

Anandamide, a Natural Ligand for the Peripheral Cannabinoid Receptor Is a Novel Synergistic Growth Factor for Hematopoietic Cells

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We recently demonstrated that the gene encoding the peripheral cannabinoid receptor (*Cb2*) may be a proto-oncogene involved in murine myeloid leukemias. We show here that *Cb2* may have a role in hematopoietic development. RNase protection analysis showed that *Cb2* is normally expressed in spleen and thymus. *Cb2* mRNA is also expressed in 45 of 51 cell lines of distinct hematopoietic lineages, ie, myeloid, macrophage, mast, B-lymphoid, T-lymphoid, and erythroid cells. The effect of the fatty acid anandamide, an endogenous ligand for cannabinoid receptors, on primary murine marrow cells and hematopoietic growth factor (HGF)-dependent cell lines was then investigated. In vitro colony cultures of normal mouse bone marrow cells showed anandamide to potentiate interleukin-3 (IL-3)-induced colony growth markedly. Whereas HGFs alone stimulate proliferation of the various cell lines in serum-free culture only weakly, anandamide enhances the proliferative response of the cell lines to HGFs profoundly. This was apparent for

responses induced by IL-3, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, and erythropoietin. Anandamide was already effective at concentrations as low as 0.1 to 0.3 $\mu\text{mol/L}$ and plateau effects were reached at 0.3 to 3 $\mu\text{mol/L}$. The addition of anandamide as single growth factor had no effect. The costimulatory effect of anandamide was not evident when cells were cultured with fetal calf serum (FCS), suggesting that FCS contains anandamide or another ligand capable of activating the peripheral cannabinoid receptor. Other cannabinoid ligands did not enhance the proliferative responsiveness of hematopoietic cells to HGFs. Transfection experiments of *Cb2* in myeloid 32D cells showed that anandamide specifically activates proliferation through activation of the peripheral cannabinoid receptor. Anandamide appears to be a novel and synergistic growth stimulator for hematopoietic cells.

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PROLIFERATION and differentiation of hematopoietic precursor cells is regulated by hematopoietic growth factors (HGFs).^{1,2} These cytokines bind and activate receptors that belong to the hematopoietin receptor superfamily.³ Receptors of this superfamily are single transmembrane proteins that, upon binding to their specific ligands, form heterodimeric or homodimeric complexes.³ Ligands that bind hematopoietin receptors are small glycoproteins, eg, interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), erythropoietin (Epo), or macrophage colony-stimulating factor (M-CSF). Another family of surface membrane receptors is the G-protein-coupled receptors (GPCRs).^{4,5} This superfamily of receptor molecules is characterized by seven hydrophobic stretches of 20 to 25 amino acids that form seven transmembrane-helices connected by alternating extracellular and intracellular loops. Up to now, more than 300 GPCRs have been identified.^{4,5} In contrast to receptors of the hematopoietin family, GPCRs are in most cases not activated by glycoproteins. Ligands of heptahelical GPCRs include amines, amino acids, peptides or proteins, nucleosides or nucleotides, fatty acid derivatives, and phospholipid

derivates.^{4,5} Little is known about the role of GPCRs in hematopoietic growth and development. We recently identified the peripheral cannabinoid receptor (*Cb2*), which encodes a GPCR, in a common ecotropic virus integration site (*Evi1*).^{6,7} In vitro transfection studies in 32D cells supported the hypothesis that *Cb2* is a proto-oncogene that is involved in leukemogenesis.⁶ In the present study, we investigated by RNase protection analysis the expression pattern of the *Cb2* gene in comparison to the gene that encodes the central cannabinoid receptor (*Cb1*)^{8,9} in different murine tissues and a panel of murine hematopoietic cell lines. We show that *Cb2* encodes a hematopoietic receptor that is expressed in myeloid, macrophage, erythroid, lymphoid, and mast cells. In contrast, *Cb1* is mainly expressed in brain and in testis.^{8,9} The effects of the fatty acid N-arachidonyl ethanolamide or anandamide, an endogenous ligand for cannabinoid receptors, on the proliferative abilities of HGF-dependent hematopoietic cell lines were investigated. The results of this study demonstrate that anandamide is a ligand that stimulates proliferation of hematopoietic cell lines in synergy with IL-3, Epo, GM-CSF, and G-CSF under serum-free conditions.

MATERIALS AND METHODS

Mouse hematopoietic cell lines. A list of murine hematopoietic cell lines used in this study is presented in Table 1. Macrophage cell lines were cultured in Dulbecco's modified Eagle's medium (GIBCO, Ghent, Belgium) plus supplements (100 IU/mL penicillin, 100 ng/mL streptomycin, and 10% fetal calf serum [FCS]). The other cell lines were cultured in RPMI-1640 medium plus supplements. Myeloid cell lines were cultured with 10 ng murine IL-3 and CTLL cells were cultured with 10 IU/mL IL-2 (Cetus, Emmerlyville, CA). The pro-B-cell line BAF3 and myeloid 32D cell line both transfected with the human G-CSF receptor gene (BAF-G and 32D [G-CSF-R], respectively) were donated by Dr I.P. Touw (Erasmus University Rotterdam, Rotterdam, The Netherlands).³⁶

RNA isolation. Mouse tissues were homogenized using an Ultratarrax T25 shearing device (IKA Labortechnik, Heiterheim, Germany). Total RNA was extracted from murine hematopoietic cell lines with guanidinium thiocyanate followed by phenol extraction.³⁷

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Table 1. *Cb2* and *Cb1* mRNA Expression in Hematopoietic Cell Lines

(A) <i>Cb2</i> ⁺ / <i>Cb1</i> ⁺			
Myeloid	Ref.	Macrophage	Ref.
32D	10	RAW 264.7	22
32Dcl3	11	RAW 309.	22
DA-1	12	WR19M.1	22
DA-3	12	Pu5-1.8	23
DA-13	12	J774	24
DA-24	12	WEHI 3	25
DA-28	12		
DA-29	12	Erythroid	
DA-31	12	RED5	26
DA-33	12	RED8	26
NFS-22	13	32D-Epo	11
NFS-36	13		
NFS-56	13	B-lymphoid	
NFS-58	13	DA-25	12
NFS-60	13	WEHI 231	27
NFS-61	13	WEHI 279	28
NFS-78	13	BAF3	29
NFS-107	13	DA-8	12
NFS-124	13		
BXH2-43	14	T-lymphoid	
BXH2-115	14	DA-2	12
14-122	15	RL12	30
C6	16	WEHI 22	31
RMB1	17		
RMB3	18	Mast cells	
ABPL-4	19, 20	ABFTL-1	21
		ABFTL-2	21
(B) <i>Cb2</i> ⁺ / <i>Cb1</i> ⁺			
Myeloid	Ref.	Macrophage	Ref.
M1	32	P388-D1	33
14-166	15		
14-259	15	T-lymphoid	Ref.
		EL4	34
(C) <i>Cb2</i> ⁺ / <i>Cb1</i> ⁺			
T-lymphoid	Ref.		
CTLL	35		

Cb2 and *Cb1* mRNA expression was determined by RNase protection (Fig 2). References are the original reports describing the cell lines.

RNA samples of several cell lines were kindly donated by Dr J. Cleveland (St Jude Children's Hospital, Memphis, TN).

RNase protection. RNase protection experiments were performed as described.³⁷ cDNA fragments were cloned into Bluescript II SK+ (Stratagene, La Jolla, CA) linearized using the proper enzymes and RNA probes synthesized with T₃ or T₇ polymerase (Promega, Leiden, The Netherlands). For each incubation, 10 µg of RNA and radiolabeled probe (25,000 cpm) were suspended in 30 µL hybridization buffer (80% deionized formamide, 40 mmol/L PIPES, pH 6.4, 0.4 mol/L NaAc, 1 mmol/L EDTA). The samples were heated to 85°C for 5 minutes and then incubated for 16 hours at 50°C annealing temperature. To these mixtures, 300 µL RNase digestion buffer (10 mmol/L Tris-HCl, pH 7.5, 5 mmol/L EDTA, and 200 mmol/L NaAc) and 1 U RNase One (Promega) was added. After 1 hour at 37°C, the reaction was stopped by the addition of

3.3 µL 10% sodium dodecyl sulfate and 20 µg carrier tRNA. The reactions were precipitated with ethanol and fractionated by electrophoresis on a 6% polyacrylamide/7 mol/L urea gel and analyzed by autoradiography. A radiolabeled GAPDH RNA fragment was used as a control.

Serum-free culture medium. A serum-free culture medium was used to study responses to HGFs and cannabinoid ligands.^{38,40} Iscove's modified Dulbecco's medium (GIBCO) was supplemented with 15 mg/mL bovine serum albumin (Cohn fraction V; Sigma, Bornem, Belgium), 10⁻⁷ mol/L sodium selenite (Merck, Darmstadt, Germany), 7.7 × 10⁻⁶ mol/L iron-saturated human transferrin (Behring Institute, Marburg, Germany), 7.8 µg/mL cholesterol (Sigma), and 2.8 µg/mL linoleic acid (Merck). In normal bone marrow colony cultures, 10 ng/mL insulin (Sigma), 10⁻⁴ mol/L β-mercaptoethanol (Merck), and 50 µg/mL of the nucleosides, adenosine, thymidine, guanosine, cytidine, uridine, 2'deoxythymidine, 2'deoxyadenosine, and 2'deoxyguanosine (Sigma) were added to the culture medium.

HGFs and cannabinoid ligands. IL-3 (10 ng/mL; donated by Dr J.N. Ihle, St Jude Children's Research Hospital, Memphis, TN), G-CSF (100 ng/mL; Amgen, Thousand Oaks, CA), GM-CSF (50 ng/mL; Genetics Institute, Cambridge, MA), and Epo (2 IU/mL; Boehringer Mannheim, Mannheim, Germany) were added to the cultures at optimal concentrations, which had been determined with normal bone marrow colony cultures.^{39,40} Cannabinoid ligands included anandamide, Δ-8-tetrahydrocannabinol (Δ⁸-THC), WIN55212-2, Cannabinol, and Cannabidiol (Sigma) and CP 55,940 (Pfizer, Groton, CT). They were added at final concentrations between 0.1 and 10 µmol/L.

Tritiated thymidine (³H-TdR) incorporation. DNA synthesis was measured essentially as described.³⁸ Five thousand cells from various cell lines were cultured in 100 µL of serum-free medium, with or without the addition of HGFs or cannabinoid ligands in 96-well round-bottom microtiter trays (Greiner, Nürtingen, Germany) for 90 hours. Four hours before harvesting, 0.1 µCi tritiated thymidine (2 Ci/mmol ³H-TdR; Amersham International, Amersham, UK) was added. Cells were harvested on nitrocellulose using a filtermate 196 harvester (Packard Instrument Co. Meriden, CT). Radioactivity was determined with a Topcount, microscintillation counter (Packard).

Colony formation by normal mouse bone marrow. Normal bone marrow cells isolated from femora and tibiae from BCBA mice were collected in Hank's Balanced Salt Solution. Fifty thousand cells were cultured in 1 mL serum-free medium with 1.2% methylcellulose. Dishes were incubated with or without 10 ng/mL IL-3 in the presence or absence of 10 µmol/L anandamide at 37°C and 100% humidity and 5% CO₂. Colonies containing 50 cells or more were scored at day 14.

RESULTS

***Cb2* and *Cb1* mRNA expression in murine tissues.** The expression of *Cb2* and *Cb1* transcripts in murine tissues was determined by RNase protection (Fig 1B). Using *Cb2* cDNA probe I (Fig 1A), an expected mRNA fragment of 185 bp was protected in spleen, thymus, and heart (Fig 1B). No *Cb2* transcripts were identified in any of the other tissues examined. RNase protection experiments using a 460-bp *Cb1* probe (Fig 1A) demonstrated *Cb1* transcripts in brain and to a lesser extent in testis (Fig 1B). No detectable levels of *Cb1* mRNA were identified in any of the other organs investigated.

***Cb2* and *Cb1* mRNA expression in murine hematopoietic cell lines.** To examine in which hematopoietic lineages the *Cb2* gene may be expressed, RNase protection experiments were performed using mRNA samples from a large panel

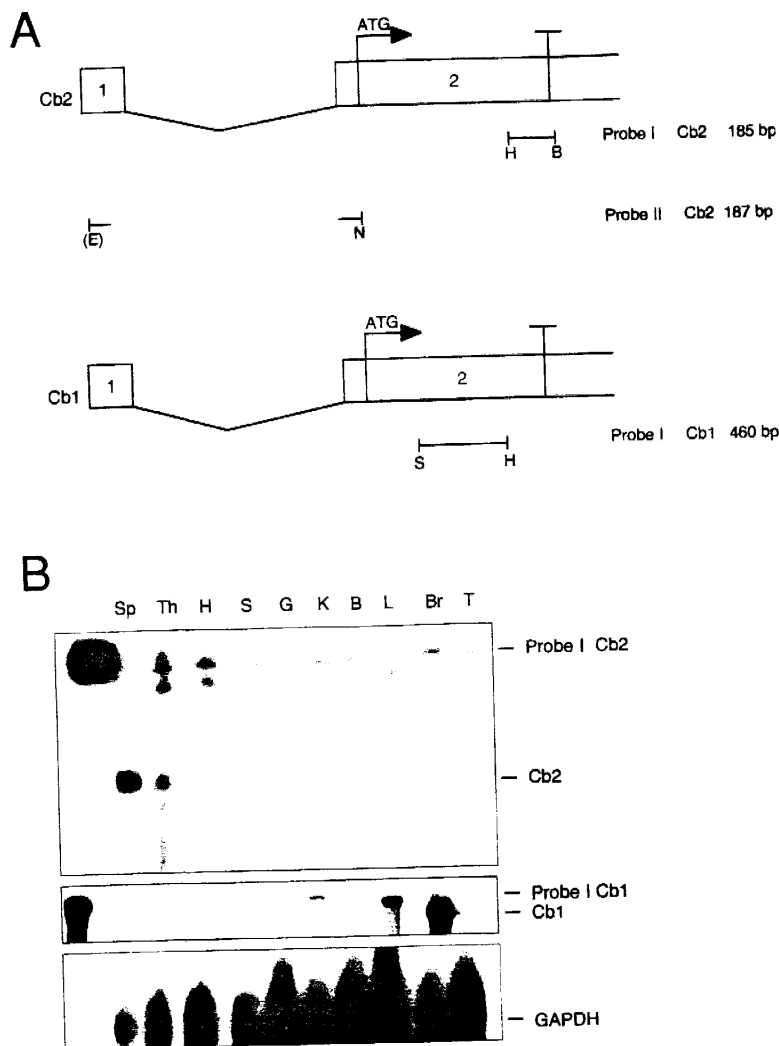


Fig 1. *Cb2* and *Cb1* mRNA expression in murine organ tissues. (A) Schematic representation of murine *Cb2* and *Cb1* mRNA (noncoding exon 1 and protein coding exon 2) and the cDNA probes used for RNase protection experiments. The shaded boxes represent open reading frames. H, *HincII*; B, *BamHI*; S, *StuI*; N, *NcoI*; (E), *EcoRI* in vector. (B) RNase protection on 10 µg of total RNA of different mouse organs using *Cb2* probe I and *Cb1* probe I (see [A]). The protected fragments were 185 bp (*Cb2*) and 460 bp (*Cb1*). Sp, spleen; Th, thymus; H, heart; S, stomach; G, gut; K, kidney; B, bladder; L, liver; Br, brain; T, testis.

of murine hematopoietic cell lines (see the Materials and Methods). Using a 187-bp *Cb2* cDNA fragment representing exon-1 (137 bp) and exon-2 (50 bp; Fig 1A), *Cb2* transcripts of the correct size were identified in 26 of 29 myeloid, 6 of 7 macrophage, 5 of 5 B-lymphoid, 3 of 5 T-lymphoid, 2 of 2 mast cell, and 3 of 3 erythroid cell lines (Table 1). An example of an RNase protection experiment is presented in Fig 2. Interestingly, a 460-bp *Cb1* transcript was shown in a murine CTLL cell line (Fig 2 and Table 1). In the CTLL cell line, no *Cb2* transcripts were identified (Fig 2). No detectable *Cb1* mRNA levels were found in any of the other cell lines (Table 1 and Fig 2). No *Cb1* or *Cb2* transcripts were found in 3 myeloid, 1 macrophage, and 1 T-lymphoid cell lines. These results show that the peripheral cannabinoid receptor is a blood cell receptor that may be expressed in all hematopoietic lineages. The central cannabinoid receptor is only incidentally expressed in hematopoietic cell lines.

Anandamide potentiates the proliferative response of myeloid cells to IL-3. The effect of a natural ligand of cannabinoid receptors, anandamide, on the proliferation of an IL-3-dependent myeloid cell line 32Dcl3 was studied in vitro.

When cultured with FCS, anandamide did not alter IL-3-induced proliferation of 32D cells (Fig 3A). However, in serum-free medium, anandamide significantly enhanced the proliferative response of 32D cells to IL-3 (Fig 3B). Anandamide as a single factor did not induce a measurable proliferative effect in 32Dcl3 cells. Tritiated thymidine incorporation (^3H -TdR) showed that as little as 0.1 to 0.3 µmol/L anandamide was sufficient to augment DNA synthesis in synergy with IL-3 in 32Dcl3, NFS-60, and NFS-78 cells (Fig 4). The maximal stimulative effect was reached at a concentration of 0.3 to 3 µmol/L anandamide. ^3H -TdR experiments with other IL-3-dependent myeloid cell lines, ie, DA-13, DA-28, DA-29, DA-31, NFS-36, NFS-56, and NFS-107, showed similar synergistic dose-response relationships between IL-3 and anandamide (data not shown).

Anandamide enhances colony growth of normal bone marrow progenitors induced with IL-3. Normal mouse bone marrow colony cultures were performed to investigate whether anandamide enhances IL-3-stimulated colony growth. In two independent experiments, a twofold elevation of colony formation was observed when the cells were cul-

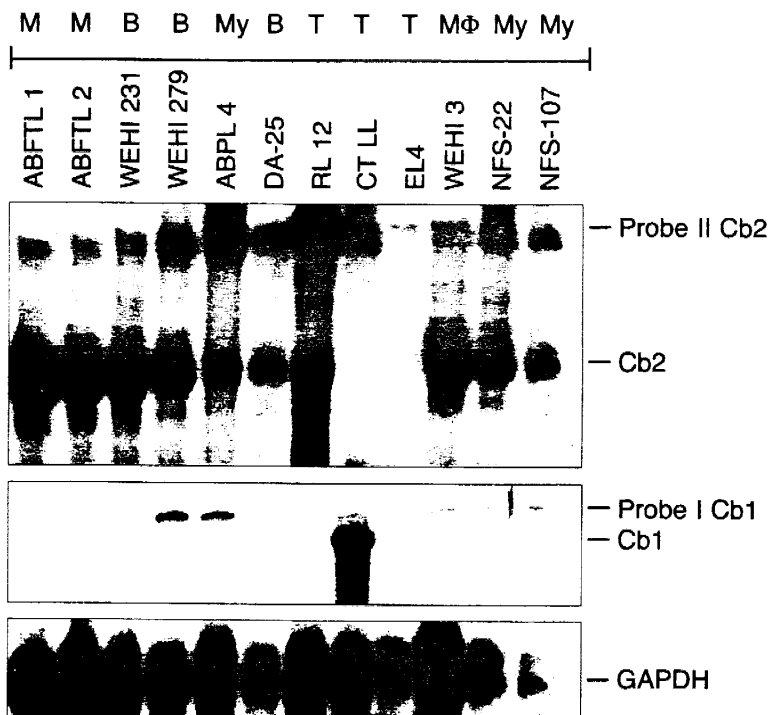


Fig 2. *Cb2* and *Cb1* mRNA expression in murine hematopoietic cell lines. RNase protection on 10 μ g of total RNA of different murine hematopoietic cell lines, using *Cb2* probe II and *Cb1* probe I (see Fig 1A). The hematopoietic phenotype of the cell lines is indicated at the top of the figure. M, mast cell; B, B lymphoid; My, myeloid; T, T lymphoid; M Φ , macrophage.

tured with IL-3 plus anandamide as compared with IL-3 alone (Table 2). Anandamide not only increased colony numbers, but also showed an effect on the size of the colonies (Fig 5). More colonies containing 250 cells or more were found in cultures with IL-3 plus anandamide. Mega-size colonies of 1,000 cells or more were found in IL-3 plus anandamide cultures, but none in colony cultures with IL-3 alone.

Effects of different cannabinoid ligands on IL-3-induced proliferation of myeloid cells. To investigate whether various synthetic molecules all capable of binding cannabinoid receptors would be capable of enhancing the proliferative effects of IL-3, 32Dcl3 cells were cultured with IL-3 plus the cannabinoid agonists CP-55,940, WIN 55212-2, Δ^8 -THC, Cannabinol, and Cannabidiol. Whereas DNA synthesis of 32Dcl3 cells was augmented significantly when costimulated with IL-3 and anandamide, no enhancement of thymidine uptake was apparent when 32Dcl3 cells were stimulated with IL-3 plus any of the other cannabinoid ligands (Fig 6).

Anandamide enhances DNA synthesis of hematopoietic cell lines in synergy with GM-CSF, Epo, and G-CSF. 3 H-TdR incorporation experiments were performed with three selected GM-CSF-responsive (NFS-36), Epo-responsive (32D-Epo), and G-CSF-responsive (BAF-G) cell lines to investigate whether anandamide would potentiate proliferation in synergy with hematopoietic growth factors other than IL-3. NFS-36 cells showed a weak response to GM-CSF alone or anandamide alone, but a significant increase of thymidine incorporation was evident when both ligands were added (Fig 7A). 32D cells cultured with Epo alone, ie, in the absence of anandamide, did not stimulate DNA synthesis at all. 32D-Epo cells became responsive to Epo in the presence of anandamide (Fig 7B). Finally, whereas G-CSF stim-

ulated some DNA synthesis of BAF-G cells, anandamide supplemented to the culture medium augmented the G-CSF effect by twofold (Fig 7C). These experiments indicate that anandamide may enhance the stimulative activity of various HGFs.

Effects of anandamide and other cannabinoid ligands on *Cb2*-overexpressing myeloid cells. To verify whether the stimulatory effect of anandamide is mediated through activation of the peripheral cannabinoid receptor, we overexpressed *Cb2* cDNA in 32D cells that express the G-CSF receptor (G-CSF-R), but do not respond to G-CSF.⁶ *Cb2*-transfected cells show high tritiated thymidine incorporation when cultured with G-CSF plus anandamide (Fig 8A). In comparison, anandamide alone or G-CSF alone did not induce DNA synthesis. Furthermore, anandamide plus G-CSF did not induce proliferation in control vector-transfected 32D (G-CSF-R) cells. These data indicate that anandamide stimulates DNA synthesis after the specific activation of *Cb2*. In addition, 3 H-TdR incorporation experiments using various concentrations of the other cannabinoid ligands showed that DNA synthesis of 32D (G-CSF-R/*Cb2*) cells was not influenced by any of the other ligands (Fig 8B). Anandamide is apparently selectively capable of stimulating hematopoietic cells through activation of the peripheral cannabinoid receptor among several cannabinoid ligands.

DISCUSSION

In this study, we show that the gene encoding the peripheral cannabinoid receptor is expressed in hematopoietic cells and appears to be important for the efficiency of stimulation of growth by a variety of HGFs.

Several studies had previously demonstrated the presence

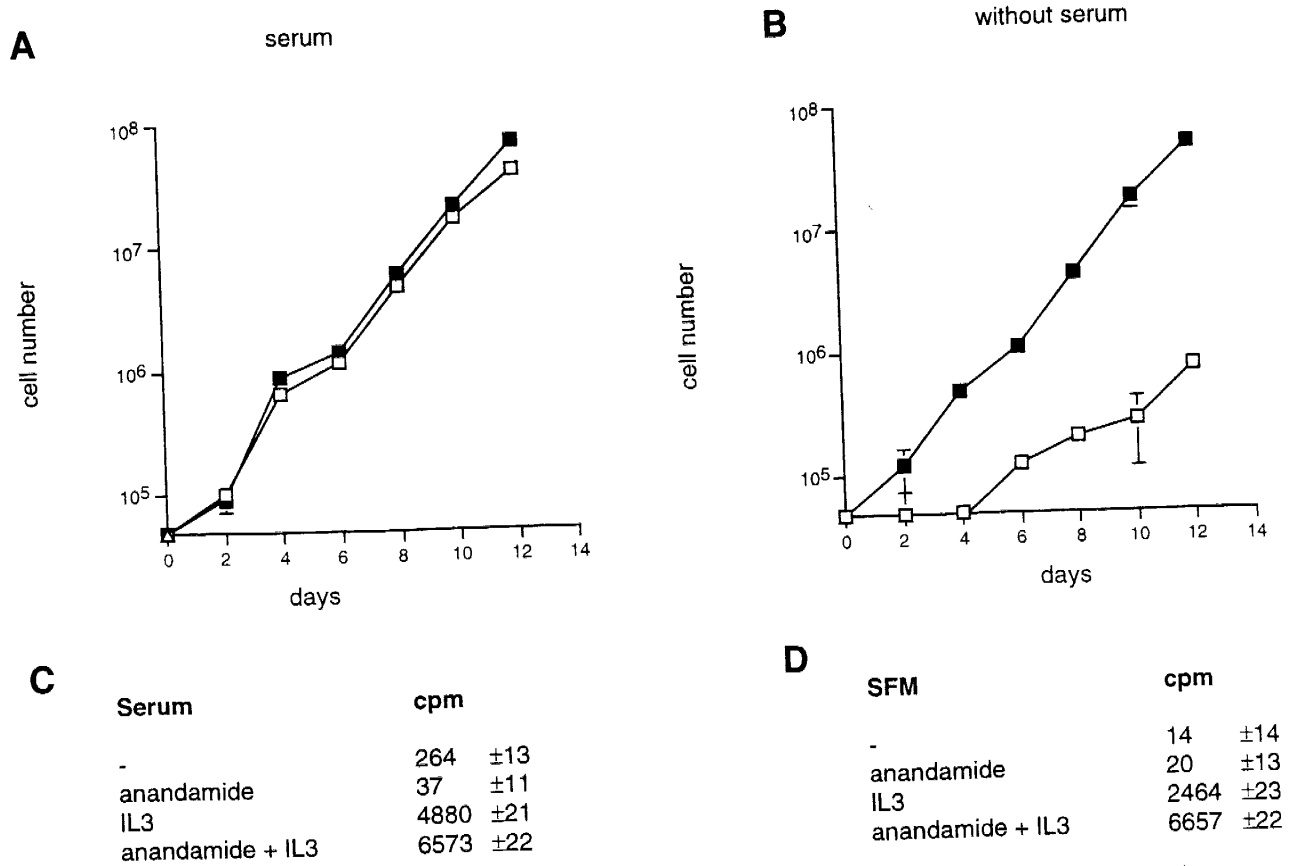


Fig 3. The effect of anandamide on the proliferation of the myeloid cell line 32Dcl3. Growth curves of 32Dcl3 cells cultured in the presence of fetal calf serum (A) or serum-free (B). Cells were cultured with no stimulus ($\square$), with 10 μ mol/L anandamide alone (Δ), with 10 ng/mL IL-3 alone ($\square$), or with IL-3 plus anandamide ($\blacksquare$). Mean cell numbers ($\pm 1 \times$ SD) of triplicate experiments are plotted against the number of days in culture. The doubling time in serum-free culture without anandamide was 75 hours and with anandamide was 29 hours. 3 H-TdR incorporation data (cpm $\pm 1 \times$ SD) of 32Dcl3 cells cultured in serum containing medium (C) or in serum-free medium (SFM) (D).

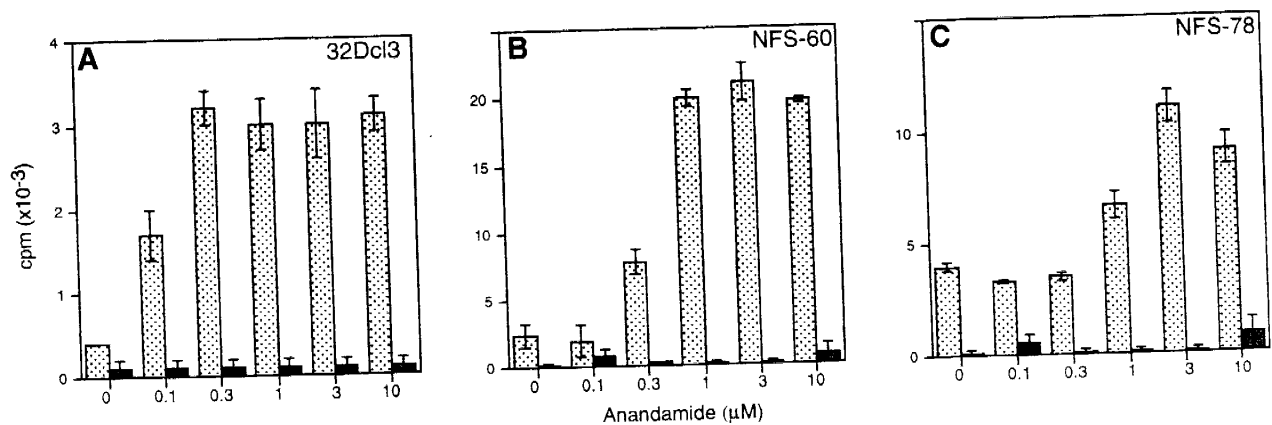


Fig 4. The effects of different concentrations of anandamide on IL-3-induced 3 H-TdR incorporation of three murine myeloid cell lines. Cells of 32Dcl3 (A), NFS-60 (B), and NFS-78 (C) were cultured with titrated concentrations of anandamide (0 to 10 μ mol/L) in the presence (10 ng/mL; $\square$) or absence of IL-3 ($\blacksquare$). The mean values $\pm 1 \times$ SD (cpm $\times 10^{-3}$) of triplicate experiments are shown.

Table 2. Effect of Anandamide on IL-3-Stimulated Colony Formation of Normal Murine Bone Marrow CFU-C (Day 14)

Stimulus	CFU-C per 10 ⁵ Cells			
	Experiment No. 1		Experiment No. 2	
	>50 Cells	>250 Cells	>50 Cells	>250 Cells
No	0	0	0	0
AN	0	0	0	0
IL-3	20	6	37 ± 8	11 ± 4
IL-3 + AN	37	17	47 ± 7	23 ± 5

In experiment no. 1, mean colony numbers of duplicate experiments are shown. In experiment no. 2, the mean and ± 1 SD of quadruplicate CFU-C counts are shown.

Abbreviations: No, no stimulus; AN, 10 μ mol/L anandamide; IL-3, 10 ng/mL IL-3.

of cannabinoid binding sites on hematopoietic cells,⁴¹⁻⁴³ but these studies had not distinguished between the central and the peripheral cannabinoid receptor. *Cb2* expression was shown in spleen and in thymus. The *Cb2* mRNA expression in heart tissue might be the result of residual blood that was not eliminated before extraction. Others demonstrated *Cb2* mRNA expression in spleen, in the myeloid cell line HL60, and in mast cell lines.^{7,44} We show here that *Cb2* may be expressed in myeloid, erythroid, B-lymphoid, T-lymphoid, macrophage, and mast cells. This finding indicates that *Cb2* encodes a hematopoietic receptor that may have a function in a broad scale of hematopoietic lineages. By applying reverse transcriptase-polymerase chain reaction, several groups demonstrated *Cb1* transcripts in hematopoietic cells.^{41,43,45,46} RNase protection studies presented here show that *Cb1* could be detected in brain and testis but not in spleen and thymus. Furthermore, *Cb1* could be detected in 1 of 51 cell lines only. These data suggest that *Cb1* may occasionally be expressed in hematopoietic cells, whereas *Cb2* is commonly expressed. These results enforce the notion that *Cb2* rather than *Cb1* encodes a hematopoietic cannabinoid receptor.

In vitro studies with murine hematopoietic cell lines and normal bone marrow precursors showed that the peripheral cannabinoid receptor has a function in the regulation of proliferation by HGFs. Stimulatory effects of anandamide on hematopoietic cell proliferation had not been reported. Anandamide enhanced the cellular proliferation induced with IL-3, GM-CSF, G-CSF, and Epo when the cells were cultured in serum-free medium. Whether anandamide synergizes with other cytokines remains open to future investigation. Most of the cell lines analyzed for *Cb2* mRNA expression in this study are HGF-independent and therefore did not allow for an extended analysis of the stimulative effects of anandamide with other HGFs. However, interestingly, when a panel of HGF independent cell lines were cultured in a serum-free medium, ie, DA-2 (T-lymphoid), DA-25 (B-lymphoid), RED-5 (erythroid), and J774 cells (macrophage), anandamide was required to induce proliferation in vitro (data not shown). This indicates that the *Cb2* may have a role in stimulation of growth of several, if not all, hematopoietic lineages. An important modification of the culture conditions used in this study is the elimination of FCS and the use of a serum-free culture system. The effects of anandamide are not evident when cells are cultured with FCS and therefore other investigators may have missed the effects of anandamide stimulation. The results of this study would suggest that anandamide or another ligand for the cannabinoid receptor is present in FCS. In fact, four other fatty acids have recently been identified to bind and activate cannabinoid receptors.^{44,47,48} The role and presence of anandamide or any of these fatty acids may explain at least in part the serum dependence of various hematopoietic cell lines and primary hematopoietic cells. The in vitro experiments show that, among a selected number of cannabinoid ligands studied, only anandamide was capable of stimulating the proliferation of hematopoietic cells synergistically with HGFs. This is remarkable, because, according to other investigators, WIN 55212-2, CP55,940, and THC bind and activate the peripheral cannabinoid receptors more efficiently than anandamide.^{7,49,50} Our findings thus suggest strongly that, in hematopoietic cells,

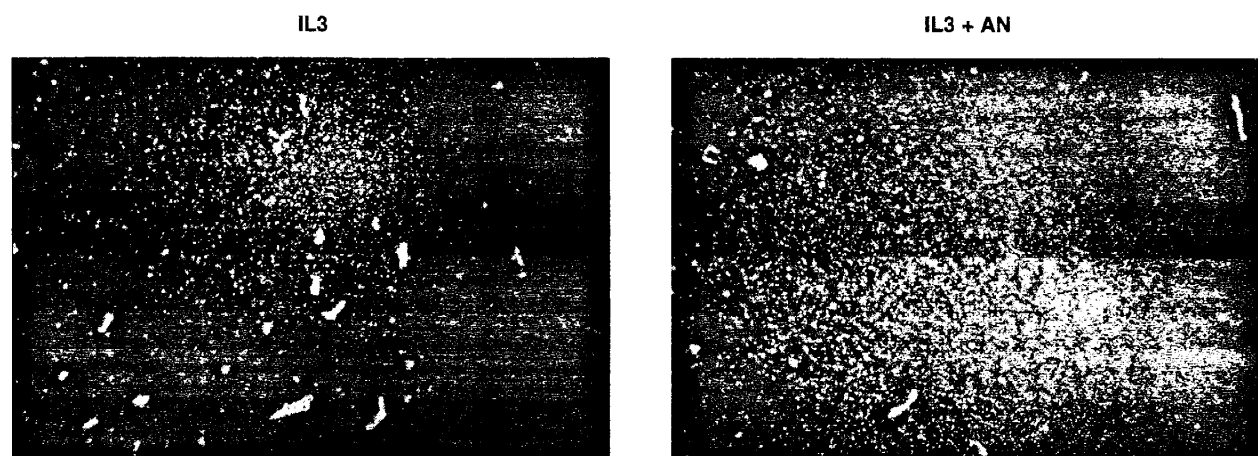


Fig 5. Effect of anandamide on the size of IL-3-induced normal bone marrow colonies. Examples of representative IL-3-induced normal bone marrow colonies after 14 days of culture with (IL-3+AN) or without anandamide (IL-3).

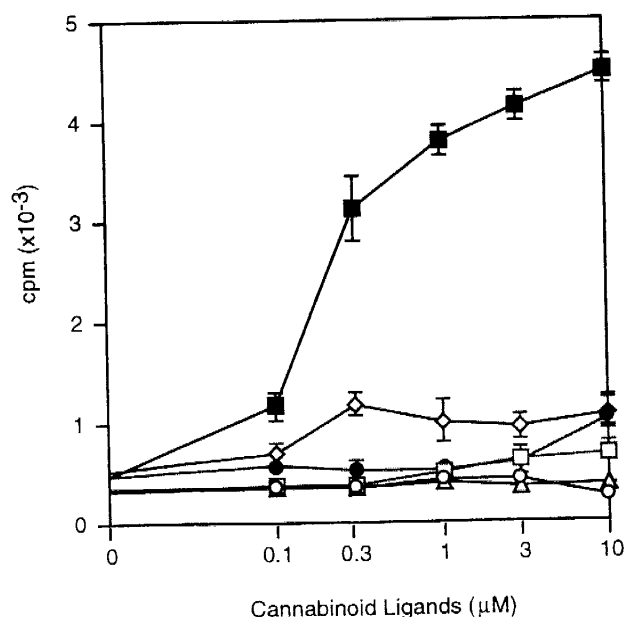


Fig 6. Dose effects of anandamide and other cannabinoid ligands on IL-3-induced ^3H -TdR incorporation of 32Dcl3 cells. Cells were cultured serum-free with IL-3 (10 ng/mL) and titrated concentrations (0 to 10 $\mu\text{mol/L}$) of anandamide (■), WIN 55212-2 (○), Δ^8 -THC (□), Cannabinol (●), Cannabidiol (Δ), and CP55,940 (◇).

anandamide acts as the most potent agonist through the latter receptors. Up to 1 to 10 $\mu\text{mol/L}$ anandamide was needed for optimal activation of proliferation, which is within the concentration range required for optimal binding.^{7,42,44,45,49} The transfection studies of *Cb2* (Fig 8) confirm that anandamide specifically activates proliferation of 32D (G-CSF-R/*Cb2*) cells through activation of the peripheral cannabinoid receptor. In contrast, the other cannabinoid ligands fail to stimulate these *Cb2*-transfected cells. In fact, when cultured with serum, the other Cb ligands inhibit proliferation (data not shown). Because the cannabinoid agonists CP55,940 and

THC have been shown to stimulate an additional nonreceptor-mediated signal transduction pathway,⁵¹ it is perhaps possible that the alternative nonreceptor-mediated signals suppress proliferation. In any case, the current available data would suggest that only anandamide is capable of stimulating proliferation synergistically with HGFs. Whether the other fatty acids are capable of stimulating proliferation will be investigated.

We have recently identified the *Cb2* gene in a common virus integration site (*Evi1*)⁶ and *Cb2* was suggested to be a proto-oncogene. Transfection of *Cb2* in 32D (G-CSF-R) cells generated G-CSF-dependent cell lines that could be maintained serum free when cultured with anandamide.⁶ The studies presented here show that activation of the *Cb2* receptor has a profound effect on the proliferation of cells. This adds further support to the notion that this receptor, when aberrantly expressed, may alter the proliferative response of hematopoietic cells and contribute to the development of leukemia. *Cb2* transgenic animals are currently being studied to investigate whether abnormal *Cb2* expression might contribute to the development of leukemia in vivo.

Cb2 receptor-mediated signal transduction by anandamide has been observed by others.⁴⁹ How activated cannabinoid receptors may stimulate proliferation synergistically with HGFs is unresolved. The costimulatory effects may occur at different levels of HGF receptor signalling. Stimulation of the peripheral cannabinoid receptor may have the following effects: (1) transactivation of the HGF receptors; (2) potentiation of HGF receptor-mediated signal transduction, eg, JAK/STAT or the p21^{ras}/MAP kinase pathways⁵²⁻⁵⁴; and (3) activation of signalling pathways that, in parallel with the HGF receptor signalling routes, enhances cell cycling. In fact, examples in support of each of those possibilities exist for seven other transmembrane receptors.⁵⁵⁻⁶² Whether these alternative mechanisms are activated by *Cb2* requires verification in future studies.

The results presented in Table 2 and Fig 5 show that the synergism between IL-3 and anandamide is also evident in normal bone marrow colony formation. A greater number

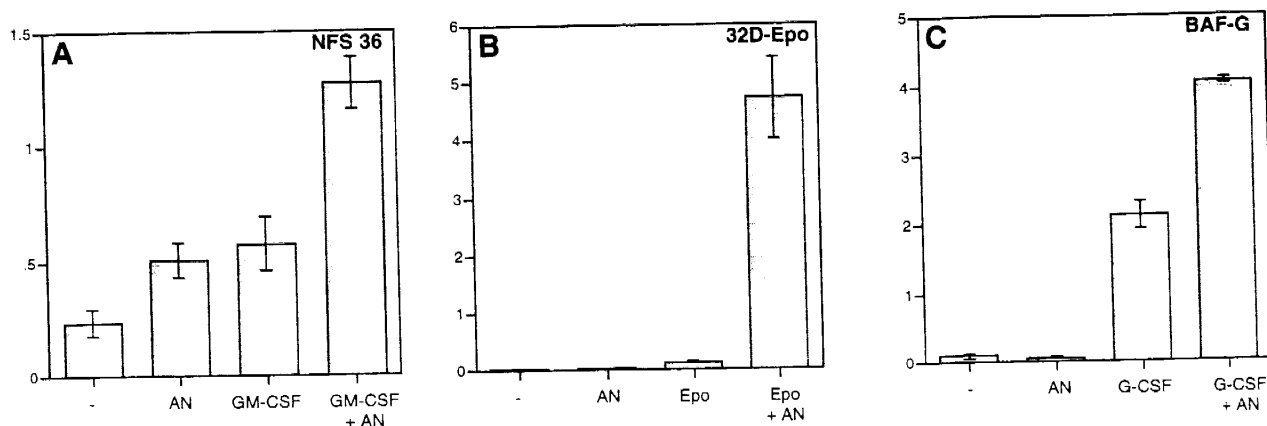


Fig 7. Synergistic activity of anandamide on GM-CSF-, Epo-, or G-CSF-stimulated thymidine incorporation. Cells were cultured with optimal concentrations of GM-CSF (50 ng/mL; [A] NFS-36), Epo (2 IU/mL; [B] 32D-Epo), or G-CSF (100 ng/mL; [C] BAF-G) in the presence of 10 $\mu\text{mol/L}$ anandamide (AN). The mean values $\pm 1 \times \text{SD}$ (cpm $\times 10^{-3}$) of triplicate experiments are shown.

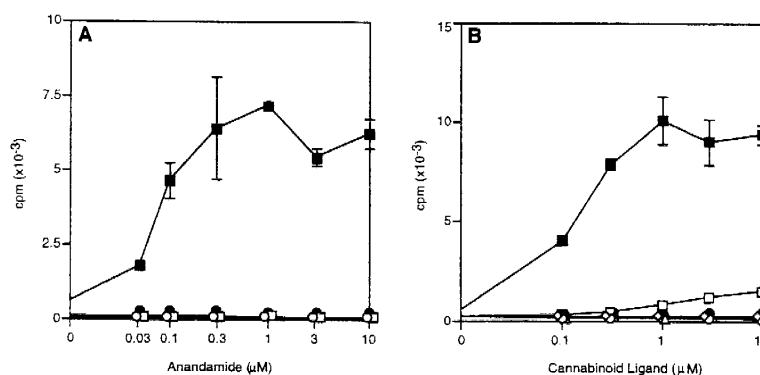


Fig 8. Effects of anandamide and other cannabinoid ligands on G-CSF-induced thymidine incorporation of Cb2-transfected 32D/G-CSF-R cells. (A) 32D/G-CSF-R were transfected with Cb2 cDNA (32D/G-CSF-R/Cb2) or with pBabe control vector (32D/G-CSF-R/pB). Cells were cultured without G-CSF with different concentrations anandamide ([○] 32D/G-CSF-R/pB; [●] 32D/G-CSF-R/Cb2) or with G-CSF plus anandamide ([□] 32D/G-CSF-R/pB; [■] 32D/G-CSF-R/Cb2). (B) 32D/G-CSF-R-Cb2 cells were cultured with G-CSF plus different concentrations of the distinct cannabinoid ligands, anandamide (■), WIN 55212-2 (○), Δ⁸-THC (□), Cannabinol (●), Cannabidiol (△), and CP55,940 (◇).

of IL-3-dependent colonies were formed in the presence of anandamide. These results indicate that, in the presence of anandamide, IL-3 stimulates the outgrowth of additional populations of precursor cells. IL-3 plus anandamide also stimulated colonies of greater size, which would indicate that the combination of the two factors augments the production of progeny from individual precursor cells as well. Whether anandamide synergizes with other HGFs in the stimulation of normal marrow precursor cells is currently under investigation.

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REFERENCES

- Clark SC, Kamen R: The human hematopoietic colony stimulating factors. *Science* 236:1229, 1987
- Metcalf D: The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. *Nature* 339:27, 1989
- Bazan JF: Structural design and molecular evolution of a cytokine receptor superfamily. *Proc Natl Acad Sci USA* 87:6934, 1990
- Gudermann T, Nurnberg B, Schultz G: Receptors and G proteins as primary components of transmembrane signal transduction. Part 1. G-protein-coupled receptors: Structure and function. *J Mol Med* 73:51, 1995
- Savarese TM, Fraser CM: *In vitro* mutagenesis and the search for structure-function relationships among G protein-coupled receptors. *Biochem J* 283:1, 1992
- Delwel R, Hol S, Vankan Y, Löwenberg B, Ihle JN, Copeland NG, Valk PJM: The cannabinoid receptor-2 is a potential oncogene in myeloid mouse leukemias. *Blood* 88:1345, 1996
- Munro S, Thomas KL, Abu-Shaar M: Molecular characterization of a peripheral receptor for cannabinoids. *Nature* 365:61, 1993
- Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI: Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* 346:561, 1990
- Gerard CM, Mollereau C, Vassart G, Parmentier M: Molecular cloning of a human cannabinoid receptor which is also expressed in testis. *Biochem J* 279:129, 1991
- Greenberger JS, Sakaakeeny MA, Humphries RK, Eaves CJ, Eckner RJ: Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. *Proc Natl Acad Sci USA* 80:2931, 1983
- Migliaccio G, Migliaccio AR, Kreider BL, Rovera G, Adamson JW: Selection of lineage-restricted cell lines immortalized at different stages of hematopoietic differentiation from the murine cell line 32D. *J Cell Biol* 109:833, 1989
- Ihle JN, Rein A, Mural R: Immunological and virological mechanisms in retroviral induced murine leukemogenesis. *Adv Viral Oncol* 4:95, 1984
- Holmes KL, Palaszinsky E, Frederickson TN, Morse HC III, Ihle JN: Correlation of cell-surface phenotype with the establishment of interleukin-3-dependent cell lines from wild-mouse murine leukemia virus-induced neoplasms. *Proc Natl Acad Sci USA* 82:6687, 1985
- Buchberg AM, Bedigian HG, Jenkins NA, Copeland NC: *Evl2*, a common integration site involved in murine myeloid leukemogenesis. *Mol Cell Biol* 10:4658, 1990
- Trakhtenbrot L, Krauthgamer R, Resnitzky P, Haran-Ghera N: Deletion of chromosome 2 is an early event in the development of radiation-induced myeloid leukemia in SJL/J mice. *Leukemia* 2:545, 1988
- Azumi JJ, Sachs L: Chromosome mapping of the genes that control differentiation and malignancy in myeloid leukemic cells. *Proc Natl Acad Sci USA* 74:253, 1977
- De Both NJ, Hagenmeijer A, Rhijsburger EH, Vermey M, Van't Hul E, Smit EME: DMSO induced terminal differentiation and trisomy 15 in myeloid cell line transformed by the Rauscher murine leukemia virus. *Cell Differ* 10:13, 1981
- Leenen PJM, Jansen AMAC, van Ewijk W: Murine macrophage cell lines can be ordered in a linear differentiation sequence. *Differentiation* 32:157, 1986
- Shen-Ong GLC, Potter M, Mushinsky JF, Lavu S, Reddy EP: Activation of the c-myc locus by viral insertional mutagenesis in plasmacytoid lymphosarcomas. *Science* 226:1077, 1984
- Dudek H, Reddy EP: Murine myeloid leukemias with aberrant myb loci show heterogeneous expression of novel myb proteins. *Oncogene* 4:1489, 1989
- Pierce JH, Di Fiore PP, Aaronson SA, Potter M, Pumphrey J,

- Scott A, Ihle JN: Neoplastic transformation of mast cells by Abelson-MuLV: Abrogation of IL-3 dependence by nonautocrine mechanism. *Cell* 41:685, 1985
22. Raschke WC, Baird S, Ralph P, Nakoinz I: Functional macrophage cell lines transformed by Abelson leukemia virus. *Cell* 15:261, 1978
 23. Ralph P, Prichard J, Cohn M: Reticulum cell sarcoma: An effector cell in antibody-dependent cell mediated immunity. *J Immunol* 114:898, 1975
 24. Ralph P, Ho M-K, Litcofsky PB, Springer TA: Expression and induction *in vitro* of macrophage differentiation antigens on murine cell lines. *J Immunol* 130:108, 1983
 25. Warner NL, Moore MAS, Metcalf D: A transplantable myelomonocytic leukemia in BALB/c mice: Cytology, karyotype and muramidase content. *J Natl Cancer Inst* 43:963, 1969
 26. Hagemeijer A, Smit EME, Govers F, de Both NJ: Trisomy 15 and other nonrandom chromosome changes in Rauscher murine leukemia virus-induced leukemia cell lines. *J Natl Cancer Inst* 69:945, 1982
 27. Lanier LL, Warner NL: Cell cycle related heterogeneity of Ia antigen expression on a murine B lymphoma cell line: Analysis by flow cytometry. *J Immunol* 126:626, 1981
 28. Warner NL, Daley MJ, Richey J, Spellman C: Flow cytometry analysis of murine B cell lymphoma differentiation. *Immunol Rev* 48:197, 1979
 29. Palacios R, Steinmetz M: IL-3 dependent mouse clones that express B-220 surface antigen, contain Ig genes in germline configuration, and generate B-lymphocytes *in vivo*. *Cell* 41:727, 1985
 30. Decleve A, Lieberman M, Ihle JN, Rosenthal NPN, Lung ML, Kaplan HS: Physicochemical, biological and serological properties of a leukemogenic virus isolated from cultured RadLV-induced lymphomas of C57BL/Ka mice. *Virology* 90:23, 1978
 31. Harris AW, Bankhurst AD, Mason S, Warner NL: Differentiated functions expressed by cultured mouse lymphoma cells. II. Theta antigen, surface immunoglobulin and a receptor for antibody on cells of a thymoma cell line. *J Immunol* 110:431, 1973
 32. Ichikawa Y: Differentiation of a cell line of myeloid leukemia. *J Cell Physiol* 74:223, 1969
 33. Koren HS, Handwerker BS, Wunderlich JR: Identification of macrophage-like characteristics in a cultured murine tumor line. *J Immunol* 114:894, 1975
 34. Shevach EM, Stobo JD, Green I: Immunoglobulin and theta-bearing murine leukemias and lymphomas. *J Immunol* 108:1146, 1972
 35. Gillis S, Smith KA: Long term culture of tumour-specific cytotoxic T cells. *Nature* 268:154, 1977
 36. Dong F, Van Buitenen C, Pouwels K, Hoefsloot LH, Löwenberg B, Touw IP: Distinct cytoplasmic regions of the human granulocyte colony-stimulating factor receptor involved in induction of proliferation and maturation. *Mol Cell Biol* 13:7774, 1993
 37. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual* (ed 2). Cold Spring Harbor, NY, Cold Spring Harbor Laboratory, 1989
 38. Salem M, Delwel R, Touw I, Mahmoud L, Löwenberg B: Human AML colony growth in serum-free culture. *Leuk Res* 12:157, 1988
 39. Ploemacher RE, van Soest PL, Boudewijn A: Autocrine transforming growth factor β 1 blocks colony formation and progenitor cell generation by hematopoietic cells stimulated with steel factor. *Stem Cells* 11:336, 1993
 40. Ploemacher RE, van Soest PL, Voorwinden H, Boudewijn A: Interleukin-12 synergizes with interleukin-3 and steel factor to enhance recovery of murine hemopoietic stem cells in liquid culture. *Leukemia* 7:1381, 1993
 41. Kaminsky NE, Abood AE, Kessler FK, Martin BR, Schatz AR: Identification of a functional relevant cannabinoid receptor on mouse spleen cells that is involved in cannabinoid-mediated immune modulation. *Mol Pharmacol* 42:736, 1992
 42. Lynn AB, Herkenham M: Localization of cannabinoid receptors and nonsaturable high density cannabinoid binding sites in peripheral tissues of the rat: Implications for receptor mediated immune modulation by cannabinoids. *J Pharmacol Exp Ther* 268:1612, 1994
 43. Bouaboula M, Rinaldi M, Carayon P, Carillon C, Delpach B, Shire D, Le Fur G, Casellas P: Cannabinoid-receptor expression in human leukocytes. *Eur J Biochem* 214:173, 1993
 44. Facci L, Dal Toso R, Burianni A, Skaper SD, Leon A: Mast cells express a peripheral cannabinoid receptor with differential sensitivity to anandamide and palmitoylethanolamide. *Proc Natl Acad Sci USA* 92:3376, 1995
 45. Schwarz H, Blanco FJ, Lotz M: Anandamide, an endogenous cannabinoid receptor agonist inhibits lymphocyte proliferation and induces apoptosis. *J Neuroimmunol* 55:107, 1994
 46. Daaka Y, Klein TW, Friedman H: Expression of cannabinoid receptor mRNA in murine and human leukocytes. In Sharp B (ed): *The Brain Immune Axis and Substance Abuse*. Vol 13. New York, NY, Plenum, 1995. p 91
 47. Barg J, Fride E, Hanus L, Levy R, Matus-Leibovitch N, Heldman E, Bayewitch M, Mechoulam R, Vogel Z: Cannabinomimetic behavioral effects of and adenylyl cyclase inhibition by two new endogenous anandamides. *Eur J Pharmacol* 287:145, 1995
 48. Lee M, Yang KH, Kaminski N: Effects of putative cannabinoid receptor ligands, anandamide and 2-arachidonyl-glycerol, on immune function in B6C3F1 mouse splenocytes. *J Pharmacol Exp Ther* 275:529, 1995
 49. Shire D, Calandra B, Rinaldi-Carmona M, Oustric D, Peseque B, Bonnin-Cabanne O, Le Fur G, Caput D, Ferrara P: Molecular cloning, expression and function of the murine *Cb2* peripheral cannabinoid receptor. *Biochem Biophys Acta* 1307:132, 1996
 50. Slipetz DM, O'Neal GP, Favreau L, Dufresne C, Gallant M, Gareau Y, Guay D, Labelle M, Metters KM: Activation of the human peripheral cannabinoid receptor results in inhibition of adenylyl-cyclase. *Mol Pharmacol* 48:352, 1995
 51. Felder CC, Veluz JS, Williams HL, Briley EM, Matsuda LA: Cannabinoid agonists stimulate both receptor- and nonreceptor-mediated signal transduction pathways in cells transfected with and expressing cannabinoid receptor clones. *Mol Pharmacol* 42:838, 1992
 52. Nicholson SE, Oates AC, Harpur AG, Ziemiecki A, Wilks AF, Layton JE: Tyrosin kinase Jak1 is associated with the granulocyte-colony-stimulating factor receptor and both become tyrosine phosphorylated after receptor activation. *Proc Natl Acad Sci USA* 91:2985, 1994
 53. Tian SS, Lamb P, Seidel AM, Stein RB, Rosen J: Rapid activation of the STAT3 transcription factor by granulocyte colony stimulating factor. *Blood* 84:1760, 1994
 54. Bashey A, Healy L, Marshall CJ: Proliferative but not nonproliferative responses to granulocyte colony-stimulating factor are associated with activation of the p21^{ras}/MAP kinase signalling pathway. *Blood* 83:949, 1994
 55. Daub H, Weiss FU, Wallasch C, Ullrich A: Role of transactivation of the receptor in signalling by G-protein-coupled receptors. *Nature* 379:557, 1996
 56. Marrero MB, Schieffer B, Paxton WG, Heerdt L, Berk BC, Delafontaine P, Bernstein KE: Direct stimulation of Jak/STAT pathway by the angiotensin II AT₁ receptor. *Nature* 375:247, 1995
 57. Wartmann M, Campbell D, Subramanian A, Burstein SH, Davis RJ: The MAP kinase signal transduction pathway is activated by the endogenous cannabinoid anandamide. *FEBS Lett* 359:133, 1995
 58. Bouaboula M, Poinot-Chazel C, Bourrie B, Canat X, Calandra

B. Rinaldi-Carmona M, Le Fur G, Casellas P: Activation of mitogen activated protein kinase by stimulation of the central cannabinoid receptor *Cb1*. *Biochem J* 312:637, 1995

59. Bouaboula M, Bourrie B, Rinaldi-Carmona M, Shire D, Le Fur G, Casellas P: Stimulation of cannabinoid receptor *Cb1* induces Krox-24 expression in human Astrocyte cells. *J Biol Chem* 270:13973, 1995

60. Bouaboula M, Poinot-Chazel C, Marchand J, Canat X, Bourrie B, Rinaldi-Carmona M, Calandra B, Le Fur G, Casellas P: Signalling pathway associated with stimulation of *Cb2* peripheral

cannabinoid receptor. Involvement of both mitogen-activated kinase and induction of Krox-24 expression. *Eur J Biochem* 237:704, 1996

61. Kato J, Matsuoka M, Polyak K, Massague J, Sherr CJ: Cyclic AMP induced G1 phase arrest mediated by an inhibitor (p27^{kip1}) of cyclin dependent kinase 4 activation. *Cell* 79:487, 1994

62. Van Biesen T, Hawes BE, Luttrell DK, Krueger KM, Touhara K, Porfiri E, Sakaue M, Luttrell LM, Lefkowitz RJ: Receptor-tyrosine-kinase- and G-mediated MAP kinase activation by a common signalling pathway. *Nature* 376:781, 1995