ENOCANNABINIODS ARE EXPRESSED IN BONE MARROW STROMAL NICHE AND PLAY A ROLE IN INTERACTIONS OF HEMATOPOIETIC STEM AND PROGENITOR CELLS WITH THE BONE MARROW MICROENVIRONMENT

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Running title: Hematopoietic stem cell niche and endocannabinoids

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Endocannabinoids are lipid signaling molecules that act via G-coupled receptors, CB1 and CB2. The endocannabinoid system is capable of activation of distinct signaling pathways on demand in response to pathogenic events or stimuli, hereby enhancing cell survival and promoting tissue repair. However, the role of endocannabinoids in hematopoietic stem and progenitor cells (HSPCs) and their interaction with hematopoietic stem cells (HSC) niches is not known. HSPCs are maintained in quiescent state in bone marrow (BM) niches by intrinsic and extrinsic signaling. We report that HSPCs express the CB1 receptors and that BM stromal cells secrete endocannabinoids, anandamide (AEA) (35 pg/10^7 cells) and 2-AG (75.2ng/10^7 cells). In response to the endotoxin lipopolysaccharid (LPS), elevated levels of AEA (75.6 pg/10^7 cells) and 2-AG (98.8 ng/10^7 cells) were secreted from BM stromal cells, resulting in migration and trafficking of HSPCs from the BM niches to the peripheral blood. Further, administration of exogenous cannabinoid CB1 agonists in vivo induced chemotaxis, migration and mobilization of human and murine HSPCs. Cannabinoid receptor knockout mice Cnr1−/− showed a decrease in side population (SP) cells while fatty acid amide hydrolase (FAAH) −/− mice, which have elevated levels of AEA, yielded increased colony formation as compared to WT mice. In addition, G-CSF-induced mobilization in vivo was modulated by endocannabinoids and was
inhibited by specific cannabinoid antagonists as well as impaired in Cannabinoid receptor knockout mice Cnr1<sup>−/−</sup>, as compared to WT mice. Thus, we propose a novel function of the endocannabinoid system, as a regulator of HSPC interactions with their BM niches, where endocannabinoids are expressed in HSC niches and under stress conditions, endocannabinoid expression levels are enhanced to induce HSPC migration for proper hematopoiesis.

**INTRODUCTION**

During postnatal life, the bone marrow (BM) supports both the self renewal and differentiation of hematopoietic stem cells (HSCs) in specialized niches (1-2). HSCs are kept in a low proliferative, relatively quiescent state in close proximity to stromal cells and osteoblasts, forming these specialized niches (3-5). The interaction of HSCs with their niches, especially the bone, is crucial to prevent exhaustion of HSCs from uncontrolled cell-cycle entry and excessive proliferation (6-8). In addition, the niche and its components protect the HSCs from stress such as accumulation of reactive oxygen species and DNA damage (3).

Endocannabinoids constitute a novel family of lipid ligands that act via specific G-protein-coupled receptors CB<sub>1</sub> and CB<sub>2</sub> (9-14). The endocannabinoid system includes the receptors (CB<sub>1</sub> and CB<sub>2</sub>), their endogenous ligands (endocannabinoids), anandamide (AEA) and 2-arachydonoyl-glycerol (2-AG), exogenous cannabinoid ligands and hydrolyzing enzymes [Fatty acid amide hydrolase (FAAH) and MGL]. Endocannabinoids were shown to be synthesized in vivo from phospholipids (15). The effects of endocannabinoids at the CB<sub>1</sub> receptors are terminated by their rapid uptake through a high affinity membrane transporter and subsequent intracellular degradation by FAAH (16).

The CB<sub>1</sub> receptor is highly expressed in the CNS, with particularly high levels in the neocortex, hippocampus, basal ganglia, cerebellum and brainstem (16-19). Endocannabinoid modulate neurotransmitter release and thus exert a wide array of actions including motor function, cognitive processes, emotion, sensory perception, endocrine functions and food intake (9). Furthermore, cannabinoid signaling via CB<sub>1</sub> receptor modulates a range of physiological functions in the adult body (19).

The CB<sub>2</sub> receptor displays a more selective pattern of expression mainly in immune cells, such as B and T lymphocytes (20). In regard to hematopoietic system, several studies have addressed the expression and function of cannabinoid receptors in mature hematopoietic and immune cells (21-23), while the effect of cannabinoids on HSPCs has not been investigated in depth. Noteworthy, the endocannabinoids promoted the growth of primary murine marrow progenitor cells (24). We also recently reported the role of cannabinoid receptors in survival of murine embryonic stem cells and their hematopoietic differentiation (25).

Here, we studied the expression of endocannabinoids in the stromal BM niches under homeostasis conditions and inflammatory response following exposure to the endotoxin LPS as well as the
expression of cannabinoid receptors in HSPCs. We report the expression of endocannabinoids in stromal BM niches and their increased secretion following exposure to LPS, resulting in migration and trafficking of HSPCs from the BM niches to the peripheral blood.

**EXPERIMENTAL PROCEDURES**

_Antibodies, chemical and biological compounds_—Rabbit polyclonal anti-CB1 antibodies (ABR-Affinity BioReagents, Golden, CO) were used for immunofluorescent staining. Rabbit polyclonal anti-CB2 antibody and URB597 were purchased from Cayman Chemical Company (Ann Arbor, MI). The specificity of murine CB1 antibodies were confirmed by FACS analysis, immuno-staining and Western blotting using mononuclear cells and HSCs derived from Cnr1/−/− mice, respectively. Anti-actin antibody was obtained from Chemicon (Temecula, CA). The human CD34 isolation kit was product of Miltenyi Biotec (Auburn, CA). PE-conjugated anti-CD34 antibody was from Pharmingen (San Diego, CA). The cannabinoid ligands Δ9-THC (THC), methanandamide, ACEA, and CP55940 were obtained from Sigma (St. Louis, MO). The cannabinoid receptor antagonists AM251 (for CB1) and AM630 (for CB2) were purchased from Tocris (Ellisville, MO). G-CSF (Neupogen) was obtained from Amgen Inc. (Thousand Oaks, CA). MethoCult 03434 (for mouse cells) and 04435 (for human cells) were obtained from StemCell Technologies (Vancouver, BC, Canada).

**Cells**—Mononuclear cells (MNCs) from human bone marrow were obtained from Cambrex Corp. (East Rutherford, NJ). Human CD34-positive cells were isolated from mononuclear cells derived from human bone marrow by using the CD34 immunomagnetic bead separation method of the mini-MACS system following the manufacturer’s guidelines (Miltenyi Biotec).

**Mice**—C57BL/6J and SJL-Ptprca Pep3b/BoyJ mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 8 weeks of age and were used in the experiments between 12 and 13 weeks of age. The Cannabinoid receptor type 1 (Cnr1/−/−) and type 2 (Cnr2/−/−) knockout (=KO) mice were kindly obtained from Dr. Tom I. Bonner (Genetics and Cellular and Molecular Regulation, National Institute of Mental Health, Bethesda, MD) and Dr. K. Dey (Vanderbilt Univ., Nashville, TN) (26-27). Cnr1/−/− and WT mice were in the background of C57BL6/129 as described previously (17). The control animals for experiments involving cannabinoid receptor knockout mice were generated by interbreeding between Cnr1/−/− and Cnr2/−/− strains, then breeding between heterozygous Cnr1+/−/Cnr2+/− offspring, and then selecting the wild-type (i.e. Cnr1+/−/Cnr2+/+) animals for further breeding as controls. FAAH/−/− mice were kindly obtained from Dr. B. Gravatt (28). All animal experiments were approved by the BIDMC Institutional Animal Care and Use Committee.
Preparation of mouse bone marrow cells, FACS and Hoechst 33342 staining - Murine bone marrow cells were harvested from C57BL/6J mice by flushing the femurs and tibias, and then placed in DMEM+ (Dulbecco's modified Eagle's medium, 2% FBS, 10 mM HEPES buffer, Gibco, Grand Island, NY). A single cell suspension was made in DMEM+ by passing the bone marrow through an 18-gauge needle. The cells were filtered through 70-micron cell strainers (Falcon) and pelleted by centrifugation. The cells were resuspended in prewarmed DMEM+, and viable cells were counted using the trypan blue dye exclusion method. Bone marrow cells were stained with Hoechst 33342 (Sigma), as described previously (29). The Hoechst-stained cells were separated using Percoll reagent (Amersham-Pharmacia, Uppsala, Sweden) to remove the red blood cells as well as the dead cells. The Hoechst-stained and separated cells were then suspended in HBSS+ (Hank’s balanced salt solution, containing 2% FBS and 10 mM HEPES buffer, Gibco) at 20 to 30×10^6 cells per ml cell density for the antibody staining. Immunostaining with anti-CB1 or anti-CB2 antibodies was followed by FACS analysis as described above, except that PE-conjugated secondary antibodies were used. The flow-cytometric analysis to determine Hoechst and PE fluorescence was performed using a dual-laser-Mo-Flo high performance cell sorter (Cytomation, Inc., Fort Collins, CO), as described previously (25).

Chemotaxis of human CD34+ cells and murine Lin−Sca1+ c-kit+ cells - CD34+ Cells (1×10^5) or murine BM cells (10^5 cells) were placed onto the filters of the Transwell inserts and the inserts were transferred in triplicate into the wells containing CP55940 (10 nM) as indicated. Cells were allowed to migrate for 4 h, and then the cells in the bottom chambers were counted. Data are presented as percentages (%) of cells migrated out of total loaded cells.

Mobilization of bone marrow HSPCs - For in vivo mobilization experiments, mice received: G-CSF (125 µg/kg) i.p. twice daily for 4 days and mice were sacrificed on day 5. LPS (2 µg/kg) was administered once i.p. and after 5 days, mice were sacrificed and analyzed. The cannabinoid ligands were dissolved in ethanol/cremophor/saline (1:1:18) vehicle and administered once daily for four consecutive days i.p: CP55940 (10mg/kg), Δ⁹-tetrahydrocannabinol (THC), or ACEA (all ligands at a dose of 10 mg/kg) either alone or in the presence of cannabinoid receptor antagonists AM251 (for CB1) (5 mg/kg), and/or AM630 (for CB2) (5 mg/kg), as indicated. URB597 (0.5 mg/kg) was injected i.p. one hour prior to the last G-CSF injection. Twenty-four hours following the last injection of cannabinoid agonists and URB597, mice were sacrificed; dissected and peripheral blood was collected by cardiac puncture. Control mice received i.p. injections of vehicle alone daily for 4 days, and twenty-four hours following the last injection, mice were sacrificed, dissected and peripheral blood was collected (0.6 ml from each mice) by cardiac puncture. White blood cells (WBC) were isolated by Ficoll separation. Briefly, blood was diluted to 6 ml and was overlaid on the 5 ml Ficoll layer. Tubes were centrifuged at 2000 rpm at 18°C for 30 min. The top layer
was removed slowly with a Pasteur pipette and the WBC layer was transferred into 8-9 ml DPBS\(^+\) (DPBS\(^+\) containing 2% FBS) in a 15 ml conical tube. Tubes were spun at 1500 rpm at 4°C for 5 to 7 min and then the pellet was resuspended in DPBS\(^+\). The cells were collected and used for the colony-formation assays using MethoCult media, according to the protocol of StemCell Technologies (Tukwila, WA). The MethoCult medium containing 1×10\(^5\) cells/ml was added into 35 mm Petri dishes and incubated at 37°C in a 5% CO\(_2\) incubator for 10-14 days. Triplicate assays were performed for each sample. Following the incubation period, the numbers of colonies were determined by light microscopy. Positive colonies were scored on the basis of an accumulation of 40 or more cells.

**HSPC assays**

The murine cells were assessed using MethoCult 03434 (Stem Cell Technologies Inc.). Human cells were assayed for CFU-GM, BFU-E, and CFU-GEMM using ingredients and cytokine combinations as noted elsewhere (30). For human CD34\(^+\) cells, we employed human methylcellulose enriched media (R\(_\alpha\)D systems), according to the protocol provided by R\(_\alpha\)D System. Colonies were scored after 14 d for human cells and after 7 d for mouse cells in a humidified atmosphere with 5% CO\(_2\) and lowered (5%) O\(_2\).

**RT-PCR analysis of CB\(_1\) and CB\(_2\) expression**

- RNA from total bone marrow MNCs or as indicated was extracted using the RNeasy Mini Kit along with DNA Shredder Kit (both from Qiagen, Valencia, CA) following the manufacturer’s protocol. A QIAshredder spin column and DNase I digestion were included in the isolation procedure to limit the possibility of PCR amplification of CB\(_1\) and CB\(_2\) from genomic DNA. cDNA and PCR amplification were performed with the BD Biosciences TITANIUM One-Step RT-PCR Kit using 200 ng of RNA as a template for first-strand synthesis. CB\(_1\) was amplified using primers: 5’-CGT GGG CAG CCT GTT CCT CA-3’ and 5’-CAT GCG GGC TTG GTC TGG-3’, which yield a product of 403 bp. CB\(_2\) was amplified using: 5’-CCA TGG AGG AAT GCT GGG TG-3’ and 5’-TCA GCA ATC AGA GAG GTC TAG-3’, which yield a product of 1100 bp. GAPDH was used as a positive control with primers: 5’-CTC ACT GGC ATG GCC TTC CG-3’ and 5’-ACC ACC CTG TTG CTG TAG CC-3’, which yield a product of 292 bp. The template was first denatured at 94°C for 2 min followed by 35 cycles (94°C for 30 sec, 58°C for 30 sec and 68°C for 1 min), followed by 68°C for 2 min in a myCycler Personal Thermal Cycler (Bio-Rad Laboratories, Inc). Aliquots (20 µl) of the PCR products were run on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide.

**Endocannabinoid Levels in Bone Marrow Stromal Cells:** 10\(^7\) cells of murine bone marrow cells were analyzed for endocannabinoid expression levels. Mixtures of the 2-AG and AEA and their denatured analogs that had been stored at -80°C were further diluted in a 20mg/ml solution of fatty acid free bovine serum albumin (BSA) to stimulate analyte-free plasma and in ethanol to make the calibration standards, quality control (QC) samples and reference samples, as
previously described (25,31). The calibration curves were constructed from the ratios of the peak areas of the analytes versus the IS.

**LC-MS Analysis for endocannabinoids** - Chromatographic separation was achieved using a Higgins Analytic Haisil C18 column (0.5x50mm, 5mm) on an ABI 4000 Q-Trap mass spectrometer with a Tempo nano-LC on the front end (Applied Biosystems Incorporated; Framingham, MA). The mobile phase consisted of 95/5 water/acetonitrile and 95/5 acetonitrile/water, with 0.1% formic acid in both, in the following gradient: initial conditions are held at 30% A for 30 seconds, increased linearly to 100% B and held from 0.75 to 4 min, then returning to initial conditions by 4.5 minutes (flow rate=10 µL/min); the autosampler was kept at 4°C to prevent analyte degradation. Eluted peaks were ionized via electrospray ionization (ESI) in MRM mode.

**Western blotting** - For the Western blotting procedure, isolated HSPCs (1×10^4) derived from bone marrow of WT, Cnr1^-/- or Cnr2^-/- mice were used to prepare protein lysates for Western blot analysis, using specific antibodies for CB1 and CB2. Actin antibody was used as internal control for loading.

**Statistical analysis** - The statistical significance of the results reported hereby was determined by a two-tailed t test. P<0.01 and P<0.05 were considered significant, as indicated.

**RESULTS**

**Expression of endocannabinoids in BM-stromal cells** - In order to study the role of endocannabinoids in hematopoietic stem cell niche, we examined the expression of 2-AG and AEA in bone marrow stromal cells. As shown in Table 1, both 2-AG and AEA were detected in stromal cells, with AEA at 35.2pg/10^7 cells and 2-AG at 75.2ng/10^7 cells. Of note, the level of 2-AG synthesis were much greater (approx.1000 fold, ng) compared to AEA (pg). However, under stress induced inflammatory responses following administration of the endotoxin LPS, enhanced expression of 2-AG and AEA (Table 1) were observed, resulting in migration and trafficking of HSPCs from the bone marrow niches to the blood circulation (Fig. 1). This mobilization was impaired in Cnr1^-/- and Cnr2^-/- mice (Fig.1A). Further, there was a synergistic effect of LPS, when co-administered with the FAAH inhibitor URB597, (which results in increased AEA levels), in mobilization of HSPCs (Fig. 1B). These results suggest that endocannabinoids induce migration and trafficking of HSPCs, and that this migration is enhanced under stress conditions.

**Expression of CB1 cannabinoid receptors in murine bone marrow cells** – Next, we evaluated the expression of CB1 receptors on murine bone marrow mononuclear cells. A total of 27.4% mononuclear cells expressed the CB1 receptor (Fig. 2A). We also assessed the expression of the CB1 receptors in Side Population (SP) as well as LSK (Lin^-Sca^-c-Kit^+) stem/progenitor cell populations obtained from mouse bone marrow. We observed that 17.6% of the SP cells expressed CB1 on their surface (Fig.
2A), and the LSK cells derived from bone marrow also expressed CB1 (Figure 2B).

Analyses of Cnr1<sup>−/−</sup>, Cnr2<sup>−/−</sup> and FAAH<sup>−/−</sup> knockout mice -. Both Cnr1<sup>−/−</sup> and Cnr2<sup>−/−</sup> mice were used on a background of C57BL/6J mice and were genotyped and characterized in our group (Fig. 3). The control mice were C57BL/6J mice. Cnr1<sup>−/−</sup> mice lack the expression of CB1 protein (Fig. 3B) and, Cnr2<sup>−/−</sup> mice lack the expression of CB2 protein (Fig. 3D) as demonstrated by Western blot analysis. Furthermore, analysis of the peripheral blood counts, including white blood cells (WBC), red blood cells (RBC) and platelets (PLT) in steady-state conditions showed no differences between the WT, Cnr1<sup>−/−</sup> and Cnr2<sup>−/−</sup> mice (Fig. 3E-Fig. 3G). Interestingly, both Cnr1<sup>−/−</sup> and Cnr2<sup>−/−</sup> mice showed a decrease in SP populations (Fig. 3H). Of note, no changes in the level of expression of CB1 or CB2 in murine HSPCs was noted in the cannabinoid knockout mice Cnr2<sup>−/−</sup> and Cnr1<sup>−/−</sup>, respectively (Fig. 3I).

Further, bone marrow cells derived from mice harboring knockout of the FAAH enzyme (FAAH<sup>−/−</sup>), and thus accumulating anandamide in sites of its natural production, produced more progenitor colonies in the CFU assay (Fig. 3J), suggesting a stimulatory role of AEA towards hematopoiesis.

Effects of cannabinoid ligands on colony formation and migration of human and murine HSPCs in vitro - To study the direct actions of cannabinoids on HSPCs, we first assessed the effects of cannabinoid agonist CP55940, yielded a significant increase in colony numbers as compared to control non-stimulated cells (Fig. 4A). Similarly, treatment of WT murine bone marrow cells with CP55940 yielded an increase in colony number (Fig. 4B).

The effect of cannabinoid ligands on the chemotaxis of human CD34<sup>+</sup> cells and murine LSK cells was then analyzed. CP55940 induced a significant increase in the migration of human CD34<sup>+</sup> cells (Fig. 4C) and murine LSK cells (Fig. 4D). Further, cannabinoid-induced migration of bone marrow cells from Cnr1<sup>−/−</sup> knockout mice was severely impaired, as compared to WT mice (Fig. 4E). These results demonstrate that cannabinoid ligands and cannabinoid receptors are involved in HSPC chemotaxis and migration.

Effects of the CB<sub>1</sub> cannabinoid on HSPC mobilization in vivo - To examine the effects of exogenous cannabinoids on the mobilization of HSPC in vivo, we employed a mouse model of HSPC mobilization from the bone marrow to the circulation, in which the number of circulating HSPC in blood is assessed by the number of colonies formed in semi-solid medium. Treatment of WT mice with either ACEA (CB<sub>1</sub> specific), or CP55940 resulted in a significant (P<0.01) increase in the number of hematopoietic colonies in peripheral blood (Fig. 5A). In addition, THC induced mobilization of HSPCs (Fig. 5B), which was inhibited by the cannabinoid receptor antagonists AM251 (selective for CB<sub>1</sub>) indicating the involvement of CB<sub>1</sub> receptors in mobilization (Fig. 5C). Knockout of Cnr1<sup>−/−</sup> and Cnr2<sup>−/−</sup> significantly (P<0.05) reduced
the effects of CP55940 to induce HSPC mobilization (Fig. 5D+5E). Of note, HSPCs mobilization by cannabinoid agonists such as CP55940 and THC yielded CFU-GM, CFU-GEMM as well as BFU-E types of colonies in vitro, suggesting that cannabinoids can mobilize multipotent progenitor cells from the bone marrow (data not shown).

Modulation of G-CSF-induced mobilization by endocannabinoids - To investigate whether endocannabinoids could modulate G-CSF-induced mobilization, we employed a model involving G-CSF. As shown in Fig. 6A, a significant reduction in the number of G-CSF-induced HSPC-derived colonies was observed following the administration of (AM251), the CB₁ receptor antagonist (Fig. 6A). Since the effects in the knockout mice may not represent the full assessment due to potential compensation by the CB₂ cannabinoid receptors, we treated Cnr₁⁻/⁻ knockout mice with the CB₂ selective AM630. The G-CSF-induced mobilization was significantly inhibited in the Cnr₁⁻/⁻ mice and in Cnr₁⁻/⁻ mice treated with AM630 (Fig. 6B). To evaluate the effects of increased endocannabinoid levels on HSPC-mobilization, we used the FAAH inhibitor, URB597 which inhibits AEA degradation, resulting in accumulation of AEA. Treatment of mice with URB597 significantly increased the incidence of HSPCs in the peripheral blood (Fig. 6C). In addition, combined treatment of G-CSF and URB597 had a synergistic effect in HSPC-mobilization, confirming the capability of endocannabinoids to modulate G-CSF-induced HSPC -mobilization (Fig. 6C).

DISCUSSION
In this study, we examined the role of the endocannabinoid system in hematopoiesis. We observed that the BM niches express and secrete endocannabinoids (AEA and 2-AG) and this secretion is significantly elevated in the presence of a stress inducer such as LPS (Table 1). In addition, these endocannabinoids can bind and activate CB₁ receptors expressed on murine HSPCs (Fig. 2). Endocannabinoids induced by LPS as well as administration of exogenous cannabinoid agonists such CP55940, induced chemotaxis migration and mobilization of HSPCs from the BM niches to the blood circulation (Fig.4). Further, G-CSF-induced HSPC mobilization was mediated by endocannabinoids and was impaired in Cnr₁⁻/⁻ mice (Fig. 6). Cnr₁⁻/⁻ and Cnr₂⁻/⁻ mice also presented a decrease in SP-side population of HSPCs, while FAAH⁻/⁻ mice, which have significant elevated levels of AEA, had an increased CFU colony formation (Fig. 3). Thus, these data collectively show that the endocannabinoid system is involved in hematopoiesis and plays a role in the interaction of HSPCs with their BM niches.

Lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, causes a marked increase in the circulating cytokines (eg. IL-1β, IL-6 and TNF-α) acting as a cytokine inducer. LPS administration in vivo induces inflammatory response and alters metabolism. This makes the use of LPS injection a valuable model in the study of the mechanisms of the
inflammatory response, which is mediated via the action of proinflammatory cytokines, affecting the homing of the stem cells to sites of inflammatory areas (38). LPS was shown to inhibit neural stem cell proliferation (39) and to induce migration of bone marrow cells in mice (40). LPS was also shown to stimulate calcium signaling via G-protein coupled receptor-mediated phospholipases C activation and generation of phosphatidylserine-specific phospholipases A2 activation (41). Therefore, we examined the effects of LPS on cellular activities that include increase in endocannabinoid levels and modulation of hematopoietic stem and progenitor cell migration.

Understanding of the signals that regulate HSCs development and the intrinsic and extrinsic mechanisms that are involved in maintenance of HSC in the bone marrow niches are crucial for proper hematopoiesis. Hematopoiesis is a life long process in which HSPCs differentiate into mature blood cells. These HSCs are valuable in a clinical setting for patients requiring hematopoietic repair (31-37). The current treatment involves hematopoietic stem cell transplantation with HSPCs obtained from mobilized peripheral blood or umbilical cord blood (31). Repopulation of hematopoiesis is a multistep process that is regulated by the ability of HSPCs to migrate, home to the appropriate marrow niches and differentiate to mature blood cells. Hence, insights into the physiological stimuli as well as external signals that induce HSPC exist from the bone marrow and traffic to peripheral blood is important for proper hematopoiesis repair. Here, we provide new evidence on the involvement of the endocannabinoid system in hematopoiesis by inducing migration and mobilization of HSPCs from the BM niches to the blood circulation following exposure to stress inducer such as LPS, or to exogenous cannabinoid agonists. The migration of HSPCs to the peripheral circulation may limit tissue damage and contribute to hematopoietic repair. Endocannabinoids may regulate hematopoiesis in the BM by maintaining important HSPC functions such as survival.

Endocannabinoids have been reported as positive or negative factors in hematopoietic cell migration and differentiation (24). Endocannabinoids were shown to directly modulate hematopoietic cell migration and differentiation as noted by increased of CFU-GEMM (24). Further, endocannabinoids play important role in endotoxic shock and inflammation (9). LPS was shown to stimulate the production of AEA in human lymphocytes, macrophages and 2-AG in rat platelets (9). LPS may induce the expression of additional endocannabinoids in the BM, in addition to 2-AG and AEA, the main two endocannabinoids express in vivo. The level of AEA in brain was reported to be 35 ± 8 pmol/g and 2-AG levels were in brain was reported to be 62 ± 1.8 nmol/g as compared to the expression in blood of AEA (2.5 ± 0.7 pmol/g) and 2-AG (10³ nmol/g) (42). Here, we report that 2-AG and AEA are found in similar levels to those reported in brain. The presence of endogenous cannabinoids in immune cells, hematopoietic cells and BM niches suggest that they play a critical physiological role, the precise nature of which remains to be characterized. Increased elevated levels of AEA and 2-AG may
further protect HSPCs from endotoxic shock and apoptosis and induce their migration from the BM niches to the blood circulation following insult. These studies also have important implication of the endocannabinoid system in HSPC homing and engraftment of HSPCs in the bone marrow. Although a very large number of HSPCs are used in the clinical procedure, the homing efficiency of HSPCs following stem cell transplantation is low. G-CSF was found to govern specific aspects of HSC localization and engraftment to the BM (43). In addition, prostaglandin E2-prostaglandin receptors and adrenergic receptors were also reported to enhance stem cell transplantation in mice (44). Here, we show that endocannabinoids, as components of the endocannabinoid system are also important in stem cell mobilization. Thus, identification of additional physiological systems that can increase homing efficiency and/or increase the nurturing capacity of the niche or increase the number of cells that can be mobilized for in vivo transplantation is crucial for HSPCs and HSCs based therapies. Based on this study, we prepare that Pharmacological intervention targeted towards the endocannabinoid system can be a novel modality for HSPCs-based therapies in patients.

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FOOT NOTE

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Abbreviations: 2-AG, 2-arachydonylglycerol; AEA, Anandamide; CB1, Cannabinoid receptor 1; CB2, Cannabinoid receptor 2; eCBs, Endocannabinoids; FAAH, Fatty acid amide hydrolase; HSPC, Hematopoietic stem and progenitor cells; HSC, Hematopoietic stem cells; LPS, Lipopolysaccharide; MNCs, Mononuclear cells; MGL, Monoacyl glycerol lipase.
FIGURE LEGENDS

FIGURE 1 (A) Induction of HSPC mobilization in wild-type (WT) versus Cnr1−/− mice. LPS was injected i.p. at a dose of 2µg/kg. Mice were sacrificed five days after injection of LPS and the incidence of PB-HPC was evaluated using colony-formation assay as described in the Methods section. These are representative experiments out of duplicate experiments involving 3 mice per treatment group. 

* P<0.01 versus control; **P<0.05 versus LPS alone.

(B) Effects of pre-treatment with URB597 on LPS-induced mobilization of HSPC. Balb/c mice were injected i.p. with URB597 one hour prior to i.p. inoculation of LPS (2µg/kg). Mice were sacrificed five days after injection of LPS and the incidence of PB-HSPC was evaluated using colony-formation assay.*P<0.05 as compared to vehicle control; **p<0.05 as compared to LPS alone.

FIGURE 2(A) Expression of CB1 in mouse bone marrow mononuclear cells, non-SP cells and SP cells. Mouse bone marrow cells were stained with Hoechst dye 33342 and then stained with anti-CB1 antibodies. The surface expression of the CB1 receptors was analyzed on total cells, nonSP cells and SP cells. This is a representative experiment out of 3 experiments.

(B): Expression of CB1 on mouse Lin- Sca+ c-Kit+ (LSK) cells: Mouse bone marrow cells were stained with antibodies against lineage markers Sca-1 and c-Kit as well as with CB1. The cell surface expression of CB1 was analyzed on the LSK cells. This is a representative experiment out of duplicate experiments.

FIGURE 3 The genotype screening and protein analysis of Cannabinoid Receptor knockout mice and wild-type mice. (A) DNA analysis of Cnr1−/− knockout mice (Wild-type mice; 1237 bp, Knockout mice; 1088 bp). Lanes 1-8=different Cnr1−/− mice. WT=wild-type mice. M=M.W. marker. (B) The protein expression of CB1 in BM derived from Cnr1−/− knockout mice (Lane 1) and wild-type mice (Lane 2). (C) The genotype screening of Cnr2−/− knockout mice. The DNA analysis of Cnr2−/− knockout mice, CB2 primer: (Wild-type: 1100 bp; Knockout mice: 850 bp). M=M.W. marker; Lanes 1-9=different Cnr2−/− mice. (D) The protein expression of CB2 in BM derived from Cnr2−/− knockout mice (Lane 1) and wild-type mice (Lane 2). WT=wild-type mice. (E-G) complete peripheral blood counts in mice devoid of CB1 or CB2 cannabinoid receptors. Groups of 4 mice from each genotype were sacrificed, and then peripheral blood was isolated through retro-orbital bleeding. Blood was analyzed for the levels of white blood cells (WBC), red blood cells (RBC) and platelets (PLT). Charts summarize the data of two experiments. Bars = the mean +S.D. (H) FACS analysis of the side population (SP) population of bone marrow cells in mice devoid of Cnr1 or Cnr2 cannabinoid receptors. Groups of four mice from each genotype were sacrificed, and then bone marrow cells were isolated by using standard procedures following the flushing of the femoral bone marrow cavity. Isolated cells were next stained with Hoechst 33342 and analyzed by FACS. This is a representative experiment of two experiments. Bars = the mean +S.D. 3I: Western blot analysis of CB1 and CB2 expression in HSPCs obtained from either WT, Cnr1−/− and Cnr2−/− mice as indicated. Actin was used as
internal control for loading. **J**: Effect of anandamide accumulation on CFU formation by bone marrow cells. 1 x 10^5 mononuclear cells from the bone marrow of wild-type (WT) or FAAH-knockout mice (FAAH-/-) were seeded onto Petri dishes and assayed for CFU colonies. The dishes were incubated at 37°C in a 5% CO2 incubator. On Day 10, colonies were counted under a light microscope. *P<0.05. Bars = the mean +S.D.

**FIGURE 4: Colony formation and migration of human and murine hematopoietic stem and progenitor cells upon cannabinoid ligand stimulation.** (A) Effects of cannabinoid ligands on the in vitro colony formation by human hematopoietic progenitor cells. 1x10^5 of CD34^+ cells were seeded onto methylcellulose based assay with or without the addition of cannabinoid ligands, as indicated. On Day 14, colonies of erythroid (BFU-E) myeloid (CFU-GM) and mixed colonies (CFU-GEMM) were counted under a light microscope. *P<0.05. Values are means ± s.d. (n=3). (B) Effects of cannabinoid ligands on the in vitro colony formation by mouse hematopoietic progenitor cells. 1x10^4 WT BM cells were seeded onto Petri dishes suspended in colony formation-supporting medium with or without the addition of cannabinoid ligands, as indicated. On Day 10 colonies were counted, *P<0.01. Values are means ± s.d. (n=6). (C) Human bone marrow CD34^+ cells were exposed to CP55940 in a Transwell assay. Y-axis shows percentage of migration from an input of 1x10^4 human CD34^+ cells. Values are means ± s.d. (n=6). *P<0.01. (D) Induction of chemotaxis of murine LSK cells by CP55940. Transwell inserts were used to evaluate the migration of LSK cells in distinct CP55940 concentrations. Cells were allowed to migrate for 4 hours and cells in the bottom chambers were then counted under a light microscope. The data are the mean ± s.d. (n=9). *P<0.001. (E) Murine WT and Cnr1^−/− bone marrow cells were added to the upper chamber in a migration assay. Cells were exposed to CP55940, or ACEA, present in the lower well, and migrated cells were counted. Y-axis represents percentage of migration from an input of 1x10^5 total murine BM cells. Values are means ± s.d. (n=6). *P<0.01 as compared to control untreated mice. **P<0.01 as compared to WT treated with the cannabinoid agonist, as indicated.

**FIGURE 5 Effects of CB1 cannabinoid agonists on the mobilization of hematopoietic progenitor cells in C57BL/6J mice.** (A) Effects of intraperitoneally (i.p.) injections of cannabinoid agonists (10 mg/kg, once daily, 4 days) on the number of circulating PB-HSC in WT mice. Following 24 hours from the last injection, peripheral blood (PB) was collected and an in-vitro colony formation assay was done. Y-axis indicates the number of colonies per 1x10^5 blood cells, per 1 ml of blood. The data are the mean ± s.d. (n=18). *P<0.05, **P<0.01. (B) Effects of multiple injections of CP55940 and THC on the number of circulating PB-HSPC. Mice either were untreated or received i.p. injections of CP55940 or THC (once daily) at a dose of 10 mg/kg or vehicle control for four consecutive days. Twenty-four hours following the last injection, peripheral blood was collected from each mouse and an in vitro colony formation assay was done. The data are the mean ± s.d. (n=9). *P<0.05 versus vehicle. (C) Effects of cannabinoid receptor inhibitors on the THC-induced mobilization of PB-HSPC. THC treatment was performed as described above. The CB1 selective receptor antagonist AM251 was applied at
a concentration of 5 mg/kg. Inhibitors were injected 30 min prior to THC treatment on each day. The data are the mean ± s.d. (n=9). *P<0.05 versus vehicle control; **P<0.05 versus THC. (D and E) Effect of CP55940 (as described above) on mobilization of WT and Cnr1−/− HSPC (Panel D) and Cnr2−/− HSPC (Panel E) *P<0.05 versus control; **P<0.05 versus WT. Values are means ± s.d. (n=18).

FIGURE 6 Modulation of G-CSF-induced mobilization by endocannabinoids. (A) Wild-type mice received i.p. injections of G-CSF twice daily for 4 consecutive days. Cannabinoid antagonists were injected i.p. into mice 30 min prior to each G-CSF injection. AM251 inhibitor (for CB1) was applied at a concentration of 5 mg/kg. Frequency of PB-HSPC was assessed as described above. Values are means ± s.d. (n=12). *P<0.01 versus control; **P<0.05 versus G-CSF alone. (B) Effects of Cnr1−/− on the G-CSF-induced mobilization of PB-HSPC, WT, and Cnr1−/− mice received i.p. injections of G-CSF and cannabinoid antagonist as indicated. The data are the mean ± s.d. (n=12). **P<0.05 versus G-CSF in WT mice. (C) URB597 (0.5 mg/kg) was injected i.p. into mice 1 hour prior to each G-CSF injection. A colony culture assay was set up as described above. The data are the mean ± s.d. (n=12). *P<0.05 versus vehicle; **P<0.05 versus G-CSF alone.
Fig. 1

A

PB Progenitors (Colonies per 1 x 10^5 PB WBC)

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Cnr2^-</th>
<th>Cnr1^-</th>
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<tr>
<td>Vehicle</td>
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B

PB Progenitors (colonies per 1 x 10^5 PB WBC)

<table>
<thead>
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<th></th>
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<td>*</td>
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<tr>
<td>**</td>
<td>30</td>
<td>20</td>
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</table>
Fig. 3

A

M     WT M       1 M        2         3         4        5         6       7       8

1237 bp

1088 bp

M     WT

1100 bp

850 bp

CB₁

CSK

1      2

CB₂

Actin

1      2

B

C

M     WT M       1 M        2         3         4        5         6      7        8   9

1500 bp

1000 bp

500 bp

1500 bp

1000 bp

500 bp

1500 bp

1000 bp

500 bp

1100 bp

850 bp

E

F

G

H

I

J

WT Cnr1⁻/⁻ Cnr2⁻/⁻

WT Cnr1⁻/⁻ Cnr2⁻/⁻

WT Cnr1⁻/⁻ Cnr2⁻/⁻

WT Cnr1⁻/⁻ Cnr2⁻/⁻

WT Cnr1⁻/⁻ Cnr2⁻/⁻

WT Cnr1⁻/⁻ Cnr2⁻/⁻

WT Cnr1⁻/⁻ Cnr2⁻/⁻

WT FAAH⁻/⁻
### Table

<table>
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<th>CFU-GM</th>
<th>CFU-GEMM</th>
<th>BFU-E</th>
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<tr>
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<td>16±5</td>
<td>38±11</td>
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<td>CP55940</td>
<td>140±18*</td>
<td>42±15*</td>
<td>60±9*</td>
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Figure 5

A

Number of colonies (per 1 x 10^5 blood cells)

B

Number of colonies (per 1 x 10^5 blood cells)

C

Number of colonies (per 1 x 10^5 blood cells)

D

Number of colonies (per 1 x 10^5 blood cells)

E

Number of colonies (per 1 x 10^5 blood cells)
Table 1: Endocannabinoid levels determined for murine-stroma cells, either untreated or treated with LPS. Note differences in units.

** p< 0.05 as compared to BM stroma cells for AEA.

* p< 0.001 as compared to BM stroma cells for 2-AG.

<table>
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<th>2-AG (ng/10^7 cells)</th>
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<td>35.2</td>
<td>75.2</td>
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<tr>
<td>BM stroma cells/LPS</td>
<td>75.6**</td>
<td>98.8*</td>
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ENOCANNABINOIDS ARE EXPRESSED IN BONE MARROW STROMAL NICHES AND PLAY A ROLE IN INTERACTIONS OF HEMATOPOIETIC STEM AND PROGENITOR CELLS WITH THE BONE MARROW MICROENVIRONMENT

Shuxian Jiang, Radoslaw Zagozdzon, Meritxell Alberich Jorda, Kalindi Parmar, Yigong Fu, John S. Williams, Jodi Anne T. Wood, Alexandros Makriyannis, Naheed Banu, Shalom Avraham, Jerome E. Groopman and Hava Karsenty Avraham

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