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Endogenous Cannabinoid Receptor Ligand Induces the Migration of Human Natural Killer Cells

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2-Arachidonoylglycerol is an endogenous ligand for the cannabinoid receptors (CB1 and CB2). Evidence is gradually accumulating which shows that 2-arachidonoylglycerol plays important physiological roles in several mammalian tissues and cells, yet the details remain ambiguous. In this study, we first examined the effects of 2-arachidonoylglycerol on the motility of human natural killer cells. We found that 2-arachidonoylglycerol induces the migration of KHYG-1 cells (a natural killer leukemia cell line) and human peripheral blood natural killer cells. The migration of natural killer cells induced by 2-arachidonoylglycerol was abolished by treating the cells with SR144528, a CB2 receptor antagonist, suggesting that the CB2 receptor is involved in the 2-arachidonoylglycerol–induced migration. In contrast to 2-arachidonoylglycerol, anandamide, another endogenous cannabinoid receptor ligand, did not induce the migration. Δ^9-Tetrahydrocannabinol, a major psychoactive constituent of marijuana, also failed to induce the migration; instead, the addition of Δ^9-tetrahydrocannabinol together with 2-arachidonoylglycerol abolished the migration induced by 2-arachidonoylglycerol. It is conceivable that the endogenous ligand for the cannabinoid receptor, that is, 2-arachidonoylglycerol, affects natural killer cell functions such as migration, thereby contributing to the host-defense mechanism against infectious viruses and tumor cells.

Key words: anandamide, 2-arachidonoylglycerol, cannabinoid, CB2 receptor, natural killer cells.

Abbreviations: 2-AG, 2-arachidonoylglycerol; FBS, fetal bovine serum; IL, interleukin; LPS, lipopolysaccharide; MAP kinase, mitogen-activated protein kinase; NK cells, natural killer cells; Δ^9-THC, Δ^9-tetrahydrocannabinol.

Δ^9-Tetrahydrocannabinol (Δ^9-THC) is a major psychoactive component of marijuana and is known to elicit a variety of pharmacological responses, mainly by acting on specific receptors, that is, the cannabinoid receptors (CB1 and CB2) (*1*). The CB1 receptor is abundantly expressed in the brain (*2*, *3*), and the CB2 receptor is predominantly expressed in several types of inflammatory cells and immunocompetent cells such as B lymphocytes, natural killer (NK) cells and macrophages/monocytes (*3–5*).

To date, two types of arachidonic acid–containing molecules, *N*-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG), have been identified as endogenous ligands for the cannabinoid receptors. Anandamide was isolated from pig brain by Devane *et al.* (*6*) in 1992. This compound has been shown to have various cannabimimetic activities *in vitro* and *in vivo* (*7–10*). However, the levels of anandamide in various living tissues are usually very low (*11*, *12*). Moreover, anandamide was found to act as a partial agonist toward the cannabinoid receptors (*13–17*). It is unnatural for an endogenous ligand to act as a partial agonist toward its own receptor. These observations led us to postulate the existence of another endogenous ligand.

In 1995, we (*18*) and Mechoulam *et al.* (*19*) identified 2-AG, a monoacylglycerol containing arachidonic acid, as another endogenous cannabinoid receptor ligand. A number of investigators have demonstrated that 2-AG exhibits a variety of cannabimimetic activities (*7–10*, *12*, *20*, *21*). Noticeably, 2-AG can be rapidly formed from phospholipids containing arachidonic acid, such as inositol phospholipids, through the combined actions of phospholipase C and diacylglycerol lipase, the combined actions of phospholipase A_1 and phospholipase C, or the combined actions of phospholipase D, phosphatidic acid phosphatase and diacylglycerol lipase in various tissues and cells upon stimulation (*8–10*, *12*, *20*, *21*). In fact, the levels of 2-AG in various mammalian tissues are markedly higher than those of anandamide (*11*, *12*, *20*). Importantly, 2-AG acts as a full agonist toward the cannabinoid receptors (*14*, *22–27*). Based on these observations, we concluded that 2-AG, and not anandamide, is the true natural ligand for the cannabinoid receptors (*12*, *20*, *21*, *23*, *24*).

There is growing evidence that 2-AG plays important physiological roles in various mammalian tissues and cells. For example, 2-AG has been suggested to be involved in the attenuation of neurotransmission by acting toward the CB1 receptor expressed predominantly in the presynaptic terminals (reviewed in Ref. *28*). We have proposed that 2-AG is a novel type of neuromodulator

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(*12*, *20*, *21*). On the other hand, the physiological roles of 2-AG as a CB2 receptor ligand remain obscure. Previously, several investigators have examined the biological effects of 2-AG on HL-60 cells (*24*, *29–32*), macrophage/monocytes (*32–35*), microglia cells (*36*) and lymphocytes (*37–39*). However, the available information is still limited. In particular, little information is thus far available concerning NK cells. It is thus of great value to investigate the physiological roles of the CB2 receptor in NK cells, and the possible biological activities of 2-AG toward NK cells, because NK cells are known to play crucial roles in innate types of immunity such as the elimination of virus-infected cells and tumor cells (*40*, *41*).

In this study, we investigated the possible physiological roles of the CB2 receptor and its endogenous ligands, especially 2-AG, in NK cells. We found that 2-AG induces the migration of KHYG-1 leukemic NK cells and human peripheral blood NK cells in a CB2 receptor–dependent manner.

MATERIALS AND METHODS

Chemicals—Arachidonic acid and essentially fatty acid–free bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Pertussis toxin (PTX) was purchased from List Biological Laboratories (Campbell, CA, USA). SR144528 was a generous gift from Sanofi (Montpellier, France). Anandamide was synthesized from ethanolamine and arachidonic acid as previously described (*23*). 2-AG was synthesized from 1,3-benzylideneglycerol and arachidonic acid (*23*). An ether-linked analog of 2-AG (2-AG ether) (2-eicosa-5′,8′,11′,14′-tetraenylglycerol) was synthesized from 1,3-benzylideneglycerol and eicosatetraenyl iodide as previously described (*23*).

Cells—KHYG-1 human leukemic NK cells (*42*) and K562 human myeloid leukemia cells (an NK-sensitive cell line) were obtained from Health Science Research Resources Bank (Sennan, Osaka). Jurkat human lymphoma cells were obtained from Riken Cell Bank (Tsukuba, Ibaraki). KHYG-1 cells were grown at 37°C in RPMI1640 medium (Asahi Technoglass Co., Chiba) supplemented with 10% fetal bovine serum (FBS) and interleukin 2 (IL-2) (50 ng/ml) in an atmosphere of 95% air and 5% CO_2. Jurkat cells and K562 cells were grown at 37°C in RPMI1640 medium supplemented with 10% FBS. Human NK cells were separated from the peripheral blood of young healthy donors as follows: 1/4 volume of 6% Dextran T-500 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in saline was added to heparinized blood to sediment the erythrocytes. The supernatant (leukocyte-rich fraction) was aspirated and centrifuged at 400 × *g* for 10 min. The cells were washed once with Ca^{2+}, Mg^{2+}-free Hanks balanced salt solution (HBSS) containing 5 mM HEPES (pH 7.4). The cells were then transferred to Lymphoprep™ (Axis Shield, Oslo, Norway) and centrifuged at 800 × *g* for 20 min. The mononuclear leukocyte fraction (the interface layer) was collected and washed with HBSS. NK cells were separated from other mononuclear leukocytes by negative selection using a MACS NK cell isolation kit (Miltenyi Biotec Gmbh, Gladbach, Germany). The purity of the human NK cells was determined before use by immunostaining for CD3 and CD56. The major $CD56^+$ subpopulations of NK cells were sorted by gating on $CD56^+$ $CD3^-$ cells. The purity of the NK cells was 90% as assessed by flow cytometry (Elite Epics Colter).

Northern Blot Analysis—Total RNAs were obtained from KHYG-1 cells, Jurkat cells and K562 cells using ISOGEN (Nippon Gene Co., Ltd., Tokyo). Poly $(A)^+$ RNAs were isolated from the total RNAs using Oligotex-dT30 Super (Takara Bio, Inc., Shiga). Poly $(A)^+$ RNAs (3 μg) from KHYG-1 cells, Jurkat cells and K562 cells were electrophoresed in a 1.0% agarose-formaldehyde gel and transferred onto a Hybond-N^+ Nylon membrane (Amersham Pharmacia Biotech). The CB2 probe (human CB2 receptor cDNA SphI/SfiI digest, 524 bp) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (BD Biosciences, Palo Alto, CA) were labeled with [α-^{32}P]dCTP (PerkinElmer Japan Co., Ltd., Kanagawa, Japan) using the Megaprime DNA labeling system (Amersham Pharmacia Biotech). Hybridization was performed at 60°C for 16 h in QuikHyb solution (Stratagene, Cambridge, UK). The filter was washed in 0.1 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate) containing 0.1% SDS at 65°C and analyzed by a bio-imaging analyzer BAS 1500 (Fuji Photo Film, Tokyo).

Migration Assay—The migration of KHYG-1 cells and human peripheral blood NK cells was assayed using Transwell™ inserts (pore size, 5 μm) and 24-well culture plates (Corning Costar, Cambridge, MA, USA). Briefly, the cells (10^6 for KHYG-1 cells and 2×10^5 for peripheral blood NK cells) suspended in 0.1 ml of RPMI1640 medium containing 0.1% BSA were transferred to the Transwell™ insert (the upper compartment). 2-AG was dissolved in dimethyl sufoxide (DMSO) and added to 0.6 ml of the RPMI1640 medium containing 0.1% BSA in the well of the culture plate (the lower compartment) (the final concentration of DMSO was 0.2%). The incubation was carried out at 37°C for 4 h in an atmosphere of 95% air and 5% CO_2. The number of KHYG-1 cells that migrated from the upper compartment to the lower compartment was counted using a hemocytometer. The number of migrated human peripheral blood NK cells ($CD56^+CD3^-$ cells) was determined by flow cytometry.

Statistical Analysis—Statistical analysis was performed by Student's *t* test. A P value <0.05 was considered to be significant.

RESULTS

Detection of the Cannabinoid CB2 Receptor in KHYG-1 NK Cells—First, we examined whether KHYG-1 NK cells contain the CB2 receptor mRNA. Figure 1 shows the results of Northern blot analysis. KHYG-1 cells contained a substantial amount of the CB2 receptor mRNA. The ratio of the CB2 receptor mRNA/GAPDH mRNA in KHYG-1 cells was almost the same as that in Jurkat cells, which are known to express a high level of the CB2 receptor (*43*). In contrast to KHYG-1 cells and Jurkat cells, K562 cells did not contain an appreciable amount of the CB2 receptor mRNA.

Effect of 2-AG on the Motility of KHYG-1 Cells—We then investigated the effect of 2-AG on the motility of KHYG-1 cells. The addition of 1 μM 2-AG to the lower

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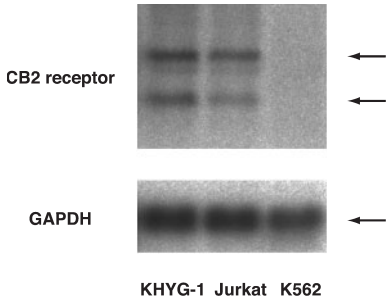

Fig. 1. **CB2 receptor mRNA levels in KHYG-1 cells, Jurkat cells and K562 cells.** Northern blot analysis of CB2 receptor mRNAs was performed using poly (A)+ RNA as described in "MATERIALS AND METHODS." CB2 receptor gene transcripts, 4.4 and 2.5 kb; GAPDH gene transcript, 1.3 kb.

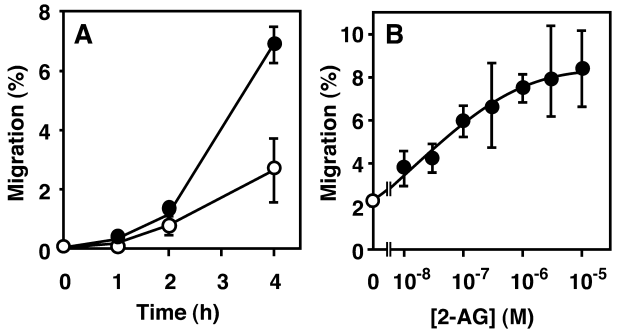

Fig. 2. **Migration of KHYG-1 cells by 2-AG.** (A) KHYG-1 cells were added to the Transwell™ inserts (the upper compartment) and 2-AG (1 μM) or vehicle (DMSO) was added to the well of the culture plate (the lower compartment). The final concentration of DMSO in the lower compartment was 0.2%. Incubation was carried out for the indicated periods of time. Closed circles, 2-AG; open circles, vehicle (DMSO) alone. (B) KHYG-1 cells were added to the inserts (the upper compartment), and various concentrations of 2-AG or vehicle (DMSO) were added to the well of the culture plate (the lower compartment). The final concentration of DMSO in the lower compartment was 0.2%. Incubation was carried out for 4 h. The migration from the upper to lower compartment was determined as described in "MATERIALS AND METHODS." The data are the means ± SD of four determinations.

compartment of the Transwell™ augmented the migration of KHYG-1 cells from the upper to the lower compartment (Fig. 2A). The number of KHYG-1 cells that migrated upon exposure to 1 μM 2-AG increased with time (Fig. 2A). The number of migrated cells also increased dose-dependently (Fig. 2B): the effect of 2-AG was observed from 10 nM and reached a peak at 10 μM.

To determine whether the cannabinoid receptors are involved in the 2-AG–induced migration of KHYG-1 cells, we examined the effects of cannabinoid receptor antagonists. We found that the addition of SR144528, a CB2 receptor–specific antagonist, to the cells suppressed the migration evoked by 2-AG (Fig. 3A). On the other hand, treatment of the cells with SR141716A, a CB1 receptor–specific antagonist, had only a slight effect on 2-AG–induced migration. These results indicate that the 2-AG–induced migration is mediated mainly via the CB2 receptor. We then examined the effect of PTX treatment on the 2-AG–induced migration of KHYG-1 cells. As depicted in Fig. 3B, pretreatment of the cells with PTX abolished the

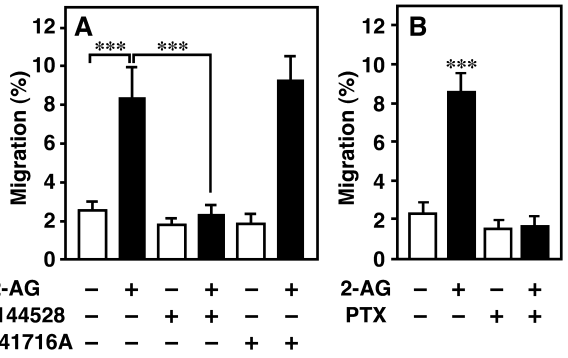

Fig. 3. **Effects of a CB2 receptor antagonist SR144528, a CB1 receptor antagonist SR141716A and PTX treatment on 2-AG–induced migration of KHYG-1 cells.** (A) 2-AG (1 μM) or vehicle (DMSO) was added to the lower compartment and SR144528 (1 μM) or SR141716A (1 μM) to both the upper and lower compartments of Transwell inserts. The final concentration of DMSO in the lower compartment was 0.4% and that in the upper compartment was 0.2%. The migration of the cells from the upper to lower compartment was examined as described in "MATERIALS AND METHODS." (B) Cells were pretreated with PTX (100 ng/ml) for 16 h and then added to the Transwell™. 2-AG (1 μM) or the vehicle (DMSO) was added to the lower compartment. The final concentration of DMSO in the lower compartment was 0.2%. The migration of PTX-treated cells from the upper to lower compartment was determined as described in "MATERIALS AND METHODS." The data are the means ± SD of four determinations. ***$P < 0.001$.

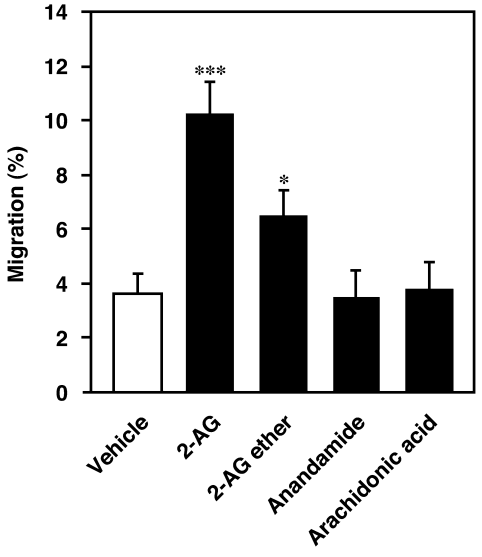

Fig. 4. **Comparison of the abilities of 2-AG, 2-AG ether, anandamide and free arachidonic acid to induce the migration of KHYG-1 cells.** The migration of cells from the upper to lower compartment in response to these compounds (1 μM each) or vehicle (DMSO) (added to the lower compartment) was examined as described in "MATERIALS AND METHODS." The final concentration of DMSO in the lower compartment was 0.2%. The data are the means ± SD of four determinations. ***$P < 0.001$, *$P < 0.05$ [compared to the control (vehicle)].

migration induced by 2-AG, indicating that Gi/o is involved in the 2-AG–induced migration.

We then compared the abilities of several structural analogs of 2-AG to induce migration. As demonstrated in Fig. 4, the highest activity was observed with 2-AG (1

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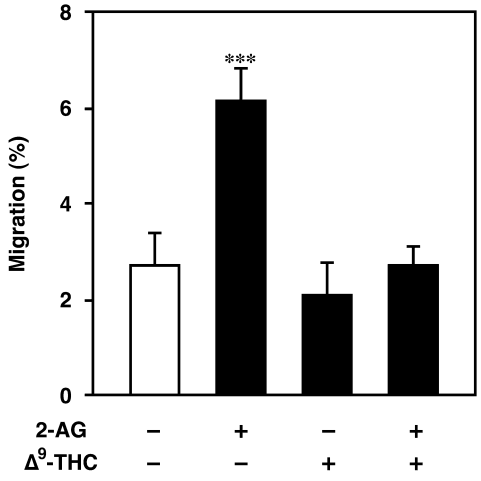

Fig. 5. **Effect of Δ9-THC on 2-AG–induced migration of KHYG-1 cells.** The migration of cells from the upper to lower compartment in response to 2-AG (1 μM) or Δ9-THC (1 μM) or 2-AG plus Δ9-THC (1 μM each) (added to the lower compartment) was examined as described in "MATERIALS AND METHODS." The final concentration of DMSO in the lower compartment was 0.4%. The data are the means ± SD of four determinations. ***$P < 0.001$ [compared to the control (vehicle)].

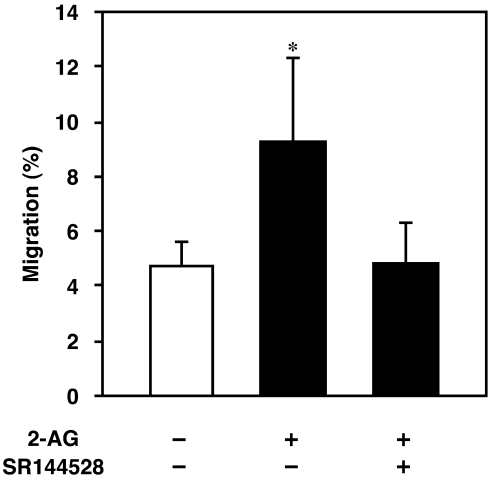

Fig. 6. **Effect of 2-AG on the motility of human peripheral blood NK cells.** The effect of 2-AG on the motility of human NK cells from the upper to lower compartment was examined using Transwell™ inserts as described in "MATERIALS AND METHODS." 2-AG (1 μM) was added to the lower compartment. SR144528 (1 μM) was added to both the upper and lower compartments. The final concentration of DMSO in the lower compartment was 0.4%. The final concentration of DMSO in the upper compartment was 0.2%. The data are the means ± SD of four determinations. * $P < 0.05$ [compared to the control (vehicle)].

μM). Although its activity was much lower than that of 2-AG, 2-AG ether (1 μM), a non-hydrolyzable ether-linked analog of 2-AG, exhibited appreciable activity. Noticeably, another endogenous cannabinoid receptor ligand, anandamide (1 μM), failed to induce the migration of KHYG-1 cells. Free arachidonic acid (1 μM) also did not exhibit appreciable activity.

Effect of Δ9-THC on the Motility of KHYG-1 Cells—Next, the effect of Δ9-THC on the motility of KHYG-1 cells was examined and the results are shown in Fig. 5. 2-AG (1 μM) provoked the migration of KHYG-1 cells, while Δ9-THC did not. Instead, the addition of Δ9-THC markedly reduced the response induced by 2-AG. These results indicate that Δ9-THC acts as an antagonist, rather than agonist, toward the CB2 receptor.

Effect of 2-AG on the Motility of Human Peripheral Blood NK Cells—We then examined the effect of 2-AG on human peripheral blood NK cells. As shown in Fig. 6, 2-AG (1 μM) enhanced the migration of peripheral blood NK cells from the upper to the lower compartment. The effect of 2-AG was abolished by treating the cells with SR144528 (1 μM), a CB2 receptor–specific antagonist, indicating that the migration is mediated *via* the CB2 receptor, as in the case of KHYG-1 cells.

DISCUSSION

The CB2 receptor is abundantly expressed in various types of inflammatory cells and immunocompetent cells such as B lymphocytes, NK cells and macrophages (*3–5*), yet details of the physiological functions of the CB2 receptor remain to be clarified. Previously, we found that 2-AG, an endogenous CB2 receptor ligand, induces a rapid transient increase in intracellular free Ca^{2+} concentration via a CB2 receptor–dependent mechanism (*24*). Employing this assay system, we examined the structure–activity relationship of a number of CB2 receptor ligands. We found that the structure of 2-AG is recognized strictly by the CB2 receptor (*24*). Importantly, 2-AG acts as a full agonist toward the CB2 receptor, whereas anandamide acts as a weak partial agonist. Gonsiorek *et al*. (*26*) also demonstrated that 2-AG is a full agonist and anandamide is a partial agonist. We have proposed that 2-AG, but not anandamide, is the intrinsic natural ligand for the CB2 receptor (*24*). We further explored possible biological activities of 2-AG and found that 2-AG induces the activation of p42/44 (*29*) and p38 mitogen–activated protein kinases (MAP kinases) (*30*) and c-Jun N-terminal kinase (*30*), augments the production of chemokines such as IL-8 and MCP-1 in HL-60 cells (*31*), and causes the migration of HL-60 cells that had been differentiated into macrophage-like cells or human peripheral blood monocytes (*32*). Several investigators have also reported that 2-AG potentiates or suppresses lymphocyte proliferation (*37*), reduces the expression of the IL-2 gene in murine T lymphocytes (*38*), inhibits the production of IL-6 in J774 macrophage–like cells (*34*), inhibits TNF-α production in LPS-stimulated mouse macrophages (*33*), and induces the migration of mouse splenocytes (*39*) and microglia cells (*36*), although, in some cases, it is not clear whether the effects of 2-AG are actually mediated through the CB2 receptor.

Despite growing information regarding the possible physiological roles of 2-AG in several types of inflammatory cells and immunocompetent cells previously mentioned, little is known concerning the effects of 2-AG on NK cell functions. In the present study, we first examined the effect of 2-AG on the motility of NK cells. We found that 2-AG induces the migration of KHYG-1 NK cells and human peripheral blood NK cells (Figs. 2–6). The 2-AG–

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induced migration of KHYG-1 cells and peripheral blood NK cells was blocked by treating the cells with SR144528 (Figs. 3A and 6), suggesting that the migration is mediated by the CB2 receptor. Gi/o was also suggested to be involved, since the PTX treatment of the cells abolished the response induced by 2-AG (Fig. 3B). Arachidonic acid and its metabolites are not involved in the 2-AG–induced migration, because free arachidonic acid failed to induce the migration (Fig. 4). This was also confirmed by the fact that 2-AG ether, a non-hydrolyzable analog of 2-AG, induces the migration of KHYG-1 cells, albeit at a lower rate (Fig. 4). To our knowledge, this is the first report showing that 2-AG stimulates NK cells in a CB2 receptor–dependent manner.

It is well known that NK cells play crucial roles in the self-defense mechanism against intracellular pathogens such as infectious viruses and in immune surveillance toward neoplastic cells (*40*, *41*). NK cells eliminate infected host cells and tumor cells by perforating their plasma membrane and inducing apoptosis (*44*). To exert their cytolytic activity effectively, NK cells must be recruited to the site of the infected or neoplastic cells. Various chemokines have been shown to induce the migration of NK cells *in vitro* (*45*, *46*), yet the mechanism by which NK cells migrate to virus-infected cells and tumor cells *in vivo* is not fully understood. 2-AG is a rather common molecule and can be formed from membrane phospholipids in various types of cells upon stimulation that triggers accelerated phospholipid turnover (*47–51*). It is possible that 2-AG, generated from target cells and/or stimulated NK cells, participates in the accumulation of NK cells around inflammatory sites in cooperation with other chemokines. Further studies are necessary to clarify whether 2-AG and the CB2 receptor are involved in the recruitment of NK cells *in vivo*.

Unlike 2-AG, Δ^9-THC, a major psychoactive constituent of marijuana, did not induce the migration of NK cells (Fig. 5). On the contrary, Δ^9-THC suppressed the migration induced by 2-AG (Fig. 5). It is thus apparent that Δ^9-THC acts as an antagonist toward the CB2 receptor in these cells. In agreement with this, we have previously demonstrated that Δ^9-THC is a weak partial agonist toward the CB2 receptor using HL-60 cells (*24*). Bayewitch *et al*. (*52*) also reported that Δ^9-THC acts as an antagonist toward the CB2 receptor. Noticeably, several investigators have demonstrated that Δ^9-THC suppresses various immune responses *in vivo*; for example, the administration of Δ^9-THC to experimental animals results in a decreased resistance to viral and bacterial infection (*53*, *54*). Δ^9-THC has also been shown to inhibit NK activity (*55*). Taken together, it is plausible that Δ^9-THC interferes with the action of 2-AG, such as the migration of NK cells, thereby inducing the suppression of innate and adaptive immune responses.

In conclusion, we found that 2-AG induces the migration of human NK cells *via* a CB2 receptor–dependent mechanism. It seems possible that the CB2 receptor and its endogenous ligand 2-AG play some essential role in NK cell–mediated innate immunity and host defense mechanism(s) against infectious viruses and tumor cells. Further detailed studies on the physiological roles of the CB2 receptor and its endogenous ligand in NK cells are thus indispensable for a full understanding of the precise regulatory mechanism of various NK cell functions.

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