Two subtypes of G-protein-coupled cannabinoid receptors have been identified to date: the CB1 central receptor subtype, which is mainly expressed in the brain, and the CB2 peripheral receptor subtype, which appears particularly abundant in the immune system. We investigated the expression of CB2 receptors in leukocytes using anti-CB2 receptor immunopurified polyclonal antibodies. We showed that peripheral blood and tonsillar B cells were the leukocyte subsets expressing the highest amount of CB2 receptor proteins. Dual-color confocal microscopy performed on tonsillar tissues showed a marked expression of CB2 receptors in mantle zones of secondary follicles, whereas germinal centers (GC) were weakly stained, suggesting a modulation of this receptor during the differentiation stages from virgin B lymphocytes to memory B cells. Indeed, we showed a clear downregulation of CB2 receptor expression during B-cell differentiation both at transcript and protein levels. The lowest expression was observed in GC proliferating centroblasts. Furthermore, we investigated the effect of the cannabinoid agonist CP55,940 on the CD40-mediated proliferation of both virgin and GC B-cell subsets. We found that CP55,940 enhanced the proliferation of both subsets and that this enhancement was blocked by the CB2 receptor antagonist SR 144528, but not by the CB1 receptor antagonist SR 141716. Finally, we observed that CB2 receptors were dramatically upregulated in both B-cell subsets during the first 24 hours of CD40-mediated activation. These data strongly support an involvement of CB2 receptors during B-cell differentiation.

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MATERIALS AND METHODS

Production of anti-CB2 receptor Abs. Synthetic peptide derived from the predicted aminoacid-sequence of the carboxylic tail of the human CB2 receptor (Y-P-D-S-R-D-L-D-L-S-D-C) and bovine serum albumin (BSA)-conjugated peptide used as immunogen were from NeoSystem (Strasbourg, France). Rabbits were injected subcutaneously with 2 mg BSA-peptide in 250 µL water and 250 µL complete Freund’s adjuvant. Animals were boosted monthly under the same conditions and blood was taken 10 days after the fifth injection. Abs directed against the C-terminal part of the human CB2 receptor were immunopurified on a Bio-Rad Affi-Gel 10 modified with the peptide (Bio-Rad, Hercules, CA) as already described. Briefly, 10 mL of immune serum was incubated overnight at 4°C with 1 mL modified gel. After extensive washings, anti-CB2 receptor Abs were eluted with 100 mmol/L
glycine-HCl, pH 1.8, and neutralized with 1 mol/L Tris-NaOH. The pooled fractions were supplemented with 10 mg/mL BSA, concentrated, and dialyzed on a Filtron microsep 30 kD (Filtron, Northborough, MA). Concentrated Abs were stored in 50% glycerol at −20°C.

Immunoblotting experiments. Membranes of wild-type hamster ovary cells (CHO-WT) and of CHO cells stably transfected with the CB2 receptor (CHO-CB2) were prepared by homogenizing cells with polytron in 5 mmol/L Tris, pH 7.4, containing 1 mmol/L EDTA, 20 µg/mL aprotinin, and 1 mmol/L 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF). The homogenate was centrifuged for 15 minutes at 2,000g. The nuclear free supernatant was centrifuged for 1 hour at 100,000g. Immunoblotting experiments were performed on the membrane pellets after electrophoresis on a 4% to 20% sodium dodecyl sulfate (SDS)-polyacrylamide gel (Novex, San Diego, CA) and transfer onto nitrocellulose filters. Proteins were electroblotted on nitrocellulose filters (Novex). Nonspecific binding was blocked with 10% casein in TBS buffer and then incubated for 3 hours at room temperature with anti-CB2 receptor Abs (1:2,000 dilution). After another washing, peroxidase-conjugated antirabbit IgG (1:8,000 dilution; Sigma, St Louis, MO) was added for 45 minutes at room temperature. After 5 extensive washes, immune complexes were detected using the ECL kit on Hyperfilm-MP (Amersham, Buckinghamshire, UK) following the supplier’s instructions.

Antibodies. The following Abs were used for flow cytometry. Phycoerythrin-conjugated human CD4, CD8, CD20, and CD38 MoAbs were purchased from Becton Dickinson (San Jose, CA). Tricolor (phycoerythrin-Cy5)-conjugated human CD3 MoAbs were from Caltag (South San Francisco, CA). Biotinylated human CD44 MoAbs were from Leinco Technologies (Ballwin, MO). Biotinylated anti-human IgD Abs were from Tagoimmunologicals (Burlingame, CA). The human purified CD77 MoAbs were from Immunotech and were stained with the biotinylated antirat IgG (mark-1) MoAbs (Immunotech). All of the biotinylated Abs were labeled with Tricolor-conjugated streptavidin (Caltag). Rabbit anti-CB2 Abs were labeled with fluorescein isothiocyanate (FITC)-conjugated donkey antirabbit IgG Abs from Jackson ImmunoResearch (West Grove, PA).

The following Abs were used for confocal laser scanning microscopy: purified human CD3, CD38 MoAbs, anti-Ki67 MoAbs, anti-human IgD MoAbs, and antifollicular dendritic cell MoAbs (HJ2) were from Dako. These MoAbs were all shown with FITC-conjugated donkey IgG Abs (1:100 dilution) for 30 minutes, washed once with the 0.1% saponin/1% BSA solution for 30 minutes. After two washes with 0.3% saponin in PBS containing 0.3% BSA, cells were incubated with FITC-conjugated donkey antirabbit IgG Abs (1:100 dilution) for 30 minutes, washed once with the 0.3% saponin/0.3% BSA solution, and washed once with PBS alone. Negative controls were performed by 1 hour of preincubation of anti-CB2 receptor Abs with the C-terminal synthetic peptide at 20 µg/mL. The fluorescence intensity mean of each subset was calculated by subtracting the fluorescence of the irrelevant controls (anti-CB2 receptor Abs + peptide) from that of the relevant labeling (anti-CB2 receptor Abs).

Tissue staining for confocal microscopy. Serial cryostat sections (9-µm thick) of tonsils were fixed in acetone for 5 minutes at room temperature. Sections were simultaneously incubated with mouse antihuman leukocyte antigen MoAbs and rabbit anti-CB2 receptor Abs under 100 µL of PBS containing 0.5% BSA. After three washes in the same buffer, sections were simultaneously stained with FITC-conjugated donkey antirat IgG Abs and Cy3-conjugated donkey antirabbit IgG Abs, both at 1:200 dilution in PBS containing 0.5% BSA. After two washes in the same buffer and one wash in PBS without BSA, sections were mounted in a solution of glycerol/PBS containing the antibleaching reagent DABCO at 50 mg/mL (Sigma). Specificity controls were performed by 1 hour of preincubation of anti-CB2 receptor Abs with the C-terminal synthetic peptide at 20 µg/mL.

Dual fluorescence analysis was performed using a laser confocal microscope (LSM410; Zeiss, Oberkochen, Germany) equipped with a Plan NEOFLUAR water immersion lens (16×; numerical aperture [NA] = 0.50). Signals were collected separately after excitations of FITC and Cy3 at 488 and 543 nm, respectively. FITC emission was collected using a transmission filter centered at 530 nm and Cy3 emission using a 590-nm long-pass filter.

RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) analysis. Subpopulations of B cells were obtained, purified, and labeled for cell surface phenotyping as described above. Cell sorting of 2×10⁶ cells of each B-cell subsets was performed using the Normal-C mode of a FacstarPlus cytometer (Becton Dickinson, Erembodegen, Belgium). Purification of each B-cell subset was checked by reanalyzing another sorting run. This procedure led to B-cell subpopulation purities ranging from 95% to 99.5%. The mRNA purification and conversion to single-strand cDNA were performed using a PolyATtract series 9600J mRNA Isolation System with CDNA Synthesis Reagents (Promega, Charbonnières, France) according to the manufacturer’s instructions.

Briefly, 10⁶ sorted cells were centrifuged and suspended in 20 µL of extraction buffer. Each sample was then transferred to the well of a V-bottom 96-well plate. After hybridization with a synthetic biotinylated oligo (dT) probe and incubation with streptavidin-coated magnetic beads, 3'-polyadenylated RNA was captured using a 96 pins Multi-Magnet (Promega, Charbonnières, France). After successive washes, purified mRNA was eluted into 20 µL of water. mRNA was converted to single-strand cDNA by adding 10 µL of reverse transcriptase master mix (containing AMV Reverse Transcriptase) in each well of the 96-well microplate. Quantitation of CB2 receptor mRNA levels was performed in the exponential phase of amplification as previously described. Indepen-
PERIPHERAL CB2 CANNABINOID RECEPTORS ARE INVOLVED IN B-CELL DIFFERENTIATION

RESULTS

Production and characterization of anti-CB2 receptor polyclonal Abs. Several rabbits were immunized with 1 of the 18 BSA-conjugated peptides corresponding to different intracellular and extracellular parts of the CB2 receptor. Among these, the peptide corresponding to the intracellular 11-aminoacid sequence of the C-terminal part was the only one that led to specific anti-CB2 receptor Abs. After five injections, Abs from immune serum were immunopurified on a gel modified with the synthetic peptide. The specificity of purified Abs was evaluated in immunoblotting experiments performed with membrane of CHO cells stably transfected with the CB2 receptor (CHO-CB2). Two bands were shown when 75 µg of protein from CHO-CB2 membranes were electrophoresed, whereas no band was observed when the same amount of protein from the wild-type cell line (CHO-WT) was analyzed (Fig 1). The major band corresponded to a molecular weight of approximately 46 kD, consistent with the deduced amino acid sequence of the CB2 receptor. For this reason, we decided to proceed with this peptide (amino acid sequence of the C-terminal part was the only one that led to specific anti-CB2 receptor Abs). After five injections, Abs from immune serum were immunopurified on a gel modified with the synthetic peptide. The specificity of purified Abs was evaluated in immunoblotting experiments performed with membrane of CHO cells stably transfected with the CB2 receptor (CHO-CB2). Two bands were shown when 75 µg of protein from CHO-CB2 membranes were electrophoresed, whereas no band was observed when the same amount of protein from the wild-type cell line (CHO-WT) was analyzed (Fig 1). The major band corresponded to a molecular weight of approximately 46 kD, consistent with the deduced amino acid sequence of the CB2 receptor.
human CB2 receptor cDNA. The minor band corresponded to a molecular weight of approximately 45 kD, which could represent a degraded receptor or another form of the receptor differently glycosylated.

The specificity of immunopurified anti-CB2 receptor Abs to recognize CB2 receptors in their native forms was achieved by studying their binding on HL60 cells transfected with the human CB2 receptor cDNA (HL60-CB2). Flow cytometric analysis showed that a positive staining was obtained in HL60-CB2 cell line but not in the wild-type cell line (HL60-WT), as shown in Fig 2A. Moreover, inhibition of the labeling was observed when anti-CB2 receptor Abs were preincubated with the C-terminal synthetic peptide confirming the specificity of anti-CB2 receptor Abs. We next examined the subcellular distribution of CB2 receptors by confocal microscopy. Figure 2B shows a localization of CB2 receptors mainly associated with the plasma membrane of HL60-CB2 cells.

Expression of CB2 receptors in mononuclear cells isolated from peripheral blood and tonsils. The expression of CB2 receptors was first assayed by flow cytometry in peripheral blood mononuclear cells isolated from three different human donors. As shown in Fig 3A, the levels of CB2 receptor expression in these cells was relatively low as compared with HL60-CB2 cells. The quantitation of CB2 receptors in leukocytes showed that B lymphocytes (CD20+), NK cells (CD56+), and CD8+ T cells expressed the highest level of CB2 receptors, followed by NK cells (CD56+);
Among T-cell subsets, T8 (CD3^+CD8^+), lymphocytes displayed a higher level of CB2 receptors than did T4 cells (CD3^+CD4^+). The preferential expression of CB2 receptors in B cells led us to investigate the in situ distribution of this molecule in the B-cell zones of secondary lymphoid organs. For this purpose, dual-immunofluorescence studies were performed on tonsil tissue sections by combining anti-CB2 receptor Abs together with several MoAbs identifying the different compartments of the B-cell follicles. In this tissue, the absence of CB2 receptor staining in interfollicular T-cell areas (CD3^+) was observed as well as a marked homogeneous labeling of the mantle zones containing the resting IgD^+ B cells (Fig 4, lines I and II, respectively). By contrast, germinal center (GC) areas displayed a heterogeneous labeling. In some cases, they appeared labeled with the same intensity as the mantle zone (Fig 4, lines II and IV), and in other cases they displayed a slight decreased CB2 receptor staining (Fig 4, lines I and V). Variations of intensity of CB2 receptor labeling observed in some GC may be associated with the presence of a particular cell subset in these areas. Indeed, when GC were full of follicular dendritic cells (FDC), as shown after labeling with anti-FDC HJ2 MoAbs, GC were entirely stained by anti-CB2 receptor Abs (Fig 4, line IV), indicating that FDC expressed the CB2 receptor. By contrast, we found that weak expression of CB2 receptors was frequently associated with the presence of Ki67^+ proliferating cells that are...
localized in the dark zones of GC in human tonsils (Fig 4, line V). These observations, which were reproducibly repeated in three different tonsils, showed that CB2 receptors appeared preferentially expressed in both follicular mantles and GC of B-cell zones. In GC, FDC expressed CB2 receptors, whereas Ki67+ cells displayed substantial lower levels of these receptors.

**Regulation of the expression of CB2 receptors during B-cell differentiation.** The low CB2 receptor expression associated with proliferating B cells suggested a subtle regulation of this receptor during B-cell differentiation. To confirm the above-noted histological observations, expression of CB2 receptors was studied in purified B cells from three different human tonsils, using anti-CB2 receptor Abs and flow cytometry. The expression of CB2 receptors was found to be modulated during B-cell differentiation (Fig 5). In CD38+ GC B cells, a decrease of CB2 receptor expression was observed and confirmed when B lymphocytes acquired CD77, corresponding to their differentiation in centroblasts. Finally, CB2 receptor expression was restored when B cells reached their terminal stages of differentiation to become memory B cells (IgD−CD44+CD38−).

These results were confirmed when the modulation of CB2 receptors was studied at the level of mRNA content during B-cell differentiation. A quantitative RT-PCR–based method was performed on 2 × 10⁶ highly purified B cells belonging to different B-cell subsets. Two analyses performed on B-cell subsets from two different donors showed that centroblasts (CD77+) displayed a fourfold loss in their CB2 receptor-mRNA content compared with virgin B cells (IgD+; Fig 5).

**Involvement of CB2 receptors in the proliferation of virgin and GC B cells induced by CD40 MoAbs.** To study the function of CB2 receptors during B-cell differentiation, tonsillar B cells were separated into two subsets corresponding to virgin (IgD−) and GC (CD38+) B cells. We first examined the effects of the CP55,940 cannabinoid agonist on the proliferation of both subsets triggered with optimal concentrations of CD40 MoAbs in the presence of CD32-L cells. CP55,940 induced a dose-dependent increase of proliferation on both subsets (Fig 6). The thymidine uptake mediated by CD40 MoAbs was increased by 27% for the virgin B cells and 20% for the GC B cells in the presence of 10 mmol/L of the cannabinoid ligand. These effects were optimal at 72 hours, although they can be detected as soon as 24 hours. They were not inhibited by the CB1 receptor antagonist SR 141716a and were totally inhibited by the CB2 receptor antagonist SR 144528 (Fig 7). Furthermore, we observed that CP55,940 was not able to induce by itself any proliferation of B-cell subsets in the absence of CD40 MoAbs (data not shown).

We then examined the regulation of CB2 receptors in virgin and GC B cells triggered by CD40 ligation. Transcripts of CB2 receptors were dramatically upregulated in both B-cell subsets. CB2 receptor mRNA content was maximum around 24 hours and returned to its basal level within 48 hours (Fig 8A). This upregulation of CB2 receptors was confirmed at the protein level using anti-CB2 receptor Abs and flow cytometry. CB2 receptors increased during the first 24 hours and was maintained at 48 hours in virgin and GC B cells (Fig 8B). This might indicate that CD40 ligation induces the transcription of CB2 receptors.

**DISCUSSION**

Both Δ⁹-THC and the chemical analog CP55,940 exert their psychoactive effects through the brain cannabinoid receptor. They activate different signaling pathways that are all inhibited by the potent and selective SR 141716a antagonist. Both cannabinoid compounds also recognize another receptor, CB2, which is mainly localized in cells of the immune system. These two receptors belong to the seven-transmembrane G-protein–coupled receptor family. Several receptors of this family, such as the chemokine receptors, display major functions in the immune system through their involvement in the traffic and activation of leukocytes as well as their implica-
tion in the infection of immune cells by the human immunodeficiency virus. It was thus relevant to postulate that the large range of pharmacological effects of cannabinoid compounds reported to date on the immune system could be mediated through the CB2 receptor.

CB2 cannabinoid receptors are rather considered as orphan receptors, because only derivatives of arachidonic acid are able to bind these receptors as putative endogenous ligands with low affinities. Signal transduction induced by cannabinoid receptors has extensively been studied in transfected or gene reporter-transformed cell lines. To contribute to the understanding of the function of CB2 receptors in the immune system, we decided to accurately study the expression of CB2 receptors in lymphocytes. We raised polyclonal Abs highly specific for the C-terminal part of this receptor. Using a semiquantitative flow cytometric assay, we found that, in human peripheral blood, the rank order of CB2 receptor expressions was B cells > NK cells > T8 cells > T4 cells, confirming our previously reported results of determination of CB2 receptor mRNA expression level in these cells. Moreover, the evaluation of the number of secondary Abs bound per cell made it possible to estimate the CB2 receptor quantity to 2,000 receptors per B cell (data not shown).

The preferential expression of CB2 receptors in the B-cell lineage led us to study their regulation during B-cell differentiation. We thus examined CB2 receptors in tonsil tissue sections by dual-color confocal microscopy and found that CB2 receptors were restricted to B-cell areas in accordance with a previous autoradiographic study of the binding of [3H]-CP55,940 to rat immune tissues. In secondary follicles, labeling by anti-CB2 receptor Abs was clearly observed in the follicular mantle, whereas in GC, heterogeneous staining was observed. In GC, CB2 receptor less staining was found to be associated with the presence of proliferating cells (Ki67+), whereas expression of CB2 receptors was found to be associated with the presence of FDC.

Identification of different B-cell subsets along the B-cell differentiation pathway in tonsils has recently been described using flow cytometric technique. We used this technique to characterize virgin B cells, GC B cells, centroblasts, and memory B cells. During B-cell differentiation process, a dramatic downregulation of CB2 receptor labeling was ob-

![Fig 6. Effect of CP55,940 on the proliferation of B-cell subsets. Virgin (A) and GC (B) B-cell subsets were induced to proliferate in the presence of different concentrations of CP55,940 for 72 hours after ligation of CD40 antigen using CD32+ L cells. Data shown are representative of two different experiments performed from two different donors. * P < .05.](image)

![Fig 7. CB2 receptors accounted for the CP 55,940-increased B-cell proliferation. The CB1 receptor antagonist SR 141716 (●) and the CB2 receptor antagonist SR 144528 (▲) were preincubated at indicated concentrations for 30 minutes with purified tonsillar B cells before the addition of 10 nmol/L CP 55,940. B cells were induced to proliferate for 72 hours after ligation of CD40 antigen. Data are expressed taking as 100% the difference between [3H] thymidine uptakes (which corresponded to 15,200 cpm) into B cells activated with CD40 MoAbs with and without 10 nmol/L CP 55,940. * P < .05.](image)
served when B cells left the virgin B-cell stage to become centroblasts. CB2 receptor expression was restored at the end of differentiation when memory B cells appeared. The polyclonal anti-CB2 receptor Abs target the intracytoplasmic CB2 receptor C-terminal tail. Therefore, the decrease in CB2 receptor staining in centroblasts may be explained either by a decrease in CB2 receptor transcription or a receptor modification at the Ab recognition site after intracellular signaling. To exclude the latter, CB2 receptor transcripts were quantitated in highly purified B-cell subsets. A decrease in CB2 receptor mRNA level was also observed in centroblasts, confirming the downregulation of CB2 receptors at the protein level during B-cell differentiation.

The original distribution of CB2 receptors among cells of the immune system and their fine modulation during B-cell differentiation suggested that these receptors may exert their function on immune cells depending on their lineages, their stages of differentiation, and their partitioning at different locations within secondary lymphoid organs. Our previous observations that cannabinoid ligands enhanced human B-cell proliferation mediated by cross-linking of surface Igs, whereas no effect was noticeable on human T-cell proliferation mediated by phytohemagglutinin (PHA), argued in favor of a B-cell lineage-specific expression of CB2 receptors. Moreover, cannabinoid agonists effects were totally inhibited by pertussis toxin, demonstrating that cannabinoid receptors are coupled to a Gi protein in B cells. To examine the pattern of expression in the mature B-cell compartment, we compared the proliferative response of virgin and GC B cells to the cannabinoid agonist, CP55,940, under CD40-MoAb challenge. Low concentrations of CP55,940 enhanced the proliferation of both subsets in the presence of CD40 MoAbs. This enhancement was mediated by CB2 receptors, because the selective CB2 receptor antagonist SR 144528A inhibited CP 55,940 effects in a dose-dependent manner, whereas the CB1 receptor antagonist SR 141716A was without any effect. Moreover, the putative endogenous CB1 cannabinoid ligand anandamide (arachidonylethanolamide) was not able to enhance the proliferation of B-cell subsets induced by CD40 MoAbs (data not shown), confirming its lack of activity on the peripheral cannabinoid receptor transfected in CHO cell line. Furthermore, stimulation of CB2 receptors by CP55,940 in the absence of CD40 MoAbs was not sufficient to induce a proliferation of B cells, indicating that CB2 receptors may act as coreceptors in the CD40-transduction pathway. The CB2 receptor-mediated enhancement of the proliferation of B cells at various stages of differentiation suggested a regulation of its expression after exposition to CD40 MoAbs. Indeed, a strong upregulation of CB2 receptors was observed in virgin and GC B cells stimulated with CD40 MoAbs. We have recently shown that CP55,940 induces the activation of p42/p44 MAPK and the expression of the growth-related gene krox-24 in a CB2 receptor-transfected cell line. The activation of MAPK after ligation of the B-cell receptor is also associated to the induction of krox-24 through the activation of p21ras pathway. Ligation of CD40 by MoAbs leads to the activation of another signaling pathway that is the stress-activated protein kinase pathway (SAPK). The fact that CB2 receptors may act as coreceptors in both signaling pathways, MAPK via surface IgM and SAPK via CD40 antigen, suggests that CB2 receptor signals may converge at a G protein regulating the activity of p21ras, which is an effector shared by the two important pathways of B-cell differentiation.

ACKNOWLEDGMENT

The authors thank Pierre Gros for a critical review of the manuscript and Catherine Carayon for her secretarial assistance.

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