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Modest and nonessential roles of the endocannabinoid system in immature hematopoiesis of mice

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Endocannabinoids are lipid mediators that signal via several seven-transmembrane domain G protein-coupled receptors. The endocannabinoid receptor CB2 is expressed on blood cells, including stem cells, and mediates the effects of cannabinoids on the immune system. The role of the endocannabinoid system in immature hematopoiesis is largely elusive. Both direct effects of endocannabinoids on stem cells and indirect effects through endocannabinoid-responsive niche cells like macrophages have been reported. Using two different CB2-deficient mouse models, we studied the role of the endocannabinoid system in immature hematopoiesis. Moreover, we utilized both models to assess the specificity of putative CB2 agonists. As heterodimerization of CB2 and CXCR4, which is highly expressed on hematopoietic stem cells, has already been described, we also assessed potential consequences of CB2 loss for CXCR4/CXCL12 signaling. Overall, no differential effects were observed with any of the compounds tested; the compounds barely induced signaling by themselves, whereas they attenuated CXCL12-induced signals in both CB2-competent and CB2-deficient cells. In vivo experiments were therefore by necessity restricted to loss-of-function studies in knockout (CB2−/−) mice: Except for mild lymphocytosis and slightly elevated circulating progenitor cells, homeostatic hematopoiesis in CB2−/− mice appears to be entirely normal. Mobilization in response to pharmacological stimuli, Plerixafor or G-CSF, was equally potent in wild-type and CB2−/− mice. CB2−/− bone marrow cells reconstituted hematopoiesis in lethally irradiated recipients with engraftment kinetics indistinguishable from those of wild-type grafts. In summary, we found the endocannabinoid system to be largely dispensable for normal murine hematopoiesis.

Hematopoiesis, that is, the continuous, lifelong process of generating short-lived mature blood cells, originates from hematopoietic stem and progenitor cells (HSPCs) [1], the proliferation of which is tightly controlled. The hematopoietic organ is physically located in the bone marrow (BM), where HSPCs interact in an intricate fashion with their environment, the niche [2]. Under the influence of the latter, HSPCs give rise to increasingly more differentiated precursors and mature blood cells, which are ultimately released into the peripheral blood [3]. The niche consists of bone-forming cells, the osteoblasts, cells of mesenchymal origin such as CXCL12-abundant reticular cells, as well as mature hematopoietic cells

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including "osteomacs" and osteoclasts. Moreover, a tight association of the niche with sympathetic nerve fibers has been demonstrated [4]. Together with extracellular matrix components, niche-derived chemokines and cytokines control HSPC survival, proliferation, and retention [5]. The interaction between the C-X-C chemokine receptor type 4 (CXCR4) expressed on the HSPC surface with its chief ligand, the chemokine CXCL12, is a key axis facilitating HSPC retention in BM. However, although the vast majority of HSPCs are retained in BM, a small number regularly circulate in the blood [6–8].

The endocannabinoid system consists of short-lived lipid mediators, the endocannabinoids, which are generated through enzymatic processing of the cell membrane phospholipid phosphatidylethanolamine, and their receptors, the G-protein-coupled receptors CB1 and CB2. It is a well-described neurotransmitter pathway [9]. However, expression of CB2 on lymphocytes and immunosuppressive effects of cannabinoid receptor agonists on lymphocytes clearly link endocannabinoid and immune systems. Anti-inflammatory effects of pharmacological endocannabinoid receptor agonists have been exploited clinically [10,11]. Integration of endocannabinoid signals by cells is complex and likely cell specific; of possible relevance within the hematopoietic system, CB2 was described to heterodimerize with CXCR4 [12] and thereby modulate CXCR4 agonist-induced signaling. Definitive evidence of direct or indirect modulation of HSPC function by endocannabinoids has thus far not been provided. Importantly, as previously shown and here confirmed, CB2 is expressed on immature hematopoietic cells in addition to mature blood cells [13].

To study endocannabinoid receptor function, both additive (pharmacological agonists) and subtractive (pharmacological antagonists) approaches have been employed, with characteristic limitations. AM1241, JWH133, and CP55940 have been utilized as selective agonists, and AM630 as a selective antagonist [14]. However, apparent cell- and species-specific differences in signaling readout after exposure to these small-molecule compounds complicated interpretation of results [15]. Availability of receptor-deficient mice gave us the opportunity to study organismic effects of lack of CB2 signaling on immature hematopoiesis for the first time. Moreover, the specificity of CB2-targeting compounds, along with their effects on the hematopoietic system, could be assessed with CB2−/− cells as the perfect negative controls. As we describe here, the four compounds were associated with virtually identical intracellular signaling events in CB2-competent and −deficient cells and are thus not suitable for exploration of CB2-mediated effects on hematopoietic cells in vitro or in vivo. CB2-deficient mice exhibit very subtle perturbations of the hematopoietic system: a mild lymphocytosis was observed in line with previous reports. Furthermore, increased numbers of homeostatically circulating HSPCs were detected. Both observations were corroborated in two different CB2-deficient mouse strains.

Methods

Mice

For all experiments, young adult mice (8–12 weeks, either sex) were used. B6.129P2-Cnr2$^{tm1Dgen/J}$ (CB2−/−) mice, described previously [16], were purchased from Jackson Laboratory (JAX stock No. 005786). Buckley CB2−/− mice [17] were a generous gift from Anne Zimmer (Haus für Experimentelle Therapie, Bonn, Germany). From CB2+/− heterozygote breedings, CB2-competent wild-type (WT) littermates of the corresponding strains were used as controls. For transplantation experiments, B6.SJL-*Ptprc*^{a}*Pep3*^{b}/BoyJ (JAX stock No. 002014; CD45.1) were used as recipients. Experiments were performed in accordance with the agreement on national animal protection and were approved by the municipal government (F27/24 and F27/1010, Darmstadt, Germany).

Hematopoietic cells

Peripheral blood was drawn from the facial vein into EDTA tubes using a 23G needle. Total cell counts were analyzed with Hemavet 950SF+ (Drew Scientific, Dallas, TX). BM cells were harvested by flushing femurs and/or tibias using phosphate-buffered saline + 0.5% bovine serum albumin. Splenic cells were obtained by blunt extrusion of the capsule.

Enumeration of hematopoietic cells

Hematopoietic cells in blood, BM, and spleen were enumerated using multiparametric flow cytometry for informative markers for mature (CD45, CD3, B220, Ter-119, CD11b, Gr-1) and immature (lineage, c-kit, sca-1, CD48, CD150) subsets, as were clonogenic assays. Antibodies used are listed in Supplementary Table E1 (online only, available at www.exphem.org); acquisition was done with FACS LSR Fortessa (Becton-Dickinson, Heidelberg, Germany) and analysis with FACS Diva 7 (BD Biosciences). Clonogenic assays were performed with cytokine-replete semisolid culture media (Methocult GF M3434, Stem Cell Technologies). A defined aliquot of white blood cells (WBCs) (50,000 BM WBCs, 200,000 spleen WBCs, and 70 μL of peripheral blood after hypotonic red blood cell [RBC] lysis) from the different tissues was plated in duplicate. Colony growth, including differentiation of colony-forming units granulocytes–macrophages (CFU-GM), blast-forming units erythroid (BFU-E), and colony-forming units granulocytes, erythrocytes, monocytes/macrophages, megakaryocytes (CFU-GEMM), was scored after 7 days using an inverted 2.5 × microscope. Cell cycle analysis was performed using Ki67 as a marker for cell proliferation. 7-Aminoactinomycin D (7-AAD) was added to distinguish between G1 and G2/S/M phases.

Isolation of mRNA and RT-PCR

Whole BM cell or immunomagnetically enriched BM c-kit+ cell mRNA was isolated using the RNeasy kit (Qiagen, Vanlo, Netherlands) according to the manufacturer's instructions. For CB2 detection on HSPC subtypes, LSK, MPP,

CMP, and HSC progenitor cells were sorted via fluorescence-activated cell sorting (FACS) as described [18], and mRNA was isolated. One microgram of RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Irvine, CA) and diluted 1:10 for the use in real-time reverse transcriptase polymerase chain reaction (RT-PCR). We used the primers for murine transcripts (CB2-fw1—TCA TTG CCA TCC TCT TTT CC, CB2-rev1—GAA CCA GCA TAT GAG CAG CA, CB2-fw2—GGA GTT CAA CCC CAT GAA GGA GTA C, CB2-rev2—GAC TAG AGC TTT GTA GGT AGG CGG G) to generate a PCR product 188 bp (1) or 385 bp (2) in length. No-reverse transcriptase samples were used as controls to exclude contamination with gDNA. Sequencing of the resulting fragments was done with Microsynth (Balgach, Switzerland).

CB2 antibody staining

BM and spleen cells were harvested as described. One million unmanipulated cells were prepared for FACS analysis and stained with two different CB2 antibodies (Davis Biotechnology, Regensburg, Germany, and Cayman Chemical, Ann Arbor, MI). Twenty micrograms of antibody was conjugated using the Alexa Fluor 488 antibody labeling kit (Thermo Fisher, Darmstadt, Germany). CB2−/− cells were used as a negative control. Data acquisition and analysis were performed as described earlier.

Signaling

Lysis-resistant BM leukocytes were starved for 2 hours at 37°C in RMPI + 0.5 % fetal calf serum. Stimulation was started by adding 10 μmol/L CXCL12, 10 μmol/L CB2 agonist (2.5 μmol/L for CP55940), or both. AM1241, CP55940, and JWH133 as agonists, as well as AM630 as an antagonist, were assessed. Phosphorylation of ERK1/2, MEK, and AKT was analyzed via FACS staining.

Mobilization

Progenitor cells were mobilized into peripheral blood using either AMD3100 (5 mg/kg once, intraperitoneally; Sigma-Aldrich, Darmstadt, Germany) or rhG-CSF (9 doses of 100 μg/kg every 12 hours, intraperitoneally; Hexal, Holzkirchen, Germany). Blood was drawn 1, 2, and 4 hours after administration of AMD3100 or 1 hour after the last administration of G-CSF, followed by CFU-C enumeration of colony-forming units cells (CFU-C) in blood as well as, where indicated, in BM and spleen. WBCs were counted at every time point. LSK, LSK-SLAM, and cell cycle analysis was performed in BM and spleen cell suspensions using flow cytometry.

Transplantation

Donor mice were sacrificed by cervical dislocation; femurs and tibias were harvested. BM cells were obtained as described above and resuspended in 0.9% NaCl at a concentration of 1 mol/mL. Recipient mice were irradiated with 9.5 Gy using a cesium source with a dose rate of 0.75 Gy/min (Biobeam 2000, Gamma-Service Medical, Leipzig, Germany). Mice then received intravenous transplants of unmanipulated (not HSPC-enriched) BM cell suspensions of wild-type or CB2−/−mice (200,000 cells/mouse) into the lateral tail vein. Engraftment kinetics were analyzed by drawing blood twice weekly. Mice were sacrificed for final analysis 14 weeks after transplantation.

Statistics

Descriptive statistics and Student t tests were calculated using Excel (Microsoft, Redmond, WA); two-way analysis of variance (ANOVA) with the Bonferroni posttest was calculated using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA); for non-normally distributed data, the nonparametric Mann−Whitney U test was calculated using SPSS (IBM, Armonk, NY). Unless stated differently, results are presented as the mean ± SEM nonsignificant [n.s.] $p \geq 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Results

Expression of endocannabinoid receptors on HSPCs

Expression of CB2 mRNA in BM and spleen cells, including the immature hematopoietic fraction, had been described by Lattin et al. [19], and was confirmed here for murine c-kit+ BM cells (Fig. 1A), whole BM cells (Fig. 1B), and HSPC subtypes (Fig. 1C). However, direct demonstration of CB2 surface expression turned out to be challenging. Thus, the polyclonal antibody from Davis Biotechnology (Regensburg, Germany), designed to be CB2 specific, stained CB2-competent and CB2-deficient BM and spleen cells with equal efficiency (Fig. 1D). Our attempt to generate a novel antibody against one of the CB2 extracellular loops was unsuccessful. For lack of suitable reagents, CB2 presence versus deficiency was therefore inferred from CB2 gene expression as well as from sequencing of the CB2 locus. The inserted stop codons were confirmed, and in silico translation predicted expression of a truncated receptor in the Buckley strain (Supplementary Figure E1, online only, available at www.exphem.org). Even if mRNA transcripts could partially be detected by Zhang et al. [16], complete loss of CB2 protein in the Deltagen strain is assumed (Supplementary Figure E2, online only, available at www.exphem.org). We excluded a possible compensatory upregulation of alternative cannabinoid receptor CB1 or GPR55 (Fig. 1A, C) in hematopoietic cells. Both CB2−/− strains are thus completely endocannabinoid receptor deficient in their hematopoietic system.

The inverse agonist AM1241 fails to modulate CB2-specific signals in hematopoietic cells.

Availability of CB2-deficient BM leukocytes allowed us to investigate potential changes in intracellular signaling responses. Presence of CB2 mRNA in a variety of hematopoietic lineages in the BM has been reported [19,20] and confirmed here by us (Fig. 1). After short serum starvation, lysis-resistant whole BM cells were stimulated using CXCL12. We assessed stimulation kinetics, expecting similar responsiveness irrespective of CB2 expression. Indeed peak activation of ERK, MEK, and AKT occurred 60 s after stimulation and returned to basal levels by 10 min

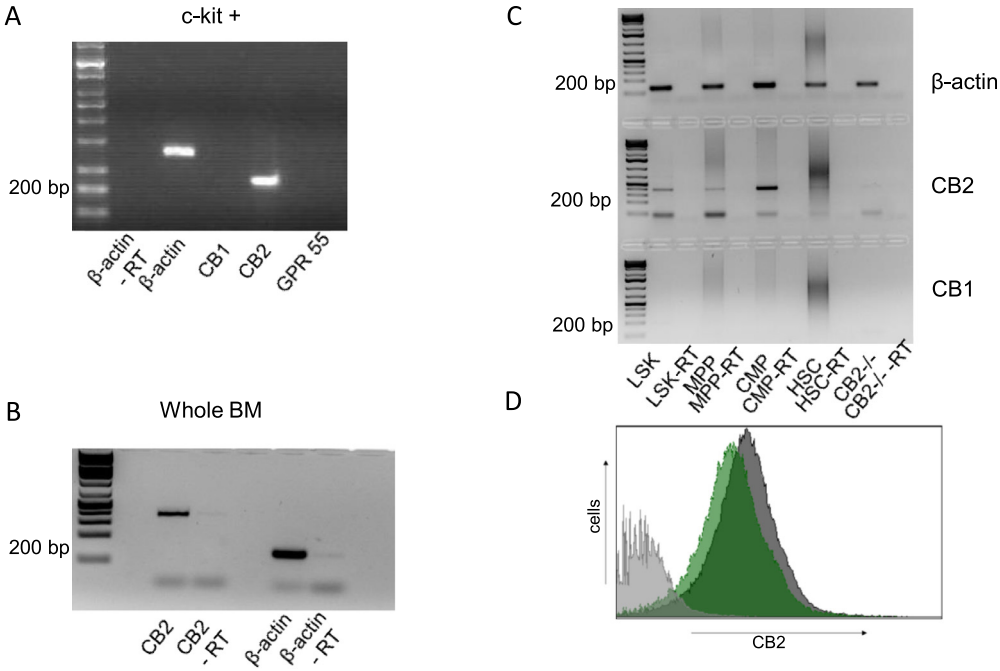

Figure 1. CB2 gene expression in BM cells. BM cells were isolated from C57Bl/6 WT mice, and mRNA was extracted and reverse transcribed into cDNA. RT-PCR verified CB2 expression, but not CB1 and GPR55 expression, in c-kit enriched (A) and CB2 expression in whole BM (B) WT cells. The negative control was performed simultaneously using a no-RT control. LSK, MPP, CMP, and HSC progenitor cells were sorted via FACS. After mRNA isolation and cDNA transcription, PCR for CB1, CB2, and β-actin transcripts was performed (C). CB2 was expressed only in WT cells irrespective of maturational stage; CB1 was expressed in cells of neither genotype. Representative flow cytometry histogram of a staining of WT (*dark gray*) and CB2−/−(*green*) BM cells with anti-CB2-antibody (Davis Biotechnology) indicating lack of CB2 specificity. Isotype staining was used as negative control (*light gray*) (D).

(Fig. 2A). Signals were very similar in magnitude and duration in CB2-deficient and CB2-competent cells. Cooperative signaling between chemokines, specifically CXCL12, and endocannabinoid receptor ligands has been reported [12]. We therefore further analyzed signaling in response to co-stimulation with the putative CB2 agonist AM1241 and CXCL12 (Fig. 2B). AM1241 partly suppressed CXCL12-induced signaling irrespective of the genetic background of the cells. On its own, AM124 modestly activated MAPK signaling 1 min after stimulation in both CB2-competent and knockout BM cells (data not shown). Similar observations were made with the agonists JWH133 and CP55940 (Supplementary Figure E3), as well as the antagonist AM630. From these results we conclude that the compounds tested here cannot be used to decipher roles of CB2 in vitro or in vivo, restricting our further experiments to comparative studies, that is, in CB2-deficient mice and littermates.

Steady-state hematopoiesis of CB2−/− mice is largely unperturbed

Mature and immature blood cells were enumerated in BM, spleen, and peripheral blood of CB2−/− or CB2-competent wild-type littermates. Immature cells were evaluated phenotypically (LSK, LSK-SLAM) as well as functionally (CFU-C as well as transplantation−reconstitution assays, see below). The only notable difference in the immature hematopoietic compartment between CB2−/− and WT mice was a 50% elevation of steady-state circulating CFU-C numbers in CB2−/−compared with WT mice (Fig. 3A). The relative distribution of different colony types was the same in both genotypes (data not shown). Mature blood cell counts were normal in BM and spleen (data not shown), whereas in blood, a 30% increase in leukocyte counts, entirely attributable to lymphocytes, was detected in CB2−/− mice (Fig. 3B), in line with previous observations [21,22]. Similar frequencies and total numbers of immature cells were detected in BM and spleen (Fig. 3C). Cell cycle analyses of the variably immature populations in spleen and marrow revealed no difference between the strains (Fig. 3D).

CB2 deficiency does not affect mobilization of HSPCs

As a mild hematopoietic stress model, we investigated mobilization kinetics in CB2−/− versus WT mice. Mice received intraperitoneal injections of the small-molecule CXCR4 antagonist AMD3100, a low-potency but rapid inducer of leukocyte (including HSPC) mobilization. In both, CB2−/− and WT mice, circulating WBCs (Fig. 4A) and CFU-C (Fig. 4B) increased to expected levels in response to AMD3100 with no difference between genotypes. As next, mice received a 5-day course of twice-daily

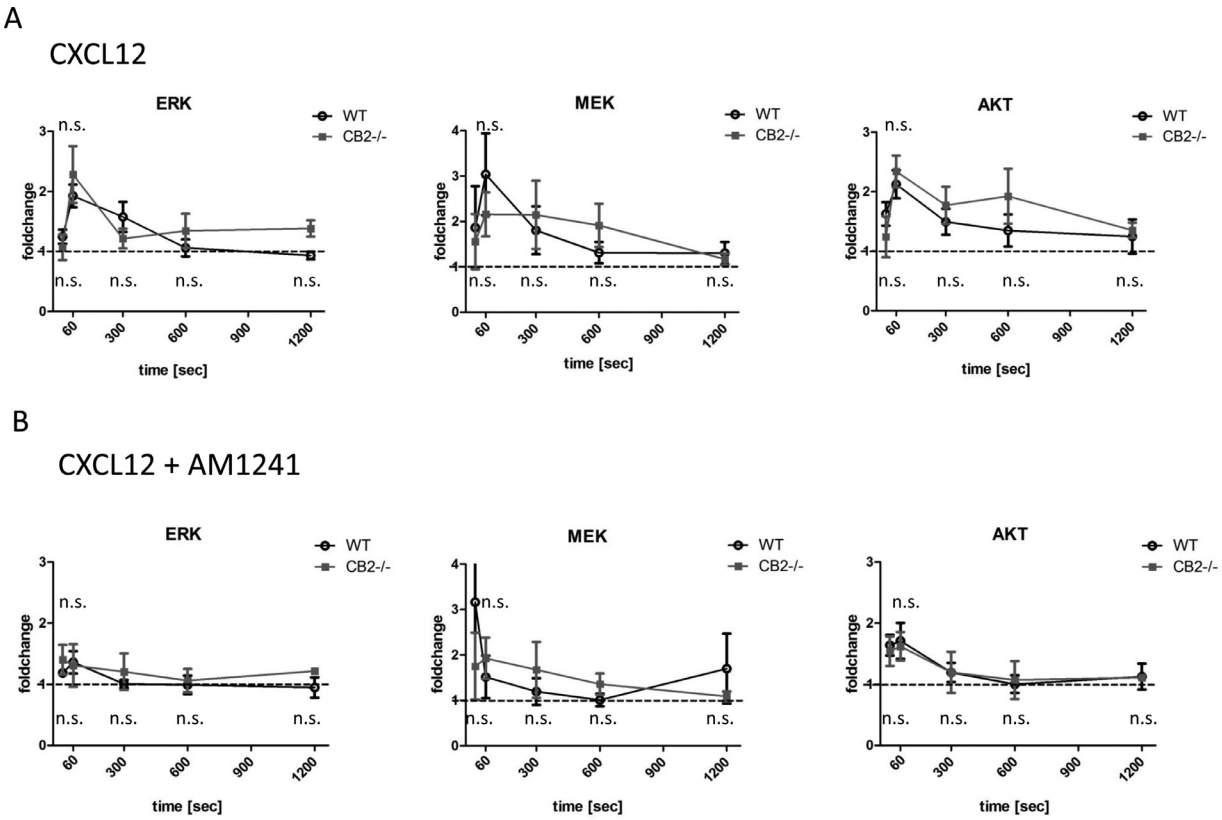

Figure 2. MAPK and AKT signaling after AM1241 stimulation. BM cells from CB2−/− and WT mice were isolated and serum starved. Stimulation of 100,000 cells of either group was performed using 10 μmol/L CXCL12 alone (A) or in combination with a 10 μmol/L concentration of the putative CB2 agonist AM1241 (B). Phosphorylation of ERK, MEK, and AKT kinases was assessed via flow cytometry staining. Genotype-specific responses were not observed, indicating lack of CB2 specificity of the compounds. Measurements were performed in duplicate in at least five independent experiments; $n \geq 5$ per time point.

G-CSF injections. One hour after the last injection, mice were sacrificed to study mature and immature hematopoietic cells in the hematopoietic compartments. In blood, WBCs and CFU-C were increased to the same level in CB2−/− versus WT mice. Progenitor cells in BM and spleen were enumerated and analyzed functionally (CFU-C) (Fig. 4C) as well as immunophenotypically (Fig. 4D). Remarkably, the characteristic depletion (approx 50%) of CFU-C content in the BM after a 5-day course of G-CSF was observed in WT but not CB2−/− mice. Although our data suggest that this effect is not due to differential cell cycle activity in immature cells (Fig. 4E), our assay may lack sensitivity to detect subtle changes.

CB2 deficiency-associated lymphocytosis is hematopoietic-intrinsic

The ability to reconstitute long-term multilineage hematopoiesis is a hallmark of the hematopoietic stem cell. To assess this property, we transplanted the low dose of 200,000 CB2−/− or WT BM cells into lethally irradiated wild-type recipients. Fourteen days after transplantation, we started monitoring engraftment kinetics. Serial differential blood count analysis, twice weekly for 8 weeks, revealed no differences between CB2−/− and WT mice except for lymphocyte counts (Fig. 5A). Fourteen weeks after transplantation, when hematopoiesis is considered to be fully established [23], significantly increased peripheral blood WBC and lymphocyte counts were detected in WT recipients repopulated with CB2−/− HSCs, similar to homeostatic CB2−/− mice, thus indicating the hematopoietic-intrinsic nature of the observed lymphocytosis. Furthermore, analysis of the immature cell compartments in BM and spleen as described before revealed no differences between both recipient groups (Fig. 5B). Neither LSK nor LSK-SLAM numbers, nor CFU-C counts (Fig. 5C), differed in WT mice reconstituted with CB2−/− versus WT cells. These results confirm that CB2-deficient HSPCs exhibit no major functional deficiencies.

Discussion

Within the hematopoietic system, expression of CB2 receptor was first described in splenic cells [24]. Shortly thereafter, CB2 was found to be expressed on immune cells in blood [20] and also shown to modulate immunologic responses in mice [9,25]. Reports of expression of CB2 receptors on HSPCs and a potential

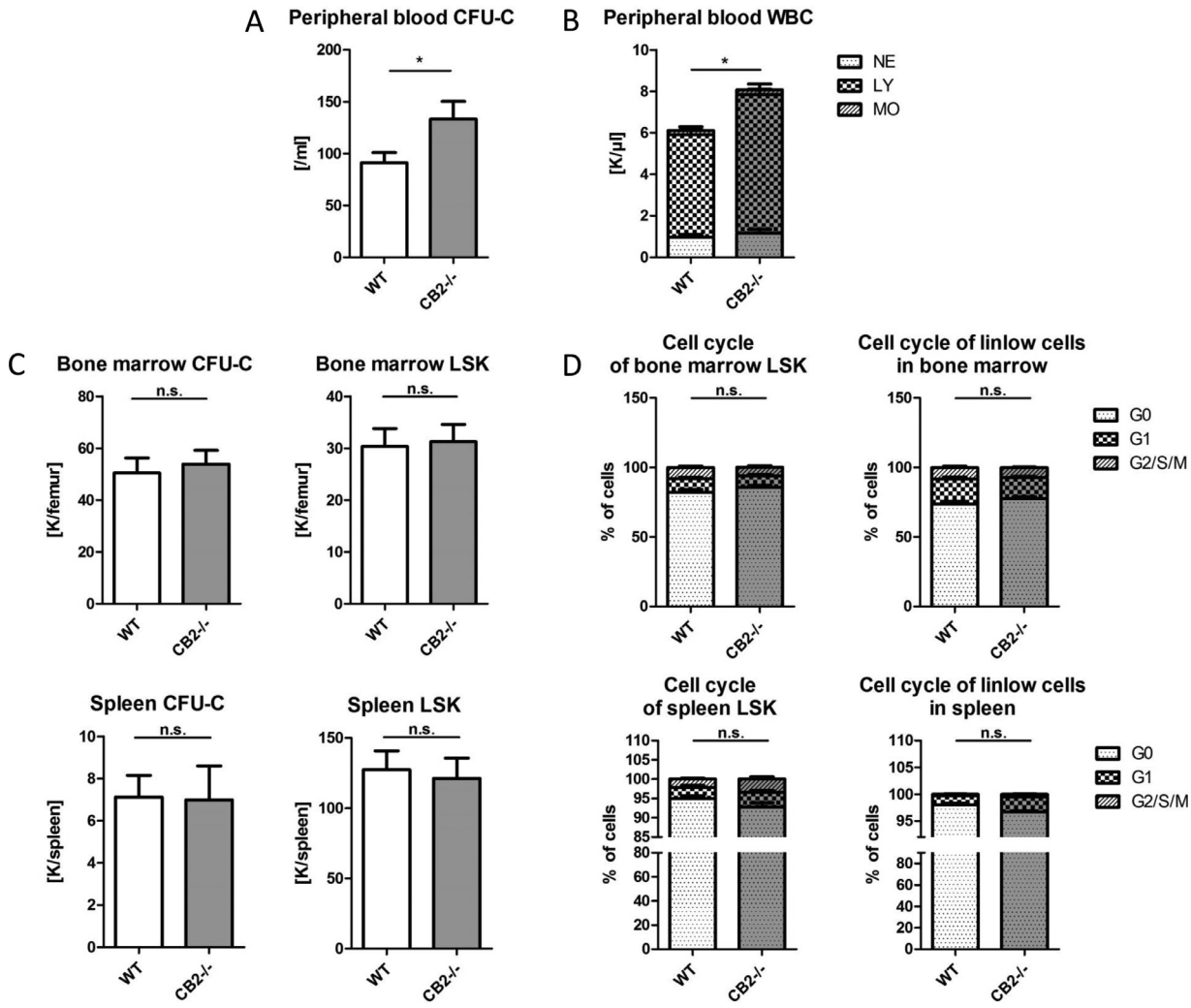

Figure 3. Steady-state analysis of CB2−/− and WT hematopoiesis. **(A)** CFU-C content in peripheral blood. **(B)** Differential blood counts in peripheral blood. **(C)** Hematopoietic progenitor cells in BM (top row) and spleen (bottom row), enumerated functionally as CFU-C (left) or phenotypically as LSK cells (right). **(D)** Cell cycle activity of LSK (left) and lineage-low (right) in bone marrow (top row) or spleen (bottom row) cells. No significant changes were observed between genotypes in BM and spleen. Except for mild lymphocytosis and elevated circulating CFU-C in CB2−/− mice, blood counts were normal throughout. $n \geq 9$ mice per group in two independent experiments.

dimerization with CXCR4 led us to the assumption that cannabinoid receptors might not only affect mature blood cells, but also be relevant for immature hematopoiesis [12]. Under steady-state conditions the individual HSPC needs to undergo only infrequent self-renewing divisions in contrast to stress hematopoiesis, where rapid HSPC expansion and generation and egress of mature blood cells are necessary. More than two decades ago, Valk et al. [26] proposed an influence of CB2 on HSPC proliferation, but the influence of CB2 on hematopoiesis has never been directly addressed. In this study, we aimed to clarify its role in steady-state and stress hematopoiesis using CB2−/− mice. As no reliably staining/performing CB2 antibodies were available, we had to rely on the knockout mouse models. Given the fact that lack of CB2 could be verified by PCR, these mice represent a suitable model for studying the role of CB2 in hematopoiesis.

In the course of this study, we first tried to distinguish CB2 knockout and CB2-competent cells using various antibodies: All antibodies tested also stained cells from CB2−/− mice, which, when tested using PCR, did not express CB2 receptor mRNA. In line with our observations, Cécyre et al. [27] had previously reported the lack of specificity of several commercial CB2 antibodies. We were able to confirm expression of CB2 in HSPCs in all studied HSPC subpopulations (LSK, MPP, CMP, and HSC) by PCR, yet could not specifically assess its function using small-molecule compounds in vitro. AM1241 and JWH133 are

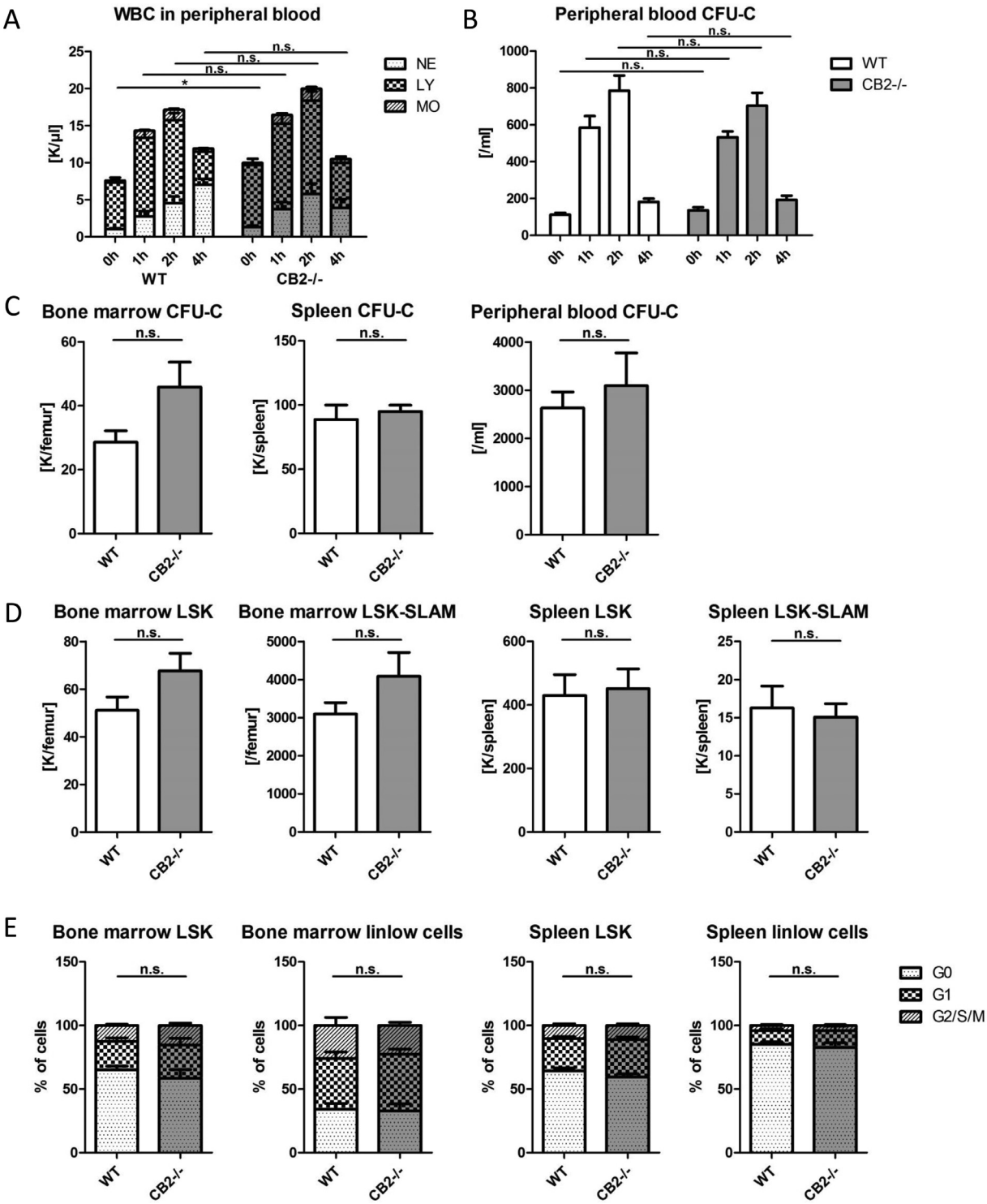

Figure 4. Blood cell mobilization in response to Plerixafor and G-CSF. **(A, B)** A single Plerixafor injection was administered intraperitoneally, and peripheral blood WBCs (A) and CFU-C (B) were enumerated at the indicated times. **(C–E)** Nine doses every 12 h of G-CSF were administered. CFU-C (C) and LSK (D) cells in bone marrow and spleen were enumerated. Cell cycle status of immature cells in BM and spleen was determined (E). Mobilization was normal except for lack of the characteristic post-mobilization depletion of HSPCs in CB2−/− mice. $n \geq 9$ mice per group in two independent experiments with at least 14 days of recovery between AMD3100 and G-CSF administration.

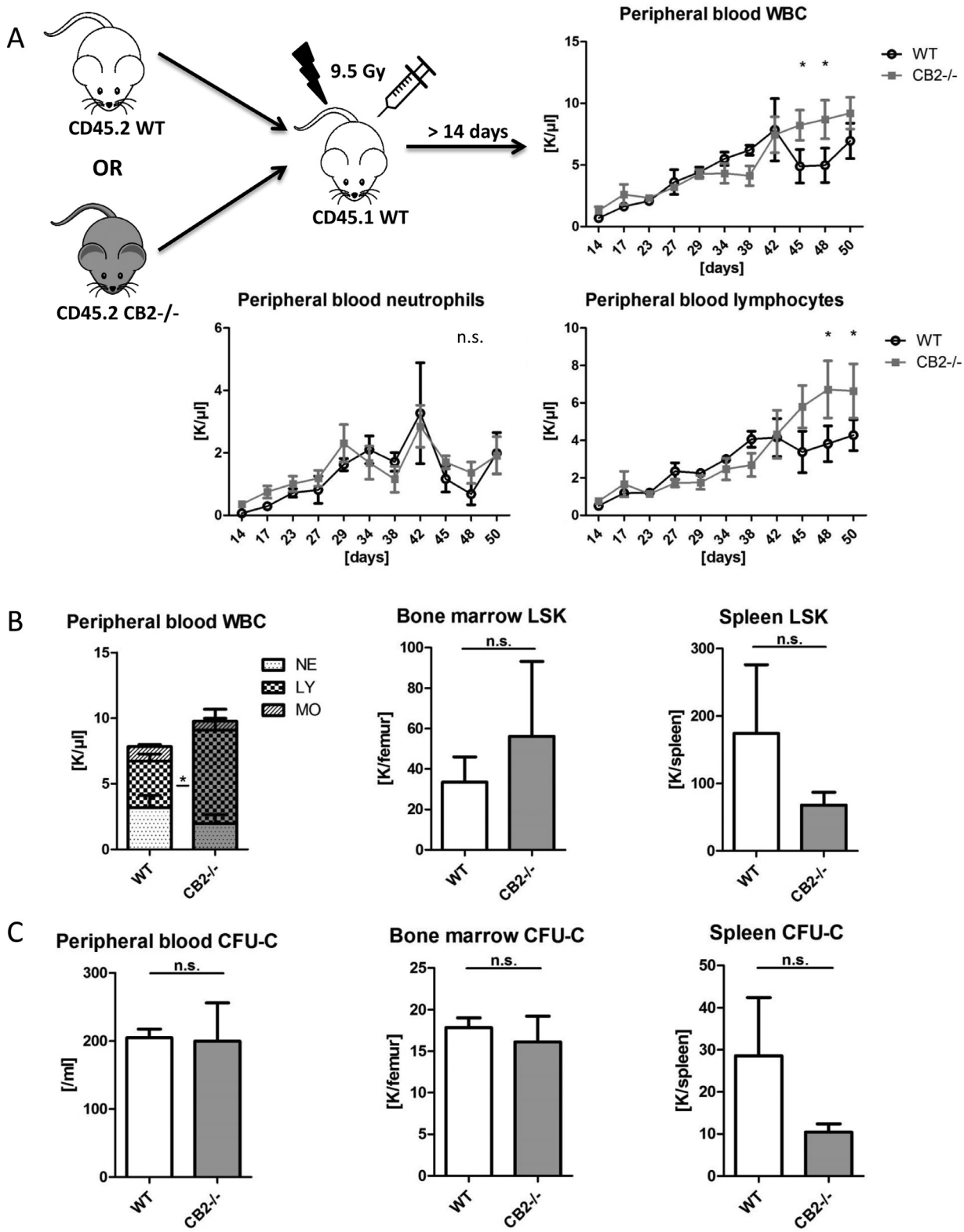

Figure 5. Repopulation of WT hosts with WT or CB2−/− hematopoietic cells. Ly5.1 mice received transplants of 0.2 mol/L CB2−/− or WT BM cells. **(A)** Hematopoietic engraftment, shown as total WBCs, neutrophils, and lymphocytes, was tracked from days 14 to 52 (A). Final analysis was performed after 14 weeks: WBCs in PB as well as LSK cells in BM and spleen **(B)** were enumerated. CFU-C in PB, BM and spleen were enumerated **(C)**. CB2−/− cells provided normal engraftment; recipients reproduced the CB2−/− phenotype. $n \geq 3$ mice per group in a single experiment.

commonly used as specific CB2 agonists, whereas AM630 is considered a specific antagonist. Yet, none of the compounds has been tested in CB2−/− versus WT hematopoietic cells. Ibrahim et al. [28] found that pretreatment with AM630 blocked the binding of AM1241 by CB2 in rat brain cells, while the exposure to a presumably specific CB1 agonist did not. As a valid negative control was not included, off-target effects of AM630 cannot be excluded, and CB2 specificity was not definitively established in that study. A functional test for AM1241 was performed by Coke et al. [12]. The authors reported reduced migration of breast cells toward a CXCL12 gradient when adding AM1241 to wild-type cells, an observation that they inferred to be CB2 mediated. Here, CB2−/− control cells were also not included. Given our results regarding the lack of specificity of CB2 agonists we can conclude that the reported effects are likely not or not solely CB2 mediated. We observed activation of ERK signaling after stimulation of BM cells with CXCL12 along with a reduction of ERK phosphorylation when the chemokine was combined with AM1241, both consistent with findings by Coke et al. [12]. However, this reduction of CXCL12-induced phosphorylation of MAP-kinases did not appear to be CB2 mediated, as it was observed in both CB2−/− and CB2-competent cells. Similarly to AM1241, the agonist CP44930 also did not show specificity for CB2. In both CB2−/− and WT cells, it induced modest prolongation of CXCL12 signaling in vitro at a concentration of 2.5 μmol/L. Higher doses were cytotoxic for both WT and CB2-deficient cells. In vivo, no pharmacologic effect on WBC counts or circulating HSPCs was discernible at doses between 0.1 and 1 mg/kg; a dose of 10 mg/kg, as previously used [29], was immediately lethal for both CB2−/− and WT mice. Absence of expression of the alternative endocannabinoid receptors CB1 and GPR55 on hematopoietic cells exclude the hypothetical possibility of compensation for CB2 deletion. Thus, the dampening effect of the small-molecule compounds tested by us either may be mediated by a different receptor or represent a ubiquitous, unspecific, non-receptor-mediated effect. Alternatively, as the knockout could only be verified by RT-PCR and based on in silico protein structure/length predictions, it is possible that a truncated protein could be present on the surface membrane, which in fact was proposed for the Buckley mouse model [16]. However, in the second, the Deltagen CB2−/− mouse model tested here, the deleted region is located close to the C-terminus and predicted to result in complete loss of CB2 [30]. Compensatory effects by alternative cannabinoid receptors could be excluded because we are showing lack of expression of CB1 and GRP55 by PCR, but the possibility of non-CB-receptor-mediated compensation cannot be ruled out. In summary, our data indicate that the supposedly CB2-targeting agonists tested here affect CB2-expressing and -deleted hematopoietic cells alike, that is, do not possess CB2 specificity in the context of (immature) hematopoietic cells.

Jiang et al. [31] proposed an effect of the cannabinoid system on regulation of the HSPC compartment. Similarly, given CB2 expression in HSPCs, we expected to find a role for CB2 receptor signaling in immature hematopoiesis. However, enumeration and characterization of progenitor cells in BM, spleen, and peripheral blood revealed only minor differences between CB2-deficient and healthy WT mice. Consistent with published data, we observed a mild lymphocytosis in both CB2−/− strains. With respect to the modest mobilization phenotype observed in homeostatic CB2−/− mice, we propose that this effect is likely an indication of endocannabinoids favoring HSPC retention. Although cooperative signaling of CXCL12 and cannabinoid agonists has been reported, we lacked the tools to reproduce this, and hence, the co-opted signaling pathway may be distinct from CXCL12/CXCR4. An alternative hypothesis would be that the HSPCs are being passively co-mobilized with the excessive lymphocyte numbers trafficking out of the bone marrow, as all reported HSPC mobilization is accompanied by a leukocytosis, often very pronounced. Of possible relevance, Pereira et al. [32] found that CB2 mediates retention of immature B cells in BM sinusoids. Inversely, then, lack of cannabinoid receptor would lead to higher B-cell counts in peripheral blood, consistent with our data for HSPCs. Schwarz et al. [22] and Schatz et al. [33] could show in the early 1990s that CB2 agonists inhibit lymphocyte proliferation in vitro, potentially explaining elevated lymphocyte levels found in CB2 knockout mice in vivo by us and others [34,35]. Our studies extend the previous characterization of the phenotype of CB2 knockout mice with the observation of increased numbers of baseline circulating immature hematopoietic cells (CFU-C). Endocannabinoids thus contribute, directly or indirectly, to HSPC retention in BM.

The only striking observation in the CB2−/− mice is in bone marrow after G-CSF treatment: Mobilization experiments revealed a lesser depletion of HSPCs in the BM of CB2−/− mice after G-CSF administration compared with WT mice (Fig. 4C). Analysis of the cell cycle on day 5 did not reveal significant differences in immature (Fig. 4E) or mature BM cell proliferation (data not shown). Possible explanations for this observation are not straightforward. Mathematically, the reduction of HSPC activity in bone marrow after G-CSF is due mostly to HSPC differentiation to precursor stage and not to egress, as only 1%−2 % of the total BM HSPC pool is mobilized into blood [3]. The possibility, therefore, that endocannabinoids elaborated

under G-CSF exert differentiation-promoting effects in CB2-competent mice, something that is not seen in the CB2−/− mice, may be entertained but cannot be tested for lack of suitable compounds. In the course of transplantation experiments no functional differences between CB2-deficient and WT BM cells were found. The mild lymphocytosis was reestablished in WT hosts reconstituted with CB2−/− BM similarly to complete CB2−/− mice, pointing toward a strictly cell-intrinsic effect. While this article was undergoing revision, Khuja et al. [36] independently came to the same results and conclusions. Importantly, the kinetics of engraftment of CB2−/− cells were indistinguishable from those of WT donor cells. Competitive transplantation experiments can decipher minor defects of stem cell function and thus would have directly addressed the competitiveness of CB2−/− HSPCs with respect to BM niche invasion/engraftment. For lack of any suggestive phenotype of conventional transplants whatsoever, such studies were not performed, possibly limiting the definitiveness of the presented work.

We here show that CB2 receptor is largely dispensable for immature hematopoiesis in mice. In two different CB2 knockout models we found no impairment of HSPC function, neither under homeostatic conditions nor under stress conditions. CB2 receptor-deficient cells repopulate lethally irradiated recipients and establish functional hematopoiesis. All CB2-targeting small-molecule compounds tested here lack receptor specificity for hematopoietic cells and therefore need to be evaluated carefully for every application, using CB2-deficient cells as negative controls.

Conflict of interest disclosure

None of the authors declare potentially competing interests with respect to this article.

Author contributions

FH, SYL, FC, SO, KD, DC, GS, and BT performed experiments. ED, EW, and DK performed experiments and analyzed data. ED and HB planned experiments, co-wrote the article, and share overall responsibility for the studies. All authors read and approved the final version of the article.

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