The peripheral cannabinoid receptor Cb2 in leukemia

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De perifere cannabinoide receptor Cb2 in leukemie

Proefschrift

Ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus Prof.dr. S. W. J. Lamberts
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 7 januari 2004 om 13:45 uur

door

Meritxell Alberich Jordà

geboren te Barcelona

Promotiecommissie

Promotor	Prof. dr. B. Löwenberg
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Copromotor	Dr. R. Delwel

The work described in this thesis was performed at the Department of Hematology, Erasmus Medical Centre Rotterdam, The Netherlands. This work was supported by grants from the Dutch Cancer Society (KWF) and the Royal Dutch Academy of Sciences (KNAW). Printing of this thesis was financially supported by the Dutch Cancer Society (KWF).

Printed by Optima Grafische Communicatie, Rotterdam

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CHAPTER 1

Introduction

1.1 Hematopoiesis

Hematopoiesis, or blood cell formation, is a highly dynamic and strictly regulated process. All blood cells are derived from a small population of precursor cells called hematopoietic stem cells (HSC), which are present in the adult bone marrow. During embryogenesis the mammalian hematopoietic system originates from mesodermally-derived cells localized in the surroundings of the yolk sac and in the aorta-gonad-mesonephros (AGM) region. In the mouse, hematopoiesis starts in the yolk sac at E7.5 (embryonic day 7.5 post coitus) and predominantly generates a transient embryonic hematopoietic system. The AGM region produces the first adult-type hematopoietic stem cells at E10, and is autonomously and exclusively dedicated to the generation of the adult hematopoietic system[1]. Blood cell formation takes place in the yolk salk and in the AGM region until day E13 and E11/E12, respectively, after which they start to degenerate. At the same time there is an increase of hematopoietic activity in the fetal liver and spleen, and definitive hematopoiesis will take place in adult bone marrow[2].

HSC have self-renewal capacity and are pluripotent, i.e. they can generate progenitor cells of all hematopoietic lineages. These progenitor cells are characterized by their irreversible lineage-commitment to either the myeloid or lymphoid lineage. They can proliferate, differentiate and terminally mature towards functional blood cells such as T and B lymphocytes, neutrophils, eosinophils, basophils, monocytes, erythrocytes or platelets (Figure 1). Generally, the more mature the cells are, the more reduced their proliferative capacity. Terminally differentiated cells detach from the bone marrow and enter circulation in the blood. Since most of these mature cells have a short lifetime, constant production of circulating cells is required. In addition, hematopoiesis has to be adjusted in response to the suddenly changing requirements caused by blood loss or infections. Homeostasis, or regulation of the blood cell formation, is the result of two different but interacting mechanisms[3]. Firstly, bone marrow stromal cells provide the cell to cell contacts that control certain aspects of hematopoiesis. These stromal cells, together with the extracellular matrix and microvascular network, provide a suitable microenvironment for hematopoiesis. Secondly, homeostasis is maintained by cytokines, chemokines and hematopoietic growth factors (HGFs).

The environment generated by the combination of stromal cells, extracellular matrix, cytokines, chemokines and adhesion molecules is crucial for migration and homing of hematopoietic cells. Mobilization of cells plays an important role in hematopoiesis, from the first stages of embryogenesis until the system has completely developed and must defend the host organism against stress conditions. For instance, distribution of hematopoietic cells in different embryonic regions is the result of active migration. Wound healing processes require mobilization of endothelial cells and fibroblasts. Leukocyte travel is crucial for defense against infection and development of an efficient immune response. Two types of migration can be distinguished, defined by the type of cellular movement: (1) chemokinesis, in which cell movement occurs in a random manner, and (2) chemotaxis, in which cell movement occurs in a unique direction determined by an increasing concentration of a stimulus, referred to as a chemoattractant.

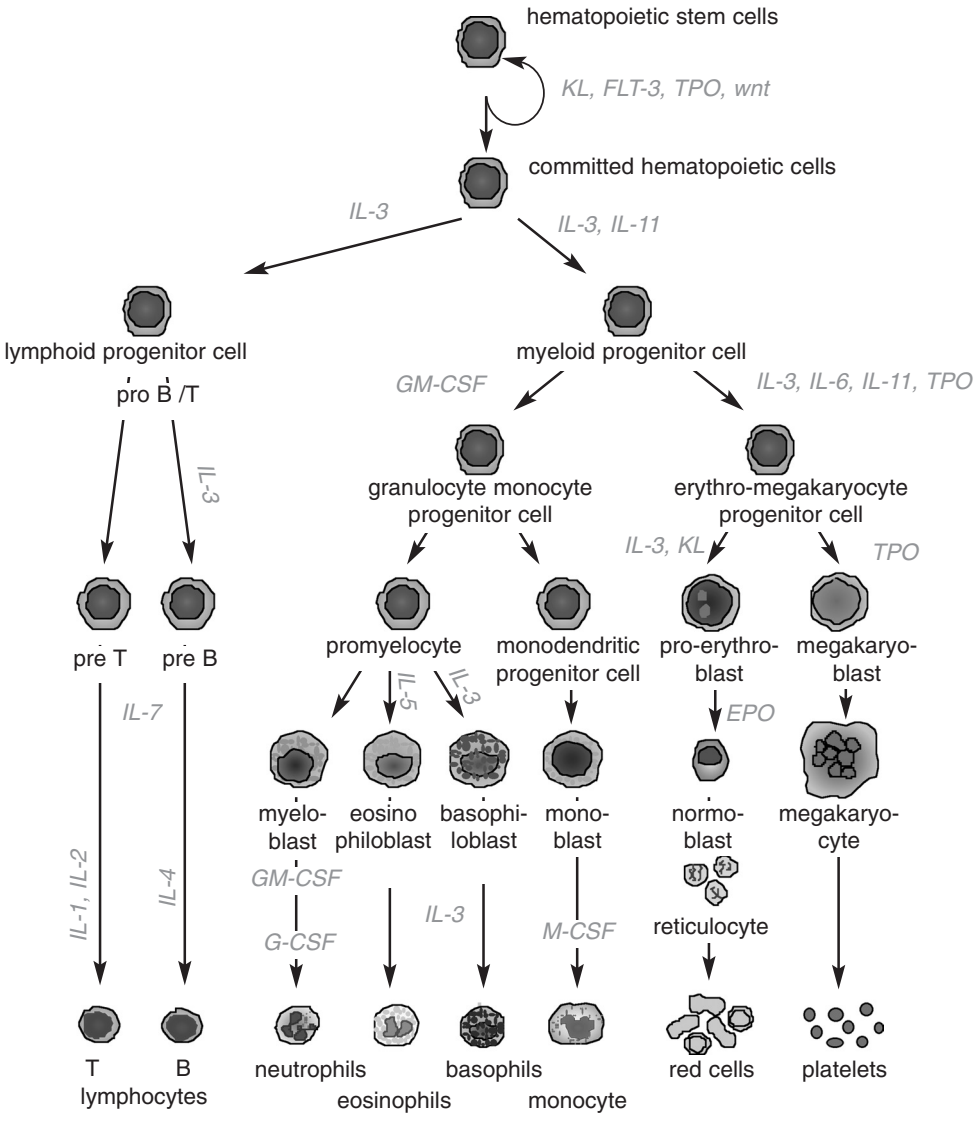

Figure 1. Schematic representation of hematopoiesis.
Hematopoietic stem cells have both self-renewal capacity and the ability to differentiate into all the distinct blood cell types. These processes require distinct hematopoietic growth factors including KL (Kit ligand), TPO (thrombopoietin), IL-3 (interleukin-3), GM-CSF (granulocyte/macrophage-colony stimulating factor), EPO (erythropoietin), G-CSF (granulocyte-colony stimulating factor) and M-CSF (macrophage-colony stimulating factor).

1.2 Hematopoietic growth factors (HGFs)

HGFs are glycoproteins involved in hematopoietic homeostasis (Figure 1). HGFs such as interleukin-3 (IL-3), IL-2, IL-7, granulocyte/macrophage-colony stimulating factor (GM-CSF) and stem cell factor (SCF), are produced by lymphocytes, monocytes, macrophages, fibroblasts, endothelial or stromal cells[4]. Specialized cells in the kidney[3,5] and liver[6,7] produce erythropoietin (EPO) and thrombopoietin (TPO), respectively. HGFs can act at the site of production or they can travel via plasma to the required areas. During normal hematopoiesis and under non-stress conditions, the HGF levels are relatively low but under certain circumstances, e.g. loss of blood or infection, they can rapidly increase. Most HGFs are multifunctional, promoting proliferation, differentiation and survival of hematopoietic cells, and also stimulating the function of mature cells. However, not all HGFs induce a positive regulatory effect. Several HGFs can act as negative regulators. For instance, TGFβ (transforming growth factor β) and MIP-1α (macrophage inflammatory protein 1α) inhibit proliferation of hematopoietic cells[8,9]. Other HGFs, such as IL-4 (interleukin-4) and TNF (tumor necrosis factor), may induce either positive or negative effects on proliferation, depending on the hematopoietic cell type and developmental stage[9-11]. HGFs can be classified into two rough groups, early-acting lineage-non-specific factors and late-acting lineage-specific factors. The first group stimulates the growth of HSC and multipotential progenitor cells, but do not induce lineage-specific differentiation. For example, IL-3, IL-11 and GM-CSF stimulate proliferation of multipotential progenitor cells but do not support terminal differentiation. The second group comprises a large variety of HGFs that support growth of lineage-specific cells and promote terminal differentiation. This group includes G-CSF, EPO, and M-CSF, which stimulate growth of lineage-committed cells and induce terminal differentiation into neutrophils, erythrocytes or monocytes, respectively.

1.3 Leukemogenesis

Defects in the blood production system may result in leukemic disease, which is characterized by an accumulation of non-functional cells. These leukemic cells are hematopoietic cells which escape from regulatory signals, allowing increased growth and enhanced survival. Such malignant cells accumulate in the bone marrow, finally entering into the peripheral blood or even other organs. Leukemias can be classified in different groups using various parameters. Based on the course of the disease, leukemias are classified as either acute and chronic. Acute leukemias are characterized by the accumulation of immature malignant cells in the bone marrow and present a highly aggressive phenotype, whereas chronic leukemias are caused by more mature, less aggressive, malignant cells. Further classification is based on the affected lineage, i.e. myeloid or lymphoid. On the basis of these criteria, blood disorders can broadly be classified as acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoid leukemia (ALL) and chronic lymphoid leukemia (CLL). Each of these groups can be subdivided into more

specific classes by means of morphology, cytochemistry, flow cytometry, cytogenetics and molecular genetics. As this thesis focuses on AML, the next paragraph will provide general information on the genetic background of this leukemia subtype.

Leukemogenesis is a multi-step process involving cooperation of several disease genes[12,13]. Since AML rarely presents an inherited predisposition, identification of AML-specific genes could not be carried out by linkage analysis, which is frequently used in other cancer types for identification of disease-related genes. The molecular defects involved in AML development are complex, and not until the last decade has a reasonable base of knowledge been accumulated. Elucidation of the genetic bases in AML has relied on cloning and characterization of acquired and recurrent chromosomal breakpoints, especially translocations, in hematopoietic progenitors. These genetic abnormalities in AML may result in aberrant expression of proto-oncogenes or inactivation of tumor suppressor genes. Translocations lead to overexpression of a proto-oncogene, which usually encodes for a transcription factor[14], or in generation of a fusion gene that results in aberrant expression of a fusion protein. Frequent translocations in AML include t(8,21), which produces AML1/ETO[15], and t(15,17), producing PML/RARα[16,17]. Activation of proto-oncogenes may also occur due to mutations[18] or viral infections[19]. Inactivation of tumor suppressor genes requires the loss of both alleles of the gene. Identification of most tumor suppressor genes is therefore based on loss of heterozygosity. Tumor suppressors usually encode for negative regulatory elements involved in the cell cycle, for instance cyclin-dependent kinase inhibitors p15 and p16[20], or the apoptosis regulator p53[21]. In addition, subtle genetic alterations, such as point mutations, deletions or insertions, have also been identified in several hematopoietic genes in AML patients.

1.4 Retroviral insertional mutagenesis and common virus integration sites

In the past decade, retroviral insertional mutagenesis has been extensively used to identify novel genes involved in leukemias and lymphomas[23-25]. Newborn mice injected with a retrovirus develop leukemias and lymphomas within a few months after infection. The viral DNA integrates into the host genome and may interfere with normal transcriptional and/or translational regulation of target genes. The nature of viral interference depends on both the orientation of the viral DNA and the site of integration in the cellular target gene. For instance, transcriptional activation of cellular genes can occur resulting in mRNA overexpression of the target gene. Alternatively, a truncated mRNA may be synthesized as a result of viral insertion in the coding region of the target gene. Complete gene inactivation may also occur. Viral integrations found in the same genomic locus in independent tumors are defined as common virus integration sites (cVIS), and they mark the position of potential proto-oncogenes. The power and reliability of retroviral insertional mutagenesis is now widely accepted, and is demonstrated by the fact that well known proto-oncogenes and tumor suppressor genes have been targeted by this technique, e.g. *N-myc*[24,26], *Evi1*[27-29], and p53[30,31].

To identify novel transforming genes involved in myeloid leukemia development we previously applied retroviral insertional mutagenesis[32-34]. Newborn NIH-Swiss mice were injected with Cas-Br-M MuLV, and a large panel of VIS was generated[25]. Interestingly, we identified insertions in the gene encoding the peripheral cannabinoid receptor *Cb2*, located in the locus *Evi11*[32,34]. Viral insertions were observed in the first intron and in the 3'non-coding region of *Cb2* (Figure 4 indicates the position and the orientation of the viral insertions in this locus). These findings suggest that *Cb2* is a proto-oncogene involved in leukemia development and that the mechanism of transformation may be related to overexpression of this receptor

The work presented in this thesis focuses on the gene encoding the peripheral cannabinoid receptor *Cb2*, a novel proto-oncogene identified as a cVIS (*Evi11*) in Cas-Br-M MuLV-induced myeloid leukemias in NIH/Swiss mice. We investigated whether and how the Cb2 receptor is involved in leukemia development, and studied the effect of Cb2 overexpression on myeloid precursor cells (Chapter 2, 3 and 4).

1.5 Cannabinoid receptors and cannabinoids: a trip through history

Cannabis sativa, popularly known as hashish or marijuana, is the most widely used drug in the world. The plant was already being used for its recreational and medicinal properties by ancient civilizations, such as the Egyptians and Hindus, who believed it had power to cure diseases, relieve pain and give vital energy. Currently *Cannabis* is still used for medical purposes, although in most countries such therapeutical use remains illegal. It has been shown to reduce tremors (in multiple sclerosis patients) and to stimulate appetite and reduce nausea (in AIDS and chemotherapy-treated cancer patients). One of the main obstacles to legalization in the medical field is the complex chemical composition of the plant, which is variable and not completely known. Marijuana contains around four hundred compounds, of which sixty belong to the cannabinoid family[35]. Its major psychoactive ingredient, Δ^9-tetrahydrocannabinol (Δ^9-THC), was isolated by Mechoulam et al in 1964, starting interest in cannabinoid research and in the development of synthetic analogs, including CP55,940, which has become one of the most extensively used cannabinoid agonists. In 1988, radiolabelled [^{3}H]CP55,940 was used to show specific cannabinoid binding sites in rat brain[36]. In 1990, Matsuda et al[51] identified and cloned the receptor responsible for CP55,940 binding. Importantly, a few years later a second cannabinoid receptor was cloned from the human promyelocytic cell line HL60[37]. These cannabinoid receptors were subsequently designated as central cannabinoid receptor Cb1, which is mainly expressed in brain, and peripheral cannabinoid receptor Cb2, mainly expressed in the immune system. The presence of cannabinoid receptors in mammals led investigators to search for endogenous ligands or endocannabinoids. The first endocannabinoid was found in brain samples and was identified as an amide of ethanolamine and arachidonate, which received the name anandamide (ananda is Sanskrit for internal bliss)[38]. To date, two more endocannabinoids have been identified, 2-arachidonoylglycerol, found

in canine gut and brain[39,40], and 2-arachidonoyl glyceryl ether, isolated from porcine brain[41].

During the last decades the number of publications involving cannabinoid receptors and/or cannabinoid ligands has been increasing drastically, as has support the existence of an endocannabinoid system. We now partially understand the molecular mechanisms of cannabinoid action on mammalian cells, but important questions remain. The work presented in this thesis provides new insights into the function of the peripheral cannabinoid receptor, Cb2, in hematopoiesis and leukemia.

1.6 Molecular biology of the cannabinoid receptors

Cannabinoid receptor genes have been cloned, sequenced and characterized in multiple vertebrates and non-vertebrate species. They exist in humans, monkeys, cats, pigs, frogs, zebra fish and *C. elegans*. Cannabinoid receptor genes have been well conserved during evolution[42-45], suggesting that they encode functionally important proteins.
Both Cb1 and Cb2 contain an N-terminal extracellular domain with multiple glycosylation sites, seven transmembrane segments, and a C-terminal intracellular domain. Cannabinoid receptors are thus members of the family of seven transmembrane G protein-coupled receptors (GPCR) (Figure 2).

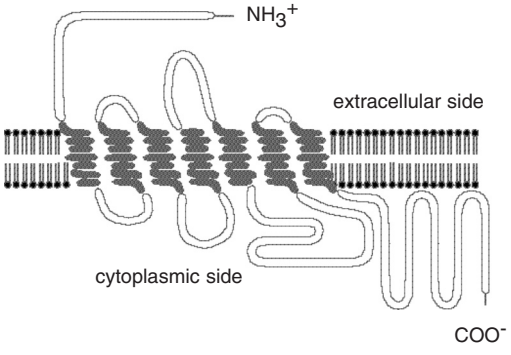

Figure 2. Schematic representation of Cb2.
Illustration of this seven transmembrane receptor located at the plasma membrane, containing an N-terminal domain on the extracellular side, three extracellular loops, seven transmembrane domains, three intracellular loops and a C-terminal tail on the cytoplasmic side.

The central cannabinoid receptor gene, *CB1*, is located on human chromosome 6q14-15 and on mouse chromosome 4 (proximal region). Although the coding sequence is located in a single exon, the presence of at least one additional exon containing a 5'-untranslated sequence has been described. The human gene encodes for a 472 amino acid protein, whereas the rat and mouse genes encode 473 amino acid proteins (Figure 3).

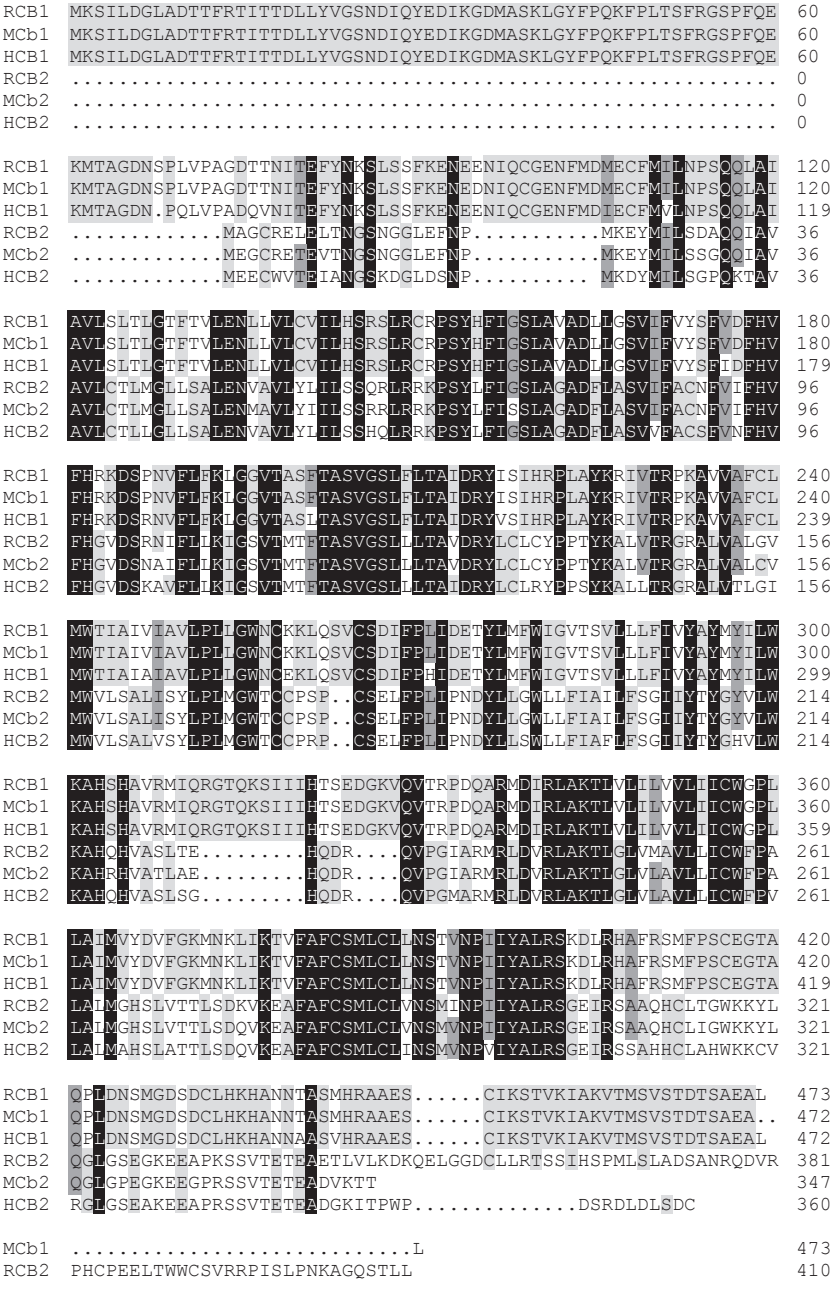

Figure 3. Protein sequences of the rat, mouse and human cannabinoid receptors.
Amino acid comparison of the rat CB1 (RCB1), mouse Cb1 (MCb1), human CB1 (HCB1), rat CB2 (RCB2), mouse Cb2 (MCb2) and human CB2 (HCB2). Identities are illustrated in black background, high similarities in dark gray and low similarities in light gray.

The central cannabinoid receptor is highly homologous across species; at the protein level the human CB1 is 96% identical to mouse Cb1. The other cannabinoid receptor, Cb2, shows more variation between species, although mouse Cb2 is 83% identical in amino acid sequence to the human CB2 (Figure 3). The Cb2 coding region is contained in a single exon of the gene (Figure 4), but available cDNA sequences indicate that there is at least one additional exon, two in mice, containing 5'-untranslated sequences. The *CB2* gene encodes for a 360 amino acid protein in humans, whereas a truncated form lacking the C-terminal 13 amino acids is present in mouse (Figure 3). The overall amino acid identity between human CB1 and CB2 is 39%. Although this might suggest significant evolutionary divergence, the amino acid identities can be as high as 82% in particular transmembrane regions, suggesting conservation of essential functional domains.

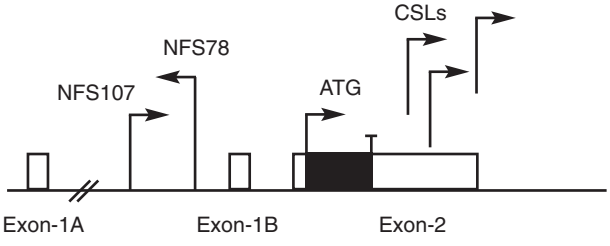

Figure 4. Genomic organisation of mouse *Cb2*.
Cb2 gene contains two exons, 1A and 1B, which do not contain protein coding sequences but splice to exon-2. The black box represents the Cb2 open reading frame. Arrows indicate the location and orientation of viral integrations in NFS78, NFS107 and several Cas-Br-M MuLV-induced primary tumors (CSLs).

1.7 Cannabinoid ligands

Cannabinoids are lipophilic compounds that can be classified according to their origin, i.e. natural ligands (extracted from the plant *Cannabis Sativa*), synthetic analogs (chemically derived) and endocannabinoids (endogenous ligands) (Table 1). These compounds have been identified as cannabinoid ligands based on their capacity to bind to cannabinoid receptors. Ligands are broadly divided into three different groups based on the effect they have on the receptor state. Similar to most ligands binding GPCRs, cannabinoid ligands can function as agonist, antagonist or inverse agonist. The fact that not all ligands bind with the same affinity to Cb1 and Cb2 (Table 1) has been very useful in studying the expression and functionality of each cannabinoid receptor subtype. Some general information about cannabinoid ligands used in this thesis is provided below (see also Table 1).

Δ^9-THC, the first cannabinoid ligand identified, was isolated from *Cannabis sativa* plants. Δ^9-THC shows significant binding to cannabinoid receptors at submicromolar concentrations, with similar affinities for Cb1 and Cb2. On CB1 it behaves as a partial agonist[46,47] although it has also been reported to behave as an antagonist in several

assays[47-49]. On CB2, the relative intrinsic activation potential of Δ^9-THC is even less that shown for CB1, and this compound may also work as a CB2 antagonist[50].

Other natural ligands, like Δ^8-THC[51], cannabidiol and cannabinol, have binding affinities for CB1 and CB2 similar to that of Δ^9-THC, without the receptor selectivity. However, significant differences have been described in their abilities to activate CB1 or CB2[52,53].

CP55,940 is a synthetic compound structurally derived from Δ^9-THC. CP55,940 presents similar binding affinities for both receptor types and behaves as a full agonist [47,54-56].

WIN55,212-2 is a synthetically derived compound with an aminoalkylindole structure. It displays high affinity for Cb1 and Cb2, and it may act as a full agonist in both receptor types[47,54-58].

Anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the two most studied endocannabinoids. They present an eicosanoid chemical structure and show similar binding affinities for both cannabinoid receptors. AEA acts as a partial agonist for CB1, and possesses even less relative intrinsic activity for CB2[47,58-60]. 2-AG is an agonist for CB1 and CB2[61-63] and exhibits higher relative intrinsic activity than AEA for both receptors[58,64,65].

SR141716 and SR144528 are diarylpyrazole compounds produced by the company Sanofi Recherche (Montpellier, France) and they display great selectivity for Cb1 or Cb2, respectively. These compounds potently block agonist-induced effects[66-70]. However, it has also been demonstrated that they can evoke inverse agonist responses[57,70-75].

Ligand	Origin	CB1 Ki value (nM)	CB2 Ki value (nM)	Reference
CB1-selective ligands				
SR141716	Synthetic	5.6	>1000	104
		1.98	>1000	104
CB2-selective ligands				
SR144528	Synthetic	437	0.6	73
		305	0.3	73
Ligands without marked selectivity				
Anandamide	Endocannabinoid	543	1940	105
		252	581	39
2-Arachidonoylglycerol	Endocannabinoid	472	1400	39
		58.3	145	63
CP55940	Synthetic	1.37	1.37	104
Δ^9-THC	Natural	35.3	3.9	104
		53.3	75.3	105
Δ^9-THC	Natural	47.6	39.3	106
WIN55,212-2	Synthetic	9.94	16.2	104
		62.3	3.3	105
Cannabinol	Natural	308	96.3	53
Cannabidiol	Natural	4350	2860	53

Table 1. Cannabinoid ligand origin and selectivity.
Ki (inhibition constant) values of certain cannabinoid ligands for Cb1- and Cb2-specific binding. Displacement was performed on [^{3}H]CP55,940, [^{3}H]R-(+)-WIN55212, or [^{3}H]HU-243.

Part of the work presented in this thesis focuses on the effects of a large panel of cannabinoid ligands on migration and neutrophilic differentiation of Cb2-expressing cells. Migration of hematopoietic cells can be studied by in vitro transwell migration. This assay is based on an upper and a lower chamber, which are separated by a microporous membrane. Cells are placed in the upper compartment and the ligand is placed in the lower chamber. After 4 hours of incubation at 37°C, cells present in the lower chamber can be collected and quantified. In this thesis, transwell assays have been performed to assess whether our receptor of interest, Cb2, is involved in migration of hematopoietic cells. We studied the effect of several agonists and antagonists on migration, and analyzed whether the observed cell movement was due to chemokinesis and/or chemotaxis (Chapter 2).
To study whether the receptor Cb2 interferes with neutrophilic differentiation of myeloid precursors the 32D/G-CSF-R cell line was used. This murine, myeloid, IL-3-dependent cell line ectopically expresses the full length human G-CSF receptor and is an excellent model to study G-CSF-induced neutrophilic differentiation. 32D/G-CSF-R cells require IL-3 for proliferation but differentiate into mature neutrophils in the presence of G-CSF. In Chapter 3, this cell system was used to analyse whether Cb2 overexpression may interfere with granulocytic differentiation of myeloid precursors. In Chapter 4 the effect of the distinct cannabinoid agonists and antagonists on neutrophilic differentiation was determined.

1.8 Cannabinoid receptor expression

Cannabinoid receptor expression has been investigated in considerable detail. Radiolabelled ligand binding studies using mainly [^{3}H]CP55,940, in situ hybridization, and immunocytochemistry have provided a clear picture of Cb1 distribution, which in general follows the same pattern in all species. Cb1 has been detected both in the nervous system and in certain peripheral tissues. High Cb1 expression has been demonstrated in the cerebral cortex, hippocampus, basal ganglia and cerebellum, whereas relatively lower levels have been found in hypothalamus and spinal cord[76-78]. In addition to the central nervous system, Cb1 is widely expressed in the peripheral nervous system, both on sensory nerve fibers and in the autonomic nervous system[79]. Moreover, *Cb1* mRNA can be found to a lower extent in peripheral tissues, including the adrenal gland, bone marrow, heart, lung, prostate, testis, thymus, tonsils and spleen[80-83]. In contrast, Cb2 has not been detected in the nervous system but rather is present in peripheral tissues, with high levels of expression in the immune system. Cb2 expression analysis has been carried out using mainly radiolabelled ligand binding assays, RT-PCR and immunocytochemistry. *Cb2* mRNA is found in spleen, thymus, tonsils, bone marrow and pancreas[37,81,82,84-86], and the rank order of *Cb2* mRNA in hematopoietic cells is as follows: B cells > natural killer cells > monocytes > neutrophils > T8 cells and T4 cells[81,82]. Cb2 protein has been localized in a large variety of immune cell types and tissues. Ligand binding assays demonstrated the presence of Cb2 protein in areas enriched for B lymphocytes, i.e. in the marginal zone

of the spleen, in the cortex of lymph nodes, in the nodular corona of Peyer's patches and in the mantle zones of secondary follicles in tonsils[37,82,87,88]. On the other hand, specific binding was absent in T lymphocyte-enriched areas, such as thymus, and in macrophage-enriched areas, such as liver and lung.

1.9 Cellular signal transduction linked to cannabinoid receptors

Cannabinoid receptors belong to the family of G protein-coupled receptors. G proteins consist of three heterologous subunits (α, β, and γ) which are stably associated to GDP during the inactive state. Upon ligand activation of the receptor, GDP is exchanged for GTP and the trimer dissociates into a Gα-GTP subunit and a G$\beta\gamma$ dimer, both capable of activating intracellular signaling pathways. Cannabinoid receptors couple specifically to Gαi proteins, meaning that receptor activation may cause a downregulation of intracellular cAMP levels[51,54]. The G$\beta\gamma$ dimer may activate additional signal transduction routes, including the MEK/ERK, p38/MAPK and JNK[89,90] pathways.
Signaling via cannabinoid receptors may thus involve multiple distinct signaling pathways, and supporting this, a large variety of routes have been linked to Cb1 and Cb2 upon receptor stimulation. Inhibition of adenyl cyclase (AC) has been reported in brain tissue and neuronal cells expressing CB1, and in human lymphocytes and mouse spleen cells expressing CB2[51,91,92]. This cannabinoid receptor-mediated inhibition of AC has been shown to be pertussis toxin-sensitive, indicating the requirement of Gαi proteins[91-93]. Additionally, cannabinoid receptor activation has been shown to stimulate tyrosine phosphorylation of focal adhesion kinase (FAK) in hippocampal slices, an effect that could be reversed by SR141716 and pertussis toxin[94]. Signal transduction via the MAPK pathway, including p38 and p42/44, has been demonstrated for CB1 as well as for CB2[95-99]. In some cases the CB1 signaling could be blocked by wortmannin, implicating PI3-K as a mediator of this pathway[97,100]. In addition, CB1 has been shown to modulate various types of K^+ and Ca^{2+} channels in a pertussis toxin sensitive manner upon agonist stimulation, also indicating involvement of Gαi proteins[101,102]. Moreover, it has been shown that CB1 can functionally interact with tyrosine kinase receptors, such as fibroblast growth factor (FGF) and insulin-like growth factor receptor, and consequently affect the intracellular signaling evoked by the tyrosine kinase receptor[57,103]. Finally, although GPCRs, may be capable of transducing extracellular signals to the intracellular network in a G protein-independent manner, there is no solid evidence indicating that the cannabinoid receptors act this way.

In Chapter 3 and 4 we analyzed which signal transduction pathway(s) may be involved in the Cb2 receptor-mediated effects. Using inhibitors of distinct signaling routes and a cAMP analog we determined whether the different pathways are crucial in mediating Cb2 effects and, moreover, we determined whether those functions require G proteins.

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CHAPTER 2

Hematopoietic cells expressing the peripheral cannabinoid receptor migrate in response to the endocannabinoid 2-arachidonoylglycerol.

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Blood, 15 April 2002; 99(8):2786-93

ABSTRACT

Cb2 is a novel protooncogene encoding the peripheral cannabinoid receptor. Previous studies demonstrated that two distinct noncoding first exons exist: exon-1A and exon-1B, which both splice to protein-coding exon-2. We demonstrate that in retrovirally induced murine myeloid leukemia cells with proviral insertion in *Cb2*, exon-1B/exon-2 *Cb2* messenger RNA levels have been increased, resulting in high receptor numbers. In myeloid leukemia cells without virus insertion in this locus, low levels of only exon-1A/exon-2 *Cb2* transcripts were present and receptors could not be detected. To elucidate the function of Cb2 in myeloid leukemia cells, a set of in vitro experiments was carried out using 32D/G-CSF-R (granulocyte-colony stimulating factor receptor) cells transfected with exon-1B/exon-2 *Cb2* complementary DNA and a myeloid cell line carrying a virus insertion in *Cb2* (ie, NFS 78). We demonstrate that a major function of the Cb2 receptor is stimulation of migration as determined in a transwell assay. Exposure of Cb2-expressing cells to different cannabinoids showed that the true ligand for Cb2 is 2-arachidonoylglycerol (2-AG), which may act as chemoattractant and as a chemokinetic agent. Furthermore, we observed a significant synergistic activity between 2-AG and interleukin-3 or G-CSF, suggesting cross-talk between the different receptor systems. Radioactive-ligand binding studies revealed significant numbers of Cb2 receptors in normal spleen. Transwell experiments carried out with normal mouse spleen cells showed 2-AG-induced migration of B220-, CD19-, immunoglobin M- and immunoglobin D-expressing B lymphocytes. Our study demonstrates that a major function of Cb2 receptor expressed on myeloid leukemia cells or normal splenocytes is stimulation of migration.

INTRODUCTION

Two distinct cannabinoid receptors have previously been identified: the central (Cb1)[1] and the peripheral (Cb2) cannabinoid receptor[2]. In retrovirally induced murine leukemia, proviral insertions frequently occur in the gene encoding *Cb2*, suggesting that the peripheral cannabinoid receptor is an oncoprotein[3,4]. The cannabinoid receptors belong to the superfamily of 7-transmembrane G protein-coupled receptors (GPCRs). Several GPCRs have been shown to be involved in cell growth and oncogenesis as the result of aberrant expression[5-7]. Examples of GPCRs with transforming ability are the α1B-adrenergic[8], thrombin[9] and serotonin 1c receptors[10] and the receptor encoded by *MAS* oncogene[11,12]. Our previous observation that *Cb2* is a common virus integration site suggests that aberrant expression of this 7-transmembrane receptor may be a critical event in transformation in certain cases of leukemia[3]. The protein-coding region of *Cb2* is located on a single exon (exon-2) of approximately 4 kilobases. Recently we identified 2 distinct 5' noncoding exons (ie, exon-1A and exon-1B) previously designated exon-1 and exon-1', respectively[3]. In the study presented here we first carried out experiments to investigate *Cb2* messenger RNA (mRNA) transcripts and protein expression in leukemic cells with or without retroviral insertion in the *Cb2* locus. Secondly, we

performed studies to determine the function of the peripheral cannabinoid receptor when overexpressed in myeloid cells.

GPCRs have been related to many functions, including cell proliferation, maturation, survival, apoptosis or migration[6,13,14]. In the present study, we investigated the function of the peripheral cannabinoid receptor when overexpressed on myeloid cells (ie, 32D/G-CSF-R [granulocyte-colony stimulating factor receptor]) in which we overexpressed exon-1B/exon-2 *Cb2* splice variant and a myeloid leukemia cell line containing a virus insertion in the *Cb2* locus, NFS 78. We also wished to determine which of the large panel of Cb2 ligands that have been identified previously is the true agonist of the receptor. We investigated the effects of natural (Δ^8-tetrahydrocannabinol [Δ^8-THC][15], Δ^9-tetrahydrocannabinol [Δ^9-THC][16], cannabinol[17] and cannabidiol[16]), synthetic (WIN 55,212-2[18] and CP55,940[19]) and endogenous (2-arachidonoylglycerol [2-AG][20,21], anandamide [AEA][22,23], N-palmitoylethanolamine [PEA][24] and N-acylethanolamine [POEA][24]) cannabinoids. We show that 2-AG is the most potent agonist for the Cb2 receptor and that a major function of 2-AG is stimulation of migration. We further studied whether 2-AG acts as a chemotactic or chemokinetic agent and whether cytokines, such as interleukin-3 (IL-3) or G-CSF, increase the stimulatory effect of 2-AG. Because the other cannabinoid ligands are also able to bind to the peripheral cannabinoid receptor, we investigated whether these compounds either synergize or antagonize with the stimulatory effect of 2-AG. Next, we investigated Cb2 receptor expression and function in normal spleen and thymus. We show that Cb2 receptors are normally expressed on $B220^+$ splenocytes and that the major function of the peripheral cannabinoid receptor on these B lymphocytes is regulation of migration as well.

MATERIAL AND METHODS

Cell lines

The myeloid cell lines NFS 58, 61, 70 and 78 were established from Cas-Br-M murine leukemia virus-initiated primary tumors[25]. The 32D/G-CSF-R cell line[26] was kindly donated by Prof. Dr. IP Touw (Erasmus University Rotterdam). The cell lines were cultured in RPMI 1640 medium (Life Technologies, Breda, The Netherlands) supplemented with penicillin (100 IU/ml), streptomycin (100ng/ml), murine IL-3 (10 ng/ml) and 10% fetal calf serum (FCS) (Life Technologies).

Ligands and cannabinoid ligands

Recombinant human stromal cell-derived factor (SDF-1α) was obtained from R&D systems (Uithoorn, The Netherlands). The Cb2 ligands used were 2-AG, AEA, WIN 55,212-2, cannabinol, cannabidiol, Δ^8-THC and Δ^9-THC from Sigma (Zwijndrecht, The Netherlands). PEA and POEA are from ICN Biomedicals (Zoetermeer, The Netherlands) and CP55,940 from Pfizer (Groton, CT). Cb1-specific antagonist SR141716 and Cb2-specific antagonist SR144528 were kindly donated by Dr. Casellas (Sanofi Recherche, Montpellier, France).

***Cb2* expression constructs and transfection of 32D/G-CSF-R cells**

Cb2 exon-1B plus exon-2, which was hemagglutinin (HA)-tagged at the 5' end (*Eco*RI/*Nco*I), was cloned as *Hin*dIII/*Bam*HI fragment into *Hin*dIII/*Bgl*II sites of pLNCX. The expression construct was transfected into 32D/G-CSF-R by electroporation (230V, 100 microfarads, and 1000 milliseconds). Following gene transfer, cells were cultured in RPMI 1640 medium supplemented as above for 48 h and then selected in neomycin at concentrations of 0.8 mg/ml. Neomycin-resistent clones were expanded. To study *Cb2* mRNA expression, ribonuclease (RNase) protection analysis was applied. Because mouse Cb2-specific antibodies are not yet available and HA antibodies are not capable of detecting HA-Cb2, Cb2 protein expression was analyzed by ligand binding (see below).

RNase protection analysis

RNase protection analysis was performed as described previously[27]. Total cellular RNA was prepared from kidney, heart, spleen, and thymus by homogenizing tissue cells in 4M guanidium thiocyanate, followed by phenol-chloroform extraction and isopropanol precipitation[28]. RNA from NFS cell lines and 32D/G-CSF-R-transfected cells was isolated using 4M guanidium thiocyanate or Trizol Reagent (Life Technologies). The RNA samples were subjected to an RNase protection assay, essentially as described by the supplier (Promega, Leiden, The Netherlands). A 249-base pair (bp) fragment (bp -147 to bp +102 of *Cb2* complementary DNA [cDNA], probe P) (Figure 1A) was cloned into a pBluescript II SK+ vector and linearized with *Hin*dIII. A radiolabeled *GAPDH* RNA fragment was used as a control[29].

Membrane preparation and [^{3}H]CP55,940 binding assays

Frozen cell and tissue pellets were kept at -80°C until use. Pellets were thawed and suspended in assay buffer (50 mM Tris.HCl [pH 7.0], 1 mM ethylenediaminetetraacetic acid, 3 mM $MgCl_2$, 100 µM phenylmethylsulforyl fluoride [pH 7.4] containing 0.1% bovine serum albumin (BSA) [Serva, Heidelberg, Germany]), and membrane suspensions were homogenized and centrifuged at 10000g for 10 min (4°C). Pellets were then resuspended in 5 ml in assay buffer, homogenized using a Potter-Elvejhem homogenizer, and resuspended in assay buffer at a final membrane concentration equivalent to 10^6 cells/ml. For binding experiments, 160 µl membrane suspension (10^6 cells/ml) was incubated in 96-well plates (flat-bottom plates, Greiner) with 20 µl [^{3}H]CP55,940 (DuPont-New England Nuclear) in concentrations ranging from 0.2 nM to 1.2 nM and 20 µl assay buffer for total binding or assay buffer containing 10^{-5}M nonlabelled CP55,940 to assess nonspecific binding. Mixtures (200 µl final volume) were incubated at 30°C for 50 min, after which suspensions were filtrated over Unifilter GF/B plates using a Filtermate-196 Harvester (Packard) and washed twice for 5 sec with 200 µl ice-cold washing buffer (50 mM Tris.HCl [pH 7.0] containing 0.25% BSA). Filtration plates were sealed at the bottom, 25 µl scintillation fluid (Microscint-O, Packard) was added per well, and radioactivity was counted in a TopCount scintillation counter

(Canberra Packard). Saturation curves produced identical affinities for [^{3}H]CP55,940 (affinity dissociation constant [K_d] ranged from 0.25 to 0.5 nM). Saturation plots were constructed by plotting specific binding (ie, total binding minus nonspecific binding) against label concentration, ranging from 0.2 nM to 1.2 nM, after which non-linear curve fitting was done to estimate B_{max} and affinity. In parallel, Scatchard plots were constructed by plotting the ratio-specific binding over free-label concentration against specific binding. Cb2 binding sites with spleen cells or thymocytes (Figure 7B) were assessed in triplicate using one radioligand concentration of CP55,940 (1nM). Nonspecific binding was determined in presence of excess nonradioactive CP55,940 (10^{-6}M), and specific binding was expressed as femtomoles (fmol)/10^6 cells (Figure 7B).

Migration assay

Migration assays were performed using 6.5 mm-diameter transwells with 5 µm pore size (Corning Costar, Amsterdam, The Netherlands). The cells used for the migration assay were NFS 58, 61 and 78 cells, transfected 32D/G-CSF-R cells, and spleen and thymus cells from male FVB mice. Mice were sacrificed by inhalation of CO_2. Spleen and thymus were isolated immediately and placed on RPMI 1640 medium. Single cell suspension was prepared using 70-µm nylon cell strainer (Falcon, New Jersey). For migration assay, 1 x 10^5 or 2 x 10^5 cells were washed twice with Hanks Balanced Salt Solution (HBSS) medium, resuspended in 100 µl of migration medium (Iscoves Modified Dulbecco Medium (IMDM) plus 0.5% Bovine Serum Albumin (BSA)) and placed in the upper chamber of the transwells with or without presence of ligand. In the lower chamber, 600 µl of migration medium with or without ligand was placed. After 4 hours incubation at 37°C and 5% CO_2, the upper chamber was removed and the number of migrated cells was determined using a CASY1/TTC cell counter (Schärfe System, Germany).

Flow cytometric analysis

Spleen cells that migrated to the lower chamber in the migration assay were immunophenotyped using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). The following rat monoclonal antibodies were used in indirect immunofluorescence assays: RA3-6B2 (anti-B220/CD45R), Ter119 (LY-76), 59-AD2.2 (anti-Thy-1) and KT3 (anti-CD3). A fluorescein isothiocyanate (FITC)-conjugated goat anti-Rat immunoglobulin (Ig) (Nordic Immunological Labs, Tilburg, The Netherlands) was used as a second-step reagent. Immunophenotyping with double or triple labeling was performed combining the mentioned antibodies with allophycocyanin-conjugated anti-B220/CD45R (RA3-6B2), phycoerythrin-conjugated anti-IgD (Souther Biotechnology, Birmingham, AL), anti-CD19 (1D3), and anti-CD11c (HL3), as well as biotinylated anti-IgM (II/41), using streptavidin-allophycocyanin as a second step (Pharmingen, San Diego, CA).

RESULTS

Cb2 expression analysis in myeloid leukemic cell lines

The protein-coding region for the peripheral cannabinoid receptor is located on exon-2 of the *Cb2* gene (Figure 1A). Two distinct *Cb2* splice variants have been identified, which both comprise the Cb2 receptor coding exon but contain different nonprotein-coding first exons: exon-1A or exon-1B (Figure 1A). To determine which splice variant is present in myeloid cell lines, RNase protection was performed using a 249-bp polymerase chain reaction product (probe P) overlapping exon-1B (101 nucleotides) and exon-2 (148 nucleotides) (Figure 1A). A protected band of 249 nucleotides corresponding to exon-1B plus exon-2 *Cb2* mRNA was identified in the myeloid cell line NFS 78 (Figure 1B), which contains a retroviral insertion in *Cb2*, whereas a 148 nucleotide exon-2-protected fragment was identified in the other myeloid cell lines (Figure 1B). As demonstrated previously, this latter protected fragment represents exon-1A/exon-2 *Cb2* transcript[3,27]. Ligand binding studies using [^{3}H]CP55,940 and Scatchard plot analysis revealed the presence of significant numbers of cannabinoid binding sites on NFS 78 cells, whereas receptors were not measurable on the other IL-3-dependent myeloid cell lines (Figure 1C).

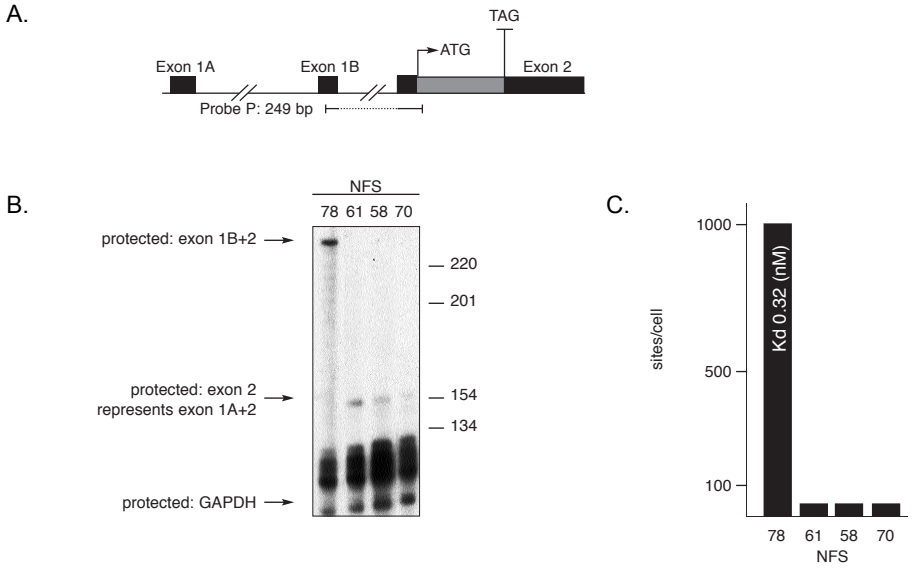

Figure 1. Expression of distinct *Cb2* mRNA splice variants and determination of receptor binding sites in mouse myeloid leukemia cell lines.

(A) Genomic organization of mouse *Cb2*. The gray box represents the Cb2 protein-coding region. Two distinct splice variants have been identified: exon-1A/exon-2 and exon-1B/exon-2. The 249 nucleotide RNA probe (Probe P) representing exon-1B/exon-2 that was used for RNase protection analysis is indicated. (B) RNase protection analysis on distinct myeloid cell lines using probe P (see panel A). (C) Cb2 receptor density and receptor affinity for CP55,940 on the NFS cell lines was assessed by saturation radioligand receptor binding experiments using [^{3}H]CP55,940. Dissociation constant (K_d) is added to the columns.

The endocannabinoid 2-AG is the true ligand for Cb2 receptor and stimulates migration of Cb2-expressing myeloid cells

Next, we wished to determine the function of the peripheral cannabinoid receptor when overexpressed in myeloid cells and investigate which of the large panel of molecules capable of binding is the true ligand for Cb2. For this purpose we used the Cb2-overexpressing myeloid cell line NFS78 as well as 2 clones of the 32D/G-CSF-R cell line in which we introduced exon-1B/exon-2 *Cb2* cDNA. High exon-1B/exon-2 *Cb2* mRNA expression was demonstrated by RNase protection (Figure 2A), and ligand binding studies demonstrated excess of [^{3}H]CP55,940 binding sites on exon-1B/exon-2 *Cb2*-transfected cells (Figure 2B). No exon-1B/exon-2 *Cb2* mRNA or ligand binding sites were detected in the vector control 32D/G-CSF-R cells (Figure 2).

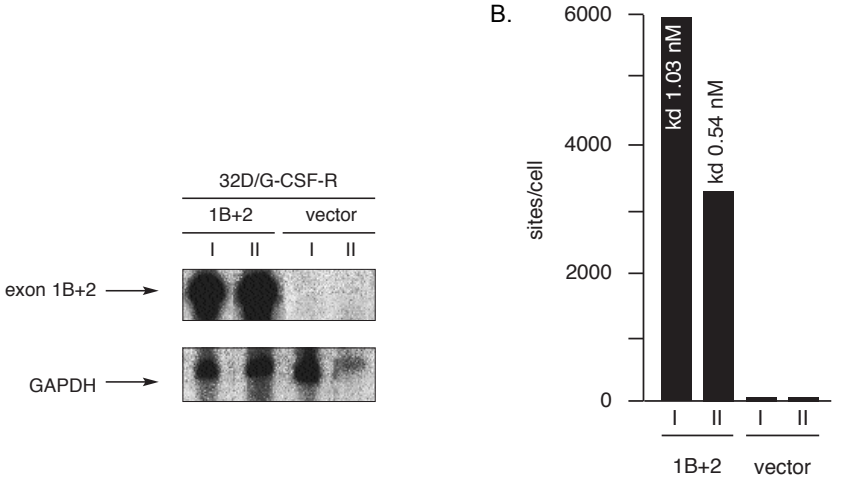

Figure 2. Messenger RNase protection analysis and ligand binding studies on transfected 32D/G-CSF-R cells.

(A) RNase protection analysis using probe P on 10 µg total mRNA from 4 distinct transfected 32D/G-CSF-R clones: 2 32D/G-CSF-R clones transfected with *Cb2* exon-1B/exon-2 cDNA and 2 clones with control vector pLNCX. (B) Cb2 receptor density and receptor affinity for CP55,940 on 2 *Cb2* exon-1B/exon-2 clones and 2 empty vector control clones. Density and affinity were assessed by saturation radioligand receptor binding experiments using [^{3}H]CP55,940. K_d is added to the columns.

Because G protein-coupled receptors may function in cell mobility, we investigated in vitro migration in transwell assays. The following cannabinoid ligands were added to the lower chamber to test their capacity to induce chemotactic migration (1 µM): AEA, 2-AG, PEA, POEA, CP55,940, Δ^8-THC, Δ^9-THC, WIN 55,212-2, cannabinol, and cannabidiol. 2-AG and, to a lesser extend, AEA stimulated migration of *Cb2*-transfected 32D/G-CSF-R (Figure 3A). None of the other ligands were capable of stimulating migration of these Cb2-

expressing cells (Figure 3A). In fact, in separate experiments using different concentrations of each of these ligands (1nM to 10 mM) 2-AG was the only potent inducer of migration at a optimal concentration of 300 nM (Figure 3B and 3C). Migration was shown to be specific because 32D/G-CSF-R parental cells as well as vector control-transfected 32D/G-CSF-R cells did not migrate in response to 2-AG (Figure 3D). Neither did we observe any migration using the cell lines NFS 58 and NFS 61 (data not shown), which were revealed to be negative in the [^{3}H]CP55,940 binding assay (Figure 1C). Moreover, addition of SR144528 (100 nM), a Cb2-specific antagonist, to the upper chamber abolished the 2-AG-mediated transwell migration of 32D/G-CSF-R/Cb2 cells completely, whereas the Cb1-specific antagonist SR141716 did not affect 2-AG-induced migration (Figure 3E).

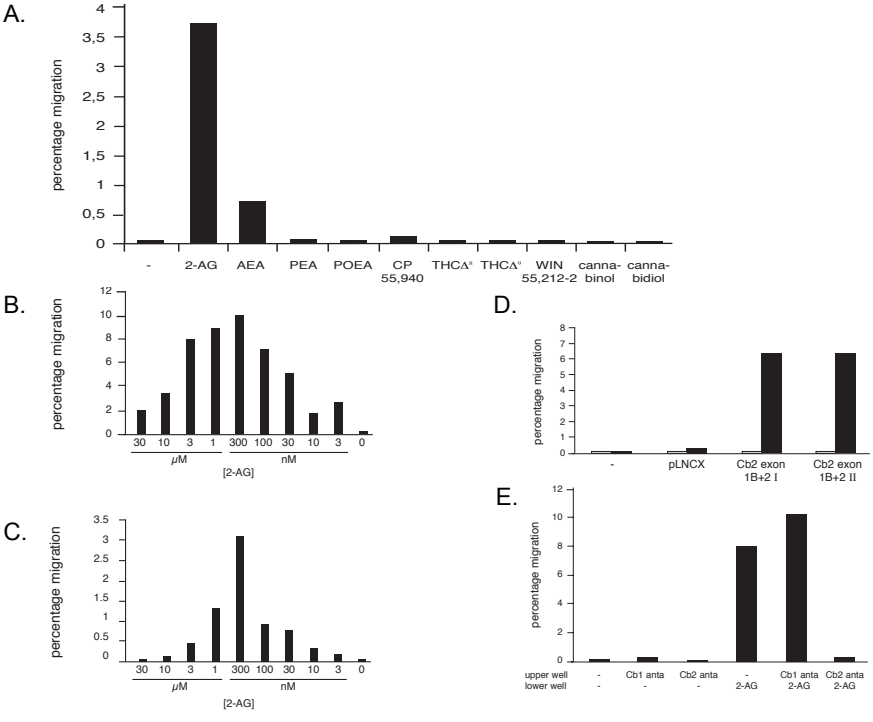

Figure 3. In vitro migration of Cb2-expressing cells following exposure to cannabinoids.
(A) Comparison of the chemoattractive effect of different cannabinoid ligands (1 μM) on *Cb2* exon-1B/exon-2-expressing 32D/G-CSF-R cells. Ligands were added to the lower chamber, and cells that migrated to the lower well were counted after 4 hours of incubation. Y-axis shows the percentage of migrated cells from an input of 1 x 10^5. (B) Effect of different concentrations of 2-AG when added to the lower chamber on the in vitro migration of Cb2-expressing 32D/G-CSF-R cells . Y-axis shows the percentage of migrated cells from an input of 1 x 10^5. (C) 2-AG titration experiment using the myeloid cell line NFS 78. Y-axis shows the percentage of migrated cells from an input of 2 x 10^5. (D) Chemoattractive effect of 300 nM of 2-AG (■) or nothing (❑) on exon-1B/exon-2 Cb2-expressing cells versus non-Cb2-expressing cells. Y-axis shows percentage of migrated cells from an input of 2 x 10^5 cells. (E) Cb2-expressing 32D/G-CSF-R cells were exposed to medium with or without 300 nM of 2-AG added to the lower well; 100 nM of Cb1-specific antagonist, SR141716, Cb2-specific antagonist, SR144528, or cells without antagonist as a control were placed on the upper well. Y-axis shows percentage of migration from an input of 1 x 10^5 cells.

2-AG stimulates chemotaxis as well as chemokinesis of Cb2-expressing myeloid cells

To investigate whether 2-AG is a chemokinetic as well as a chemotactic agent, in vitro migration experiments were carried out with 2-AG added to the lower chamber, the upper chamber, or both. When 2-AG was added to the upper and lower chamber simultaneously, approximately 50% migration was observed as compared to the chemotactic experiment (ie, with 2-AG added to the lower well only) (Figure 4). No cells migrated when 2-AG was added to the upper well only. In comparison, 32D/G-CSF-R/Cb2 cells, which express the CXC chemokine receptor 4 (CXCR4) endogenously, migrated when the CXCR4 ligand SDF-1α was added to the lower chamber. However, no chemokinetic mobility was observed when SDF-1α was added to both chambers (Figure 4).

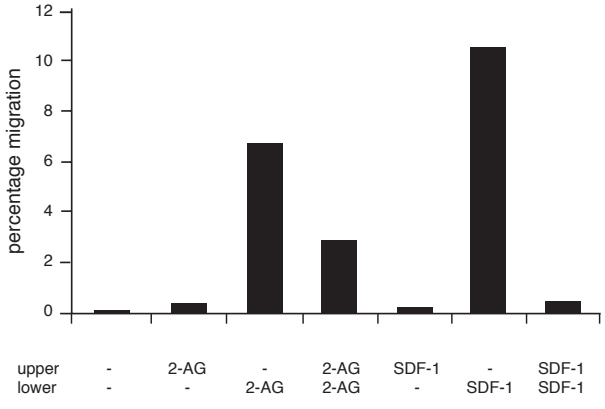

Figure 4. Chemotaxis versus chemokinesis of Cb2-expressing cells in response to 2-AG or SDF-1 using a transwell assay.

Cb2-transfected 32D/G-CSF-R cells, which naturally express CXCR4, were placed in the upper well with or without 2-AG or SDF-1α. Migration medium with or without ligand was placed in the lower well. Y-axis shows percentage of migration from an input of 1 x 10^5 cells.

Natural and synthetic cannabinoids inhibit 2-AG-induced migration, whereas endocannabinoids have no effect

Because the endocannabinoids (AEA, PEA and POEA) as well as the natural (Δ^8-THC, Δ^9-THC) and the synthetic cannabinoid ligands (CP55,940, WIN 55,212-2) are capable of binding to the peripheral cannabinoid receptor, we wondered whether these compounds could either synergize with 2-AG or antagonize the effect of this ligand in a migration assay. The synthetic cannabinoids CP55,940 and WIN 55,212-2 as well as Δ^8-THC and Δ^9-THC completely abolished 2-AG-induced chemoattraction of *Cb2*-transfected 32D/G-CSF-R cells when added either to the lower (Figure 5A) or the upper chamber in a transwell assay (data not shown). On the other hand, AEA, PEA and POEA did not affect 2-AG-induced chemoattraction (Student t test, $p>0.05$) of cells when either added to the lower (Figure 5B) or to the upper chamber in a migration assay (not shown).

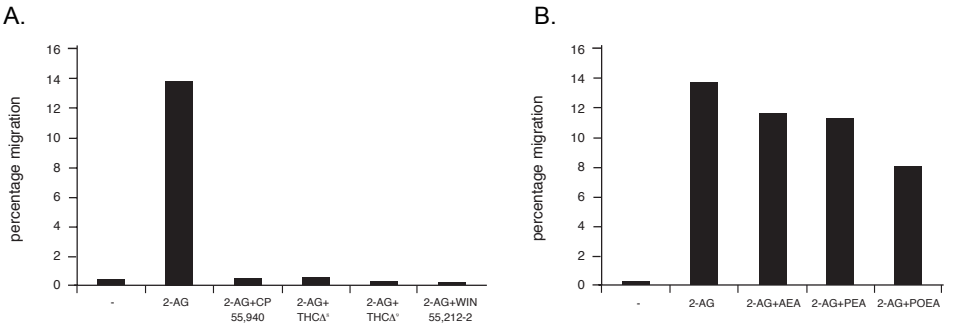

Figure 5. Effect of endocannabinoids and natural and synthetic cannabinoid ligands on the 2-AG-induced mobilization of cells.
(A) *Cb2*-transfected 32D/G-CSF-R cells were exposed to 2-AG or 2-AG plus CP55,940, Δ^9-THC, Δ^8-THC or WIN55,212-2 in a chemotactic experiment. Y-axis shows percentage of migration from an input of 2 x 10^5 cells. (B) *Cb2*-transfected 32D/G-CSF-R cells were placed in the upper well, and migration medium containing 2-AG or 2-AG in combination with AEA, PEA and POEA was added to the lower chamber. Y-axis shows percentage of migration from an input of 2 x 10^5 cells.

Effects of 2-AG in combination with IL-3 or G-CSF on migration of Cb2-expressing cells

To study whether Cb2 receptor ligands may synergize with other ligands that can activate 32D/G-CSF-R cells, we carried out migration experiments using 2-AG in combination with IL-3 or G-CSF. No migration was observed when 32D/G-CSF-R/Cb2 cells were exposed to IL-3 or G-CSF alone in a transwell assay. However, addition of 2-AG with IL-3 or G-CSF showed a significant increase in the migration rate of these cells as compared to experiments using 2-AG as a single agent (Figure 6). The same effect was observed when NFS 78 cells were exposed to 2-AG and IL-3 (data not shown).

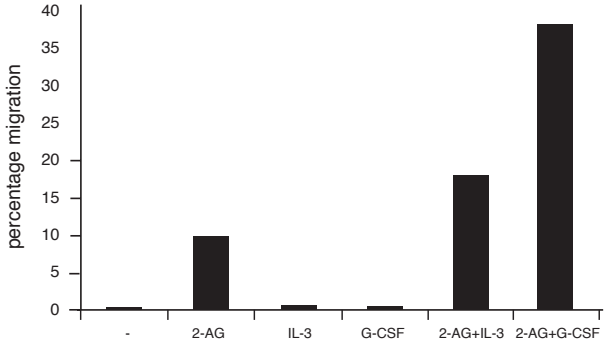

Figure 6. Synergy between 2-AG and IL-3 or G-CSF in migration stimulation.
Cb2-transfected 32D/G-CSF-R cells were added to the upper chamber in a migration assay. Cells were exposed to migration medium present at the lower well containing 2-AG with or without IL-3 or G-CSF. Y-axis shows percentage of migration from an input of 2 x 10^5 cells.

Distinct *Cb2* mRNA splice variants and protein levels in mouse spleen and thymus

The functional assays carried out so far were performed with cells that overexpress Cb2 receptor. Before elucidating whether naturally expressing Cb2 cells migrate following 2-AG exposure as well, we first investigated which hematopoietic organs express Cb2. RNase protection analysis was carried out on thymus and spleen mRNA using probe P (Figure 1A). A protected band of 249 nucleotides, corresponding to exon-1B plus exon-2 *Cb2* mRNA, was identified in spleen (Figure 7A), whereas a 148 nucleotide exon-2-protected fragment, representing exon-1A/exon-2 *Cb2* transcript, was identified in spleen and thymus (Figure 7A). *Cb2* mRNA was not detectable in the other tissues investigated[27] (Figure 7A). To study the presence of Cb2 binding sites in spleen and thymus a binding assay was carried out using a saturated concentration of 1 nM of [^{3}H]CP55,940 (determined on NFS 78 [Figure 1C]). The experiment in Figure 7B demonstrates that [^{3}H]CP55,940 binding is observed in spleen and not in thymus of normal FVB mice, which is in agreement with previous studies[30,31].

Migration of Cb2-expressing spleen cells following 2-AG stimulation

To investigate whether naturally expressing Cb2 cells migrate following 2-AG exposure as well, spleen cells from FVB mice were studied using a transwell assay. A titration experiment showed optimal migration of spleen cells in the presence of 300 nM of 2-AG (data not shown). Moreover, this migration could be blocked by addition of Cb2-specific antagonist to the upper well but not by addition of Cb1-specific antagonist (Figure 7C). No significant migration was evident of thymocytes in transwells (data not shown). Immunophenotyping by flow cytometric analysis of the 2-AG-induced migrated spleen cells compared with spontaneously migrated spleen cells revealed that the cells were B220$^+$. Double staining revealed that these B220$^+$ cells expressed CD19, IgM and IgD (Figure 7D). In addition, these cells were CD11c$^-$, indicating that the spleen-migrated cells were B lymphocytes and not B220$^+$/CD11c$^+$ dentritic cells (Figure 7D).

DISCUSSION

In the study presented here, we demonstrate that two distinct *Cb2* mRNA splice variants exist in the mouse, each composed of the same protein-encoding exon-2 but with a different nonprotein-coding first exon. In most myeloid leukemia cell lines *Cb2* exon-1A/exon-2 is expressed, which correlates with low protein expression. On the other hand, in mouse leukemia NFS 78, retroviral insertion has occurred 5' of exon-1B,[3,32] resulting in the expression of high levels of *Cb2* exon-1B/exon-2 mRNA and the appearance of Cb2 proteins on the cell surface. Cb2 receptors are also present on normal spleen cells, which express both *Cb2* splice variants: exon-1A/exon-2 and exon-1B/exon-2. On the other hand, thymocytes only express *Cb2* exon-1A/exon-2 splice variants, which are not accompanied by the presence of detectable numbers of ligand binding sites. These data suggest that in the cells studied here, Cb2 protein may be translated from exon-1B/exon-2 transcripts rather than from exon-1A/exon-2 mRNAs.

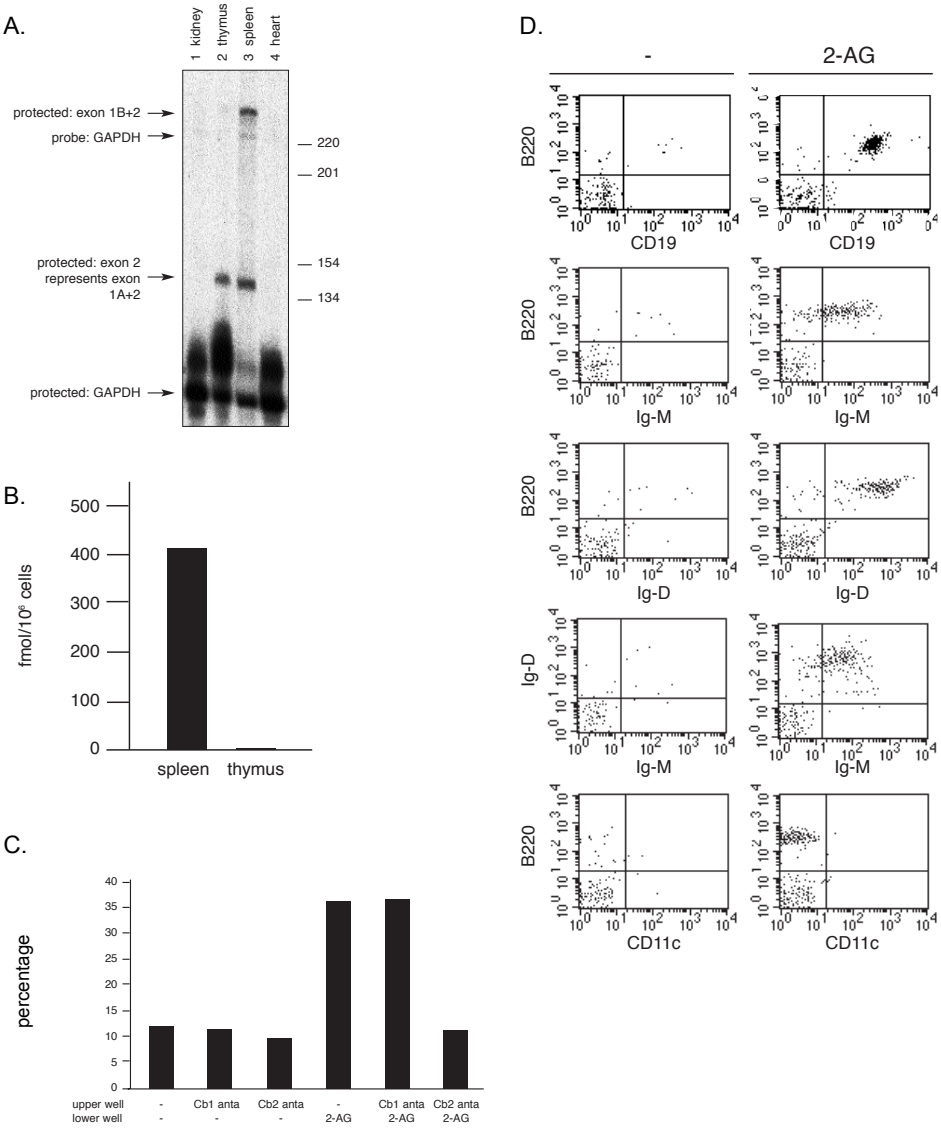

Figure 7. Cb2 expression and function in normal spleen and thymus.
(A) RNase protection on 10µg of total mouse mRNA isolated from several organs. The protected fragments were 249 nucleotides (exon-1B/exon-2 *Cb2* mRNA protected) or 148 nucleotides (exon-2 *Cb2* mRNA-protected, representing exon-1A/exon-2). A *GAPDH* probe was used for normalization of the signals. (B) Receptor density (fmol/10^6 cells) on spleen cells and thymocytes was assessed by measuring specific binding of [^{3}H]CP55,940 (1nM) (See "Materials and Methods"). (C) Effect of Cb1-specific antagonist SR141716 or Cb2-specific antagonists SR144528 on spontaneous or 2-AG-induced migration of spleen cells. Antagonists and 2-AG were placed in the wells as indicated under the figure. Y-axis shows percentage of migration from an input of 2 x 10^5 spleen cells. (D) Immunophenotyping of 2-AG-induced migrated spleen cells versus spontaneous spleen-migrated cells using flow cytometry.

These results may be explained by a regulatory mechanism of translation involving the noncoding first exons of *Cb2*. Although several mechanisms of translational control involving noncoding mRNA have been described,[33-36] the function of the 2 first exons in *Cb2* is currently unknown. Whether and how these nonprotein-coding exons may regulate protein expression is subject to future investigations.

Oncogenic transformation by GPCRs may be caused either by structural alteration of the receptor itself or by deregulated presentation of the ligands[8,37,38]. We previously demonstrated that retroviral insertion in the *Cb2* locus occurs frequently in myeloid leukemias in mice[3,32]. The data presented here suggest that proviral insertion in *Cb2* in myeloid leukemia cells may result in the selective expression of particular splice variants and overexpression of the peripheral cannabinoid receptor. GPCRs have been related to a variety of cellular functions, including cell proliferation, differentiation, survival, and migration[39,40]. To study the role of the peripheral cannabinoid receptor when overexpressed on myeloid cells, we investigated the effects of cannabinoid ligands on the myeloid cell line NFS78 and on 32D/G-CSF-R cells transfected with the *Cb2* exon-1B/exon-2 splice variant. We demonstrate that stimulation of Cb2-overexpressing cells by its potent agonist 2-AG induces migration in vitro at nanomolar concentrations. Given the role of Cb2 receptor in migration in vitro, we investigated whether 2-AG may act as a chemotactic as well as a chemokinetic agent. In contrast to SDF-1α, which is a chemoattractant that acts via CXCR4[41-45], 2-AG may stimulate both chemotaxis (directional migration) and chemokinesis (random migration), a behaviour that has been described for other receptors as well[46,47]. Interestingly, a significant increase of migration was observed when Cb2-expressing 32D/G-CSF-R cells were exposed to 2-AG in combination with IL-3 as well as with G-CSF. This set of experiments demonstrates that activation of the peripheral cannabinoid receptor by the endocannabinoid 2-AG may occur in many ways, such as by chemoattraction, chemokinesis, and in synergy with cytokines. Therefore, aberrant Cb2 expression on myeloid leukemia cells may result in changes in mobilization and, subsequently, altered homing of the leukemic precursors in vivo. In future experiments we plan to investigate the in vivo mobility of Cb2-expressing bone marrow cells using *Cb2* transgenic mice[48] or bone marrow precursor cells that have been infected with viral vectors carrying the *Cb2* gene.

We previously demonstrated that AEA is a potent inducer of proliferation in synergy with IL-3, granulocyte-macrophage-CSF or G-CSF[27]. However, we and other investigators suggested that although AEA may be an activator of Cb2, this fatty acid has also a non-receptor-mediated stimulating effect[27,49-51]. We could not detect an effect of 2-AG on survival or proliferation of Cb2-expressing cells (data not shown). However, this does not exclude the possibility that under different conditions or in combination with other cytokines this receptor may have an effect on these functions. In fact, synergy between cytokines and chemokines has been previously observed in proliferation, differentiation, and survival assays as well as in migration experiments[27,52-55]. Our findings showing increased migration when cells were stimulated with 2-AG and IL-3 as well as 2-AG and G-CSF strengthen the idea to further investigate the role that 2-AG may have in combination with cytokines in proliferation, differentiation, or survival of leukemic progenitor cells.

Several cannabinoid ligands from different origin (natural, synthetic, and endogenous) have been proposed as the true cannabinoid ligands[19,20,22,56]. In the present study we compared the effects of 2-AG with that of the other endocanabinoids (AEA, PEA, POEA), the natural cannabinoids (Δ^8-THC, Δ^9-THC, cannabinol, cannabidiol), and the synthetic molecules (WIN55,212-2, CP55,940). Among the endocannabinoids, 2-AG was the most potent inducer of migration, supporting the idea that 2-AG is the true endogenous ligand for the peripheral cannabinoid receptor[56-59]. AEA, which was the first endogenous cannabinoid ligand isolated,[22] shows similar binding affinity to the Cb2 receptor as 2-AG does[59]. However, AEA only weakly stimulated migration of Cb2-expressing cells. This observation is in agreement with other sudies demonstrating that AEA acts at the most as a partial agonist for the Cb2 receptor[59]. The fact that the other endocannabinoids (PEA, POEA) did not stimulate migration or interfere with 2-AG-induced migration may be explained by much lower affinity of these compounds for the peripheral cannabinoid receptor[60]. Neither CP55,940, which has been considered a potent cannabinoid ligand and an inducer of migration[31,61], nor any of the other synthetic or natural cannabinoids were capable of stimulating cell mobility. In fact, we observed that most of the synthetic and natural cannabinoids interfere with 2-AG-stimulated Cb2 receptor activation and function. This observation may be in agreement with previous findings suggesting that cannabinoids may one way or the other impaire the immune response[62]. This interference may include impairment of macrophage functions[63,64], induction of an imbalance in T-cell CD4/CD8 ratio[65], alteration of immunoglobin production[66-68], downregulation of natural killer cell activity[69,70], or perturbation in macrophage/T-cell cooperation[64,71]. It will therefore be of relevance to exactly define which cells express Cb2 and repond to its endocannabinoid and further dissect the function of Cb2 receptor in the immune response. In this study we demonstrate that spleen cells migrating in response 2-AG were $B220^+$, $CD19^+$, IgM^+, and IgD^+, which is an agreement with previous studies showing that Cb2 receptor is mainly expressed on B lymphocytes[31,72,73]. Furthermore, our data show that the 2-AG-induced migration could be completely blocked by SR144528, a Cb2-specific antagonist, but not by SR141716, a Cb1-specific antagonist, indicating that stimulation of migration occurs exclusively via Cb2 receptors. Previously, the function of the peripheral cannabinoid receptor has also been related to B-cell differentiation, as proposed by Carayon et al[31]. These observations together with our findings suggest a role of the peripheral cannabinoid receptor in attraction, mobilization, or activation of B cells during the immune response.

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CHAPTER 3

The peripheral cannabinoid receptor Cb2, a novel onco-protein, induces a reversible block in neutrophilic differentiation.

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Blood, 15 February 2003; 101(4):1336-1343

ABSTRACT

We previously identified a novel common virus integration site, *Evi11*, by means of retroviral insertional mutagenesis. We demonstrated that the gene encoding the peripheral cannabinoid receptor (*Cb2*) is the potential target, suggesting that *Cb2* is a proto-oncogene. To elucidate a role for this G protein-coupled receptor (GPCR) in leukemic transformation we generated a *Cb2-EGFP* cDNA construct that was introduced into 32D/G-CSF-R cells. These cells require interleukin 3 (IL-3) to proliferate in vitro, whereas in the presence of granulocyte-colony stimulating factor (G-CSF) they differentiate towards mature neutrophils. We demonstrate that 32D/G-CSF-R/Cb2-EGFP cells migrate in a transwell assay in reponse to the Cb2 ligand 2-arachidonoylglycerol (2-AG), indicating that the fusion protein was functional. When cultured in the presence of G-CSF neutrophilic differentiation of Cb2-EGFP expressing 32D/G-CSF-R cells was completely blocked. Moreover, a Cb2-specific antagonist fully recovered the G-CSF-induced neutrophilic differentiation of 32D/G-CSF-R/Cb2-EGFP cells. To investigate which signal transduction pathway(s) may be involved in the block of neutrophilic maturation, differentiation experiments were carried out using specific inhibitors of signaling routes. Interestingly, full rescue of G-CSF-induced neutrophilic differentiation was observed when cells were cultured with the mitogen-induced extracellular kinase (MEK) inhibitors, PD98059 or U0126, and partial recovery was detected with the phosphoinositide 3-kinase (PI3-K) inhibitor LY-294,002. These studies demonstrate that the Cb2 receptor is an oncoprotein that blocks neutrophilic differentiation when overexpressed in myeloid precursor cells. Cb2 appears to mediate its activity through MEK/ERK and PI3-K pathways.

INTRODUCTION

Cb2, the gene encoding the peripheral cannabinoid receptor has previously been identified as a common virus integration site (cVIS) in retrovirally-induced leukemias, suggesting that it is a proto-oncogene[1,2]. The peripheral as well as the central cannabinoid receptor (Cb1) are seven transmembrane proteins and belong to the family of G protein-coupled receptors (GPCRs)[3,4]. Previous studies have shown that the rank order of *Cb2* mRNA in hematopoietic cells is as follows: B cells> natural killer cells> monocytes> neutrophils> T8 cells> T4 cells[5,6]. Moreover we and others have shown that Cb2 protein is normally expressed in areas enriched for B lymphocytes, that is, in the marginal zone of the spleen, the cortex of lymph nodes, the nodular corona of Peyer's patches, and the mantle zones of secondary follicles in tonsils[3,6-8] (Rayman and Delwel; Unpublished observation). We recently described a role of the Cb2 receptor in migration of hematopoietic cells[9]. Using transwell assays we observed strong migration of Cb2-expressing spleen cells in response to 2-arachidonoylglycerol (2-AG), the endogenous ligand for the Cb2 receptor. This migration could be completely abolished by a Cb2-specific antagonist, SR144528, whereas the Cb1-specific antagonist SR141716 had no effect. Immunophenotyping revealed that the 2-AG responding cells express B220, CD19, IgM

and IgD, suggesting a role for this GPCR in chemoattraction, mobilisation and/or activation of splenic B lymphocytes during the immune response[9].

In acute myeloid leukemia (AML), immature myeloid precursor cells accumulate in marrow and blood (for review see Löwenberg et al[10]). Although several genes involved in leukemia development have been identified by cloning of breakpoints at chromosomal translocations[11-13], little is known about the genetic defects and aberrant signaling causing a block of myeloid differentiation. Retroviral insertional mutagenesis has been extensively used by different research groups in the past 2 decades and several novel disease genes involved in leukemia and lymphoma development have been identified by means of this procedure[2,14-17]. Our previous observation that the *Cb2* gene is a frequent proviral target in Cas-Br-M MuLV-induced myeloid leukemias suggests a role for this receptor in myeloid transformation[1,2,18].

In the present study we investigated whether overexpression of Cb2 receptor in myeloid precursor cells could interfere with neutrophilic development, the major characteristic of myeloid leukemia. For this purpose we generated a *Cb2-EGFP* fusion construct that was cloned into the viral vector pLNCX and introduced into 32D/G-CSF receptor cells (32D/G-CSF-R). This cell system has been demonstrated to be a powerful in vitro model to study molecular mechanisms involved in granulocytic differentiation[19-21]. 32D/G-CSF-R cells proliferate in vitro in the presence of interleukin 3 (IL-3) and are capable to fully differentiate towards mature neutrophils upon granulocyte-colony stimulating factor (G-CSF) stimulation. Cb2 expression levels are low in the 32D/G-CSF-R cells, as has been shown by radiolabelled ligand binding studies[9]. To determine exogenous Cb2 protein levels, *Cb2-EGFP* fusion constructs were generated and transfected into 32D/G-CSF-R cells. After demonstrating that the Cb2-EGFP fusion protein was expressed on the surface membrane of 32D/G-CSF-R cells and fully functional, we investigated whether overexpression of Cb2 could affect G-CSF-induced neutrophilic differentiation. We studied whether activation of the receptor by agonists or inactivation by antagonists could influence neutrophilic development. Using specific signal transduction inhibitors, we investigated through which intracellular signaling pathway Cb2 might alter normal development. We demonstrate that (1) G-CSF-induced granulocytic differentiation of 32D/G-CSF-R is fully blocked by Cb2-EGFP overexpression, (2) addition of the Cb2-specific antagonist SR144528 fully recovers neutrophilic differentiation, and (3) the differentiation arrest conferred by Cb2 receptor may involve activation of MEK/ERK (mitogen-induced extracellular kinase/extracellular signal-related kinase) and PI3-K (phosphoinositide 3-kinase) signaling routes.

MATERIALS AND METHODS

Cannabinoid ligands, cytokines and inhibitors of intracellular signaling

The Cb2 cannabinoid ligand 2-arachidonoylglycerol (2-AG) was obtained from Sigma (Zwijndrecht, The Netherlands). Cb1-specific antagonist SR141716 and Cb2-specific antagonist SR144528 were kindly donated by Dr. Casellas (Sanofi Recherche, Montpellier, France). Murine IL-3 was obtained from an IL-3-producing Chinese hamster

ovary (CHO) cell line and G-CSF was from Amgen (Thousand Oaks, CA). Dibutyryl cyclic adenosine monophosphate (AMP) (dbcAMP), LY-294,002 (PI3-K inhibitor), SB203580 (p38/MAPK [mitogen-activated protein kinase] inhibitor) and U0126 (MEK inhibitor) are from Kordia Life Science (Leiden, The Netherlands) whereas PD98059 (MEK inhibitor) was obtained from Omnilabo International (Breda, The Netherlands). The inhibitors were dissolved in dimethyl sulfoxide (DMSO) and added to the cultures at concentrations indicated and refreshed daily.

In vitro proliferation and neutrophilic differentiation of 32D/G-CSF-R cells

The 32D/G-CSF receptor (32D/G-CSF-R) cell line[19] was cultured in RPMI 1640 medium (Life Technologies, Breda, The Netherlands) supplemented with penicillin (100 IU/ml), streptomycin (100ng/ml), 10% Fetal Calf Serum (FCS) and murine IL-3 (10 ng/ml) or human G-CSF (100 ng/ml). Cell counting was performed using a CASY1/TTC cell counter (Schärfe System, Germany) and the cell density was readjusted to 2 x 10^5 cells/ml daily. Morphological analysis was determined by microscopy on May-Grünwald-Giemsa stained cytospins (Shandon Holland, Amsterdam, The Netherlands).

Cb2-EGFP expression construct and infection of 32D/G-CSF-R cells

The primers 5'-CAAAGCCCATCCATGGAG-3' and 5'-AAGGATCCGTGGTTTTCACATCAG-3' were used to amplify the coding region of *Cb2*, mutate the TAG stop codon and introduce a *Bam*HI restriction site at the 3' end. The polymerase chain reaction (PCR) product was cloned in TA vector and subcloned as an *Eco*RI/*Bam*HI fragment into pEGFP-N1. *Cb2-EGFP* fusion construct was obtained by *Eco*47III/*Not*I digestion of this latter construct and cloned as a blunt fragment into the *Hpa*I site of pLNCX (Clontech, Palo Alto, CA). The *Cb2-EGFP* fusion construct was verified by nucleotide sequencing. The expression constructs were transfected into Phoenix cells type E (gift from G. Nolan, Stanford, CA) and the viral supernatants were used for infection of 32D/G-CSF-R cells. Single clones were obtained using limiting dilution in 96-well microtiter trays (Becton Dickinson, Mountain View, CA) and infected clones were selected on 0.8 mg/ml G418 (Gibco, Breda, The Netherlands). To determine *Cb2-EGFP* mRNA expression Northern blot analysis was performed[2]. Blots were hybridized using a *Not*I/*Eco*RI enhanced green fluorescence protein (EGFP) cDNA probe of 760 bp. Cb2-EGFP fusion protein expression was analyzed by Leica DMRXA microscopy (Leica Microsystems, Rijswijk, The Netherlands) and flow cytometric analysis of EGFP fluorescence (Figure 1).

Flow cytometric analysis

32D/G-CSF-R/Cb2-EGFP and 32D/G-CSF-R/EGFP clones were analyzed by flow cytometric analysis (FACScan flow cytometer, Becton Dickinson, Mountain View, CA). Cells (1 x 10^6) were washed twice with Phosphate Buffered Saline (PBS) and resuspended in 500 µl Hank's Balanced Salt Solution (HBSS) containing 0.5% Bovine Serum Albumin (BSA). Dead cells were excluded from the analysis by addition of 7-AAD (7-aminoactinomycin D; Eugene, Leiden, The Netherlands). EGFP fluorescence was determined and the amount of protein was related to the peak channel (Figure 1).

Migration assay

Migration assays were performed using 5 µm pore size and 6.5 mm diameter transwells (Corning Costar, Amsterdam, The Netherlands) as previously described[9]. In brief, cells were washed twice with HBSS medium, resuspended in 100 µl of migration medium (Iscove's Modified Dulbecco's Medium (IMDM) plus 0.5% BSA) and placed in the upper chamber of the transwells. In the lower chamber, 600 µl of migration medium with or without 300 nM 2-AG were placed. After 4 hours of incubation at 37°C and 5% CO_2 the upper chamber was removed and the numbers of migrated cells were determined using a CASY1/TTC cell counter (Schärfe System, Germany). Cb1- and Cb2-specific antagonists (100 nM) were added to the upper chamber when tested.

Tritiated thymidine (^{3}H-TdR) incorporation assay

DNA synthesis was determined by ^{3}H-TdR incorporation as described before[22]. Briefly, 1 x 10^4 cells were incubated in 100 µl of RPMI 1640 medium supplemented with 10% FCS, murine IL-3 (10 ng/ml) or human G-CSF (100 ng/ml), and in the presence or absence of 2-AG (300 nM), Cb1- or Cb2-specific antagonist (100 nM). A quantity of 0.25 µCi (0.00925 MBq) ^{3}H-TdR (Amersham International, Amersham, UK) was added to each well 4 hours before cell harvesting. Cells were harvested on nitrocellulose using a filtermate 196 harvester (Packerd Instrument Co, Meriden, CT) and ^{3}H-TdR incorporation was measured by Topcount liquid scintillation counter (Packerd Instrument Co, Meriden, CT).

RESULTS

32D/G-CSF-R/Cb2-EGFP cells migrate in response to the natural Cb2 ligand 2-AG

32D/G-CSF-R cells were infected with retrovirus carrying *Cb2-EGFP* or *EGFP* constructs. Following G418 selection 8 Cb2-EGFP and 4 EGFP-expressing 32D/G-CSF-R clones were obtained. Expression of *Cb2-EGFP* or *EGFP* RNA in those 32D/G-CSF-R transfected cells was demonstrated by Northern blot analysis (data not shown). Cb2-EGFP and EGFP protein expression was assessed by means of fluorescence microscopy and flow

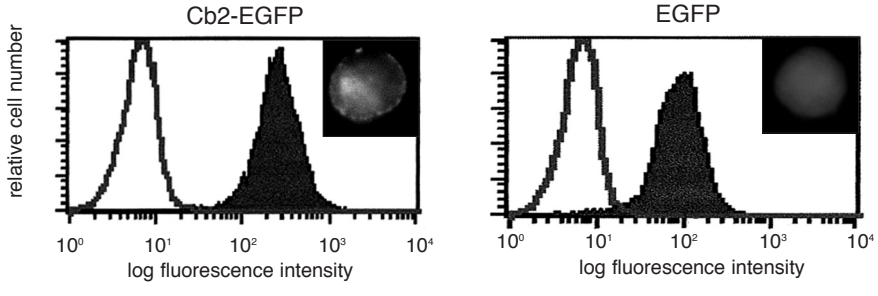

Figure 1. Cb2-EGFP and EGFP expression in 32D/G-CSF-R cells.
Flow cytometric analysis of a 32D/G-CSF-R clone overexpressing Cb2-EGFP and a 32D/G-CSF-R control clone overexpressing EGFP. Upper right inserts show cell fluorescence distribution in the transfected cells by microscopy.

cytometric analysis. Figure 1 shows 2 representative clones. Although high levels of EGFP were present in both clone types, membrane fluorescence was only observed on Cb2-EGFP-expressing cells (Figure 1; inserts).

To investigate whether the Cb2-EGFP fusion protein had retained the normal Cb2 function, migration assays were performed. Figure 2A shows that the 8 32D/G-CSF-R/Cb2-EGFP clones migrate with high efficiency following 2-AG exposure, whereas the 4 EGFP control clones did not migrate in response to 2-AG. The migration levels of the Cb2-EGFP-expressing clones are comparable to those observed previously using Cb2-expressing 32D/G-CSF-R cells[9]. Moreover, 2-AG-induced migration could be completely blocked by the addition of Cb2-specific antagonist SR144528 to the upper chamber in a transwell assay, whereas the Cb1-specific antagonist SR141716 had no effect (Figure 2B). These experiments indicate that EGFP fused to the C-terminus of Cb2 does not interfere with the normal function of the peripheral cannabinoid receptor.

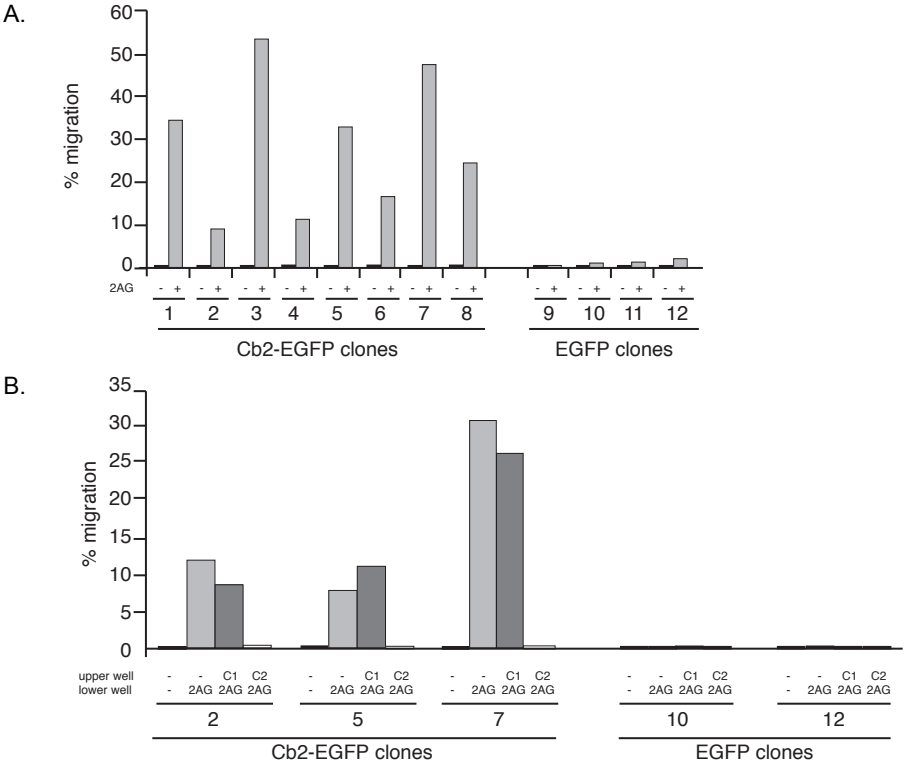

Figure 2. In vitro migration of Cb2-EGFP-expressing cells following 2-AG stimulation.
(A) Cb2-EGFP-expressing 32D/G-CSF-R cells (clones 1-8) and EGFP control 32D/G-CSF-R cells (clones 9-12) were exposed to 300 nM 2-AG (+) or nothing (-) in a transwell assay. The y-axis shows percentage of migration from an input of 2 x 10^5 cells. (B) Effect of Cb1-specific antagonist SR141716 (C1) or Cb2-specific antagonist SR155528 (C2) on 2-AG-induced migration of 3 Cb2-EGFP expressing clones (nos 2,5 and 7) and 2 EGFP control clones (nos 10 and 12). The y-axis shows percentage of migration an input of 2 x 10^5 cells.

Overexpression of Cb2-EGFP in 32D/G-CSF-R cells blocks G-CSF-induced neutrophilic differentiation

To study whether overexpression of Cb2 receptor in 32D/G-CSF-R cells affects neutrophilic development, 8 Cb2-EGFP-expressing clones and 4 EGFP control clones were cultured in the presence of G-CSF. Morphological analysis showed a complete block of neutrophilic differentiation of 32D/G-CSF-R/Cb2-EGFP clones cultured with G-CSF (Figure 3A). Differential countings on day 9 of culture revealed that the majority of these cells presented a blast-like morphology (Figure 3B). In contrast, EGFP-expressing 32D/G-CSF-R control cells fully matured towards neutrophilic granulocytes in response to G-CSF (Figure 3A-B). Flow cytometric analysis revealed high Cb2-EGFP or EGFP fluorescence during the 9 days of culture in the presence of G-CSF (data not shown).

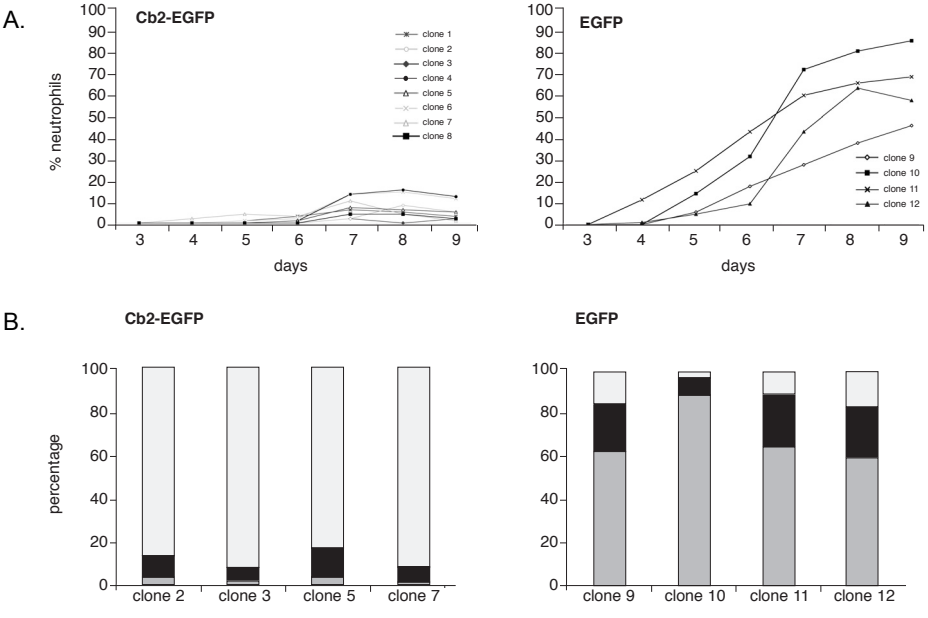

Figure 3. G-CSF-induced differentiation response of Cb2-EGFP- and EGFP-expressing 32D/G-CSF-R clones.

(A) 8 Cb2-EGFP-expressing clones and 4 EGFP control clones were cultured during 9 days in the presence of G-CSF (See Material and Methods). The y-axis shows the percentage of neutrophils and the x-axis the days of culture. (B) Differential countings of 4 Cb2-EGFP-expressing clones and 4 EGFP control clones at day 9 of G-CSF culture. White represents blast cells, black represents intermediately matured granulocytic forms and gray indicates terminally differentiated neutrophilic granulocytes.

Strikingly, the maturation arrest of 32D/G-CSF-R/Cb2-EGFP cells already occurred without the addition of Cb2 ligand. Inclusion of 2-AG to the G-CSF-supplemented cultures had no additional effect on granulocytic differentiation (data not shown).

Cb2-specific antagonist reestablishes G-CSF-induced neutrophilic differentiation of Cb2-EGFP-expressing 32D/G-CSF-R clones

We next studied whether interfering with the Cb2 receptor using the cannabinoid antagonists would restore the neutrophilic differentiation of Cb2-EGFP-expressing clones. The Cb2-specific antagonist SR144528 was added at different concentrations to G-CSF-supplemented cultures in 2 different Cb2-EGFP clones. One representative experiment is shown in Figure 4A, demonstrating that 1 nM of Cb2-specific antagonist was already capable of partially restoring differentiation of Cb2-EGFP-overexpressing cells, whereas higher concentrations resulted in a complete recovery of neutrophilic differentiation of these cells.

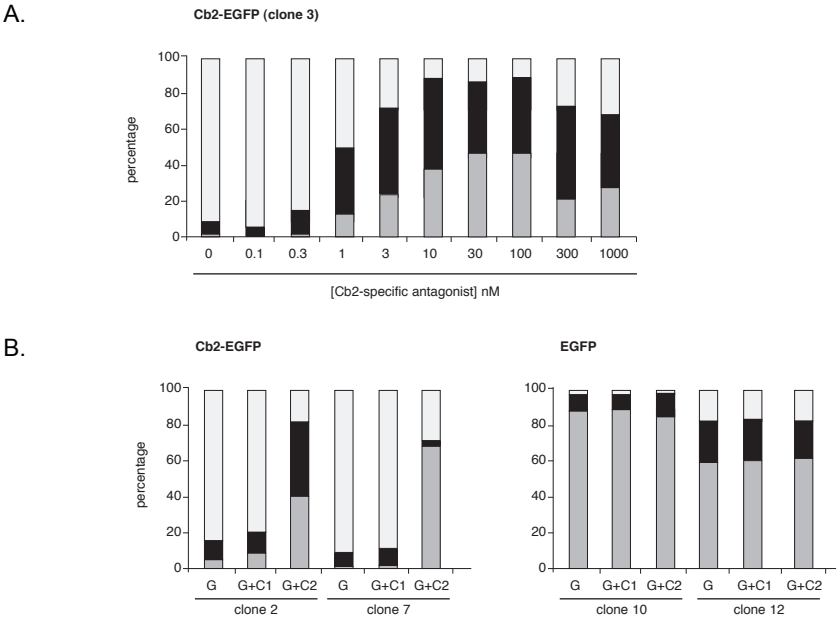

Figure 4. Effect of Cb2-specific antagonist SR144528 on G-CSF-induced differentiation.
(A) Cb2-specific antagonist titration experiment when added to the G-CSF cultures in differentiation assays. Countings were carried out at day 9 of culture. (B) Effect of Cb1-specific (C1) and Cb2-specific (C2) antagonist (100 nM) on 2 Cb2-EGFP and 2 EGFP clones cultured for day 9 in the presence of G-CSF (G). White represents blast cells, black represents intermediate forms, and gray indicates neutrophils.

All 8 32D/G-CSF-R/Cb2-EGFP clones, of which 2 are shown in Figure 4B and Figure 5, revealed a complete recovery of neutrophilic differentiation when cultured with G-CSF and 100 nM of Cb2-specific antagonist SR144528. In contrast, the Cb1-specific antagonist SR141716 (100 nM) did not restore granulocytic maturation of 32D/G-CSF-R/Cb2-EGFP cells (Figure 4B and 5). Neither Cb1- nor Cb2-specific antagonists exerted differentiation-inducing effects on EGFP-expressing control clones (Figure 4B and 5).

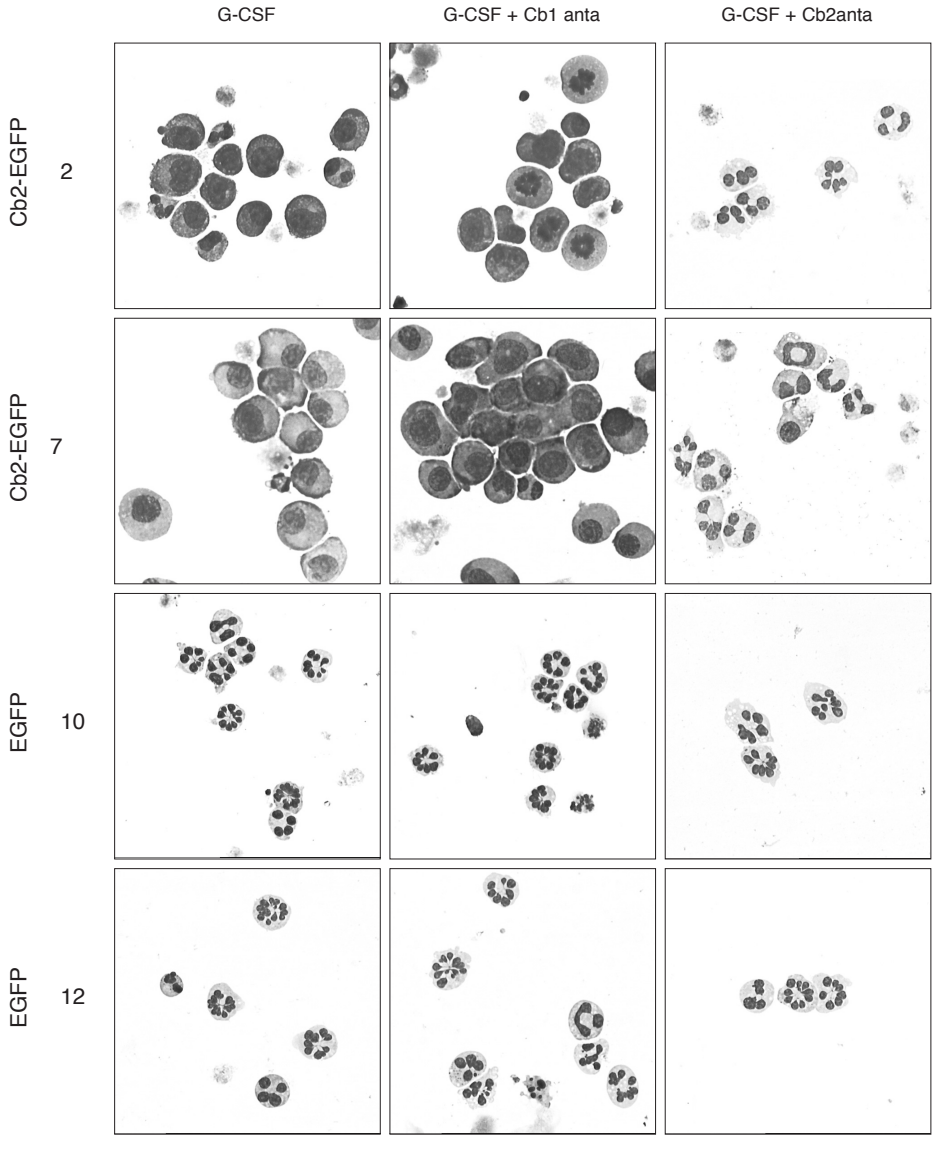

Figure 5. Cell morphology of 32D/G-CSF-R cells cultured in different conditions.
Morphological analysis of 2 Cb2-EGFP-expressing clones and 2 EGFP control clones cultured for 9 days in the presence of G-CSF, G-CSF plus Cb1-specific antagonist or G-CSF plus Cb2-specific antagonist. Original magnifications x 63.

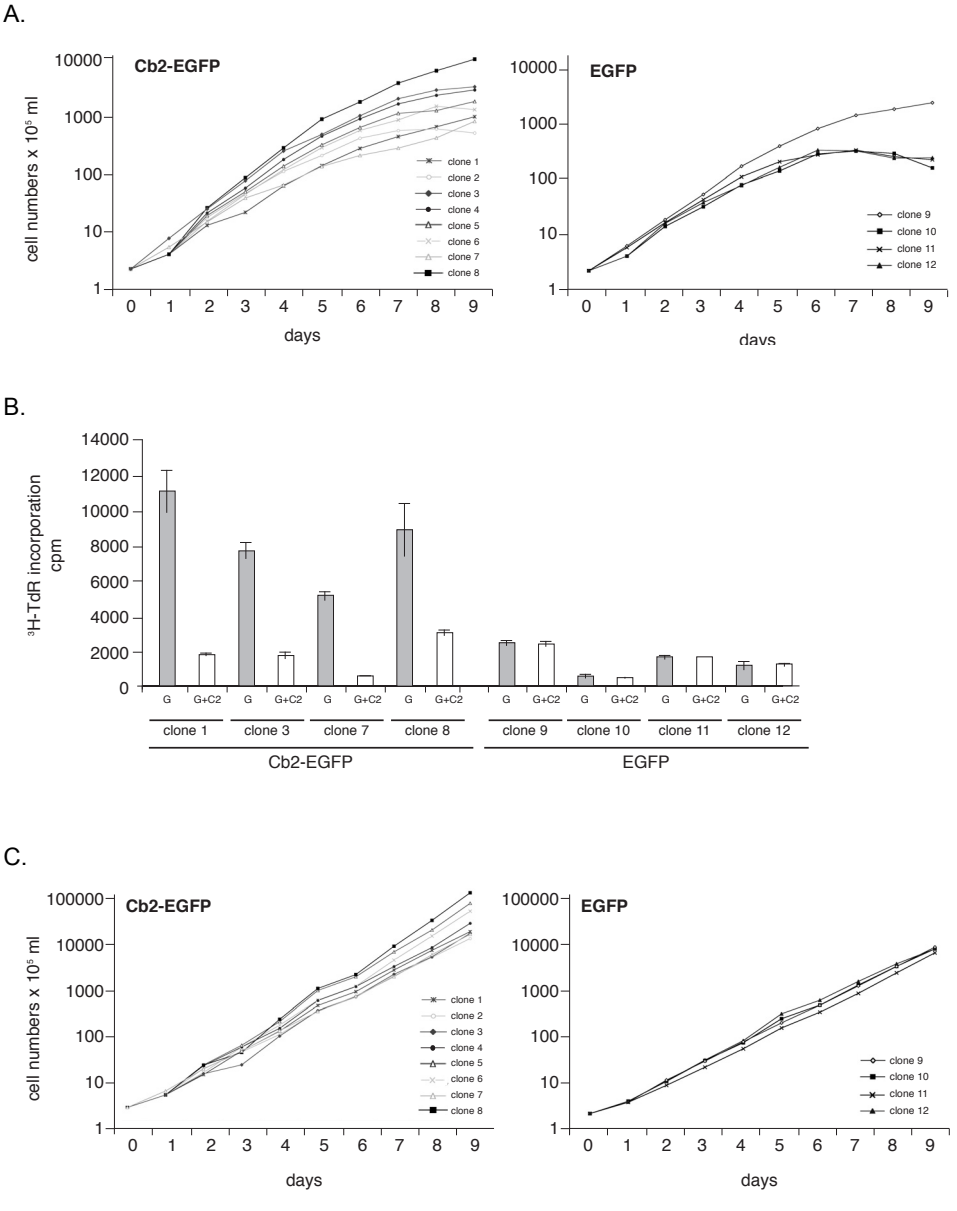

Figure 6. G-CSF and IL-3 proliferative response of Cb2-EGFP- and EGFP-expressing 32D/G-CSF-R clones.

(A) Different proliferation curves of 8 Cb2-EGFP-expressing clones and 4 EGFP control clones stimulated with G-CSF. The y-axis indicates the number of cells x 10^5/ml. (B) [^{3}H]-TdR incorporation of Cb2-EGFP-expressing cells or EGFP control cells at day 7 of culture in G-CSF (G) with or without Cb2-specific antagonist (C2). The y-axis represents [^{3}H]-TdR incorporation in counts per minute. (C) Proliferation curves of 8 Cb2-EGFP and 4 EGFP-expressing clones stimulated with IL-3 during 9 days of culture. The y-axis indicates the number of cells x 10^5/ml.

The G-CSF-induced proliferative response of Cb2-EGFP-expressing 32D/G-CSF-R cells is slightly altered

Since Cb2-EGFP-expressing 32D/G-CSF-R clones are blocked in neutrophilic differentiation, we wondered whether the blasts that accumulate when cultured with G-CSF still had proliferative capacity. Although the 8 Cb2-EGFP-expressing and the 4 EGFP-expressing 32D/G-CSF-R clones show a similar G-CSF proliferative curve, the data suggest that Cb2-EGFP-expressing 32D/G-CSF-R clones stop growing at a later time point than control clones (Figure 6A). Indeed, ^{3}H-TdR incorporation experiments carried out with cells harvested at day 7 of G-CSF culture showed that Cb2-EGFP-expressing 32D/G-CSF-R clones were still proliferating, whereas EGFP-expressing clones were not (Figure 6B). Moreover, Cb2-EGFP-expressing 32D/G-CSF-R cells cultured with G-CSF plus Cb2-specific antagonist SR144528 harvested at day 7 behaved like EGFP-transfected control cells; that is, they did not incorporate ^{3}H-TdR. The same cells cultured in the presence of Cb1-specific antagonist SR141716 did not lose their proliferative response (data not shown). Cells from Cb2-EGFP-expressing or control clones harvested at day 8 or 9 of culture in the presence of G-CSF did not show any ^{3}H-TdR incorporation (data not shown).

No significant differences were found in the IL-3 response of Cb2-EGFP-expressing clones versus EGFP-expressing clones (Figure 6C). No effect of 2-AG or of the antagonists was observed on the IL-3-induced proliferative response of Cb2-EGFP- or EGFP-expressing 32D/G-CSF-R clones (data not shown).

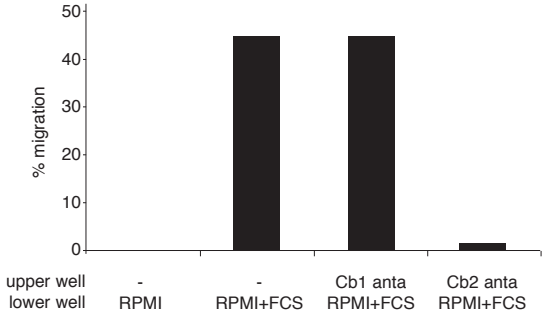

Figure 7. Effect of FCS on migration of Cb2-EGFP-expressing 32D/G-CSF-R cells.
32D/G-CSF-R/Cb2-EGFP cells were exposed to RPMI medium containing or not serum during a transwell assay. A quantity of 100 nM of Cb1- or Cb2-specific antagonist was placed in the upper well. The y-axis indicates the percentage of migration from an input of 2 x 10^{5} cells.

A potent Cb2 agonist is present in the culture medium

2-AG, a potent Cb2 ligand, is usually required to induce migration of Cb2-expressing cells (Figure 2 and Alberich Jordà[9]). However, the addition of 2-AG was not required in the G-CSF containing cultures to block G-CSF-induced neutrophilic differentiation. We hypothesized that a ligand activating the Cb2 receptor had been present in the cultures. This

potent agonist could either have been present in the serum added to the cultures or endogenously produced by the cells (autocrine stimulation). To test whether the serum contained a Cb2-specific agonist, we assessed the effect of the culture medium on migration of 32D/G-CSF-R/Cb2-EGFP cells. Cb2-EGFP-expressing cells showed a significant migration when RPMI plus 10% FCS was added to the lower chamber in a transwell assay (Figure 7). Moreover, this migration could be completely blocked by addition of Cb2-specific antagonist SR1445283 to the upper well, but not by addition of Cb1-specific antagonist SR141716 (Figure 7).
Transwell experiments using conditioned medium obtained from serum-free cultures of the different 32D/G-CSF-R clones showed no migration of Cb2-EGFP-expressing cells (data not shown). These data indicate that the block of neutrophilic differentiation of 32D/G-CSF-R/Cb2-EGFP cells was caused by a potent Cb2-agonist, which was present in the serum used for these cultures.

Inhibitors of the MEK/ERK and PI3-Kinase pathways restore neutrophilic differentiation of Cb2-EGFP-expressing 32D/G-CSF-R cells

To determine which signal transduction pathway(s) may be involved in the Cb2 receptor-mediated block of neutrophilic differentiation we investigated the effect of distinct small molecules capable of interfering with specific signaling routes. Interestingly, addition of the MEK inhibitors, 10 μM U0126, or 25 μM PD98059, fully restore the G-CSF-induced neutrophilic differentiation of Cb2-EGFP-expressing 32D/G-CSF-R cells (Figure 8).
Furthermore, the PI3-Kinase inhibitor LY-294,002 (5 μM) was capable of partially recover maturation of these cells when cultured with G-CSF (Figure 8B). On the other hand, SB203580 (5 μM), an inhibitor of p38/MAPK, had no promoting effect on neutrophilic differentiation (data not shown). We also investigated whether Cb2 interferes with differentiation via a protein kinase A (PKA)-dependent signaling mechanism. Addition of dbcAMP (100 μM) to the G-CSF cultures did not influence maturation of 32D/G-CSF-R/Cb2-EGFP clones (Figure 8B). The data suggest that the block of neutrophilic differentiation in myeloid precursor cells caused by the Cb2 receptor may involve activation of both MEK/ERK and PI3-K pathways.

DISCUSSION

We previously described that the gene encoding for the peripheral cannabinoid receptor Cb2 is located in a common virus integration site (*Evi11*) in murine myeloid leukemia, indicating that aberrant expression of this GPCR may be a critical event in leukemia development[1,2]. However, by which mechanism this GPCR may be involved in leukemic transformation has remained elusive. The objective of the present study was to determine the effect of Cb2 overexpression in myeloid precursor cells. We demonstrate here that overexpression of Cb2-EGFP completely suppresses G-CSF-induced neutrophilic differentiation of 32D/G-CSF-R cells. The addition of the Cb2-specific antagonist fully recovered neutrophilic differentiation of these cells, indicating that the block of differentiation is a Cb2

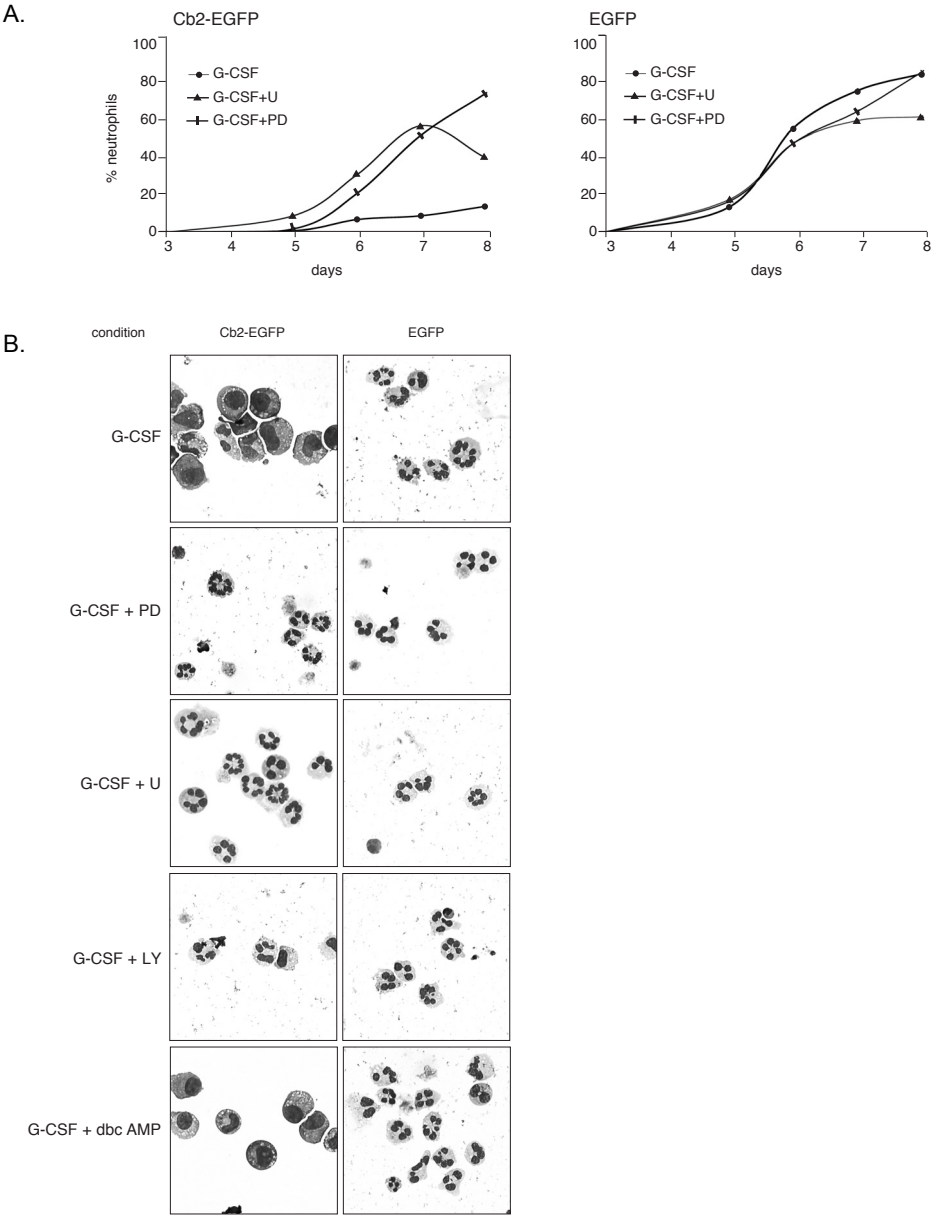

Figure 8. Effect of signal transduction pathway inhibitors on the G-CSF differentiation response of 32D/G-CSF cells.

(A) Cb2-EGFP-expressing cells and EGFP control cells were cultured for 9 days in the presence of G-CSF plus U0126 (U), PD98059 (PD). At the y-axis the percentage of neutrophils is shown, and at the x-axis the day of culture is indicated. (B) Morphological analysis of a Cb2-EGFP-expressing clone and an EGFP control clone cultured in the presence of G-CSF plus PD98059, U0126, LY-294,002 or dbcAMP. Pictures were taken at day 8 of culture except for incubations with U0126 (day 6). Original magnifications x 63.

receptor-specific effect. The data demonstrate that stimulation of Cb2 receptors when overexpressed on myeloid precursors turns on signals that interfere with normal neutrophilic differentiation.

A major characteristic of myeloid leukemia is a differentiation arrest of the granulocytic lineage[10]. G-CSF regulates proliferation, survival and differentiation of myeloid progenitor cells[23,24]. Multiple gene products or their mutants have been implicated in impairment of G-CSF-regulated neutrophilic development. Alterations in the G-CSF-R, signaling molecules (e.g. MEK, STAT1/3, SHP-2, Grb2, shc) or transcription factors (e.g. Evi1, C/EBPα, Hoxa9, Hoxb8, Meis) have been reported to alter neutrophilic differentiation[19,21,25-31]. Here, we describe the first example of a GPCR that interferes with G-CSF-induced neutrophilic differentiation. Several GPCRs have been implicated in transformation when overexpressed in NIH 3T3 fibroblasts model, for example, the *Mas*-oncogene[32], thrombin receptor[33] and serotonin 1c receptors[34]. Introduction of *Cb2* cDNA into NIH 3T3 cells did not result in oncogenic transformation (Delwel et al; unpublished observation), suggesting another mechanism of transformation by this GPCR. Overexpression of the other oncogenic GPCRs in 32D/G-CSF-R cells may reveal whether the Cb2 receptor indeed uniquely mediates a block of neutrophilic maturation or whether a more general GPCR-related process causes tranformation of myeloid precursor cells.

G proteins consist of three heterologous subunits (α, β, and γ) which are stably associated to guanosine diphosphate (GDP) only during inactive state. Upon ligand activation of the receptor, GDP is exchanged for guanosine triphosphate (GTP) and the trimer dissociates into a Gα-GTP subunit and a G$\beta\gamma$ dimer, both capable of activating intracellular signaling pathways[35-37]. Cb2 receptor belongs to the family of GPCRs that couple to Gαi/o $\beta\gamma$ proteins. Following receptor activation, the Gαi/o subunit inhibits adenylyl cyclase (AC) activity resulting in decreased level of intracellular cAMP[38-40]. Interference of this particular intracellular process, by addition of dibutyryl cyclic AMP to the G-CSF cultures did not restore granulocytic differentiation of Cb2-EGFP-expressing 32D/G-CSF-R cells. Thus, downregulation of cAMP levels following Cb2 activation appears not responsible for the block of neutrophilic differentiation and suggests involvement of another signaling pathway. The G$\beta\gamma$ dimer has been shown to mediate mitogenic-mediated protein kinase (MAPK) activation[41-44], thereby influencing cell proliferation and differentiation. We observed restoration of neutrophilic development by addition of U0126 or PD98059 inhibitors to the G-CSF cultures, suggesting that overactivation of MEK/ERK pathway following Cb2 stimulation is a major cause of the block of granulocytic differentiation. Furthermore, at day 7 of culture in the presence of G-CSF Cb2-EGFP-expressing cells still incorporated tritiated thymidine, whereas control cells did not. These observations are in agreement with previous studies showing that activation of MEK/ERK is a critical event in G-CSF-induced proliferation and is not required for differentiation[26,45].

The MEK/ERK pathway has been implicated in signaling by multiple GPCRs, including SDF-1[46], somatostatin[47], and opioid receptors[48]. Expression of each of these receptors has been demonstrated on hematopoietic precursor cells[49-51], but under these physiological conditions interference with neutrophilic differentiation has not been reported. GPCR overexpression and subsequent uncontrolled MEK/ERK activation may be required for

myeloid transformation. Multiple GPCR transfectants of 32D/G-CSF-R should be generated to answer the question of whether sustained MEK/ERK activation by GPCR stimulation is sufficient to cause a block of neutrophilic differentiation.

Several GPCRs can activate MAPK by signaling via PI3-K[52-54]. In the presence of LY-294,002, granulocytic development in response to G-CSF is partially recovered as well, suggesting the involvement of PI3-K in the block of neutrophilic differentiation evoked by activation of the peripheral cannabinoid receptor. PI3-K has been related with mitogenic signaling through a variety of growth factors[55], including G-CSF[56]. Activation of PI3-K via G-CSF-R has been shown to correlate with enhanced proliferation, suggesting that activation of PI3-K by Cb2 receptor will keep the Cb2-EGFP-expressing cells with a higher growth potential. This would be in agreement with our findings presented in Figure 6. It is possible that activation of multiple signaling pathways, including PI3-K and MEK/ERK, are required to interfere with G-CSF-induced neutrophilic differentiation. Because PI3-K may be involved in cell survival[57,58], we tested whether Cb2-expressing 32D/G-CSF-R cells, cultured in the presence of the endocannabinoid 2-AG but in the absence of cytokines, showed altered survival or proliferation. Although PI3-K signaling may be involved in the Cb2-induced block of neutrophilic differentiation, we did not observe any effect of Cb2 receptor activation on survival or proliferation under the conditions tested.

Several cannabinoid ligands from different origin have been proposed as the true ligand for the peripheral cannabinoid receptor[59-62]. We recently compared the capability of these different ligands to induce migration of Cb2-expressing cells. Using transwell assays we demonstrated that 2-AG is the most potent inducer of migration[9]. Since 2-AG or other cannabinoid ligands were not added to the cultures presented here, we hypothesized that a Cb2 ligand was present in the serum containing medium or produced by the cells themselves. We demonstrate in Figure 7 that significant levels of Cb2 ligands were present in the serum. It is unclear whether culture medium contains 2-AG or another yet-to-be identified stimulus for Cb2. The fact that specific Cb2 agonist(s) are present in the serum used in our studies suggests that Cb2 ligands may be present in vivo in sufficient quantities. This finding may allow studies to investigate the effect of Cb2 overexpression in bone marrow progenitor cells in vivo. Introduction of *Cb2* gene into primitive progenitor cells followed by transplantation into sublethally irradiated mice should reveal whether high Cb2 expression causes a myeloproliferative disorder or myeloid leukemia.

Our observations raise the question whether human CB2 receptor or other GPCRs may be abnormally expressed in certain cases of human AML. High-throughput AML patient screens using cDNA array technology are currently in progress, which may identify such cases. Exposure of those leukemia samples to GPCR antagonists, specific MEK/ERK inhibitors or combinations thereoff may unravel the importance of those receptors and downstream signaling routes in myeloid differentiation abnormalities and possibly open new ways for treatment of AML.

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CHAPTER 4

The peripheral cannabinoid receptor Cb2, frequently expressed on AML blasts, either induces a neutrophilic differentiation block or confers abnormal migration properties in a ligand-dependent manner.

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Submitted

ABSTRACT

Cb2, the gene encoding the peripheral cannabinoid receptor, is a common virus integration site in murine myeloid leukemias. Here we show that this G protein-coupled receptor (GPCR) is frequently expressed in human AML but absent in normal myeloid cells. We demonstrate that Cb2 is transforming when introduced into myeloid precursors. Cells migrate upon stimulation by the endocannabinoid 2-arachidonoylglycerol but show a differentiation block upon exposure to another ligand, i.e CP55,940. Both effects depend on MEK/ERK activation and Gαi protein interaction, although the conserved G protein interacting DRY-motif in Cb2, is only critical for chemotaxis. This is the first example of a transforming GPCR in myeloid precursors that, in a ligand-dependent manner, causes two distinct effects known to be critical in oncogenic transformation.

INTRODUCTION

Using retroviral insertional mutagenesis we recently demonstrated that *Cb2*, the gene encoding the peripheral cannabinoid receptor, is located in a common virus integration site (*Evi11*) in Cas-Br-M MuLV-induced myeloid leukemias, suggesting that *Cb2* is a proto-oncogene involved in transformation[1,2]. *Cb2* encodes a seven transmembrane (7TM) protein, belonging to the family of Gαi protein-coupled receptors (GαiPCRs)[3]. This receptor is normally expressed in areas enriched for B lymphocytes, i.e. the marginal zone of the spleen, the cortex of lymph nodes, the nodular corona of Peyer's patches, and the mantle zones of secondary follicles in tonsils[3-6]. Cb2 is involved in both B cell differentiation and splenic B lymphocyte migration, suggesting a role for this receptor in the immune response[5,7]. The natural activator of Cb2 has been demonstrated to be 2-arachydonoylglycerol (2-AG)[7-11], although a number of alternative Cb2 ligands have been reported (for review see Howlett et al[12]).

Acute myeloid leukemia (AML) is characterized by an accumulation of immature non-functional cells in the bone marrow and blood[13]. Myeloid leukemia is considered to be a multigenic disease involving cooperation between several disease genes[14-16]. The genetic abnormalities in AML may result in aberrant expression of proto-oncogenes or inactivation of tumor suppressor genes. These abnormalities allow leukemia cell escape from regulatory signals, resulting in altered proliferation, aberrant survival and maturation arrest. Our previous observation that Cb2 is overexpressed in myeloid cell lines containing a retroviral insertion near *Cb2* suggests that *Cb2* may be involved in leukemic transformation in certain murine leukemias. In the present study, we demonstrate that CB2 is aberrantly expressed in several human myeloid cell lines and primary AML samples, whereas normal bone marrow precursor cells do not express this GPCR.

We generated a *Cb2-EGFP* fusion construct[17] that was introduced into both murine normal bone marrow cells and 32D/G-CSF-R cells. 32D/G-CSF-R cells proliferate in vitro

in the presence of interleukin 3 (IL-3) and terminally differentiate into mature neutrophils upon granulocyte-colony stimulating factor (G-CSF) exposure. Furthermore, this cell line is a useful in vitro model to study the molecular mechanisms involved in granulocytic differentiation[18-20] and to functionally analyze transforming genes causing a block in this differentiation[17,21]. The Cb2-EGFP fusion protein appears fully functional, as Cb2-expressing marrow cells and 32D/G-CSF-R/Cb2 cells migrate in response to the endocannabinoid 2-AG. In the present study we assessed whether 2-AG was capable of inducing a neutrophilic differentiation block of 32D/G-CSF-R/Cb2 cells. We demonstrate that the endocannabinoid 2-AG, although a potent stimulator of migration of Cb2-expressing cells, could not block G-CSF-induced neutrophilic differentiation. We tested if another potent cannabinoid ligand CP55,940[22,23], could affect neutrophilic differentiation of Cb2-expressing cells. Interestingly, CP55,940 failed to induce migration but evoked a complete arrest of neutrophilic differentiation. Next, we demonstrated that MEK/ERK signaling is involved in both Cb2 functions whereas downregulation of the intracellular cAMP levels is only required for migration.

Classical signaling by GPCRs is based on the transduction of extracellular signals to downstream effectors via intracellular, heterotrimeric G protein complexes, which comprise α, β and γ subunits[24,25]. The recruitment of G proteins to GPCRs may require several motifs present in 7TM receptors. A well-characterized domain involving G protein recruitment and activation is the so-called DRY motif[26-29]. The DRY (asp-arg-tyr) box is a highly conserved region in 7TM receptors, located N-terminally in the second intracellular loop of most GPCRs. In the present study, we generated two different DRY mutants, i.e. DRA-Cb2 and DAY-Cb2, and analyze whether the DRY motif present in Cb2 is crucial for migration and/or block of differentiation.

MATERIAL AND METHODS

Cannabinoid ligands, cytokines and inhibitors of intracellular signaling

The Cb2 ligands 2-arachidonoylglycerol (2-AG), anandamide (AEA), WIN 55,212-2, cannabinol, cannabidiol, Δ^8-tetrahydrocannabinol (Δ^8-THC) and Δ^9-tetrahydrocannabinol (Δ^9-THC) were obtained from Sigma (Zwijndrecht, The Netherlands). N-palmitoylethanolamine (PEA) and N-acylethanolamine (POEA) were from ICN Biomedicals (Zoetermeer, The Netherlands) and CP55,940 from Pfizer (Groton, CT). Cb1 inverse agonist SR141716 and Cb2 inverse agonist SR144528 were kindly donated by Dr. Casellas (Sanofi Recherche, Montpellier, France). Recombinant human stromal cell-derived factor (SDF-1) was obtained from R&D systems (Uithoorn, The Netherlands). Murine IL-3 was obtained from an IL-3 producing CHO cell line and G-CSF was from Amgen (Thousand Oaks, CA). Dibutyryl cyclic AMP (dbcAMP) and U0126 (MEK inhibitor) were from Kordia Life Science (Leiden, The Netherlands), whereas PD98059 (MEK inhibitor) was obtained from Omnilabo International (Breda, The Netherlands). The inhibitors were dissolved in DMSO, added to the cultures at the indicated concentrations, and refreshed daily.

***Cb2-EGFP* expression construct, site directed mutagenesis and infection of 32D/G-CSF-R cells**

A *Cb2-EGFP* fusion construct was generated and cloned into pLNCX (Clontech, Palo Alto, CA) as described previously[17]. A QuikChangeTM Site-Directed Mutagenesis Kit was used to mutate the DRY motif present in the Cb2-EGFP receptor as indicated by the supplier (Stratagene Europe, Amsterdam, The Netherlands). The primers 5'-GCTGTTGACCGCGCCCTATGTCTGTG-3' and 5'-CACAGACATAGGGCGCGGTCAA-CAGC-3' were used to mutate the wt DRY motif into a DRA motif, and the primers 5'-CCGCTGTTGACGCCTACCTATGTCTG-3' and 5'-CAGACATAGGTAGGCGTCAA-CAGCGG-3' to mutate the wt DRY motif into a DAY motif (Figure 8A). The Cb2-EGFP and the DRY mutant constructs were verified by nucleotide sequencing. These constructs were then transfected into Phoenix cells type E (gift from G. Nolan, Stanford, CA) and the viral supernatants used for infection of 32D/G-CSF receptor (32D/G-CSF-R) cells. Single clones were obtained using limiting dilution in 96 well microtiter trays (Becton Dickinson, Mountain View, CA) and infected clones selected on 0.8 mg/ml G418 (Gibco, Breda, The Netherlands). Cb2-EGFP fusion protein and DRY-mutant expression was analyzed by both Leica DMRXA microscopy (Leica Microsystems, Rijswijk, The Netherlands) and flow cytometric analysis of EGFP fluorescence.

Flow cytometric analysis

32D/G-CSF-R cells transduced with either the Cb2-EGFP[17], the Cb2-EGFP DRA mutant, the Cb2-EGFP DAY mutant or an EGFP control were analyzed by flow cytometric detection of EGFP fluorescence (FACScan flow cytometer, Becton Dickinson, Mountain View, CA) as described previously[17].

The myeloid cell lines HL60, NB4, U937, MV11, KG1, KG1A, ME and K562, as well as primary AML samples and $CD34^+$ cells, were also analyzed by immunofluorescence. Bone marrow AML samples at diagnosis and samples from healthy volunteers were obtained after informed consent. Blasts from AML patients and healthy bone marrow specimens were isolated from the samples by Ficoll-Hypaque (Nygaard, Oslo, Norway) centrifugation[69]. The cells were then cryopreserved as described[70]. Normal umbilical cord blood $CD34^+$ cells were purified using a magnetic cell sorting system (MACS cell isolation Kits, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). In brief, cells were thawed, washed twice in RPMI1640 medium (Life Technologies, Breda, The Netherlands) and cultured in this medium suplemented with penicillin (100 U/ml), streptomycin (100 ng/ml) and 10 % fetal calf serum (Life Technologies, The Netherlands) for one hour at 37°C and 10% CO_2. After washing, cells were incubated on ice with the polyclonal N-terminal anti-CB2 antibody (1:50) (Affinity Bioreagents Inc, CO, USA) for 1 hour, followed by 30 min incubation with the FITC-conjugated secondary rabbit antibody (1:200) GAR-FITC/IgG (Nordic Immunological Labs, Tilburg, The Netherlands). In case of dual staining, cells were additionally incubated for 30 minutes with phycoerythrin (PE)-conjugated primary or secondary control antibodies (IgG/GAR-FITC and IgG1-PE). CD34 PE and CD14 PE were obtained from Becton Dickinson (NJ, USA), CD33 PE and IgG1 PE were from Beckman Coulter (CA, USA), and CD66 PE was obtained from CLB Laboratories (Amsterdam, The

Netherlands). Cells were washed twice with Phosphate Buffered Saline (PBS), resuspended in 500 μl PBS containing 0.5% BSA (Bovine serum albumin) and analyzed using a FACScan flow cytometer (Becton Dickinson Mountain View, CA, USA).

Ligand binding analysis

[^{3}H]2-AG was synthesized from 1,3-dibenzyloxy-2-propanol and [^{3}H]arachidonic acid (200 Ci/mmol, ARC Inc., St. Louis, MO), as reported[71], and [^{3}H]CP55,940 (5-(1,1'-dimethyheptyl)-2-[1R,5R-hydroxy-2R-(3-hydroxypropyl) cyclohexyl]-phenol; 126 Ci/mmol) was from NEN DuPont de Nemours (Köln, Germany). Membrane fractions were prepared from the various clones (100 x 10^6/test) as reported[72], and were used in rapid filtration assays with the synthetic cannabinoid [^{3}H]CP55,940. Apparent dissociation constant (K_d) and maximum binding (B_{max}) values of [^{3}H]CP55,940 were calculated from saturation curves through nonlinear regression analysis utilizing the Prism 3 program (GraphPAD Sofware for Science, San Diego, CA). Binding of [^{3}H]2-AG was evaluated with the same filtration assays used for [^{3}H]CP55,940, and apparent K_d and B_{max} values were calculated through nonlinear regression analysis of saturation curves[72]. In all experiments, non-specific binding was determined in the presence of 10 μM nonlabeled agonist. Data reported are the mean (± S.D.) of at least three independent determinations, each in duplicate. Statistical analysis was performed by the nonparametric Mann-Whitney test utilizing the InStat 3 program (GraphPAD Software for Science).

In vitro neutrophilic differentiation of 32D/G-CSF-R cells

The 32D/G-CSF receptor (32D/G-CSF-R) cell line[19] was cultured in RPMI 1640 medium (Life Technologies, Breda, The Netherlands) supplemented with penicillin (100 IU/ml), streptomycin (100ng/ml), 10% Fetal Calf Serum (FCS) and human G-CSF (100 ng/ml) for nine days. Cell counting was performed using a CASY1/TTC cell counter (Schärfe System, Germany) and the cell density was readjusted to 2 x 10^5 cells/ml daily. Morphological analysis was done by microscopy on May-Grünwald-Giemsa stained cytospins (Shandon Holland, Amsterdam, The Netherlands).

Migration assay

Migration assays were performed using 5 μm pore size and 6.5 mm diameter transwells (Corning Costar, Amsterdam, The Netherlands) as previously described[7]. In brief, cells were washed twice with Hank's Balanced Salt Solution (HBSS) medium, resuspended in 100 μl of migration medium (Iscove's Modified Dulbecco's Medium (IMDM) + 0.5% BSA) and placed in the upper chamber of the transwells. 600 μl of migration medium with or without ligand was placed in the lower chamber. After 4 hours of incubation at 37°C and 5% CO_2 the upper chamber was removed and the numbers of migrated cells were determined by a CASY1/TTC cell counter (Schärfe System, Germany). Cb1 and Cb2 inverse agonist (100 nM) were added to the upper chamber when tested. PD98059, U0126 and dbcAMP were added to the cells, incubated 30 min at 37ºC, and then transferred to the upper well.

Cb2-EGFP retroviral vectors, virus production and infection of murine bone marrow progenitor cells

Cb2-EGFP was obtained by *Eco*47III/*Not*I digestion from pEGFP-N1 vector and subcloned as a blunt fragment into the *Hpa*I site of the pBabe retroviral vector. Correct insertion of *Cb2-EGFP* was verified by nucleotide sequencing. The expression constructs were transfected into Phoenix cells type E (gift from G. Nolan, Stanford, CA) and the virus-containing supernatants were used for infection of bone marrow progenitor cells as described previously[21]. Briefly, bone marrow cells were harvested from the femurs and tibiae of normal FVB male mice and crushed in a mortar with HBBS plus 5% FCS. Cells were passed through a 70 µM filter, resulting in a monocellular suspension. After depletion of adherent cells, immature progenitors were separated using a percoll density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) and prestimulated two days in Cell Gro medium (Boehringen Ingelheim Bioproducts Partnership Heidelberg, Germany) supplemented with murine IL-3 (10 ng/ml), thrombopoietin (TPO), human Flt3-ligand and murine stem cell factor (SCF). Retroviral infection was performed in culture dishes (Falcon 1008, Becton Dickinson) coated with 12 µg/ml retronectin during two consecutive days. Transduction efficiency was determined by FACS analysis of EGFP fluorescence. To study migration, cells were cultured in Cell Gro medium supplemented as before plus 2.5 µg/ml puromycin (Sigma, Zwijndrecht, The Netherlands) for 4-5 days and then used in a migration assay. Bone marrow suspension cultures were performed in RPMI 1640 medium supplemented with 10% FCS, 2.5 µg/ml puromycin (Sigma, Zwijndrecht, The Netherlands) and human G-CSF (100 ng/ml). Cultures were carried out in the presence or absence of CP55,940, the Cb1 inverse agonist SR141716, the Cb2 inverse agonist SR144528 or combinations of these agents. Cell counts were performed every 3-4 days and cytospins were prepared for morphological analysis.

RESULTS

CB2 is frequently expressed on human acute myeloid leukemia cells but is absent from normal myeloid precursors

Cb2 is frequently targeted in murine retrovirally-induced leukemia, resulting in overexpression of this receptor[1,7]. To investigate whether CB2 may be involved in human malignancies as well, we studied expression of this receptor on malignant and normal myeloid precursor cells using specific antibodies and flow cytometric analysis. High receptor levels were observed in HL60, NB4 (Figure 1A), U937 and MV11 (data not shown). The cell lines KG1, KG1a, K562 and ME did not show CB2 protein expression (data not shown). High CB2 expression was observed on AML blasts in 14/30 patient samples. Two typical examples are demonstrated in Figure 1B. Flow cytometric analysis of $CD34^+$ purified fractions from normal bone marrow revealed no expression of CB2 on these cells (Figure 1B). Moreover, double labeling of normal marrow cells using CB2 specific antibodies in combination with CD34, CD33, CD66 or CD14, revealed no detectable CB2 levels on myeloid cells at any differentiation stage (Figure 1C). These data suggest that CB2 expression on myeloid leukemia cells in humans, as well as in mice, is an abnormal feature.

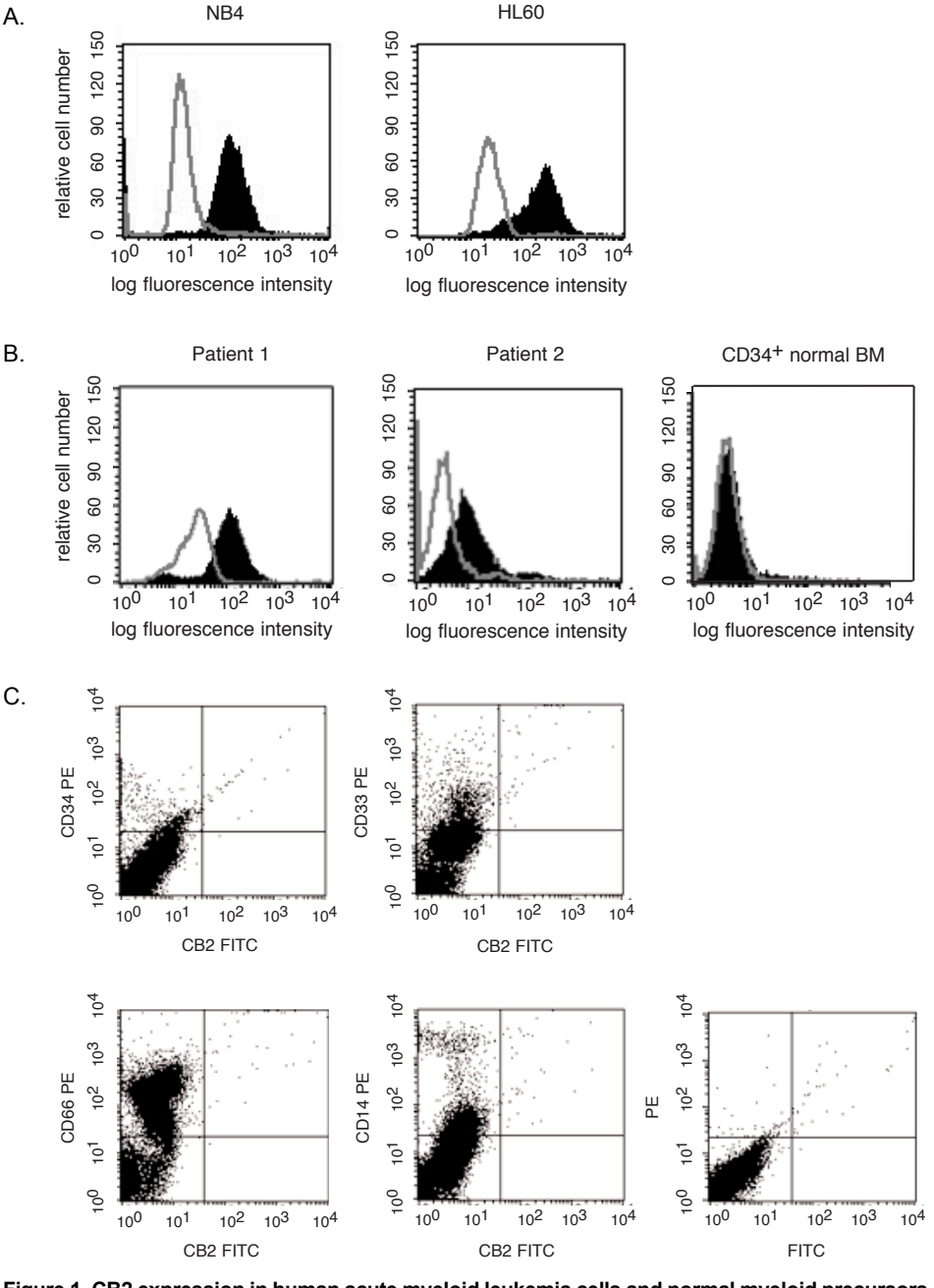


Figure 1. CB2 expression in human acute myeloid leukemia cells and normal myeloid precursors. (A) Flow cytometric analysis of representative CB2 positive cell lines. Staining was performed using a CB2 N-terminal antibody followed by FITC-conjugated secondary rabbit antibody. (B) CB2 cell surface expression analysis of primary AML patient samples and normal $CD34^+$ bone marrow cells using the CB2 N-terminal antibody. (C) Immunophenotyping of normal total bone marrow using flow cytometric analysis.

CP55,940 decreases neutrophilic differentiation, while 2-AG induces migration, of Cb2-expressing bone marrow precursors

To study the mechanism of transformation by this GPCR we introduced the *Cb2* gene, fused in frame to EGFP as previously described[7], into percoll separated normal murine bone marrow cells. Cb2-expressing bone marrow cells migrated well in response to the endocannabinoid 2-AG, whereas control infected marrow cells did not (Figure 2). 2-AG-induced migration could be fully abolished by addition of Cb2 inverse agonist, whereas a Cb1 inverse agonist did not affect migration. In vitro culture with IL-3 or G-CSF revealed no effect of 2-AG on proliferation or differentiation of Cb2-expressing marrow cells (data not shown).

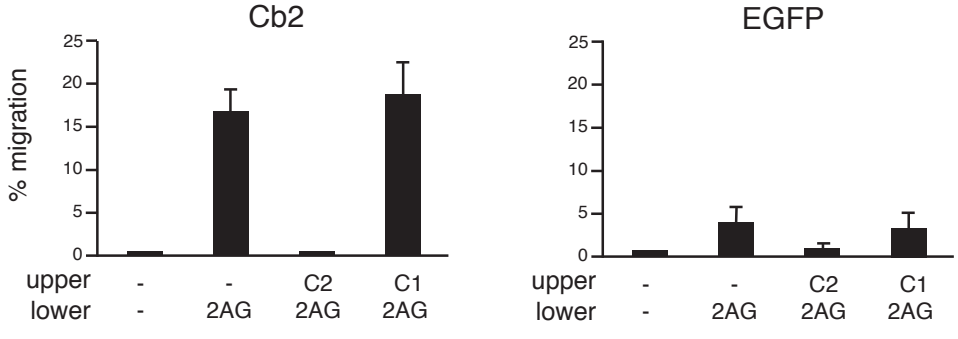


Figure 2. 2-AG-induced migration of Cb2- or EGFP-transduced murine bone marrow cells. Cb2- or EGFP-transduced bone marrow precursors were exposed to medium with or without 300 nM 2-AG. Cells were placed in the upper well in the presence or absence of 100 nM of either a Cb2 (C2) or Cb1 (C1) inverse agonist. Data represent the mean values of three independent experiments.

We next studied whether another well known Cb2 agonist, CP55,940, had an effect on marrow precursors expressing Cb2. A modest but significant decrease in neutrophilic differentiation of Cb2-expressing cells was observed when cells were cultured in suspension with G-CSF plus CP55,940, as compared to cultures with G-CSF alone (Table 1). Moreover, addition of a Cb2 inverse agonist led to recovery of mature neutrophils (Table 1), whereas the Cb1 inverse agonist had no effect (data not shown). No differences were observed when EGFP-transduced marrow cells were cultured with G-CSF with or without CP55,940 and/or inverse agonists (Table 1).

Cb2	Experiment 1			Experiment 2			Experiment 3		
	Immature[a]	Premature[b]	Mature[c]	Immature[a]	Premature[b]	Mature[c]	Immature[a]	Premature[b]	Mature[c]
G[d]	18	30	52	15	17	68	5	11	84
G+CP[e]	17	39	44	29	32	39	13	30	57
G+CP+C2[f]	8	11	81	9	21	70	3	10	87

EGFP	Experiment 1			Experiment 2			Experiment 3		
	Immature[a]	Premature[b]	Mature[c]	Immature[a]	Premature[b]	Mature[c]	Immature[a]	Premature[b]	Mature[c]
G[d]	6	11	83	17	17	66	7	6	87
G+CP[e]	5	16	79	13	25	62	2	15	83
G+CP+C2[f]	10	20	70	1	20	73	2	8	90

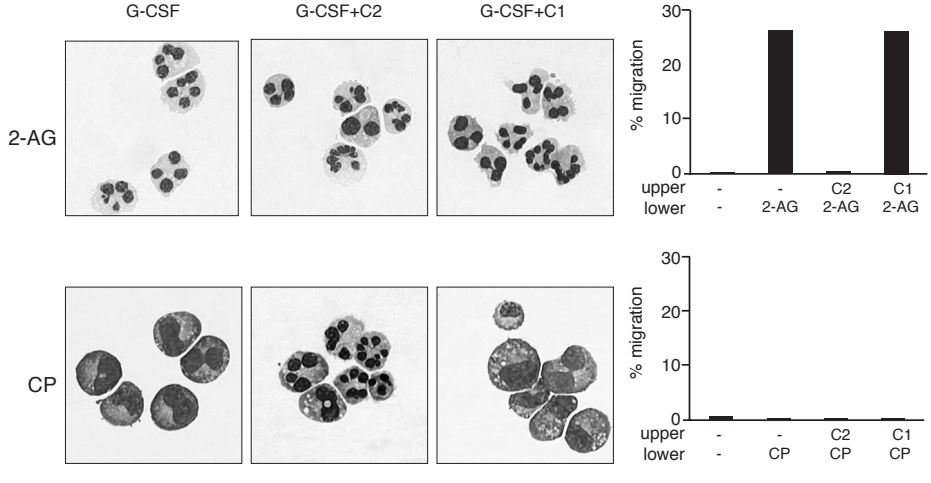
[a]Percentage of myeloblast and promyelocytes
[b]Percentage of myelocytes and metamyelocytes
[c]Percentage of band and segmented neutrophils
[d]G-CSF (100 ng/ml)
[e]G-CSF+100 nM of CP55,940
[f]G-CSF+100 nM of CP55,940+1 μM of Cb2 inverse agonist

Table I. Morphologic analysis of Cb2- and EGFP-transduced murine bone marrow cells cultured for 6 days.

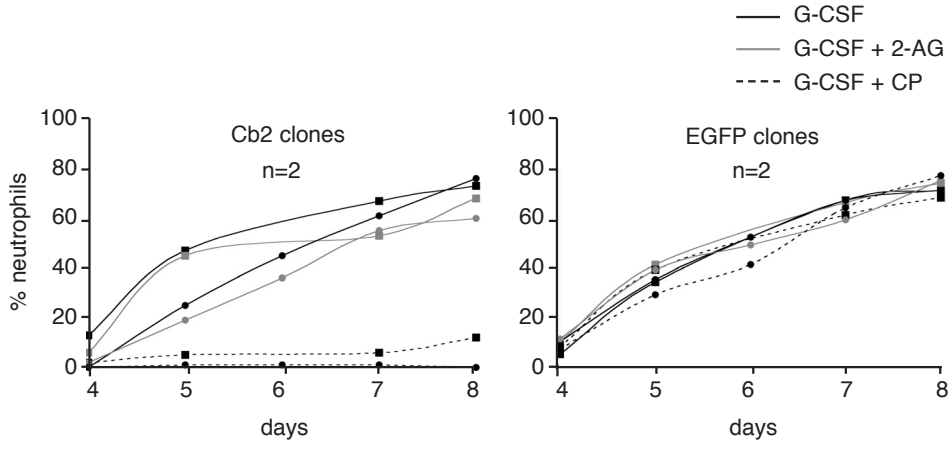
The endocannabinoid 2-AG stimulates migration while CP55,940 induces a full block of neutrophilic differentiation in Cb2-expressing 32D/G-CSF-R cells

To further analyze the effects of Cb2 and its distinct ligands in detail, *Cb2-EGFP* or *EGFP* constructs were introduced into 32D/G-CSF-R cells. 8 Cb2-expressing clones and 8 EGFP control clones were cultured in the presence of G-CSF and various concentrations of 2-AG (100 nM-1 µM). 2-AG did not affect neutrophilic differentiation at any of the concentrations tested (Figure 3A and B). On the other hand, 2-AG was shown to be an efficient stimulator of 32D/G-CSF-R/Cb2 cell migration, as determined by transwell assay (Figure 3A). This effect was receptor specific, as 2-AG-induced migration was fully counteracted by the Cb2 inverse agonist SR144528 but not by the Cb1 inverse agonist SR141716 (Figure 3A). In contrast, the Cb2 ligand CP55,940 fully blocked G-CSF-induced neutrophilic differentiation of Cb2-expressing 32D/G-CSF-R clones (Figures 3A and B). CP55,940 did not affect maturation of EGFP control 32D/G-CSF-R cells (Figure 3B). Addition of Cb2 inverse agonist to the G-CSF/CP55,940 containing cultures completely restored neutrophilic differentiation of Cb2-expressing 32D/G-CSF-R cells, whereas the Cb1 inverse agonist had no effect (Figure 3A). Titration experiments revealed that pM concentrations of CP55,940 were sufficient to significantly block differentiation (Figure 3C). Using 100 nM CP55,940 and different concentrations of Cb2 inverse agonist we observed that neutrophilic differentiation of Cb2-expressing cells could be recovered in a dose dependent manner (Figure 3D). In contrast to 2-AG, CP55,940 could not induce migration of Cb2-expressing cells (Figure 3A).

A.



B.



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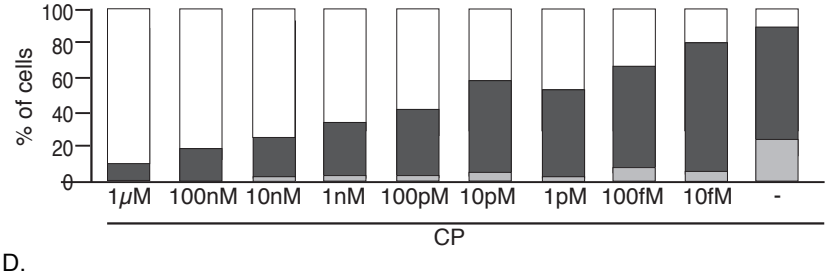

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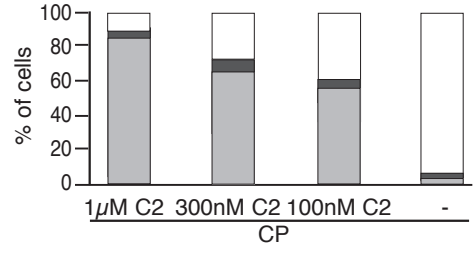


Figure 3. Effects of distinct cannabinoids on the G-CSF-induced neutrophilic differentiation and migration of 32D/G-CSF-R cells.

(A) Morphologic analysis of May-Grünwald-Giemsa stained cytospins of one representative Cb2-expressing clone cultured in G-CSF and 100 nM 2-AG or CP55,940 (CP), in the presence or absence of 1μM Cb2 (C2) or Cb1 (C1) inverse agonist. In vitro migration of 32D/G-CSF-R/Cb2 cells upon 2-AG or CP stimulation. 100nM C2 or C1 were added to the upper well when tested. (B) Two representative Cb2- and two representative EGFP-expressing clones were cultured in G-CSF and 100 nM 2-AG or CP. (C) Differential counts of a representative CP titration experiment in the presence of G-CSF (day 8 of culture). White bars represent blast cells, black bars intermediate forms and gray bars terminally differentiated neutrophils. (D) Differential counts of a 32D/G-CSF-R/Cb2 clone cultured with G-CSF, 100 nM of CP and different concentrations of C2.

CP55,940-mediated block of differentiation and 2-AG-induced migration are pertussis toxin (PTX) sensitive

As the Cb2 receptor belongs to the GαiPCR sub-family we studied whether Gαi proteins were necessary for Cb2 to either stimulate migration or block neutrophilic differentiation. We observed full differentiation of Cb2-expressing 32D/G-CSF-R cells when PTX (100 ng/ml) was added to the G-CSF/CP55,940 cultures (Figure 4A and B). Addition of PTX to the EGFP control clones had no effect on the neutrophilic differentiation of these cells (Figure 4A and B). Moreover, 2-AG-induced migration of 32D/G-CSF-R/Cb2 cells was completely abolished by 300ng/ml PTX (Figure 4C).

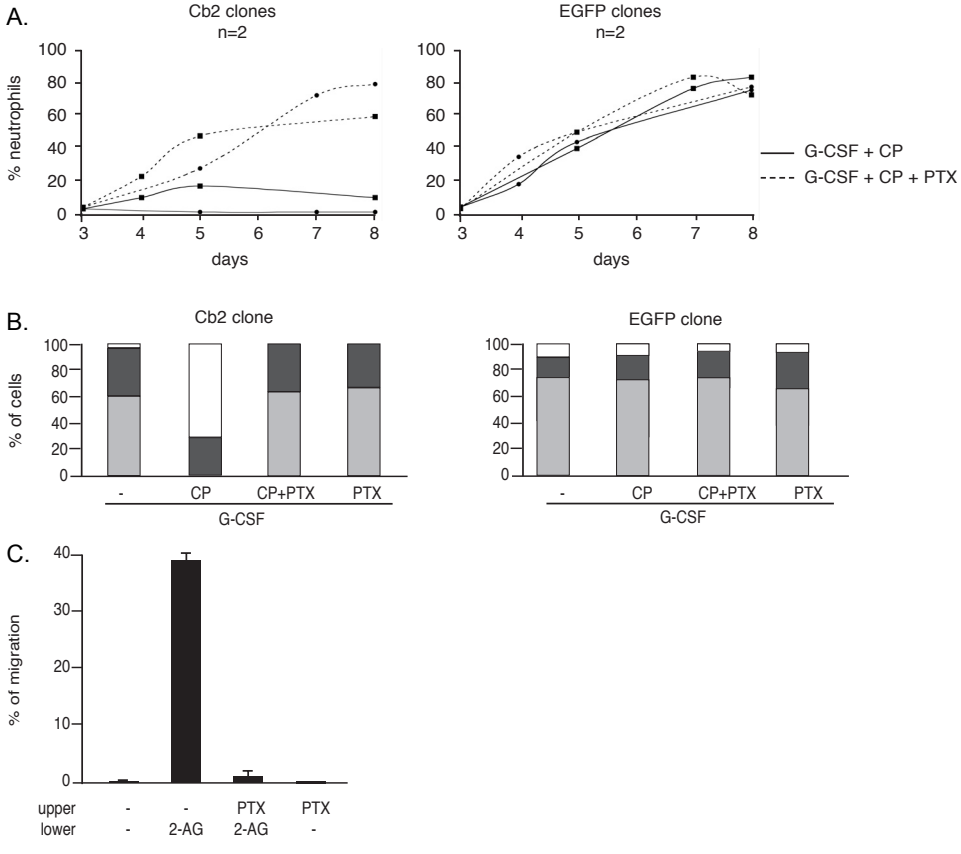


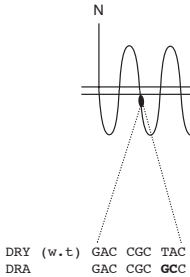
Figure 4. Effect of pertussis toxin (PTX) on the CP55,940-evoked block of differentiation and the 2-AG-induced migration of 32D/G-CSF-R cells.

(A) Two representative Cb2- and EGFP-expressing 32D/G-CSF-R clones were cultured for 8 days in the presence of G-CSF plus CP55,9940 (CP) with or without PTX (100 ng/mL). (B) Differential counts of a representative Cb2- and EGFP-expressing 32D/G-CSF-R clones at day 7 of culture in the presence of G-CSF with or without CP and PTX. White bars represent blast cells, black bars intermediate forms and gray bars terminally differentiated neutrophils. (C) Effect of 300 ng/ml PTX on 2-AG-induced migration of 32D/G-CSF-R/Cb2 cells. PTX was added to the cells and pre-incubated for 1 hour at 37°C before placing the cells in the upper chamber of a transwell assay. Values indicate the average of three reprensentative clones.

Mutation of the DRY-motif in Cb2 causes a reduced migration response to 2-AG but does not affect CP55,940-mediated block of differentiation

To assess whether the Cb2 DRY motif is important in recruiting and activating G proteins during CP55,940-mediated block of differentiation and/or 2-AG-induced migration, 32D/G-CSF-R cells were infected with retrovirus carrying different *Cb2-EGFP* DRY-mutants. Distinct constructs, i.e. *Cb2-DRY* (wt), *Cb2-DRA* (mutant) and *Cb2-DAY* (mutant), were generated (Figure 5A), and introduced into 32D/G-CSF-R cells for analysis in transwell and differentiation assays. Following G418 selection, 6 32D/G-CSF-R clones carrying each construct were obtained. Expression of the distinct Cb2-EGFP variants was analyzed by fluorescence microscopy and flow cytometric analysis. A representative clone for each transfected construct is shown in Figure 5B. Equal levels of fluorescence were detected in all clones and receptor membrane distribution was similar (Figure 5B). Binding of 2-AG and CP55,940 to the different clones was assessed by a ligand binding assay. Figure 5A shows that receptor levels (B_{max}), as well as affinities (K_d), for 2-AG and CP55,940 on 32D/G-CSF-R cells were comparable among clones transduced with the distinct constructs.

A.



		Binding of CP55,940		Binding of 2-AG	
		K_d	B_{max}	K_d	B_{max}
DRY (w.t)	GAC CGC TAC	409±27	1145±33	94±17	517±35
DRA	GAC CGC **GC**C	390±42	1210±56	119±30	315±32
DAY	GAC **GC**C TAC	349±72	1025±86	99±23	487±42

B.

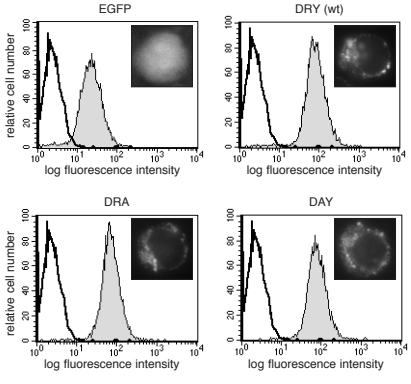


Figure 5. Mutation of the DRY motif in Cb2 and generation of 32D/G-CSF-R/Cb2-mutant clones. (A) Location of the wt DRY motif in Cb2, and of the two mutated forms, DRA and DAY. Introduced mutations are indicated in bold. Right box shows the results of ligand binding assays performed in distinct 32D/G-CSF-R clone types. The dissociation constant (K_d) of CP55,940 is expressed in pM, whereas the K_d values of 2-AG are expressed in nM. Maximum binding (B_{max}) is expressed as fmol/mg protein. Data were pooled from independent experiments performed on two clones of the same cell type. (B) Flow cytometric analysis of representative 32D/G-CSF-R clones expressing the distinct constructs. Upper right inserts show cell fluorescence distribution in the infected cells by microscopy. Original magnification x 63.

Cb2 mutants cultured in the presence of G-CSF plus CP55,940 showed a block in neutrophilic differentiation (Figure 6A and B), comparable to the differentiation block observed with the non-mutated Cb2 wt transduced cells. This block of differentiation was reversible with the Cb2 inverse agonist, but not by addition of Cb1 inverse agonist (Figure 6B). In contrast, the 2-AG-induced migration of 32D/G-CSF-R/Cb2-mutants was significantly reduced in comparison to the non-mutated Cb2 control clones (Figure 6C). The reduced levels of 2-AG-induced migration could still be abolished by addition of Cb2, but not Cb1, inverse agonist (Figure 6C).

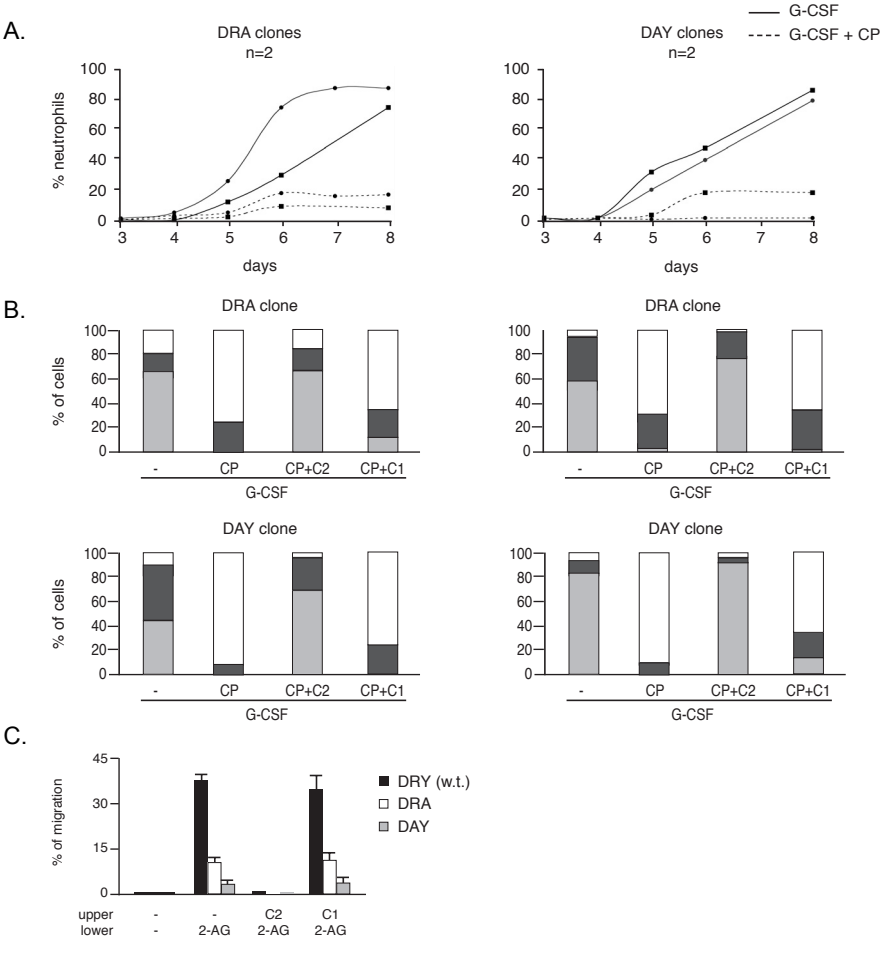


Figure 6. 32D/G-CSF-R/Cb2-mutants in neutrophilic differentiation and migration.
(A) 2 Cb2 DRA and 2 Cb2 DAY clones cultured in G-CSF with or without CP55,940 (CP).(B) Four representative 32D/G-CSF-R clones expressing Cb2 mutants cultured in G-CSF with or without CP, Cb2 (C2) and Cb1 (C1) inverse agonist (100 nM). Counts were carried out on day 8 of culture. White bars represent blast cells, black bars intermediate forms and gray bars terminally differentiated neutrophils. (C) In vitro migration of cells containing a DRY, DRA or DAY motif. Cells were exposed to medium with 300 nM 2-AG or control medium. 100 nM C1 or C2 were added to the upper chamber. The percentage of migration is the average of three representative clones.

dbcAMP interferes with migration but not with the neutrophilic differentiation block of Cb2-expressing 32D/G-CSF-R cells

Since activation of GαiPCRs inhibits adenyl cyclase activity, we first investigated the effect of dbcAMP on the two distinct functions of Cb2 in 32D/G-CSF-R/Cb2 cells. Addition of dbcAMP to the G-CSF and CP55,940 containing cultures did not recover neutrophilic differentiation of 32D/G-CSF-R/Cb2 cells (Figure 7A and B). dbcAMP did not alter neutrophilic maturation of EGFP control clones (Figure 7A). Increasing concentrations of dbcAMP partially blocked 2-AG-induced migration of Cb2-expressing 32D/G-CSF-R cells (Figure 7C). Thus, downregulation of intracellular cAMP levels seems to be partly responsible for Cb2-mediated migration but appears unimportant for the block of neutrophilic differentiation following Cb2 receptor stimulation.

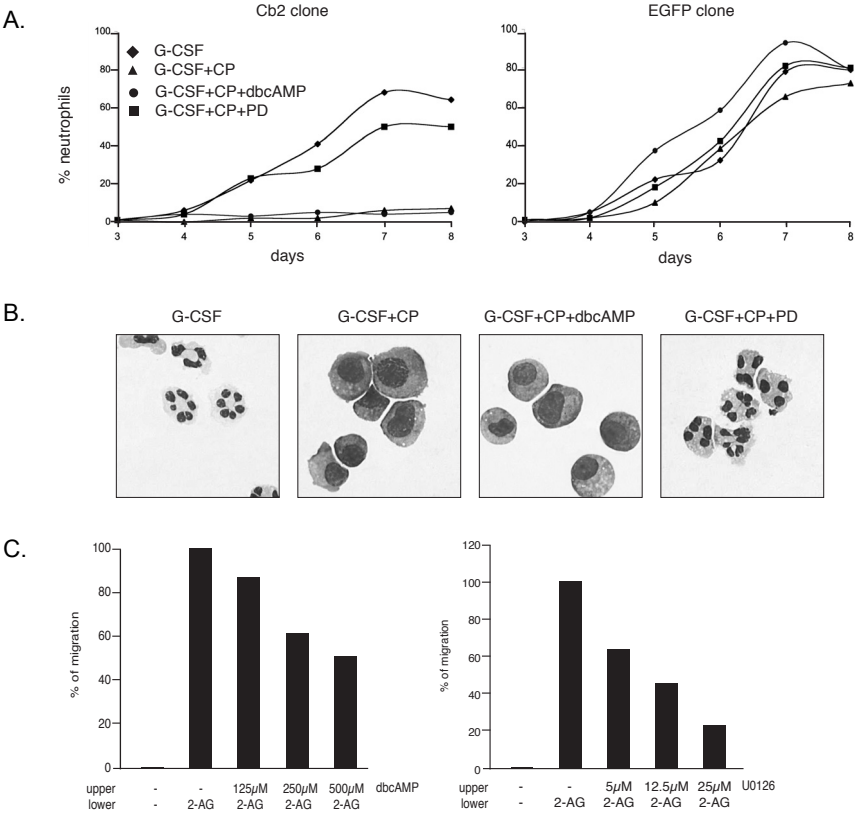


Figure 7. Effects of dbcAMP and MEK/ERK inhibitors on 2-AG-induced migration and the CP55,940-stimulated block of differentiation.

(A) A representative Cb2 and EGFP 32D/G-CSF-R clone cultured with G-CSF, with or without 100 nM CP55,940 (CP), 100 μM dbcAMP, or 25 μM PD98059 (PD). (B) Pictures of May-Grünwald-Giemsa-stained cytospins (day 8) of a representative Cb2-expressing 32D/G-CSF-R clone cultured under the different conditions. Original magnifications x 63. (C) Effects of different concentrations of dbcAMP and U0126 on the 2-AG-induced (300 nM) migration of a representative Cb2-expressing 32D/G-CSF-R clone. The y-axis indicates the percentage of migrated cells in relation to the non-treated cells.

Interference of both the CP55,940-mediated block of differentiation and the 2-AG-induced migration by MEK/ERK pathway inhibitors

We next studied whether signaling via the MEK/ERK pathway is critical for the distinct Cb2-mediated effects. MEK inhibitors, PD98059 (Figure 7A and B) or U0126 (data not shown), fully recovered neutrophilic differentiation of 32D/G-CSF-R/Cb2 cells cultured with G-CSF plus CP55,940. MEK inhibitors did not alter differentiation of EGFP control clones (Figure 7A). Addition of U0126 to transwell assays revealed a dose dependent inhibition of 2-AG-induced migration of Cb2-expressing 32D/G-CSF-R cells (Figure 7C). The same results were observed when the cells were exposed to PD98059 in a transwell assay (data not shown). This effect appeared highly specific, as stimulation of migration of 32D/G-CSF-R cells by SDF1, the ligand for CXCR4, could not be inhibited by U0126 (data not shown). These data indicate that MEK/ERK signaling is critical in 2-AG-induced migration as well as for CP55,940-induced block of neutrophilic differentiation.

DISCUSSION

The peripheral cannabinoid receptor gene, *Cb2*, encodes a seven transmembrane (7TM) G protein-coupled receptor (GPCR)[3]. Using retroviral insertional mutagenesis we identified *Cb2* as the target gene in the *Evi11* locus, indicating that *Cb2* may be a proto-oncogene involved in leukemogenesis[1,2]. We previously observed that Cb2 is highly expressed in myeloid cell lines containing a retroviral insertion in *Cb2*[1,7], and in this report we showed that CB2 is overexpressed in several human myeloid leukemia cell lines. Interestingly, CB2 is frequently overexpressed in AML blasts, whereas normal bone marrow fractions are CB2 negative. It is unclear why CB2 is so highly expressed in particular AML samples and cell lines. Real-time PCR studies revealed high levels of *CB2* mRNA in the CB2 positive cell lines and not in the negative lines, suggesting altered transcription (unpublished observation). The cause of these differences in transcription remains to be elucidated. When overexpressed in myeloid precursors, Cb2 induces a block in neutrophilic development[17] and stimulates migration of Cb2-expressing cells in vitro[7]. The endocannabinoid 2-AG is the most potent agonist capable of inducing migration of cannabinoid receptor expressing cells[7,9-11]. Here we report that 2-AG has no effect on the G-CSF-induced differentiation of 32D/G-CSF-R/Cb2 cells in vitro, whereas another well described ligand CP55,940[22,23], stimulates a neutrophilic differentiation block. The fact that the Cb2 inverse agonist SR144528 could fully counteract the two functions demonstrates receptor specificity. To our knowledge, this is the first example of a GPCR that, when overexpressed in myeloid precursors cells, causes two different effects that depend on the nature of the ligand.

Normal murine bone marrow precursors aberrantly expressing Cb2 show a moderate but reproducible impairment of maturation when cultured with G-CSF plus CP55,940. Leukemia is a multigenic disease, meaning that a combination of genetic defects is required to obtain a full leukemia[15,16]. For instance, we previously demonstrated that aberrant Cb2 expression frequently coincides with aberrant expression of Evi1[30,31], another transfor-

ming gene shown to be involved in the impairment of neutrophilic development[32-34]. The observation that in 32D/G-CSF-R cells Cb2 overexpression causes a complete block of neutrophilic differentiation suggests cooperation between *Cb2* and other genetic defects present in this myeloid precursor cell line. Interestingly, retroviral insertions at the *Evi1* locus and Evi1 overexpression in 32D cells[35] has been reported. It would be of interest to study whether overexpression of Cb2 in combination with Evi1 in normal marrow precursors would lead to a more severe block of neutrophilic differentiation.

AML is characterized by an accumulation of immature myeloid precursors in the bone marrow and blood[13]. Therefore, the block of differentiation observed in Cb2-expressing cells should be considered a leukemic event. Whether altered migration and/or homing of Cb2-expressing myeloid precursor cells may contribute to leukemia development is conceivable but remains presently unclear. The Cb2 mutants fully capable of inducing a block in differentiation, but lacking the ability to migrate, may be excellent tools to address these questions. Transplantation of murine bone marrow cells transduced with *Cb2* wt or *Cb2* mutants into irradiated recipients may clarify whether altered migration and/or differentiation arrest are critical in the development of a myeloproliferative disease or leukemia in vivo.

The experiments presented in this manuscript demonstrate that Cb2 may evoke two distinct biological effects depending on the ligand used for stimulation: (1) migration upon 2-AG presentation and (2) a block in neutrophilic differentiation following exposure to CP55,940. How the interaction of distinct Cb2 ligands with Cb2 results in activation of different processes in the same cell remains to be addressed. Several examples exist of a receptor interacting with distinct ligands. The cytokines IL-3, IL-5 and GM-CSF interact with different receptor complexes that all share a common beta-receptor chain. The specificity of these distinct ligands for the different complexes is determined by the ligand specific receptor alpha chains[36,37]. Similarly, IL-2, IL4, IL-7, IL-9 and IL-15 each interact with unique receptor complexes that all share a common gamma chain[38,39]. The distinct effects that we observed upon 2-AG or CP55,940 stimulation may be explained by stimulation of two distinct receptor complexes which both contain Cb2. It has been generally believed that GPCRs function as monomers. However, GPCRs may be involved in high molecular weight complexes formed by homo- as well as hetero-dimers. Interestingly, dimer formation may affect receptor-ligand binding, signaling and function[40-42]. Another intriguing observation is that agonist presentation can either promote or decrease receptor dimerization in several GPCRs[43-45]. In addition, receptor heterodimerization between GPCRs may result in the generation of novel ligand binding sites[46], or novel ligand binding properties[47]. GPCRs may even interact with non-G protein-coupled receptors, such as tyrosine kinase receptors[48]. In this respect it is interesting to note that functional cooperation between CB1 and both the fibroblast growth factor receptor and insulin-like growth factor 1-receptor has been demonstrated . Our observation that binding of distinct ligands to Cb2 receptors evoked varied effects, may be explained by the presence of Cb2 in distinct complexes which depend on ligand identity. Alternatively, Cb2 may act as a monomer with one agonist and as a di- or multimere with the other.

Cb2 belongs to the family of GPCRs[3,51,52], suggesting that signaling upon receptor stimulation may require G proteins. The inhibition of migration by PTX administration, as well as the inefficient chemotaxis of cells expressing DRY-Cb2 mutants, indicate that Cb2 requires G protein activity to induce migration upon 2-AG stimulation. In addition, most chemokine receptors belong to the subgroup of GαiPCRs[53-55], suggesting that adenyl cyclase inactivation and cAMP downmodulation via Gαi proteins may be critical for induction of migration. The decrease of 2-AG-induced migration of 32D/G-CSF-R/Cb2 cells following dbcAMP administration demonstrates that this pathway is critical for Cb2-mediated cell motility. On the other hand, our data show that downregulation of the intracellular cAMP levels is not the only pathway involved in migration of Cb2-expressing cells. Transwell studies using MEK inhibitors demonstrate that this route plays a critical role in 2-AG-stimulated chemotaxis as well. Previous studies demonstrated that stimulation of the cannabinoid receptors Cb1 and Cb2 results in activation of the ERK pathway[56-58]. Although critical for Cb2-induced chemotaxis, MEK/ERK signaling is not a prerequisite for GPCR-induced migration in general. For instance, SDF-1-induced migration of 32D/G-CSF-R/Cb2 cells, which endogenously express the CXCR4 receptor, is insensitive to the addition of MEK inhibitors (data not shown). We clearly demonstrate that 2-AG-induced migration involves the MEK/ERK pathway, but the mechanism of activation remains unclear. Multiple examples show that MEK/ERK signaling route may be activated through the βγ complex[59], although G protein-independent manners of ERK activation have been proposed for GPCRs[59,60] as well. In summary, Cb2-mediated migration depends on at least two distinct signaling pathways that both appear to be indispensable (Figure 8).

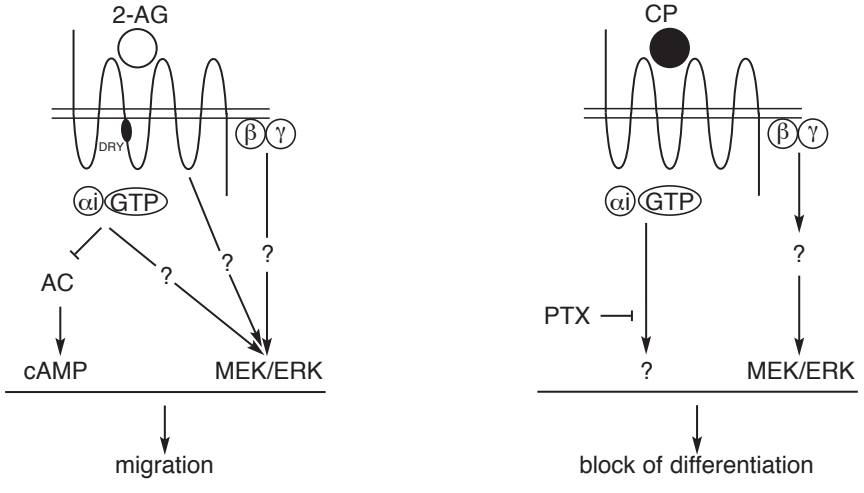


Figure 8. Schematic representation of Cb2 signaling.
Signal transduction pathways linked to Cb2 leading to the 2-AG-induced migration and the CP55,940-evoked block of neutrophilic differentiation.

As with migration induction, our data suggest that multiple pathways are involved in the Cb2-induced block of differentiation (Figure 8). MEK/ERK signaling has been shown to be critical, since MEK inhibitors fully recovered G-CSF-induced differentiation in the presence of CP55,940. Previously, we also demonstrated involvement of PI3-K in the Cb2-mediated block of differentiation[17]. On the other hand, intracellular cAMP downregulation is unimportant for this effect. Moreover, the DRY motif, which is critical for the 2-AG-induced migration, was completely unnecessary for the induction of a maturation arrest. These findings would suggest that G protein signaling is dispensable for the Cb2-induced differentiation block. However, we demonstrated that the CP55,940-evoked block in differentiation of 32D/G-CSF-R/Cb2 cells could be fully reversed by the addition of PTX. Therefore, these data suggest that activated Cb2 may induce the proper signals via G proteins. However, as we observed that the DRY motif is unnecessary for the CP55,940-induced block of differentiation, we suggest involvement of another currently unknown G protein interaction domain in Cb2.

Multiple GPCRs have been previously reported to possess transforming abilities, including the α1B-adenergic[61], thrombin[62], and serotonin 1 C receptors[63] and the receptor encoded by the *Mas* oncogene[64,65]. In contrast to Cb2, which interacts with Gαi subunits, a large number of these previously identified transforming GPCRs can associate with Gαs subunits[66]. An interesting question to be addressed is whether transformation of myeloid precursor cells by Cb2 is a feature unique for this particular GPCR or whether the peripheral cannabinoid receptor is a paradigm for a novel class of transforming GPCRs. The fact that stimulation of migration as well as interference with differentiation by Cb2 are PTX sensitive, showing involvement of Gαi, may indicate that other Gαi interacting GPCRs with transforming abilities may exist. Interestingly, using retroviral insertional mutagenesis we and others recently identified, among a large panel of novel leukemia disease genes, four GPCR encoding genes[67,68] (unpublished observation). Three of those genes encode GPCRs that may interact with Gαi subunits, namely endothelial differentiation gene 3-R (Edg3-R), chemokine-R7 (CCR7) and vomeronasal1-R (V1-R). Introduction of these transforming receptors, or other potentially interesting GPCRs, into 32D/G-CSF-R cells is a valid approach to address the idea of Cb2 as a paradigm for a novel class of transforming GPCRs.

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CHAPTER 5

General discussion

5.1 *Cb2* is a proto-oncogene involved in leukemic transformation

The peripheral cannabinoid receptor gene *Cb2* has previously been identified as a common virus integration site in retrovirally-induced leukemias, suggesting that it is a proto-oncogene[1,2]. The major part of the work presented in this thesis was performed to determine the effect of Cb2 overexpression in myeloid precursor cells. We demonstrated that Cb2, when introduced into either the in vitro myeloid differentiation model 32D/G-CSF-R, or normal bone marrow precursor cells, transforms hematopoietic cells. Cb2 interferes with G-CSF-induced neutrophilic differentiation, a hallmark for myeloid leukemia, in the presence of the synthetic ligand CP55,940. Moreover, Cb2-expressing cells migrate upon stimulation by 2-AG, a potent endocannabinoid. Taking these observations into a leukemic context, we hypothesize that Cb2-expressing leukemia cells are unable to differentiate into neutrophils and migrate abnormally. Interestingly, Cb2 is the first example of a GPCR that interferes with G-CSF-induced neutrophilic differentiation. Future studies to determine if Cb2-mediated transformation of myeloid precursor cells represents a unique mechanism or whether it serves as a paradigm for transformation in general will be discussed in this chapter.

5.2 Identification of critical domains in Cb2

5.2.1 Cb1 stimulates migration of myeloid precursor cells but does not induce a block in neutrophilic differentiation

The identification of critical domains in Cb2 required for transformation of hematopoietic cells may come from the introduction and analysis of the central cannabinoid receptor Cb1 in myeloid precursors. Cb1 is mainly expressed in brain and is the closest homologue of Cb2. High homology has been observed within the transmembrane domains, regions that form the binding pocket for the cannabinoid ligands. Cb1 and Cb2 respond to some extent to the same compounds. For instance, 2-AG and CP55,940 bind with similar affinity to both cannabinoid receptors[3]. These similarities between the cannabinoid receptors provide us with a valuable tool to further study how Cb2 interferes with G-CSF-induced neutrophilic differentiation. In a preliminary study *Cb1* was fused to *EGFP*, cloned into pLNCX, and introduced in 32D/G-CSF-R cells following the same procedure described for Cb2 (see Chapters 3 and 4 for details). Interestingly, we observed that 32D/G-CSF-R/Cb1-EGFP clones migrated in response to 2-AG in a transwell assay, but not to any other cannabinoid (unpublished observation, Figure 1A). Moreover, this 2-AG-induced migration could be completely abolished by Cb1-, but not by Cb2-, inverse agonist, indicating that the effect was Cb1 receptor-mediated (Figure 1B). In contrast to Cb2-expressing 32D/G-CSF-R cells, Cb1-expressing cells did not show a block in neutrophilic differentiation when cultured with G-CSF plus different concentrations of CP55,940 ligand (unpublished observation, Figure 2) or any other cannabinoid (data not shown). These results suggested the existence of critical domain(s), required for the neutrophilic differentiation block with Cb2,

which are absent in Cb1. Identification of these regions will be an important step towards elucidation of the mechanism of transformation by Cb2 or other transforming GPCRs.

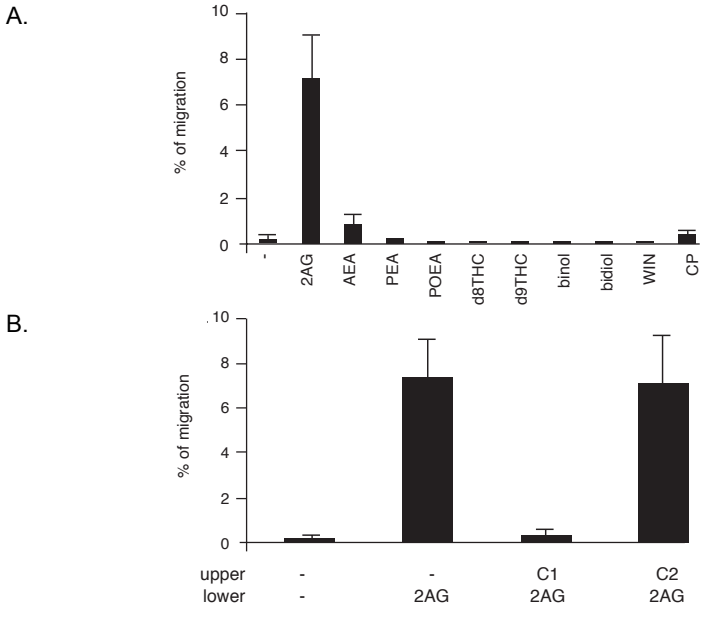

Figure 1. In vitro migration of Cb1-expressing 32D/G-CSF-R cells following exposure to cannabinoids.

(A) 1 µM of various cannabinoid ligands was added to the lower chamber in migration assays, and cells that migrated to the lower well were counted after 4 hours of incubation. Values represent the average of two independent clones. (B) 32D/G-CSF-R/Cb1 cells were exposed to 300 nM of 2-AG. A quantity of 100 nM of Cb1 (C1) or Cb2 (C2) inverse agonist was added to the upper chamber of the transwell assay when tested. The percentage of migrated cells corresponds to the average of three independent clones.

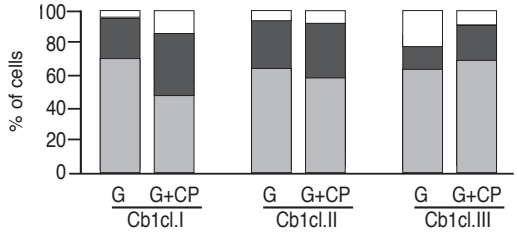

Figure 2. Effect of CP55,940 in the G-CSF-induced neutrophilic differentiation of 32D/G-CSF-R/Cb1 clones.

Three representative Cb1-expressing 32D/G-CSF-R clones were cultured in the presence of G-CSF with or without 100 nM CP55,940 for 9 days. Differential counts were carried out on day 8 of culture. White represents blast cells, black represents intermediately matured granulocytic forms and gray indicates terminally differentiated neutrophils.

5.2.2 Cannabinoid receptor chimeras

Chimeric GPCRs have been generated to define the role of specific domains in 7TM receptors. The intracellular, transmembrane and extracellular domains in these receptors are well defined, and can therefore be easily exchanged with corresponding regions from other GPCRs. Examples of GPCR chimeras that have succesfully been generated to carry out functional analysis of particular domains are secretin/VPAC1[4], dopamine1A/dopamine1B[5], melanocortin1/melanocortin3[6] and CB1/CB2[7,8]. Ho et al successfully generated human CB1/CB2 chimeric receptors which showed similar expression levels and agonist affinity as the wt receptors, but presented differences in phospholipase C activation[7]. Chin et al used CB1/CB2 chimeras to identify critical regions in the transmembrane domain that contribute to subtype specificity in ligand binding[8]. These results suggest that Cb1/Cb2 and Cb2/Cb1 chimeras may be powerful tools to identify critical domains present in Cb2 that are responsible for the CP55,940-induced neutrophilic differentiation block. Therefore, we may swap the distinct intracellular regions of Cb1 and Cb2 to identify which domains are critical for the CP55,940-mediated maturation arrest. Chimeric receptors may be generated in which all four intracellular regions, i.e. the three intracellular loops and the C-terminal intracellular region, may be exchanged at the same time (Figure 3), as well as chimeras in which only one region may be swapped.

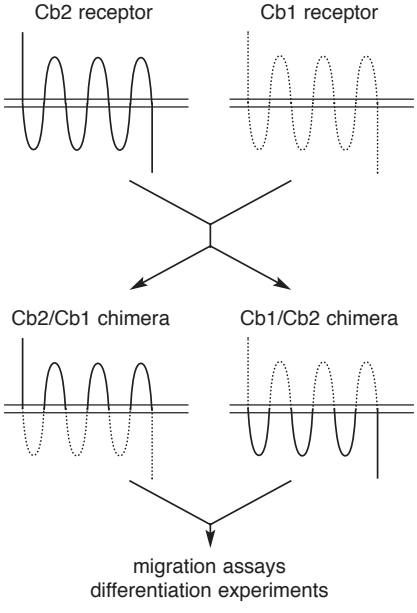

Figure 3. Generation of chimeras between Cb1 and Cb2 receptors.
Schematic representation of the seven transmembrane cannabinoid receptors, as well as Cb1/Cb2 and Cb2/Cb1 chimeric receptors. The chimeras may be tested in migration assays and differentiation experiments as described in Chapters 2, 3 and 4.

These chimeric proteins may be introduced into 32D/G-CSF-R cells and functionality assessed in migration and differentiation assays. These experiments should determine whether we can generate a Cb1 receptor capable of fully blocking neutrophilic differentiation, or whether we can alter the Cb2 receptor such that it cannot interfere with differentiation. These studies would reveal whether particular intracellular domains in Cb2 are critical for CP55,940-induced neutrophilic differentiation interference. However, it is possible that other regions in the transmembrane domains or in the extracellular loops are involved in the Cb2-induced differentiation block. In that case, swapping of those domains should be carried out as well. Once the critical regions are defined, we could further determine crucial amino acids by applying alanine scanning in those regions and generating the corresponding mutants. Presence of the critical regions and/or amino acids in GPCRs in addition to Cb2 may suggest transforming abilities in those receptors as well.

5.2.3 Critical role of the DRY motif in 2-AG-induced migration but not in CP55,940-mediated block of neutrophilic differentiation

Several conserved domains present in GPCRs have been described and linked to receptor biology, e.g. receptor expression, agonist binding, internalisation and receptor-G protein interaction. In Chapter 4 we demonstrated that the DRY motif, Asp-Arg-Tyr, located at the boundary of the transmembrane helix 3 and the second intracellular loop, is crucial for 2-AG-induced migration but not for the CP55,940-evoked block in neutrophilic differentiation of Cb2-expressing cells. Mutational analysis of several GPCRs has shown that this triplet of amino acids plays an important role in G protein recruitment, activation and signaling[9-12]. As the CP55,940-induced block of neutrophilic differentiation was pertussis toxin sensitive, we hypothesize that regions other than DRY may be involved in recruitment and activation of G proteins leading to the block of neutrophilic differentiation. Mutational analysis of other known motifs present in Cb2, e.g. $NPX_{2-3}Y$ in the 7TM region or the junction between the third intracellular loop and the 6TM domain, may reveal critical sequences in this receptor for mediating a differentiation block.
Sequence alignment of previously identified transforming GPCRs to Cb2 and identification of conserved boxes, preferably in transmembrane regions, intracellular loops or the C-terminal tail, may be an alternative strategy to identify motifs involved in G protein recruitment, activation and signaling in the CP55,940-mediated effect. Mutation of the identified domains, transfection into a suitable cell system, and measurement of intracellular cAMP levels and phosphatidylinositol turnover may determine whether those regions represent novel domains required in Cb2 to couple the G proteins that mediate the block in neutrophilic differentiation.

5.3 Cb2 receptor homo/hetero-oligomerisation in signaling

Recent studies have shown that GPCRs can form high molecular weight complexes composed of homo- as well as hetero-dimers. Dimerization may regulate the receptor function in different manners, e.g. by altering receptor-ligand binding, signaling, internalization or trafficking[13-15]. In some cases, GPCR homodimerization may depend on the agonist, that may either promote receptor dimerization[16-18], or decrease the level of dimer formation[19,20]. In other cases a receptor may bind to a particular ligand as a monomer, whereas a homodimeric receptor complex may form new binding sites for another ligand[21]. Furthermore, receptor heterodimerization between GPCRs has been described for several receptors, resulting in either generation of a novel ligand binding site[22], or in novel ligand binding properties[23]. GPCRs can also physically interact with non-G protein-coupled receptors, such as tyrosine kinase receptors[24,25]. However, physical interactions between cannabinoid receptors and other receptor types has not yet been demonstrated. Interestingly, CB1 functionally cooperates with the fibroblast growth factor receptor (FGF) or with the insulin and insulin-like growth factor 1 (IGF-1) receptor[26,27]. Bouaboula et al demonstrated that activation of the MEK/ERK signaling pathway following insulin or IGF-1 exposure could be counteracted by the Cb1 inverse agonist. In Chapter 3 and 4 we demonstrated that the Cb2-mediated effects could be reversed by MEK inhibitors, suggesting involvement of MEK/ERK signaling in 2-AG-induced migration and CP55,940-mediated block of neutrophilic differentiation. It is possible that Cb2 is part of a receptor complex, for example including tyrosine kinase receptors, and that MEK/ERK is activated by this complex following ligand binding. Exposure to the Cb2 inverse agonist may interfere with MEK/ERK signal transduction through this receptor complex. We observed that some serum batches are capable of inducing migration and/or a block in neutrophilic differentiation, suggesting that serum may contain Cb2 ligands. However, these effects might also be explained by the presence of non-cannabinoid compounds which may activate Cb2-containing complexes by stimulating receptors other than Cb2. In fact, preliminary results indicate that neither 2-AG nor CP55,940 are able to directly induce ERK phosphorylation in 32D/G-CSF-R (unpublished observation). The identification of the serum components that stimulate MEK/ERK activation and the differentiation block, may provide important information about the constituents of this particular receptor complex and therefore about the role of Cb2 within this complex.

5.4 GPCRs in malignant transformation

The first link between GPCRs and cellular transformation was provided by the isolation and characterisation of the *mas* oncogene[28]. Young et al suggested that this GPCR was tumorigenic when overexpressed in NIH 3T3 fibroblasts in an agonist-dependent manner. Later, similar results were described for serotonin 1c[29] and muscarinic acetylcholine receptors[30], which ectopic expression confered a transformed phenotype to NIH 3T3 cells. Later, activating mutations were identified in GPCRs genes and linked to transfor-

mation. These mutated genes cause transformation in a ligand-independent way. Examples of activating mutations have been reported for the α_{1B}-adrenoreceptor[31] and the thyroid-stimulating hormone receptor (TSHr)[32]. In some cases, such mutations can also contribute to transformation, when persistently stimulated by agonists released from tumors. For example, this has been shown for both gastrin-releasing peptide (GRP) receptor in prostate cancer and cholecystokinin receptors (CCK1/2) in pancreatic hyperplasia and carcinoma[30,33]. We observed that overexpression of wt Cb2 evolves in a leukemic phenotype in myeloid precursor cells. Overexpression of other oncogenic GPCRs in 32D/G-CSF-R cells may reveal whether Cb2 indeed uniquely mediates a block of neutrophilic maturation or whether a more general GPCR-related process causes transformation of myeloid precursor cells.

We may also consider the possibility of transformation due to mutations in *Cb2*. We recently generated Cb2 mutants in which we altered the DRY motif, a conserved domain involved in G protein recruitment and activation (unpublished observation). Interestingly, mutation of the DRY motif into a VRY motif shows a novel and unique phenotype. 32D/G-CSF-R cells transfected with this VRY-Cb2 mutant do not migrate upon 2-AG stimulation (Figure 4A). This observation may be in agreement with the work of Rhee et al showing that mutation of the D amino acid produces a significant loss of cannabinoid binding[34]. However, 32D/G-CSF-R/Cb2-VRY mutant clones respond to CP55,940 with a neutrophilic differentiation block (Figure 4B). This far these data are identical to the results obtained using the Cb2 DAY- and DRA-mutants described in Chapter 4. Interestingly, and in contrast to cells expressing Cb2 DAY- and DRA-mutants, we could not restore differentiation by adding Cb2 inverse agonist to the cultures (Figure 4C). Moreover, the Cb2 inverse agonist by itself, when added to the G-CSF cultures, induced a block of neutrophilic development (Figure 4C). These data indicate that this particular mutation may alter ligand binding to and affinity for Cb2, and consequently affect Cb2 function. These observations suggest that certain mutations in the DRY motif of Cb2 may cause a leukemic phenotype by allowing different responses to ligand. It would be of interest to determine whether other mutations in Cb2 result in altered migration and/or differentiation in the absence of agonist. Introduction of random mutations in *Cb2* and analysis of the effect in the 2-AG-induced migration and the CP55,940-mediated block of differentiation may be a valid approach to determine crucial mutations in specific motifs.

Most of the currently known transforming GPCRs are associated with Gαs subunits, whereas Cb2 is coupled to Gαi subunits. Using retroviral insertional mutagenesis, we and others identified among a large panel of novel disease genes four GPCR-encoding genes, i.e. endothelial differentiation gene 3-R (Edg3-R), melanocortin1-R (MC1R), chemokine-R7 (CCR7) and vomeronasal1-R (V1-R)[35,36] (Erkeland et. al. in preparation). Interestingly, these receptors bind to different G proteins subtypes, i.e. Edg3-R to Gαi, Gq/11 and G12/13[37], MC1R to Gαs[38], CCR7 to Gαi and Gαq[39], and V1-R to Gαi[40], suggesting that transformation by 7TM receptors may not be restricted to the GαsPCR family. Therefore, introduction of already known or novel identified disease genes encoding GPCRs into 32D/G-CSF-R cells represents an excellent way to determine whether GPCRs, other than the previously identified transforming GαsPCRs, may represent a group of receptors with

transforming capacities. Interestingly, these experiments might provide an excellent tool to determine whether Cb2 is transforming in a classical manner or represents a novel mechanism of transformation.

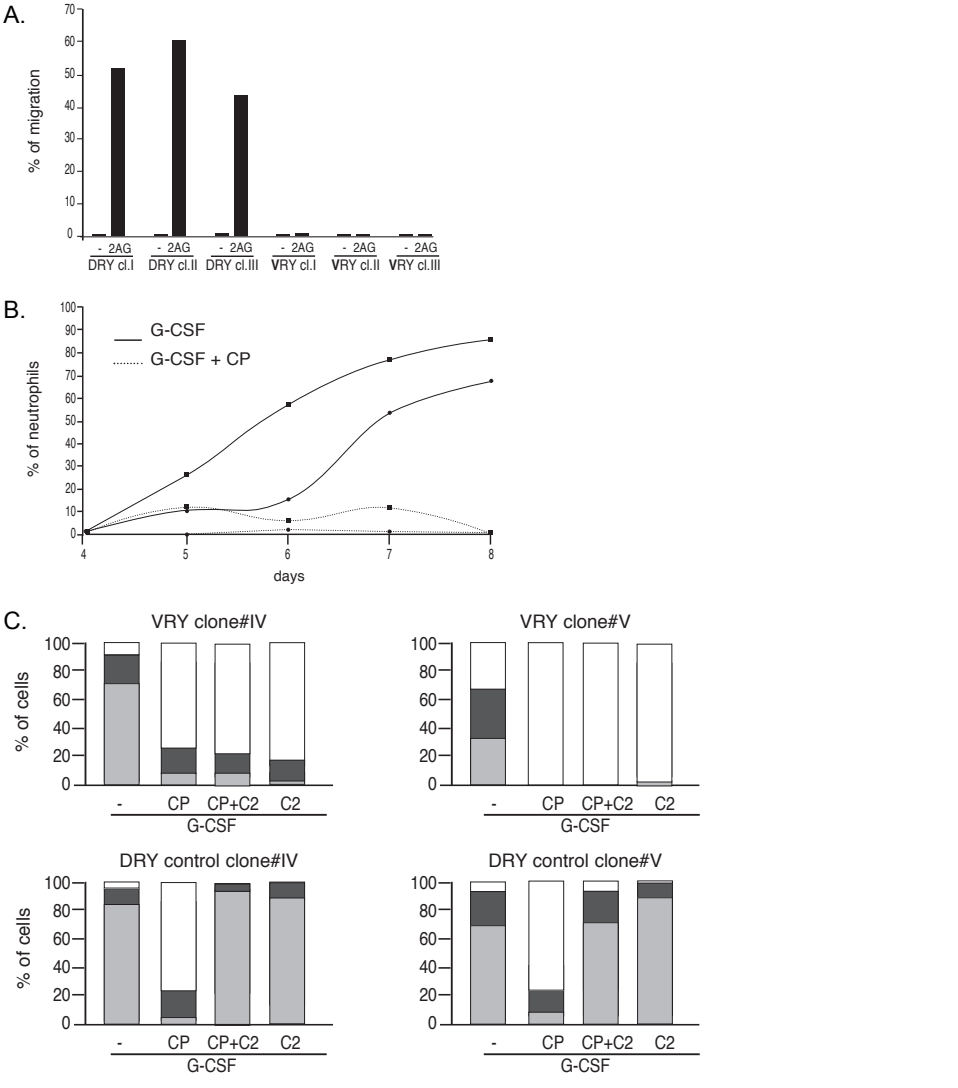

Figure 4. Functional analysis of 32D/G-CSF-R/Cb2-VRY mutants in 2-AG-induced migration and G-CSF-evoked neutrophilic differentiation.

(A) Three representative Cb2 wt DRY clones and three representative Cb2 VRY mutant clones were exposed to 300 nM of 2-AG in a transwell assay. (B) Two representative 32D/G-CSF-R clones expressing VRY Cb2 mutant were cultured in the presence of G-CSF with or without CP55,940 (100 nM). (C) Two representative 32D/G-CSF-R/Cb2 VRY mutant clones and two wt clones were cultured in G-CSF with or without 100 nM CP55,940 and Cb2 (C2) inverse agonist (100 nM). Counts were carried out at day 8 of culture. White represents blast cells, black represents intermediately matured granulocytic forms and gray indicates terminally differentiated neutrophils.

5.5 CB2 in human disease

We recently reported that normal murine bone marrow precursor cells do not express Cb2 receptor[41]. Similarly, in Chapter 4 we demonstrate that human bone marrow $CD34^+$ cells do not express CB2 protein on the surface membrane, but that several human leukemic cell lines, i.e. NB4, HL60, MV11 and U937 highly express CB2. In addition, data from AML patient sample analysis demonstrate that approximately 50% of AML cases are positive for CB2 protein when analyzed by flow cytometry, suggesting a possible role of CB2 in human AML. Introduction of CB2 into human $CD34^+$ cells may help to determine whether overexpression of this GPCR confers a leukemic phenotype in human precursors as well. A block in differentiation of $CD34^+$ cells due to overexpression of CB2 receptor may further support the hypothesis that this GPCR is involved in certain cases of AML. Analysis of a large panel of AML patient samples using cDNA micro-arrays would be of help to determine in which cases CB2 is overexpressed, as well as to study overexpression of other GPCRs.
Furthermore, preliminary data discussed in the previous paragraph suggest that point mutations in Cb2 might also lead to transformation (see Section 5.4). Sequence analysis may determine whether mutations in the DRY motif in human CB2 exist in certain AML cases. Because the DRY motif is crucial for G protein recruitment and signaling in most GPCRs, it may be of interest to set up a high throughput screening protocol to analyze DRY mutations in multiple GPCRs in human AML.

5.6 Concluding remarks and future directions

The work presented in this thesis demonstrates that the peripheral cannabinoid receptor *Cb2*, originally identified as a cVIS in retrovirally-induced murine leukemias, is a proto-oncogene. The experiments performed here describe a possible mechanism by which Cb2 may be involved in leukemic transformation. We observed that overexpression of Cb2 in murine precursor cells induces a block in neutrophilic differentiation, a hallmark in AML. Moreover the data suggest that aberrant expression of this receptor may alter migration/homing upon receptor stimulation. Strikingly, these two Cb2-mediated effects are induced by two distinct ligands in vitro. We demonstrated that the endocannabinoid 2-AG is a potent inducer of migration of Cb2-expressing cells, whereas the synthetic ligand CP55,940 evokes a neutrophilic differentiation block. Since we can mimic this effects by using serum in our experiments, it is possible that potent endocannabinoid ligands, still to be discovered, are naturally produced. However, and as discussed previously in this chapter, it is also possible that distinct non-cannabinoid ligands are present in the serum, activating Cb2-containing complexes, and leading the different Cb2 effects.
In conclusion, Cb2 is the first example of a GαiPCR that, when overexpressed in myeloid precursor cells, induces a neutrophilic differentiation block. Since leukemogenesis is a multi-step disease involving the cooperation of multiple genes, another important issue to be addressed is the identification of those cooperating genes. Previous studies demon-

strated that virus integrations in *Evi11* frequently coincide with insertions in *Evi1* and *Evi12*[42,43]. Generation of *Cb2* transgenic mice may be a valid in vivo approach to address this issue. Crossing of *Cb2* transgenic with *Evi1* transgenic mice (Spensberger and Delwel, unpublished data), or other candidate-gene transgenic mice, may determine whether and how these two proto-oncogenes may collaborate to produce a full leukemia.

As we observed that CB2 is expressed in a high portion of human AML cases, another question yet to be addressed is whether CB2 expression can be used as a prognostic marker in human AML. Interestingly, GPCRs like CB2 are major targets for drug therapy in the pharmaceutical industry. It would be of interest to study if Cb2 inverse agonist is capable of restoring neutrophilic differentiation in those human AML samples in which CB2 is overexpressed. Once the involvement of CB2 or other GPCRs in human AML is further established, specific drugs could be designed to interfere with these receptors.

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SUMMARY

Summary in English

Acute myeloid leukemia (AML) is a blood cell disorder characterized by an accumulation of immature blasts in bone marrow and blood. Human AML is frequently characterized by non-random chromosome translocations resulting in the generation of specific transforming fusion genes and fusion proteins, of which a significant number has been cloned, e.g. AML1-ETO fusion gene in AML with a t(8;21) translocation or PML-RAR in cases with translocation t(15;17). However, in approximately 40 - 50% of AML cases no chromosomal abnormalities are evident, indicating that other more subtle mutations are responsible for the leukemic transformation of myeloid precursor cells. Moreover, AML, like other cancers, is a multigenic disease resulting from an accumulation of multiple genetic aberrations. Thus even in cases with well-characterized translocations, additional genetic defects have likely contributed to the development of AML. The identification and functional analysis of novel disease genes in AML is a major goal of our research group.

One approach utilized to identify novel disease genes in leukemia is retroviral insertional mutagenesis. Mice injected with murine leukemia viruses (MuLVs) develop leukemia following proviral insertion into or near potential disease genes. Viral insertions found in a particular locus in independent tumors are called common virus integration sites, cVIS, and mark the locations of potential proto-oncogenes or tumor suppressor genes. The mouse strain and the type of retrovirus used will determine the kind of leukemia that will develop. We used NIH/Swiss mice injected with Cas-Br-M MuLV which develop frequently myeloid leukemias. Using this combination, we previously identified the cVIS *Evi11* and demonstrated that the gene encoding the peripheral cannabinoid receptor Cb2 is the likely target gene. *Cb2* encodes a seven transmembrane receptor that belongs to the G protein-coupled receptor (GPCR) family and is predominantly present on B lymphocytes. The main objective of the work presented in this thesis is to determine whether *Cb2* is indeed a proto-oncogene and, if so, by which mechanism it may transform hematopoietic precursor cells.

A brief introduction to hematopoiesis and leukemogenesis, followed by a concise explanation of retroviral insertional mutagenesis and the identication of the cVIS *Evi11* is given in **Chapter 1**. This chapter additionally provides a general overview of the central and the peripheral cannabinoid receptors, Cb1 and Cb2.

In **Chapter 2** we demonstrate *Cb2* mRNA and protein expression in retrovirally-induced murine myeloid leukemic cell lines. *Cb2* contains two distinct noncoding first exons, exon-1A and exon-1B, both of which splice to protein-coding exon-2. We demonstrate that in myeloid leukemia cells with proviral insertion in *Cb2*, exon-1B/exon-2 *Cb2* mRNA levels increase, resulting in high receptor numbers, and that cell lines without virus insertion in *Cb2* present low levels of only exon-1A/exon-2 *Cb2* transcripts and receptors could not be detected. We next investigated the function of Cb2 using both a myeloid cell line carrying a virus insertion in *Cb2* and 32D/G-CSF-R (granulocyte-colony stimulating factor receptor) cells transfected with exon-1B/exon-2 *Cb2* cDNA. Using transwell assays we

observed that in these cells a major function of Cb2 is migration stimulation. Exposure of Cb2-expressing cells to various cannabinoid ligands showed that the endogenous agonist for Cb2 is the fatty acid 2-arachidonoylglycerol (2-AG). mRNase protection analysis and radioactiveligand binding studies on murine tissues revealed the presence of both *Cb2* splice variants and Cb2 protein in normal spleen. Transwell experiments carried out with spleen cells showed that 2-AG induces migration of B220-, CD19-, immunoglobin M- and immunoglobin D-expressing B lymphocytes.

In **Chapter 3**, we further explored the role of Cb2 in leukemic transformation. *Cb2* was fused to *EGFP* (enhanced green fluorescence protein) to facilitate receptor identification, and the fusion construct was introduced into 32D/G-CSF-R cells. We demonstrate that 32D/G-CSF-R/Cb2-EGFP cells migrate in a transwell assay in response to 2-AG, indicating that the fusion protein was functional. The 32D/G-CSF-R cell line is an excellent model to study myeloid differentiation as, in the presence of G-CSF, 32D/G-CSF-R cells differentiate into mature neutrophils. Experiments described in this chapter were carried out to study the effect of the Cb2 receptor on G-CSF-induced neutrophilic differentiation of 32D/G-CSF-R cells. We show that Cb2 induces a complete block in neutrophilic differentiation that could be abolished by addition of the Cb2 inverse agonist to the cultures. Furthermore, we reported that full rescue of G-CSF-induced neutrophilic differentiation was observed when cells were cultured with the MEK inhibitors PD98059 or U0126, while partial recovery was detected with the PI3-K inhibitor LY-294,002.

In **Chapter 4** we demonstrate that aberrant expression of Cb2 on hematopoietic precursor cells can lead to various ligand-specific effects. Cb2-expressing myeloid precursors migrate upon stimulation by the endocannabinoid 2-AG and are blocked in neutrophilic differentiation upon exposure to the alternative ligand, CP55,940. Experiments were performed to determine the intracellular mechanisms involved in both the migration and the differentiation block. We demonstrate that both effects depend on the activation of Gαi proteins and require the MEK/ERK pathway. Moreover, down regulation of intracellular cAMP levels appears to play an important role in migration induction, but is not required for the Cb2-mediated maturation arrest. Next, we sought to determine whether the highly conserved G protein-interacting DRY motif, present in the second intracellular loop of Cb2, is crucial for this GPCR. The experiments we present here indicate that the DRY motif is critical for migration, but is unimportant for the block of neutrophilic differentiation. The studies presented in this chapter also reveal that Cb2 is normally absent on human normal bone marrow myeloid precursors, but is frequently present on blast cells isolated from human AML samples.

Chapter 5 compromises a general discussion of several topics that arise from this thesis. First, distinct aspects illuminating the transformation mechanisms utilized by the Cb2 receptor are discussed. This is followed by a paragraph concerning the relevance of GPCRs in malignant transformation and by a section discussing the importance of CB2 in human AML . The last part of chapter 5 contains concluding remarks and future directions for this project.

Samenvatting in Nederlandse

Acute myeloide leukemie (AML) is een bloedziekte die gekenmerkt wordt door een opeenhoping van onrijpe blasten in beenmerg en bloed. AML in de mens wordt vaak gekenmerkt door niet-willekeurige chromosomale translocaties waaruit specifieke transformerende fusiegenen en fusie-eiwitten voortvloeien, waarvan een beduidend aantal gekloneerd is, bijv. het AML-ETO-fusiegen in AML waarbij sprake is van een t(8;21) translocatie, of PML-RAR in gevallen met een translocatie t(15;17). Echter, in ongeveer 40-50% van alle gevallen van AML zijn er geen duidelijke chromosomale afwijkingen aanwezig, wat aangeeft dat andere, meer subtiele mutaties verantwoordelijk zijn voor de leukemische transformatie van myeloide voorlopercellen. Bovendien is AML, net als andere vormen van kanker, een multigene ziekte die ontstaat door een opstapeling van meerdere genetische afwijkingen. Dus zelfs in gevallen met goed omschreven translocaties is het waarschijnlijk dat additionele genetische defecten een bijdrage hebben geleverd aan het ontstaan van AML. De identificatie en functionele analyse van nieuwe ziektegenen in AML is een van de voornaamste doelen van onze onderzoeksgroep.
Een benadering die gebruikt is om nieuwe ziektegenen in leukemie te identificeren, is retrovirale insertie mutagenese. Wanneer muizen geïnjecteerd worden met muizenleukemievirus (MuLV), ontwikkelen zij leukemie nadat provirus in of nabij potentiële ziektegenen is geïntegreerd. Een locus waarbij in meerdere onafhankelijke tumoren virale inserties worden gevonden, worden common virus integratie sites (cVIS) genoemd en markeren de plaats van potentiële proto-oncogenen of tumor suppressorgenen. De keuze van de muizenstam en het type retrovirus die gebruikt worden, bepalen de vorm van de leukemie die zich ontwikkelt. Wij hebben NIH/Swiss muizen geïnjecteerd met Cas-Br-M MuLV, waardoor heel vaak myeloide leukemieën ontstaan. Gebruik makend van deze combinatie hebben wij eerder de cVIS *Evi11* geïdentificeerd en aangetoond dat het gen dat codeert voor de perifere cannabinoide receptor het vermoedelijke targetgen is. *Cb2* codeert voor een zeven-transmembraan-receptor die behoort tot de familie van G-eiwit gekoppelde receptoren (GPCR) en die voornamelijk aanwezig is op B-lymfocyten. Het voornaamste doel van het in dit proefschrift beschreven werk is te bepalen of *Cb2* inderdaad een proto-oncogen is en, wanneer dat het geval is, te bepalen via welk mechanisme het hematopoietische voorlopercellen kan transformeren.
Een korte introductie in de hematopoiese en leukemogenese wordt in **Hoofdstuk 1** gegeven, gevolgd door een beknopte uitleg van retrovirale insertie mutagenese en de identificatie van de cVIS *Evi11*. Daarbij wordt in dit hoofdstuk ook een algemeen overzicht gegeven van de centrale en de perifere cannabinoide receptoren, Cb1 en Cb2.
In **Hoofdstuk 2** tonen wij expressie van *Cb2* mRNA en eiwit aan in retroviraal geïnduceerde myeloide muizenleukemie-cellijnen. *Cb2* bevat twee verschillende, niet-coderende eerste exonen, exon-1A en exon-1B, die beide naar het eiwit-coderende exon-2 kunnen splicen. Wij bewijzen dat in myeloide leukemiecellen met een provirale insertie in *Cb2* exon-1B/exon-2 *Cb2* mRNA niveaus toenemen, wat resulteert in hoge receptor aantallen, en dat cellijnen zonder virus insertie in *Cb2* lage hoeveelheden van alleen exon-1A/exon-2 *Cb2* transcripten hebben, waarbij geen receptoren konden worden

aangetoond. Vervolgens is de functie van Cb2 onderzocht, waarbij we gebruik hebben gemaakt van zowel een myeloide cellijn met een virus integratie in *Cb2*, als 32D/G-CSF-R (granulocyte colony-stimulating factor receptor) cellen die getransfecteerd waren met exon-1B/exon-2 *Cb2* cDNA. Met behulp van transwell assays hebben wij gezien dat stimulatie van migratie een zeer belangrijke functie van Cb2 is in deze cellen. Blootstelling van cellen met Cb2-expressie aan verschillende cannabinoide liganden laat zien dat het vetzuur 2-arachidonoylglycerol (2-AG) de endogene agonist voor Cb2 is. mRNAse protectie analyse en radioactieve ligand-binding studies op muizenweefsels tonen de aanwezigheid van zowel *Cb2*-splicevarianten als Cb2-eiwit in normaal miltweefsel. Transwell experimenten uitgevoerd met miltcellen laten zien dat 2-AG migratie induceert van B-lymfocyten die B220-, CD19-, immunoglobine M- en immunoglobine D tot expressie brengen.

In **Hoofdstuk 3** hebben we de rol van Cb2 in transformatie verder onderzocht. Cb2 werd gefuseerd met EGFP om identificatie van de receptor te vergemakkelijken, en het fusieconstruct werd in 32D/G-CSF-R cellen gebracht. Wij bewijzen dat 32D/G-CSF-R/Cb2-EGFP cellen in een transwell assay migreren in repons op 2-AG, wat aangeeft dat er sprake is van een functioneel fusie-eiwit. De 32D/G-CSF-R cellijn is een uitstekend model om myeloide differentiatie te bestuderen aangezien 32D/G-CSF-R cellen in aanwezigheid van G-CSF differentiëren naar volwassen neutrofiele cellen. De in dit hoofdstuk beschreven experimenten werden uitgevoerd om het effect van de Cb2-receptor op G-CSF-geïnduceerde differentiatie van 32D/G-CSF-R cellen te bestuderen. We laten zien dat Cb2 een complete blokkade van de neutrofiele differentiatie veroorzaakt, die opgeheven kan worden door toevoeging van de Cb2 inverse agonist aan de kweken. Daarnaast beschrijven wij volledige G-CSF-geïnduceerde neutrofiele differentiatie van 32D/G-CSF-R cellen met Cb2 die werden gekweekt in aanwezigheid van de MEK inhibitors PD98059 of U0126, terwijl partieel herstel optreedt met de PI3-K inhibitor LY-294,002.

In **Hoofdstuk 4** tonen wij aan dat aberrante expressie van Cb2 in hematopoietische voorlopercellen tot verschillende ligand-specifieke effecten kan leiden. Myeloide voorlopercellen met expressie van Cb2 migreren na stimulatie door het endocannabinoide 2-AG en de neutrofiele differentiatie wordt geblokkeerd na blootstelling aan het alternatieve ligand, CP55,940. Experimenten werden uitgevoerd om de intracellulaire mechanismen die betrokken zijn bij zowel de migratie als de differentiatieblokkade te onderzoeken. Wij bewijzen dat beide effecten afhankelijk zijn van de activatie van Gαi eiwitten en dat ze de MEK/ERK pathway nodig hebben. Bovendien lijkt downregulatie van intracellulaire cAMP niveaus een belangrijke rol te spelen in migratie inductie, maar dit is niet vereist voor de Cb2-gemedieerde uitrijpingsstilstand. Vervolgens hebben wij getracht te bepalen of het zeer geconserveerde G-eiwit interagerende DRY motief, aanwezig in de tweede intracellulaire loop van de Cb2 receptor, cruciaal is voor de functies van deze GPCR. De experimenten die wij hier laten zien, wijzen erop dat het DRY motief beslissend is voor migratie, maar onbelangrijk voor de blokkade van neutrofiele differentiatie. De onderzoeken die gepresenteerd worden in dit hoofdstuk maken ook duidelijk dat Cb2 normaalgesproken afwezig is op normale humane myeloide voorlopercellen in het beenmerg, maar dat deze receptor vaak aanwezig is op blastcellen die geïsoleerd zijn uit humane AML samples.

Hoofdstuk 5 bevat een algemene discussie over verschillende onderwerpen die naar boven komen uit dit proefschrift. Ten eerste worden verschillende aspecten bediscussieerd die de transformerende mechanismen, benut door de Cb2-receptor, belichten. Dit wordt gevolgd door een gedeelte met betrekking tot de relevantie van GPCRs in maligne transformatie en door een sectie waarin het belang van CB2 in humane AML besproken wordt. Het laatste deel van hoofdstuk 5 bevat conclusies en ideeën voor de toekomst voor dit project.

Resum en Català

La leucèmia mieloide aguda (LMA) és un desordre de les cèl·lules sanguínies caracteritzat per una acumulació de blasts immadurs a la medul·la òssia i a la sang. La LMA humana es caracteritza freqüentment per translocacions cromosòmiques no aleatòries que resulten en la generació d'específiques fusions genètiques malignes, de les quals un número considerable han estat clonades, per exemple la fusió genètica AML1-ETO en LMA amb una translocació t(8;21) o PML-RAR en casos amb translocació t(15;17). De totes maneres, en aproximadament 40 - 50% dels casos de LMA no hi ha anormalitats cromosòmiques evidents, indicant que altres mutacions més subtils són responsables de la transformació leucèmica de cèl·lules precursores mieloides. A més a més, la LMA, com altres càncers, és una malaltia multigenètica que resulta de l'acumulació de diverses aberracions genètiques. Per tant, fins i tot en els casos amb translocacions ben caracteritzades, sembla ser que defectes genètics addicionals contribueixen al desenvolupament de LMA. La identificació i l'anàlisi funcional de nous gens malignes en LMA és el principal objectiu del nostre grup de recerca.

Un mètode utilitzat per identificar nous gens malignes en leucèmia és mutagenesis per inserció de retrovirus. Ratolins injectats amb virus causants de leucèmia (MuLVs) desenvolupen aquesta malaltia a causa de la inserció de provirus dins o a prop de possibles gens malignes. La identificació d'insercions víriques en locus específics en tumors independents es coneixen com a llocs comuns d'integració vírics, cVIS, i marquen la localització de possibles proto-oncogens o gens supressors de tumors. El tipus de ratolí i de retrovirus empleats determinaran el tipus de leucèmia a desenvolupar-se. Nosaltres vam fer servir ratolins NIH/Swiss que van ser injectats amb Cas-Br-M MuLV i van desenvolupar frequentment leucèmia mieloide. Fent servir aquesta combinació, prèviament vam identificar el cVIS *Evi11* i demostrat que el gen que codifica el receptor canabinoide perifèric Cb2 és possiblement el gen implicat. *Cb2* codifica un receptor amb set dominis transmembrana que pertanyen a la família de receptors associats a proteïnes G i és present principalment en limfocits B. El principal objectiu del treball presentat en aquesta tesis és determinar si *Cb2* és un proto-oncogen i, en tal cas, per quin mecanisme pot transformar cèl·lules hematopoiètiques precursores.

Una breu introducció a l'hematopoiesis i la leucèmia, seguida per una explicació concisa sobre mutagènesis per inserció de retrovirus i la identificació del cVIS *Evi11* és presentada al **Capítol 1**. En aquest capítol es facilita addicionalment un resum general dels receptors canabinoide perifèric i central, Cb1 i Cb2.

Al **Capítol 2** demostrem expressió de mRNA *Cb2* i proteïna Cb2 en línies cel·lulars murines amb leucèmia mieloide induïda per retrovirus. *Cb2* conte dos diferents primers exons, exó-1A i exó-1B, els quals convergeixen en l'exó-2 que codifica la proteïna. Nosaltres hem demostrat que en cèl·lules leucèmiques mieloides amb inserció viral a *Cb2* hi ha un augment dels nivells de mRNA *Cb2* variant exó-1B/exó-2, que resulta en un alt nombre de receptors. Les línies cel·lulars sense inserció de virus a *Cb2* presenten només baixos nivells de transcripts exó-1A/exó-2 i receptors no van ser detectats. A continuació hem investigat la funció de Cb2 fent servir una línia cel·lular mieloide que té una inser-

ció viral a *Cb2* i cèl·lules 32D/G-CSF-R (receptor del factor stimulador de colònies granulocítiques) infectades amb *Cb2* cDNA variant exó-1B/exó-2. Fent servir proves amb transwells hem observat que en aquestes cèl·lules una funció principal de Cb2 és l'estimulació de la migració. La presentació de diferents lligams a cèl·lules que expressen Cb2 ha mostrat que l'agoniste endogen per Cb2 és l'àcid gras 2-arachidonoylglycerol (2-AG). Anàlisi de protecció de mRNA i estudis d'associació de lligam radioactiu en teixits de murins va revelar la presència tant de mRNA *Cb2* com de proteïna Cb2 en melses. Experiments amb transwells van ser duts a terme amb cèl·lules de la melsa i vam mostrar que 2-AG estimula la migració de B limfocits que expressen B220, CD19, immunoglobina M i immunoglobina D.

Al **Capítol 3** seguim explorant el paper de Cb2 en la transformació leucèmica. Cb2 va ser fusionat a EGFP per tal de facititar l'identificació del receptor, i el construct de fusió va ser introduït a cèl·lules 32D/G-CSF-R. Observem que cèl·lules 32D/G-CSF-R/Cb2-EGFP migren en experiments amb transwells en resposta a 2-AG, indicant que la proteïna de fusió era funcional. La línia cel·lular 32D/G-CSF-R és un model excel·lent per estudiar la diferenciació mieloide de cèl·lules, doncs en la presència de G-CSF aquestes cèl·lules es diferencien en neutròfils madurs. Experiments que es descriuen en aquest capítol van ser duts a terme per estudiar l'efecte del receptor Cb2 en la diferenciació neutrofílica de les cèl·lules 32D/G-CSF-R. Nosaltres mostrem que Cb2 indueix un bloqueig total de la diferenciació neutrofílica, i que l'efecte pot ser evitat afegint Cb2 agoniste invers als cultius. A més a més, demostrem que una recuperació completa de la diferenciació neutrofílica induïda per G-CSF va ser observada quan les cèl·lules van ser cultivades amb els inhibidors de MEK, PD98059 o U0126, mentre que una recuperació parcial va ser detectada amb l'inhibidor de PI3-K, LY-294,002.

En el **Capítol 4** demostrem que una expressió aberrant de Cb2 en cèl·lules hematopoiètiques precursores pot derivar en diferents efectes segons el lligam. Cèl·lules precursores mieloides que expressen Cb2 migren al ser estimulades per l'endocannabinoide 2-AG i presenten un bloqueig de la diferenciació neutrofílica en la presència d'un altre lligam, CP55,940. Experiments van ser fets per determinar el mecanisme intracel.lular involucrat en la migració i en el bloqueig de la diferenciació cel·lular. Nosaltres hem demostrat que les dues funcions depenen de l'activació de proteïnes Gαi i necessiten de la ruta MEK/ERK. A més a més, la reducció dels nivells intracel·lulars de cAMP sembla ser important en l'inducció de migració, però no és necessària per al bloqueig de diferenciació dut a terme per Cb2. A continuació, determinem si el motiu DRY, altament conservat, interactiu amb proteïnes G i present al segon llaç intracel.lular del receptor Cb2, és crucial per aquest GPCR. Els experiments que presentem aquí indiquen que el motiu DRY és crític per a la migració, però que no és important per al bloqueig de la diferenciació neutrofílica. Els estudis presentats en aquest capítol també revelen que CB2 és normalment absent en cèl·lules humanes mieloides precursores de la medul·la òssia, però que freqüentment es present en cèl·lules blàstiques aïllades de mostres humanes de pacients amb LMA.

El **Capítol 5** engloba una discussió general sobre diferents tòpics que sorgeixen durant aquesta tesis. Primer, diferents aspectes referents al mecanisme de transformació utilitzat

pel receptor Cb2 són discutits. Continua amb un paràgraf sobre la rellevància de GPCRs en la transformació maligne i amb una secció on es discuteix la importància de CB2 en la LMA humana. L'última part del capítol cinquè conté unes observacions finals així com futures direccions per aquest projecte.

LIST OF ABBREVIATIONS

AEA	anandamide
2-AG	2-arachidonoylglycerol
AML	acute myeloid leukemia
ALL	acute lymphoblastic leukemia
BSA	bovine serum albumine
cAMP	cyclic adenosine monophosphate
Cb1	central cannabinoid receptor
Cb2	peripheral cannabinoid receptor
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CSL	Cas-Br-M MuLV swiss leukemias
cVIS	common virus integration site
ERK	extracellular signal-regulated protein kinase
Evi	ecotropic virus integration
FACS	fluorescense-activated cell sorting
FCS	fetal calf serum
G-CSF	granulocyte-colony stimulating factor
G-CSF-R	granulocyte-colony stimulating factor receptor
GPCR	G protein-coupled receptor
G protein	guanine-nucleotide-binding protein
HGF	hematopoietic growth factor
HSC	hematopoietic stem cell
IL	interleukin
MAPK	mitogen-activated protein kinase
MuLV	murine leukemia virus
PI3K	phosphatidylinositol 3-kinase
THC	tetrahydrocannabinol
7TM	seven transmembrane receptor
VIS	virus integration site
wt	wild type

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Submitted

Curriculum vitae

Meritxell Alberich Jordà was born on 9 July 1975 in Sant Boi de Llobregat, Barcelona, Spain. She graduated from the secondary school Escola Esportiva Llor, Sant Boi de Llobregat, with a specialization in science. She studied biology at the Faculty of Biology, in the University of Barcelona (Barcelona) and obtained her diploma in 1999. In September 1998 she was awarded a European Erasmus Grant and moved to Leiden, The Netherlands, to perform a practical period in the Clusius Laboratorium. The title of her project was "Influence of the nod proteins nodZ, nodX, nodL and nolL on the nodulation process of Lotus japonicus". From May 1999 to January 2004 she has been working as a Ph.D student at the Institute of Hematology, Erasmus Medical Center, Rotterdam, The Netherlands, where she performed the work described in this thesis.

Meritxell Alberich Jordà werd op 9 juli 1975 geboren in Sant Boi de Llobregat, Barcelona, Spanje. Na het behalen van haar eindexamen middelbare school in de richting wetenschap aan de Escola Esportiva Llor, Sant Boi de Llobregat, begon zij aan haar studie biologie aan de Universiteit van Barcelona, waar zij in 1999 afstudeerde. In september 1998 kreeg zij een Europese Erasmus Beurs en verhuisde naar Nederland om in Leiden onderzoek te verrichten aan het Clusius Laboratorium. Haar project was getiteld "Influence of the nod proteins nodZ, nodX, nodL and nolL on the nodulation process of Lotus japonicus". Vanaf mei 1999 tot januari 2004 was zij als assistent in opleiding verbonden aan het Instituut Hematologie van Erasmus Medisch Centrum in Rotterdam, waar zij werkte aan het in dit proefschrift beschreven onderzoek.

La Meritxell Alberich Jordà va néixer a Sant Boi de Llobregat, Barcelona. Es va graduar de B.U.P. i C.O.U., branca de ciències, a l'Escola Esportiva Llor. Va estudiar biologia a la Facultat de Biologia, a la Universitat de Barcelona, i va obtenir el títol de Llicenciada en biologia l'any 1999. Al Setembre de 1998 li va ser atorgada una beca europea Erasmus, i es va traslladar a Leiden (Holanda) on va dur a terme una estada pràctica al Clusius Laboratorium. El títol del projecte era "Influence of the nod proteins nodZ, nodX, nodL and nolL on the nodulation process of Lotus japonicus". Desde maig del 1999 a gener del 2004 ha estat treballant a l'Institut d'Hematologia, Erasmus Medical Center, Rotterdam (Holanda), com a estudiant de doctorat, on ha realitzat la feina descrita en aquesta tesis.

GRACIAS!

I don't know anymore how many times I started this section and two minutes later quit it. I thought it would be the easiest part of the thesis, but somehow it was extremely difficult. It feels like saying goodbye to all of you: lab, boss, colleagues, friends, Dutch weather and the last five and a half years of my life. 5.5 years…not bad, taking into account I came to Holland for just few months!

Ruud Delwel, you are the first one in this long list, and definitely the one I have to thank the most. Thanks for giving me the opportunity of doing the Ph.D. with you, and for being the best supervisor I ever had and an excellent person. You were always enthusiastic, motivating and never too busy for a talk.

To my promotor, Bob Löwenberg, thanks for believing in me from the first day. Thanks as well for your comments during the Friday morning work discussions and the "Refereeravonden".

Prof. dr. I. P. Touw, Prof. dr. R. Kanaar and Prof. dr. J. A. Grootegoed, members of the small committee, I would like to acknowledge you for the critical reading of the thesis and your comments. Ivo, I also want to thank you for all the discussions we had during the last years, and to let you know that I learned a lot from your comments.

Marieke Joosten and Marieke Mossink: writing down thanks is not enough to express what I want to say. You have been my colleagues, my friends, my Dutch family and my "paranimfen". Thanks for always supporting me and giving good advice. Wherever I'll be in the future I'll keep you in my heart! Keep in touch!

Sandra Verbakel, I would like to thank you for helping me in the lab, I have been very lucky to have you around. I also want to say thanks for all the experiments you did for the Cb2 project. Sandra, working with you has been "heel gezellig". Marjolein Tas, Eva de Wee, Christopher Dicke, and Ineke van Ostaijen, thanks for all the work you did. I'll always remember all the good times we had in the lab. And of course, Chris and Eva, all the good ones out of the lab!!

Old and new Delwel's group, thanks for creating such a friendly working environment. Antoinette van Hoven, Marion van de Broek and Yolanda Vankan, three excellent technicians, always ready to help, thank you. Dominik Spensberger, I want to thank you for all discussions (and coffees) we had together, good luck with the end of your thesis. Eric van den Akker, good experience in Ananda Village, I had a great time! To Fokke Lindeboom I want to thank you for all the talks (not always work-related) we had. Sahar Khosravani, thanks for sharing the computer with me, especially at the end of the thesis, when I was in front of it all day long. Nazik Rayman, Mojca Jongen-Lavrencic and Claudia Erpelinck, I had a nice time working with you, thanks.

Gert-Jan van de Geijn, colleague and friend, I want to thank you for all the time we spent together in and out of the lab. Thanks for being there at difficult times. Bart Aarts, time for a terrace? I enjoyed all the chats we had and I appreciate all the support I got from you. Joanna Prasher and Matthew Schmolesky, thanks for the fun we had together and, hopefully, the fun we will have somewhere in the future. Joanna, I want to thank you for your comments on the thesis, muchas gracias. David Whyatt, thanks for your support, advice

and the interesting discussions we had. I have learned many things from you. Stefan Erkeland, long time sharer of the office, thanks for all the discussions we had, and of course, the gezellig!! Peter Valk, thanks for "fishing" Cb2, talking to you has always been very encouraging.
To Marieke von Lindern I would like to thank you for your critical comments, ideas and suggestions. It was a pleasure having you in the lab, including those late evenings when you still had enthusiasm to have a talk. Martine Parren-van Amelsvoort, Emile van den Akker, Thamar van Dijk and Walbert Bakker, thanks for your western-tips and helping me to find the right antibody.
Elwin Rombouts, Trui Visser, Monique Verstegen, Hans Hoogerbrugge, and Peter van Geel, thanks for giving me good advice and providing me with all kinds of material that I needed. Jan van Kapel, I want to thank you for fixing all the computer-related problems I had and taking time to teach me how to use the computer properly. Ineke van de Kraats wil ik bedanken voor de al het sterilisatie werk en de gezellige tijd die we samen in de keuken hadden.
I want to thank Rudi Hendriks, Kirsten van Lom, Pierre Casellas, and Mauro Maccarrone for their collaborations.
Karola van Rooyen, thanks for the design of the figures, and the good time I had preparing them. Of course, EDS was great fun! Egied Simons, they were few but intensive days. I really want to thank you for the good job you did in such a short time.
Ans Mannens and Eveline van Heese, our two efficient secretaries, thanks a lot for being so helpful and always having time to give me a hand. Jeanne Vlasveld, I want to thank you all the letters, paper work and calls you did for me during the preparation of the thesis.
Pieter Admiraal, I want to thank you for all the good times we had together and everything you did for me. Thanks for the unconditional support you gave me during my first years in Holland. Cristina Pacios, gracias por ser mi supervisora en Leiden y darme la oportunidad de trabajar contigo. Pero incluso más importante, gracias por ser mi amiga madrileña! Este último año te he echado de menos en Holanda. And Clusius people, Herman Spaink, Guido Bloemberg and Kees Breek, it was good working with you, and I especially want to thank Herman and Guido for encouraging me to do the Ph.D.
Oriol Pascual, gràcies per tot el que em viscut junts: visites al parc, sopars, classes de ball, cines, rises,… en fi, una relació de lagartijillas productiva i sostenible. Molta sort amb el teu doctorat! I ara els de Barcelona, que no per ser més lluny han estat menys importants. Noemí i Laia, us podeu imaginar com d'important heu estat aquests últims anys? Visites, emails, viatges, concells, recolçament,… Gràcies per ser unes amigues excel.lents, fins i tot en la distància. Laia, merci per les correccions! Xavi, et vull agrair que sempre hagis tingut una estoneta per mi, temps per una xerrada i un cafè. Com t'he trobat a faltar! Miquel, Mònica, Thaïs i Glòria, gràcies per animar-me i recolçar-me.
Finalment vull donar les gràcies al papa, la mama, la iaia i al Let. Merci per ser sempre al meu costat i ajudar-me a conseguir els meus objectius. Us estimo!

Meritxell