CB1 and CB2 receptor expression in G-CSF-untreated and G-CSF-treated groups compared with MSCs. MNCs but not MSCs expressed CB1 and CB2 receptors based on qRT-PCR and flow cytometry. AEA- and 2-AG-stimulated HSC migration was blocked by eCB receptor antagonists in an in vitro migration assay. In conclusion, components of the eCB system and their interaction with Adr β subtypes were demonstrated on HSCs and MSCs of G-CSF-treated and G-CSF-untreated healthy donors in vitro, revealing that eCBs might be potential candidates to enhance or facilitate G-CSF-mediated HSC migration under stress conditions in a clinical setting. © 2018 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.

Hematological malignancies are currently treated with hematopoietic stem cell transplantation (HSCT) [1]. Rapid and sustained recovery of hematopoietic functions after HSCT correlates with the number of CD34⁺ hematopoietic stem cells (CD34⁺ HSCs) infused [2]. CD34⁺ HSCs reside mainly in the bone marrow (BM) microenvironment(s) and mobilize to the peripheral blood (PB) after administration of growth factors or antagonists, such as granulocyte colony-stimulating factor

(G-CSF). The release mechanism of these cells from the hematopoietic microenvironment is still not completely understood [3,4]. Infection and stress increase HSC migration through lipopolysaccharide (LPS) release [5] and activation of the sympathetic nervous system via β -adrenergic receptors (Adr β 1, Adr β 2, and Adr β 3) [6,7] in a circadian rhythm [8]. However, they may not be the only regulators of HSC migration [9]. Chemokine stromal derived factor-1 (SDF-1, also termed CXCL12) and its major receptor, CXCR4, are crucial in mediating both retention and mobilization of HSCs. G-CSF and the CXCR4 antagonist AMD3100 are the only U.S. Food and Drug Administration-approved agents for patients when HSCs fail to mobilize [10]. G-CSF-based migration requires a multiday dosing regimen and is associated

Offprint requests to: Petek Korkusuz, MD, PhD, Department of Histology and Embryology, Faculty of Medicine, Hacettepe University, Ankara 06100, Turkey; E-mail: petek@hacettepe.edu.tr

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with some morbidity and rare but serious complications [11].

Endocannabinoids (eCBs) are endogenous lipid mediators generated by many cell types both in the brain and peripheral tissues and activate the cannabinoid receptors CB1 and CB2. The eCBs, in particular anandamide (AEA; full agonist for CB1 and CB2) and 2-arachidonoylglycerol (2-AG; full agonist for CB2, weak agonist for CB1), are modulators of cell proliferation, differentiation, apoptosis, and migration [12,13]. Cannabinoid receptor expression was reported on macrophage, erythroid, B-lymphoid, T-lymphoid, and mast cell lines [14] and rodent BM- HSCs and mesenchymal stem cells (MSCs) [15]. Cannabinoid-receptor-mediated cell migration has been investigated in murine myeloid leukemia cells [16], mice BM mononuclear cells (MNCs) [17], and BM- HSCs [18], embryonic kidney cells [19], mouse microglial cells [20], and endothelial cells [21].

The sympathetic activation and the subsequent beta-adrenergic system involvement is well described in BM under physiologic and stress conditions, but it is not the only/efficient stimulator for HSC mobilization. eCBs functioning as neurotransmitters and paracrine factors may play role in HSC mobilization similar to mobilization induced as a result of stress-induced sympathetic hyperactivity in the human BM microenvironment. Therefore, the utility of eicosanoid-based therapeutic strategies including cannabinoids is being investigated but still requires further investigation for improving HSC mobilization [6–8,22,23]. Here, we assessed the following: (1) if healthy donor HSCs and MSCs release eCBs (AEA and 2-AG) and express CB1 and CB2 receptors, (2) if HSCs migrate toward eCBs and MSCs, and (3) if there is a difference between cells obtained from donors after G-CSF treatment.

The aim of the present study was to elucidate the role of the eCB system in the human BM microenvironment on migration of HSCs and the effects of G-CSF thereon. We found expression of eCBs in stromal BM microenvironments and cannabinoid receptors in HSCs. HSC migration in the presence of the eCB ligands (AEA and 2-AG) increased compared with controls and decreased in presence of the eCB receptor antagonists AM281 and AM630. Therefore, eCBs may be a novel candidate to enhance or facilitate/accelerate G-CSF-mediated HSC mobilization in a clinical setting.

Methods

Cell culture

Use of human material was approved by the Hacettepe University Local Ethical Committee (GO13/170-17). Age-matched BM aspirations were obtained from G-CSF-untreated ($n = 10$) or treated ($n = 10$) healthy donors (average ages: 15.1 ± 10.2 years in G-CSF-untreated donors; 12.4 ± 7.6 years for the G-CSF-treated group) scheduled to serve as donors for transplantation purposes. MNCs were obtained by density gradient centrifugation using Biocoll (#L6113/5, Merck-Millipore, Germany). CD34⁺ HSCs were isolated using magnetic activated cell sorting using a human CD34

MicroBead Kit (#130-046-702, Miltenyi Biotec, Germany). MNCs were plated in culture flasks in cell proliferation medium (DMEM-LG #11885084; Invitrogen) supplemented with 10% fetal bovine serum (#10500064; Invitrogen), 1% L-glutamine (#25030081; Invitrogen), 10,000 U/mL penicillin–streptomycin (#15140122; Invitrogen) to obtain MSCs. Third-passage human MSCs were characterized with respect to their morphology, adherence to the culture plate, expression of surface antigens, and differentiation assays ($n = 10$) [24–27].

LC-ESI-MS/MS

2-AG and AEA levels were measured in BM, PB plasma, and MSC supernatant by liquid chromatography–tandem electrospray ionization–mass spectrometry (LC-ESI-MS/MS) (#LCMS-8030, Shimadzu, Japan) following the optimization protocol with standards (protocol adopted from Bradshaw et al [28]). The daily calibration curves were used for quantification of AEA and 2-AG; all samples were just prepared before the run and analyzed twice. The solvent controlled validation results are shown as [Supplementary Fig. E1](#) (online only; available at www.exphem.org). The experiment was performed with 10 independent samples for each group. Polymeric sorbent-based solid-phase extraction (SPE) cartridges (Strata-X0.5 mL) were used for high-throughput sample preparation. Briefly, 250 μ L of BM and PB plasma or 7 mL of the supernatant of 7×10^6 MSCs were passed through the cartridges activated with methanol. Cartridges were washed with a water:methanol mixture (45:55, v/v) and elution of 2-AG (#8923, Sigma-Aldrich, Germany) and AEA (#A0580, Sigma-Aldrich, Germany) was performed with 2 mL of MeOH. The calibration curves of 2-AG and AEA were constructed with the peak area of the analyte versus the concentration. The chromatographic separation was achieved on a C₁₈ column (Hypersill ODS4, 50 \times 3.0 mm, 2.1 μ m) using a mobile phase consisting of acetonitrile containing 0.1% formic acid and water containing 0.1% formic acid at 0.3 mL/min flow rate.

Flow cytometry

CB1, CB2, and Adr β 1, Adr β 2, and Adr β 3 levels were measured in BM-MSCs, BM-MNCs, and BM- CD34⁺ HSCs of G-CSF-treated and G-CSF-untreated donors by flow cytometry (FC). The experiment was performed with 10 independent samples for each group. In addition, CD34⁺ peripheral blood stem cells (PBSCs) collected from G-CSF-stimulated donors after apheresis were assessed. Live BM-MNCs were selected by density gradient centrifugation method [29,30]. Cells were permeabilized using permeabilizing solution 2 (#340973, BD Biosciences, USA) before eCB receptor labeling by FC. Direct immunofluorescence assay was performed using rat-anti-human CB1 (#ab92764, Abcam, UK), CB2 (#10010712, Cayman, USA), Adr β 1 (#ABIN 669358, Antibodies Online, USA), Adr β 2 (#81578, Santa Cruz Biotechnology, USA), and CD34 and CD38 (#560710 and #555462, BD Biosciences, USA). Indirect immunofluorescence assay was done for Adr β 3 with rabbit-primary anti-human Adr β 3 (#1472, Santa Cruz Biotechnology) and rabbit FITC-conjugated anti-goat secondary antibody (#ab97099, Abcam). After exclusion of death cells by gating, live cells were measured with a FACSAria flow cytometer (Becton Dickinson, USA) and analyzed using BD FACS-Diva software version 6.1.2 with 10,000 list mode events recorded for each sample by ruling out the background labeling with isotype controls.

qRT-PCR

CNR1, *CNR2* (genes for CB1 and CB2, respectively), *ADRB1*, *ADRB2*, and *ADRB3* (genes for Adr β 1, Adr β 2, and Adr β 3, respectively) expression levels were measured in MNCs and MSCs of both groups by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The experiment was performed with six independent samples for each group and three repeats for each sample. Total RNA was isolated from MNCs and MSCs using the RNeasy mini RNA isolation kit (#74104, Qiagen, USA) following the manufacturer's instructions. Total RNA concentrations and ratios were determined by spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific, USA) and stored at -80°C . cDNA was generated from 200 ng of total RNA using the ProtoScript[®] First Strand cDNA synthesis kit (#E6300, New England BioLabs, USA) following the manufacturer's instructions. qRT-PCR was performed with the PowerUp[™] SYBR[®] Green Master Mix (#A25741, Thermo, USA) using the ViiA[™] 7 Real-Time PCR System (Thermo, USA). Relative mRNA expression analysis was calculated by delta delta Ct method and ViiA[™] 7 Software (version 1.2.4).

Transwell assay

For Transwell assays CD34⁺ PBSCs were used. To set a migration standard, CD34⁺ PBSCs were placed onto prewetted filters of the polycarbonate membrane Transwells with a 5 μm pore size (#3421, Corning, USA) with 100 μL of DMEM-HG (#D5671, Sigma-Aldrich, Germany) medium. SDF-1 (200 ng/mL; American Research Products, USA) and norepinephrine (NE, 1 $\mu\text{g}/\text{mL}$; Sigma-Aldrich, Germany) were added to the lower wells of the coculture system with or without their antagonists (AMD3100, 25 $\mu\text{g}/\text{mL}$; SR59230A 2.5 $\mu\text{mol}/\text{L}$; both Sigma-Aldrich, Germany).

For the migration assay itself, CD34⁺ PBSCs were placed onto prewetted filters of Transwells with 100 μL of high-glucose Dulbecco's modified Eagle medium (DMEM-HG). DMEM-HG containing SDF-1, the cannabinoid agonists AEA and 2-AG (both at 30 nmol/L, 1 $\mu\text{mol}/\text{L}$, 50 $\mu\text{mol}/\text{L}$; Sigma-Aldrich, Germany) with or without AM281 and AM630 (both at 10 $\mu\text{mol}/\text{L}$; Tocris Bioscience, UK) were added to the lower wells alone or combined with LPS-stimulated or LPS-unstimulated MSCs in 600 μL of DMEM-HG. Cells were allowed to migrate for 4 h at 37°C in a humidified atmosphere with 5% CO_2 . Filters were then removed from the chambers and counting was performed with Turk's solution. All Transwell assay experiments were performed with six independent PBSC donors and three repeats for each donor.

Statistical analysis

Descriptive results were presented as mean \pm SEM and median (minimum–maximum). Normality of the distribution of variables

in every study group was evaluated by the Shapiro–Wilk test and differences between study groups (with nonparametric distribution) were assessed by Wilcoxon's test. p values < 0.05 were considered statistically significant.

Results

MSCs from G-CSF-treated and G-CSF-untreated donors are phenotypically and morphologically similar

MSCs showed plastic adherence and fibroblastic morphology; positive expression for CD73, CD44, CD90, and CD29; and were negative for hematopoietic markers, including CD34 and CD45, in the G-CSF-untreated and G-CSF-treated group, respectively (Figs. 1A and 1B). Expression of MSC markers was similar in the G-CSF-untreated and G-CSF-treated groups ($p \geq 0.05$; Fig. 1B). Adipogenic differentiation was confirmed by morphology and the amount of Oil Red O (Figs. 1C and 1E). Osteogenic differentiation was confirmed by morphology using Alizarin Red S and the production of calcium phosphates (Figs. 1D and 1E). G-CSF-treated and G-CSF-untreated MSCs exhibited similar adipogenic and osteogenic differentiation capacity ($p \geq 0.05$). The morphological, differentiation, and immunophenotypic characteristics confirmed stromal and multipotential nature of the MSCs used in this study [31].

Endogenous AEA and 2-AG was found in BM and PB plasma and MSC supernatant

MSCs secrete 2-AG and AEA in culture supernatants. AEA was detected in PB and BM plasma of G-CSF-treated and G-CSF-untreated groups at similar levels (Table 1). However, the 2-AG level in PB of the G-CSF-treated group was significantly higher compared with the BM plasma samples ($p = 0.01$; Table 1). The 2-AG level was higher in PB plasma compared with AEA in both groups, but the difference was not statistically significant (Table 1). MSCs of G-CSF-treated and G-CSF-untreated groups exhibited a similar profile of AEA and 2-AG secretion with PB and BM cells (Table 1).

BM-MNCs and CD34⁺ HSCs express CB1 and CB2 receptor and Adr β subtypes

MNCs and CD34⁺ cells expressed significantly higher levels of CB1 and CB2 receptors compared with MSCs of those groups ($p = 0.001$ for all groups) by FC (Fig. 2). CB1

Table 1. MSCs secrete AEA and 2-AG; the PB and BM niches contain AEA and 2-AG

	G-CSF-Untreated Group		G-CSF-Treated Group	
	AEA (nmol/L)	2-AG (nmol/L)	AEA (nmol/L)	2-AG (nmol/L)
PB Plasma	80.99 (20.1–188.93)	131.08 (42.58–1631.76)	62.09 (58.12–178.21)	1655.23 (325.41–1816.14)*
BM Plasma	77.76 (7.69–286.42)	70.38 (9.27–402.84)	17.42 (5.1–534.3)	30.7 (5.94–400.99)
MSC Supernatant	5.62 (1.07–10.63)	2.24 (0.77–6.13)	3.92 (1.44–8.67)	1.45 (0.63–12.88)

AEA and 2-AG levels were measured by LC/ESI/MS-MS. Data are presented by median (minimum–maximum). In the G-CSF-treated group, the level of 2-AG was found to be higher in PB plasma than in BM plasma ($*p < 0.05$, $n = 10$ for each group; *comparison of PB vs BM for G-CSF-treated group).

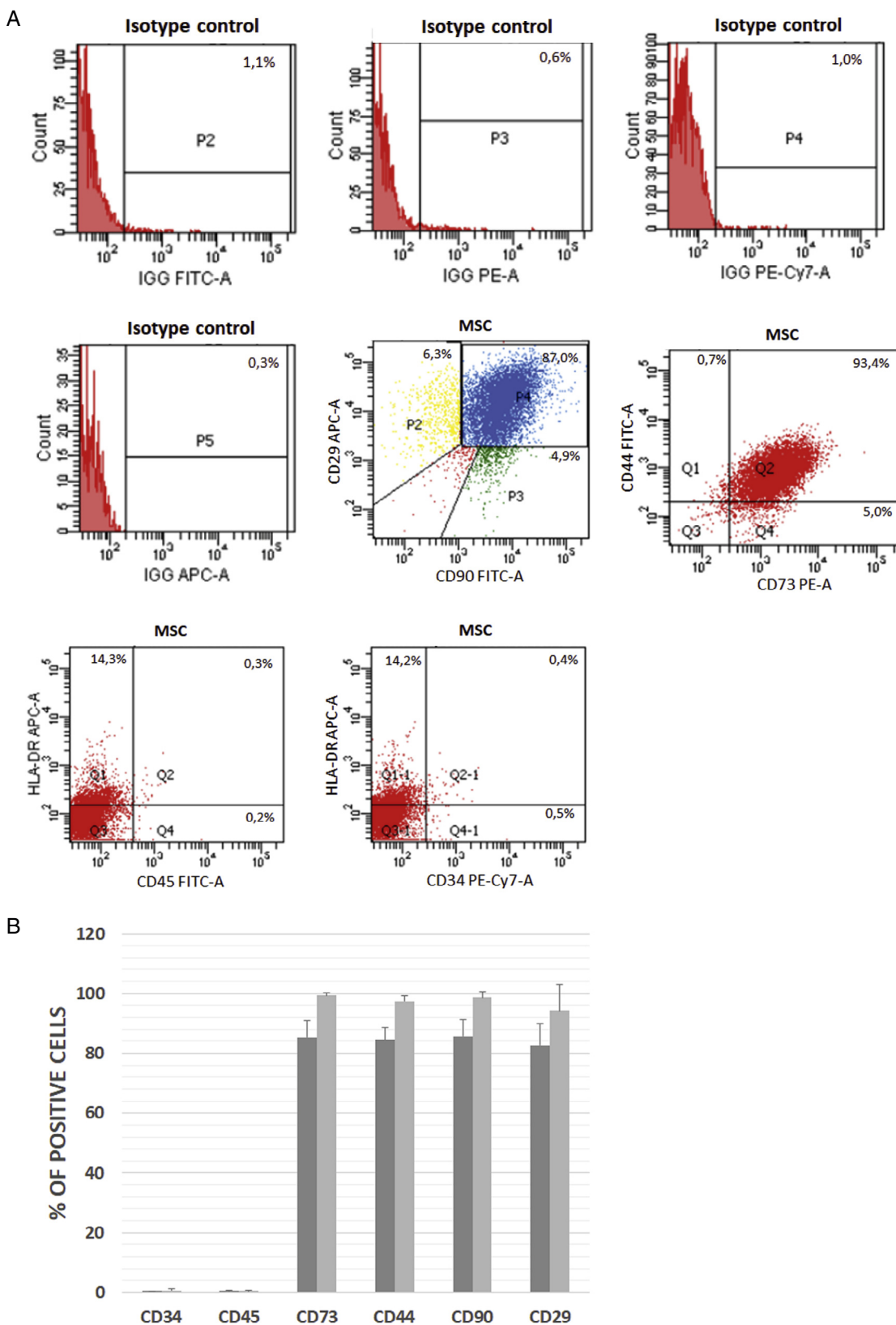


Figure 1. Characterization of human BM-MSCs. Stromal and multipotential nature of MSCs were confirmed morphologically, immuno-phenotypically, and through differentiation. (A) Representative histograms showing specific markers for MSCs by FC. FITC = fluoro-isothiocyanate; PE = phycoerythrin; APC = allophycocyanin; PE-Cy7 = PE-Cyanin 7. Shown are conjugated antibodies or their isotypes. (B) Bar graphic showing the percentage of negative (CD34, CD45) and positively (CD73, CD44, CD90, CD29) labeled MSCs in G-CSF-untreated and G-CSF-treated groups ($n = 10$ for each group). (C) Oil Red O (ORO) staining for adipogenic cells (inverted microscope, Olympus, 10 \times) and (D) Alizarin Red S (ARS) for osteogenic cells (inverted microscope, Olympus, 10 \times) are positive after 21 days of differentiation in appropriate media ($n = 10$ for each group). (E) The amount of Ca²⁺ and ORO dye for osteogenic and adipogenic differentiation of MSCs respectively ($n = 10$ for each group) was measured semiquantitatively.

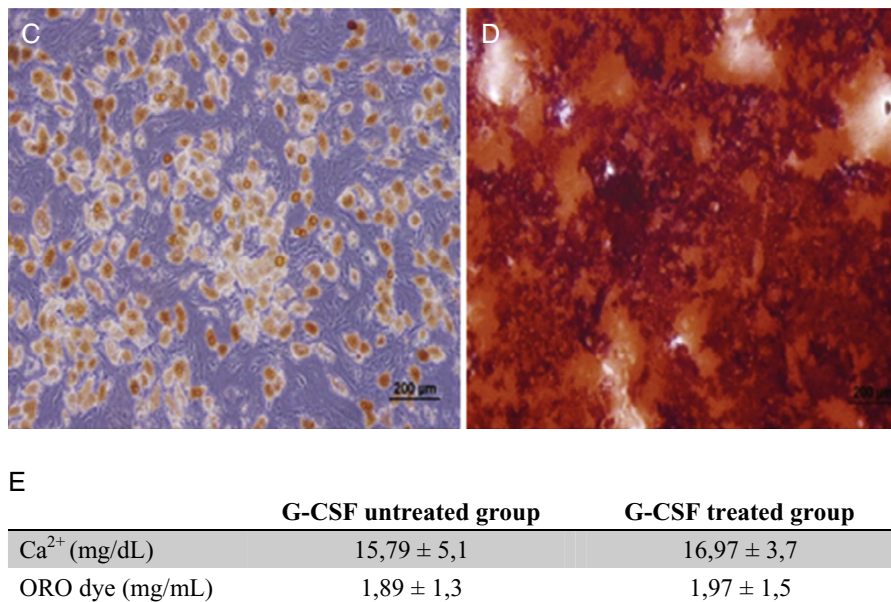


Figure 1. (continued)

receptor expression was highest on CD34⁺ HSCs. This difference was significant for the G-CSF-untreated group only ($p = 0.02$; Fig. 2A). Data were confirmed by qRT-PCR, with which CB1 and CB2 gene expressions were detected in MNCs, but not in MSCs (Figs. 2B and 2C). CB2 expression of MNCs was higher in the G-CSF-untreated group compared with the treated group ($p = 0.04$; Fig. 2B).

MNCs, CD34⁺ HSCs, and MSCs expressed various subtypes of Adr β subtypes by FC; however, MNCs and MSCs mainly expressed Adr β 2, as confirmed by qRT-PCR (Fig. 2). MNCs and CD34⁺ HSCs exhibited significantly higher expression of Adr β 2 ($p = 0.02$ and $p = 0.004$ for MNC; $p = 0.001$ and $p = 0.001$ for CD34⁺ HSCs in the G-CSF-untreated and G-CSF-treated groups, respectively) compared with MSCs by FC (Fig. 2A). CD34⁺ HSCs exhibited higher expression for Adr β 2 and Adr β 3 in all groups compared with MNCs, but the difference was only significant for Adr β 3 ($p = 0.001$ and $p = 0.013$ for MNC; $p = 0.001$ and $p = 0.002$ for MSC in the G-CSF-untreated and G-CSF-treated groups, respectively) (Fig. 2A). Increased Adr β 2 expression was found in G-CSF-treated MNCs compared with untreated ($p = 0.02$) MNCs by qRT-PCR (Fig. 2B). Differences in expression of Adr β 1 as measured by FC were not found.

CD34⁺ PBSC migration toward SDF-1, NE, AEA, and 2-AG is blocked by specific antagonists

CD34⁺ PBSCs were characterized by high expression of CD34 ($87.9 \pm 4.5\%$) and CD38 ($35.5 \pm 12.4\%$) and dual expression of CD34/CD38 ($33.0 \pm 12.7\%$) before the Transwell assay. The cells were also checked for Adr β subtypes and CB1 and CB2 receptor expression (Fig. 3A). To test CD34⁺ PBSC migration, we established a Transwell/coculture assay (Fig. 3B), in which we showed migration of CD34⁺ PBSCs toward

SDF-1 and NE. SDF-1 and NE antagonists (AMD3100 and SR59230A, respectively) inhibited migration (Fig. 3C). CD34⁺ PBSCs effectively migrated toward AEA and 2-AG. CB1 and CB2 receptor antagonists specifically blocked this effect (Fig. 3D). The CD34⁺ PBSCs exhibited significantly higher migration to 30 nmol/L and 50 μ mol/L doses of AEA, respectively, compared with SDF-1 ($p = 0.027$ and $p = 0.028$, respectively; Fig. 3D). AM281 and AM630 significantly inhibited migration of these doses of AEA ($p = 0.028$ for 30 nmol/L and $p = 0.027$ for 50 μ mol/L, respectively; Fig. 3D). The CD34⁺ PBSCs exhibited significantly higher migration toward all doses of 2-AG compared with SDF-1 ($p = 0.027$, $p = 0.027$ and $p = 0.028$, respectively; Fig. 3D). AM281 and AM630 significantly inhibited CD34⁺ PBSC migration at all doses of 2-AG ($p = 0.027$ and $p = 0.027$ for 30 nmol/L, $p = 0.028$ and $p = 0.027$ for 1 μ mol/L, $p = 0.028$ and $p = 0.026$ for 50 μ mol/L, respectively; Fig. 3D).

PBSCs migration toward MSCs is blocked by CB receptor and β -AR antagonists

CD34⁺ PBSCs effectively migrated toward LPS-stimulated and LPS-unstimulated MSCs in the coculture system (Figs. 3B and 3E). However, the LPS-stimulated MSCs exhibited higher eCB-receptor-mediated migration stimulation compared with unstimulated cells. The CB1 antagonist AM281 ($p = 0.03$), the CXCR4 antagonist AMD3100 ($p = 0.05$), and the Adr β inhibitor SR59230A ($p = 0.03$) all significantly blocked migration toward LPS-stimulated MSCs (Fig. 3E). The difference was not significant for the CB2 antagonist AM630 (Fig. 3E). Inhibition of migration toward LPS-unstimulated MSCs was not significant. The LPS-only control group revealed a response at the baseline with almost no migrating CD34⁺ PBSCs.

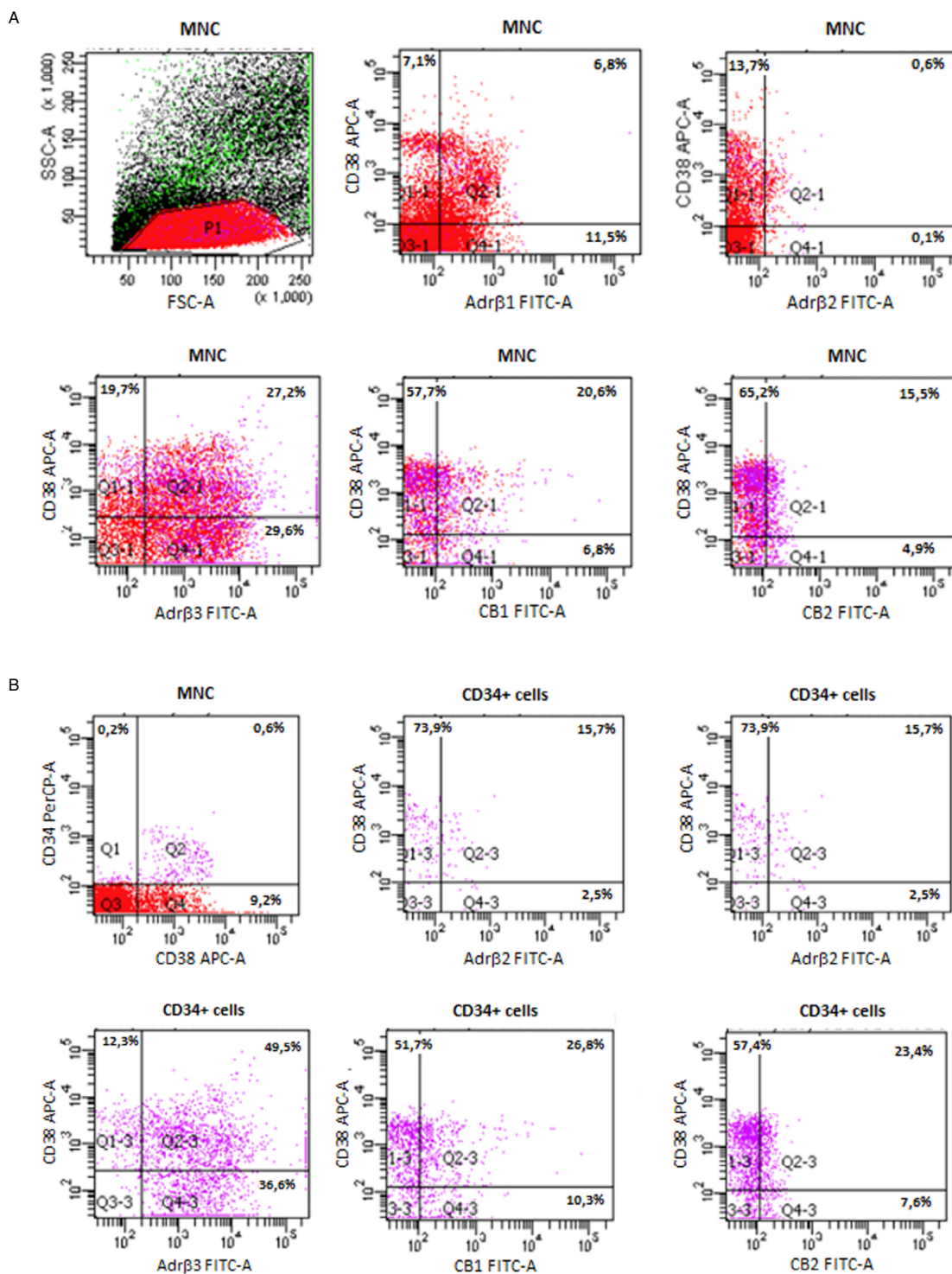


Figure 2. BM-MNCs and CD34⁺ HSCs express CB1, CB2, and various subtypes as shown by FC and qRT-PCR. (A) Representative histograms showing specific markers for MNCs obtained after density gradient centrifugation method. Monocyte/lymphocyte/stem cell fraction (as gated in P1) of the MNCs were used for receptor analysis ($n = 10$ for each group). (B) Representative histograms showing specific markers for CD34⁺ HSCs (Q1 + Q2 quadrants on the first graphic) by FC ($n = 10$ for each group). (C) Representative histograms showing specific markers for BM MSCs by FC. MSCs were gated according to their own FSC/SSC plot ($n = 10$ for each group). (D) Bar graphic showing the percentage of MNCs, MSCs, and CD34⁺ HSCs expressing Adrβ1, Adrβ2, and Adrβ3 and CB1 and CB2 receptors as determined by FC. The MNCs and CD34⁺ HSCs express significantly higher levels of CB1 and CB2 receptors in G-CSF-untreated and G-CSF-treated groups compared with MSCs. CD34⁺ HSCs exhibit higher expression of Adrβ2 and Adrβ3 in all groups compared with MNCs, but the difference is only significant for Adrβ3 ($n = 10$ for each group). (E,F) qRT-PCR analysis revealing CB1 and CB2 gene expressions on MNCs, but not on MSCs. MNCs and MSCs mainly express Adrβ2 ($n = 6$ for each group). FITC = fluoro-isothiocyanate; PE = phycoerythrin; APC = allophycocyanin; PE-Cy7 = PE-Cyanin 7. Shown are conjugated antibodies or their isotypes ($\#p < 0.05$).

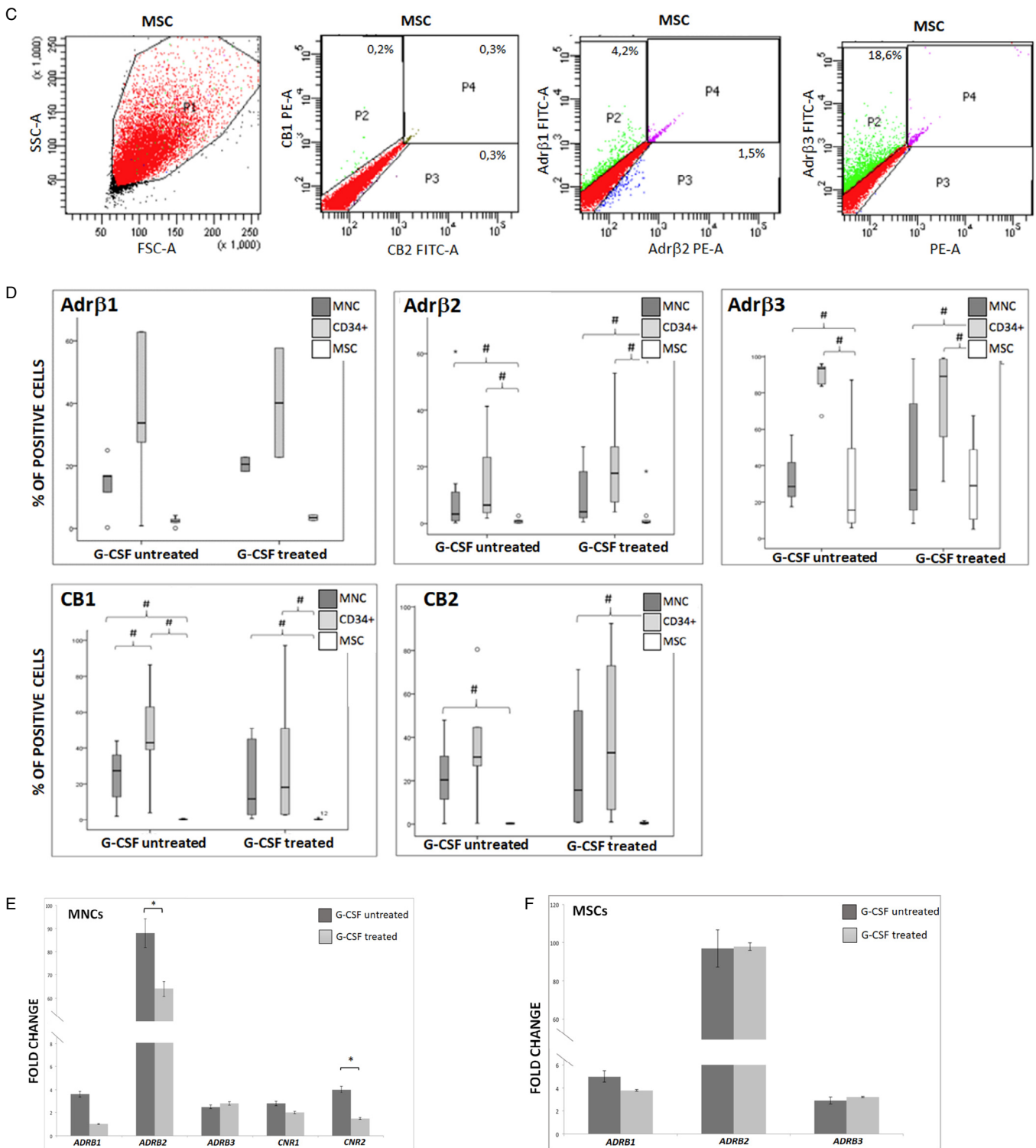


Figure 2. (continued)

Discussion

Secretion of eCBs in the stromal BM microenvironment and cannabinoid receptors on HSCs was assessed in G-CSF-treated and G-CSF-untreated donors in this study. We showed that eCBs act as mobilizers of CD34⁺ PBSCs through CB1

and CB2 receptors in vitro. Our results demonstrated that the eCBs AEA, and 2-AG secreted by MSCs increase human CD34⁺ PBSC migration dose dependently toward LPS-stimulated MSCs. MSCs play important roles in the BM microenvironment and modulate HSC mobilization and

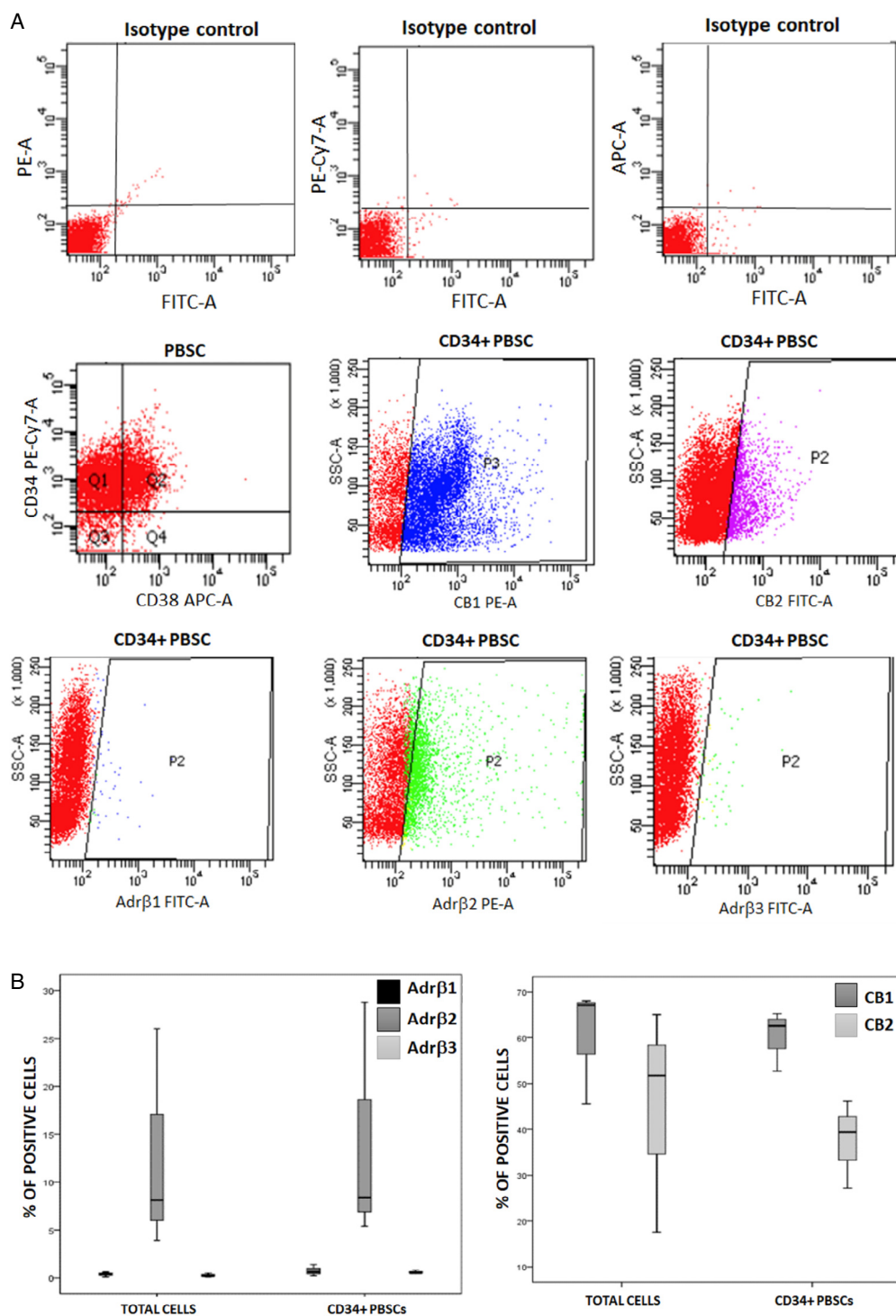


Figure 3. AEA and 2-AG stimulate CD34⁺ PBSC migration to MSCs and this migration effect is blocked by Adrβ and CB receptor antagonists. **(A)** Representative histograms showing isotype controls and specific markers for CD34⁺ PBSCs (Q1 + Q2 quadrants on fourth graphic) by FC ($n = 6$ for each group). **(B)** Characterization of PBSCs and CD34⁺ cells from apheresis product for the β-AR and CB receptors by FC ($n = 6$). The cells expressed Adrβ subtypes and the CB receptors. **(C)** Experimental design for Transwell migration assay. The Transwell/coculture system allows CD34⁺ PBSC migration toward SDF-1, norepinephrine, AEA, 2-AG, or MSCs, respectively. **(D)** CD34⁺ PBSC migration toward SDF-1 and NE are inhibited by the specific antagonists AMD3100 and SR59230A, respectively ($n = 6$). **(E)** Migration of CD34⁺ PBSCs to the eCBs AEA and 2-AG. CD34⁺ PBSCs exhibited significantly higher migration to 30 nmol/L and 50 μmol/L doses of AEA and 30 nmol/L, 1 μmol/L, and 50 μmol/L doses of 2-AG compared with SDF-1. This migration effect is blocked by CB antagonists ($*p < 0.05$, $n = 6$). **(F)** CD34⁺ PBSCs effectively migrated toward LPS-stimulated (LPS⁺) and unstimulated (LPS⁻) MSCs. The LPS-only control group is not presented because it is at the base line. The migration effect is blocked by the CB1 antagonist AM281, the CXCR4 antagonist AMD3100, and the Adrβ blocker SR59230A significantly ($*p < 0.05$, $n = 6$). FITC = fluoro-isothiocyanate; PE = phycoerythrin; APC = allophycocyanin; PE-Cy7 = PE-Cyanin 7. Shown are conjugated antibodies or their isotypes.

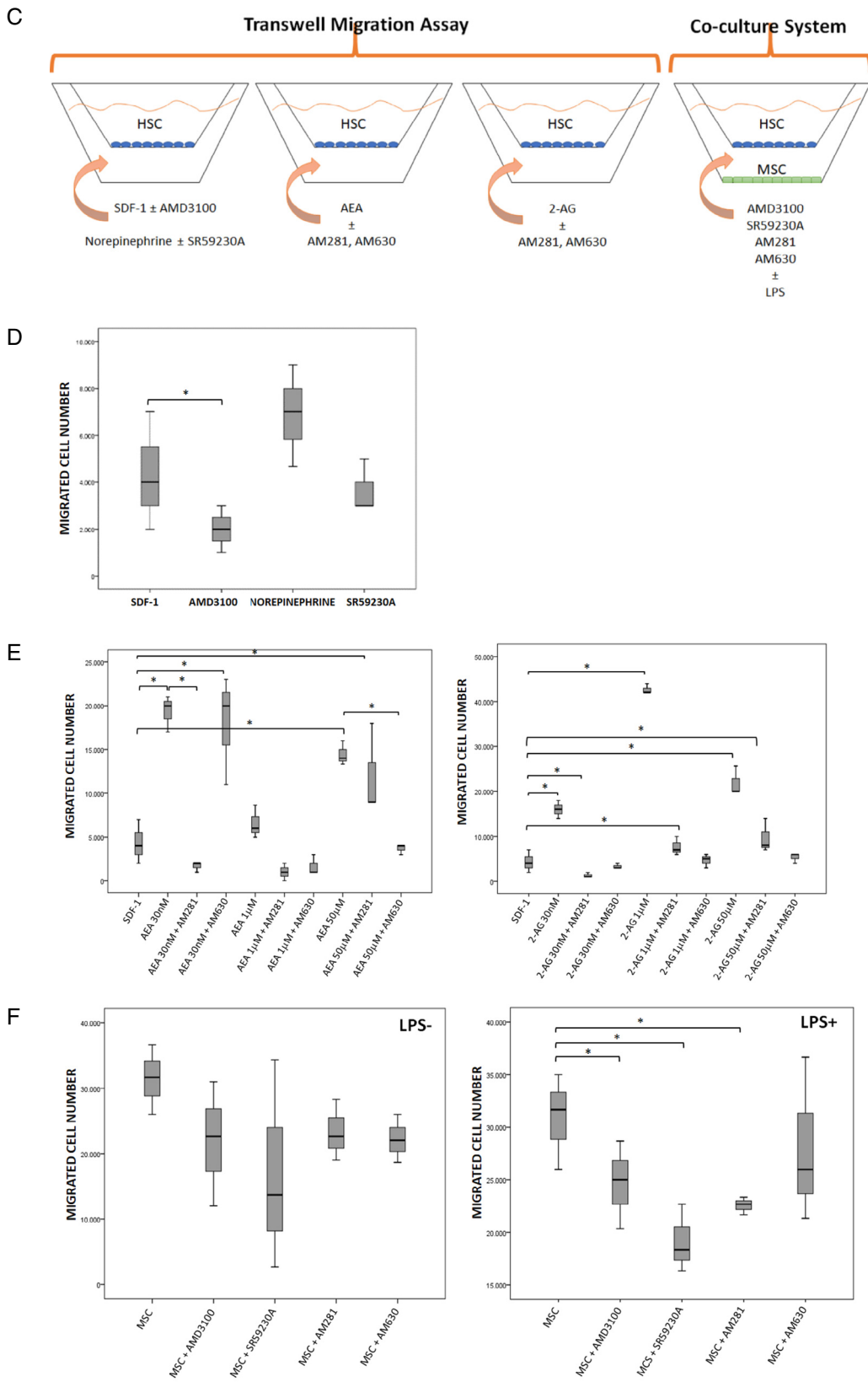


Figure 3. (continued)

survival [32] under stress conditions, which results in sympathetic Adr β stimulation [6,7]. In this study, AEA and 2-AG caused CD34⁺ PBSC migration, reflecting migration via the β -adrenergic system. BM-HSCs and MSCs express Adr β 2 and Adr β 3 [33]. Adr β signaling and LPS release favor the positive effect of G-CSF on HSC mobilization [6,34].

PB and BM plasma and MSC supernatants contained AEA and 2-AG. AEA and 2-AG levels in the PB plasma of healthy controls were found previously to be 0.8 ± 0.12 and 19.0 ± 2.61 nmol/L, respectively, using LC-ESI-MS/MS [35]. Levels of AEA and 2-AG in PB were previously found to be 0.56 ng/mL and 2.0 ng/mL in healthy donors [36]. We report levels of AEA and 2-AG of approximately 50 times higher compared with findings by Jean-Gilles et al. [35] and Quercioli et al. [36]. However, others reported a range from pmol/L to μ mol/L levels for AEA and 2-AG at a different matrix [37] (Human Metabolite Data Base, www.hmdb.ca). AEA levels (0.36 ± 0.14 ng/mL) and 2-AG (6.26 ± 2.10 ng/mL) were also measured in PB plasma of patients during traumatic stress exposure and posttraumatic stress disorder [38]. Our study on AEA and 2-AG levels in PB plasma of G-CSF-treated and G-CSF-untreated donors correlated well with that study. eCB levels were not examined in BM plasma of humans or other species previously. AEA levels in BM and PB plasma were generally equivalent between G-CSF-treated and G-CSF-untreated donors in our study. The 2-AG level was higher in PB plasma compared with BM plasma in the G-CSF-treated group. The 2-AG level in PB increased approximately 10-fold after G-CSF treatment compared with the untreated group. This may suggest a potential function for 2-AG during G-CSF treatment.

Rossi and colleagues [39] reported a gradual decrease in AEA and 2-AG levels secreted by human BM-MSCs from passage 1 (AEA: 5 pmol/mg protein and 2-AG: 11 pmol/mg protein 2-AG, at passage 1). In our study, we used passage 1 MSCs to assess CD34⁺ PBSC migration. Human adipose tissue-derived MSCs have been reported to secrete AEA and 2-AG (AEA: 3.5 pmol/mg protein and 2-AG: 7.3 pmol/mg protein) [40]. These findings were consistent with our results showing AEA and 2-AG secretion by BM-MSCs. Therefore, endogenous eCBs found in BM plasma samples are likely to be secreted by MSCs.

Here, we report for the first time the distribution of eCB receptor and Adr β subtypes on human BM and PB cells simultaneously with or without stimulation by G-CSF. We found significantly higher CB1 and CB2 receptor expression on MNCs and CD34⁺ HSCs compared with MSCs. There are limited studies reporting distribution of CB1 and/or CB2 receptors on HSCs [14,15]. Although CB1 receptor expression is reported in a single murine T-lymphoid cell line, CB2 receptor expression was found in a multitude of myeloid, macrophage, erythroid, B/T-lymphoid, mast cell lines [14]. In rodents, CB1 and CB2 receptors were detected in BM-MSCs and HSCs [15].

Here, Adr β subtypes were detected on MNCs, CD34⁺ HSCs, and MSCs of both G-CSF-treated and G-CSF-untreated samples at similar levels. CD34⁺ HSCs displayed the highest level of Adr β 2 expression. These Adr β distribution data correlate with previous data [6,7,22,23,41,42]. Adr β 2 was determined by FC and confocal microscopy in mouse BM-HSCs (Lin⁻, Sca1⁺, CD117⁺) [22]. Functional studies assessing Adr β antagonists showed that Adr β 2 and Adr β 3 stimulation increase migration of rat hematopoietic progenitor cells (HPCs) [23], mouse HSCs, and HPCs [6]. Exogenous NE administration promotes human CD34⁺ HPC proliferation and migration via Adr β 2 [7].

Activation of Adr β 2 and Adr β 3 on BM-MSCs downregulate SDF-1 and regulate G-CSF-induced migration of HPCs in mice [41]. Monitoring of SDF-1 levels in the BM can be used directly as an indicator for mobilization of HSCs to the PB [42]. Therefore, we tested migration of CD34⁺ PBSCs toward NE and SDF-1. We showed that CD34⁺ PBSCs exhibited significantly higher migration toward AEA and 2-AG compared with SDF-1. eCBs stimulate HSC migration via both CB1 and CB2 receptors. In our study, we determined our dose range for AEA and 2-AG according to their K_i values for CB1 and CB2 receptors and the related literature [16,17,19–21,43]. AEA (1 μ mol/L) and 2-AG (300 nmol/L and 1 μ mol/L) act as chemoattractants when applied to genetically CB2-overexpressing murine myeloid leukemia cells in a Transwell assay [16]. 2-AG and AEA (both at 0.25 μ mol/L) stimulated rapid migration of 8- to 12-week-old mice BM-MNCs in vitro [17]. HU-210, WIN55212-2, and AEA (at 50 μ mol/L), three CB1 receptor agonists with distinct chemical structures, induced migration of human *CB1*-gene-overexpressing HEK293T cells [19]. 2-AG (50 μ mol/L) and AEA (1 μ mol/L) triggered migration of CB1 and CB2 receptor expressing newborn mouse neopallia microglial cells and BV-2 cells (a murine microglial cell line) in a Boyden chamber assay in vitro [20]. The nonpsychoactive cannabinoid agonist abnormal cannabidiol (abn-cbd) was shown to stimulate HUVEC migration in Transwells and the effect was inhibited by the selective antagonist O1918 (30 μ mol/L) [21]. A nonselective CB1 and CB2 agonist CP55940 (dose not indicated) addition to single dose of G-CSF treatment increased granulocyte-macrophage colony-forming unit mobilization significantly compared with G-CSF alone in BALB/c mice [18].

We report that the migratory effect of AEA (a full agonist for CB1 and CB2) and 2-AG (a full agonist for CB2 and a weak agonist for CB1) [12,13] was generally inhibited by CB1 and CB1 receptors separately. Conversely, AEA activated CB2 receptors mainly when applied at 50 μ mol/L and its effect was blocked with AM630, possibly due to the high dose activation ability for and a shift to CB2 receptors.

In our first set of experiments, we showed higher Adr β 2 and CB2 receptor expression in G-CSF-treated MNCs compared with untreated cells. We also demonstrated that AEA and 2-AG mediate CD34⁺ PBSC migration toward MSCs in a coculture system. LPS-stimulated MSCs exhibited higher

eCB-receptor-mediated migration stimulation effect to CD34⁺ PBSCs compared with unstimulated. Those findings were not shown previously. Human BM-MSCs endogenously secrete AEA and 2-AG, which in turn induces CD34⁺ PBSC migration in vitro.

Our study results are limited due to the small number of human samples used due to ethical considerations. Second, because our assay does not simulate a 3D BM niche accurately, the true mobilizing effects of eCBs on HSCs should be tested further in vivo. These limitations, however, do not constrain future in vivo and clinical studies because statistical accuracy was validated at the beginning of the study. We have demonstrated that the effects of the components of the eCB system and other mobilizing agents on the interaction of HSCs with MSCs should be further assessed in animal models and in clinical trials. In particular, the lower ADR β 2 and CB2 receptor expression pattern in G-CSF-treated MNCs should be confirmed with high numbers of fresh donor samples immediately after treatment.

Rapid and sustained recovery of hematopoietic functions after HSCT correlates with the number of CD34⁺ HSCs infused. New solutions should be explored to increase the number of CD34⁺ HSCs. The sympathetic activation and the subsequent beta-adrenergic system involvement is well described, but it is not the only/efficient stimulator for HSC mobilization. Therefore, the utility of eicosanoid-based therapeutic strategies including cannabinoids requires investigation [8,18]. If the mechanism of mobilization of HSCs by eCB agonists or antagonists can be controlled or regulated, then this may result in the development of clinically applicable new mobilization strategies and a better understanding of BM niche dynamics and HSCT strategies.

In conclusion, we found an important role for both the eCB systems and β -adrenergic systems in the migration of HSCs and demonstrate interactions between the HSCs and MSCs of G-CSF-treated and G-CSF-untreated healthy age-matched donors. The eCB system works well in both G-CSF-treated and G-CSF-untreated donors. Therefore, cannabinoid agonists may be strong candidates for new potential therapies of various hematological diseases.

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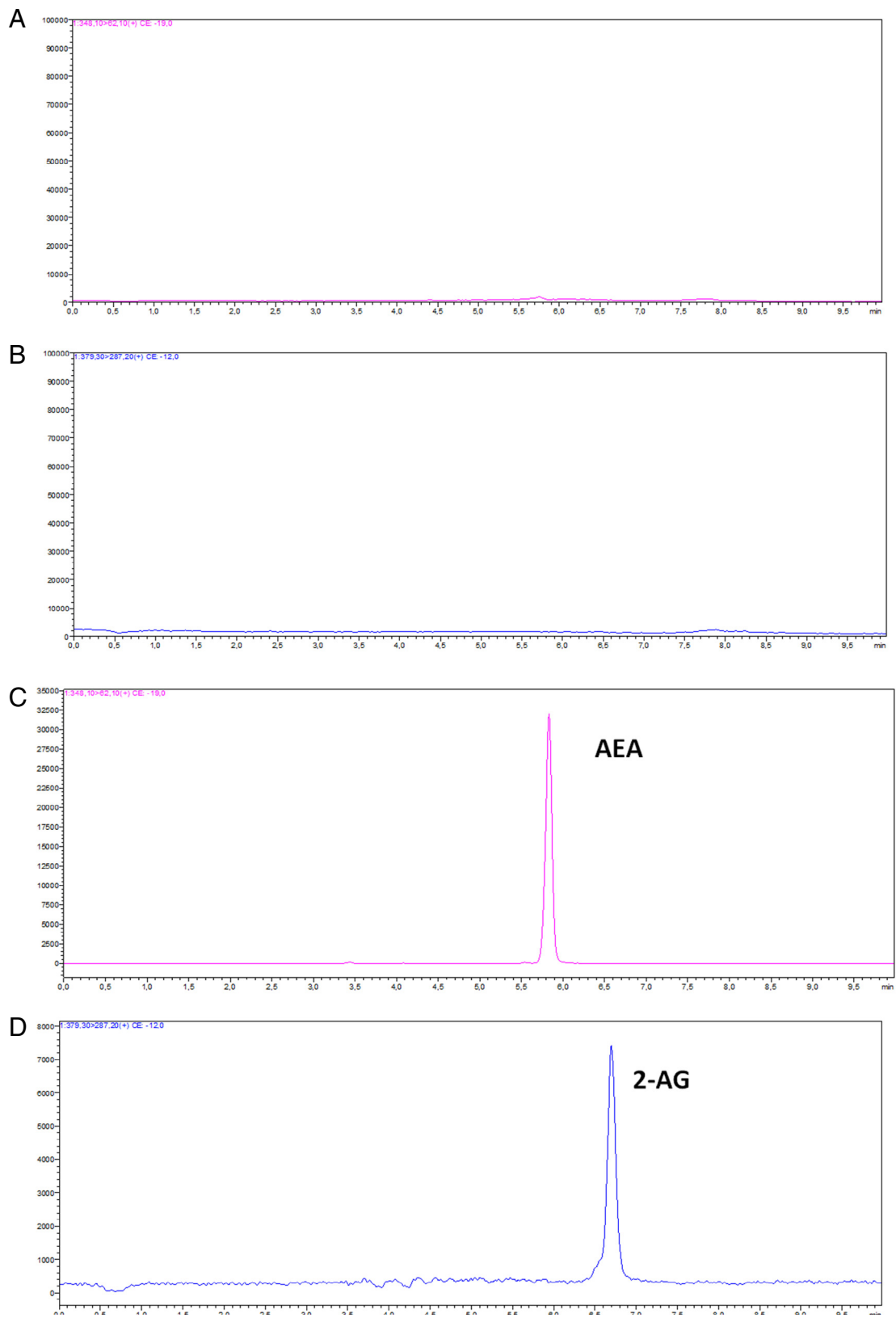
Medicine. Optimization of the LC-ESI-MS/MS method was done by Assoc. Prof. Erol Şener at the Department of Analytical Chemistry, Anadolu University Faculty of Pharmacy.

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Supplementary data



Supplementary Figure E1. The solvent controlled calibration peaks are shown for AEA and 2-AG. (A) Solvent only for AEA, (B) Solvent only for 2-AG, (C) AEA in solvent, (D) 2-AG in solvent