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Presence and regulation of the endocannabinoid system in human dendritic cells

Isabel Matias[1], Pierre Pochard[2], Pierangelo Orlando[3], Michel Salzet[4], Joel Pestel[2] and Vincenzo Di Marzo[1]

[1]*Endocannabinoid Research Group,* [1]*Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Comprensorio Olivetti, Pozzuoli (Napoli), Italy;* [2]*Inflammatory Reaction and Allergic diseases Department, INSERM unit, Pasteur Institute, Lille, France;* [3]*Istituto di Biochimica delle Proteine ed Enzimologia, Consiglio Nazionale delle Ricerche, Comprensorio Olivetti, Pozzuoli (Napoli), Italy;* [4]*Laboratoire de Neuroimmunité des Annélides, UMR 8017 CNRS, Université des Sciences et Technologies de Lille, Villeneuve d'Ascq, France*

Cannabinoid receptors and their endogenous ligands, the endocannabinoids, have been detected in several blood immune cells, including monocytes/macrophages, basophils and lymphocytes. However, their presence in dendritic cells, which play a key role in the initiation and development of the immune response, has never been investigated. Here we have analyzed human dendritic cells for the presence of the endocannabinoids, anandamide and 2-arachidonoylglycerol (2-AG), the cannabinoid CB_1 and CB_2 receptors, and one of the enzymes mostly responsible for endocannabinoid hydrolysis, the fatty acid amide hydrolase (FAAH). By using a very sensitive liquid chromatography-atmospheric pressure chemical ionization-mass spectrometric (LC-APCI-MS) method, lipids extracted from immature dendritic cells were shown to contain 2-AG, anandamide and the anti-inflammatory anandamide congener, *N*-palmitoylethanolamine (PalEtn) (2.1 ± 1.0, 0.14 ± 0.02 and 8.2 ± 3.9 pmol·10^{-7} cells, respectively). The amounts of 2-AG, but not anandamide or PalEtn, were significantly increased following cell maturation induced by bacterial lipopolysaccharide (LPS) or the allergen Der p 1 (2.8- and 1.9-fold, respectively). By using both RT-PCR and Western immunoblotting, dendritic cells were also found to express measurable amounts of CB_1 and CB_2 receptors and of FAAH. Cell maturation did not consistently modify the expression of these proteins, although in some cell preparations a decrease of the levels of both CB_1 and CB_2 mRNA transcripts was observed after LPS stimulation. These findings demonstrate for the first time that the endogenous cannabinoid system is present in human dendritic cells and can be regulated by cell activation.

Keywords: anandamide; 2-arachidonoylglycerol; cannabinoid; receptor; fatty acid amide hydrolase.

The Δ^9-tetrahydrocannabinol (THC), the major psychoactive component of *Cannabis sativa*, has been reported to have beneficial effects on the treatment of nausea, glaucoma, hypertension, migraine, neurological disorders (i.e. epilepsy, Huntington's disease, Tourette's syndrome, dystonia and Parkinson's disease) and pain [1], and to play a down-regulatory role on the immune system [2]. Indeed, cannabinoids exhibit immunosuppressive properties and *in vitro* they weaken humoral immunity [3,4], cell-mediated immunity [5,6] and cellular defenses against infectious agents [7,8]. A modulation of the cytokine network and a decrease of T- and B-cell proliferation have been described *in vitro* [9]. A reduction of the cytolytic activity of natural killer cells and of antigen presentation was also observed, again *in vitro* [9].

The endocannabinoid system, comprising membrane receptors for THC, endogenous ligands for these receptors, and proteins for their biosynthesis and inactivation, is present to a large extent in mammalian immune tissues. The cannabinoid CB_2 receptor, cloned by Munro *et al.* [10] from a human promyelocytic leukemia (HL60) cell cDNA library, appears to be the predominant cannabinoid receptor in the immune system, while it is not expressed in the brain. High CB_2 expression is observed in B cells and in natural killer cells, and may be related to the established alteration of the function of these cells by cannabinoids. CB_2 is also expressed to a lesser extent in monocytes, neutrophils and T cells. The brain cannabinoid receptor, CB_1, is also expressed in immune cells such as like lymphocytes [11], splenocytes [12] and T cells [13].

Anandamide was the first endogenous cannabinoid receptor ligand to be discovered in 1992 [14]. Other 'endocannabinoids' were reported later, i.e. 2-arachidonoyl-glycerol (2-AG) [15,16] and noladin ether [17]. Endocannabinoids have been found in immune cells like macrophages [18–21] and RBL-2H3 basophilic leukemia

Correspondence to V. Di Marzo, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Comprensorio Olivetti, Pozzuoli (Napoli), Italy.
Fax: + 39 081 8041770, Tel.: + 39 081 8675093,
E-mail: vdimarzo@icmib.na.cnr.it

Abbreviations: 2-AG, 2-arachidonoylglycerol; PalEtn, *N*-palmitoylethanolamine; FAAH, fatty acid amide hydrolase; THC, Δ^9-tetrahydrocannabinol; LPS, lipopolysaccharide; LC-APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; MACS, magnetic cell sorting.

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cells [22]. After stimulation with either lipopolysaccharide (LPS) or platelet activating factor, macrophages and lymphocytes are able to produce a higher amount of anandamide and/or 2-AG [21,23–26]. IgE-dependent stimulation of RBL-2H3 cells also leads to the formation of anandamide and of its congener *N*-palmitoylethanolamine (PalEtn) [22], which exerts anti-inflammatory actions via nonCB_1, nonCB_2–mediated mechanisms [27]. Endocannabinoids have various effects on immune cell function, some of which (e.g. modulation of cytokine release from macrophages and inhibition of lymphocyte proliferation) resemble those of THC, while some others (e.g. stimulation of hematopoietic cell proliferation) are exerted via noncannabinoid receptor-mediated mechanisms (reviewed in [28,29]).

After cellular uptake, mediated by one or more yet to be characterized specific membrane transporters, the degradation of endocannabinoids occurs via the fatty acid amide hydrolase (FAAH) [30] in neuronal as well as immune cells, such as RBL-2H3 basophilic leukemia cells [22], U937 monocytic cells [31], macrophages [24,32], mast cells [33], and platelets [34]. FAAH, a serine hydrolase and a member of the amidase family, is an integral membrane protein that is responsible for the inactivation of anandamide and, to some extent, 2-AG [35,36].

Dendritic cells, derived from bone marrow stem cells, are the most potent antigen-presenting cells of the immune system. They play a central role in the initiation of primary immune response and in the enhancement of secondary immune response [37,38]. Immature dendritic cells localized in peripheral tissues are able to take up antigens (i.e. viruses, bacteria, parasites, cancer cells) and, subsequently, to migrate through afferent lymphatics to the T cell-rich zone of draining lymph nodes. During migration, immature dendritic cells undergo an additional maturation step and become able to present processed antigens in association with major histocompatibility complex II antigens [39], and to stimulate naive T cells [40]. Dendritic cells are involved in the polarization of the immune response towards a Th1 (large production of interferon-γ) or a Th2 (sustained production of interleukins-4 and -5, as observed in allergies) profile.

Despite the key pivotal role in the immune response played by dendritic cells, nothing is known about their capability to produce, respond to and degrade endocannabinoids. Indeed as dendritic cells can be derived from monocytes, and as monocytes were previously described to express the endocannabinoid system, we investigated the presence and regulation of endocannabinoids, cannabinoid receptors and FAAH in immature and mature dendritic cells obtained by stimulation with either the bacterial agent LPS or the mite allergen, Der p 1.

MATERIALS AND METHODS

Materials and animals

Deuterated anandamide, PalEtn and 2-AG were synthesized from [$^2H_4$]palmitic acid and [$^2H_8$]arachidonic acid and ethanolamine or glycerol as described previously [22]. Rats (Strain CD, Charles River, France) were anaesthetized before their brain and spleen were removed and placed in nitrogen.

Antibodies

Rabbit antihuman CB_1 and CB_2 polyclonal antibodies and also the corresponding blocking peptides were from Cayman. The CB_1 antibody was raised against the N-terminal (amino acids 1–14) extracellular region of human and rat CB_1 receptor. The CB_2 antibody was raised against a sequence between the N-terminal and the first *trans*-membrane domain of the protein of the human and rat CB_2 receptor. The specificity of the CB_1 and CB_2 antibodies was described in McIntosh *et al.* [41] and in Shire *et al.* [42], respectively. Rabbit anti-human and rat FAAH polyclonal antibody, kindly provided by M. Maccarrone (Department of Experimental Medicine and Biochemical Sciences, University of Rome-Tor Vergata, Italy), was elicited against the conserved FAAH sequence VGYYETDNYTMPSPAMR [26].

Isolation of human monocytes and differentiation into dendritic cells

Dendritic cells were generated *in vitro* from peripheral blood mononuclear cells (PBMC) as described previously [43]. Blood from healthy donors was centrifuged (120 ***g***, 15 min) and platelet rich plasma was discarded. Blood cells were further diluted in Roswell Park Memorial Institute medium (RPMI 1640) and layered over a Ficoll gradient (Pharmacia) (v/v). After centrifugation (400 ***g***, 30 min), two fractions were obtained: a top leukocyte band containing mononuclear cells (monocytes and lymphocytes) and a lower band containing polymorphonuclear leukocytes (granulocytes) and the red cells. The PBMC were recovered, washed with RPMI and counted. After a further centrifugation, the cell pellet was resuspended in $NaCl/P_i$ containing BSA and EDTA for $CD14^+$ monocyte purification by magnetic cell sorting (MACS) micro beads (Miltenyl Biotech, Germany), as described by the manufacturer.

Briefly, CD14 microbeads were developed for human cell separation based on the expression of the CD14 antigen. The CD14 antigen is expressed in high amounts in monocytes and/or macrophages and in low amounts in granulocytes. For monocyte purification, 10×10^6 enriched PBMC were incubated for 30 min on ice with 20 μL MACS micro beads coated with antibodies directed against CD14 membrane marker, washed and applied onto a column placed in the magnetic field of a MACS separator (Miltenyi Biotec, Paris, France). After elution of the CD14-negative cells by two washings with $NaCl/P_i$/BSA/EDTA buffer, the column was removed from the magnetic field and the $CD14^+$ monocytes were collected, washed twice in RPMI 1640 medium before plating (2×10^6 cells; 2 mL per well) into six-well flat-bottomed culture plates in RPMI 1640 medium supplemented with 1% Tiacarpen (0.2 $mg \cdot mL^{-1}$; SmithKline Beecham) and 10% fetal bovine serum (Life Technologies). To allow monocyte differentiation into immature dendritic cells, $CD14^+$ cells were cultured for 6 days at 37 °C in humidified 5% CO_2 in air, into six-well flat-bottomed culture plates in RPMI medium supplemented with granulocyte-macrophage colony stimulating factor (Peprotech, London, UK) (20 $ng \cdot mL^{-1}$), and interleukin-4 (R&D Systems) (200 $U \cdot mL^{-1}$).

For dendritic cell activation, LPS (1 $\mu g \cdot mL^{-1}$) or the Der p 1 antigen (a major allergen of the house dust mite

Dermatophagoides pteronyssinus) (500 ng·mL^{-1}) was added to the culture medium for 24 h. Cell cultures were further harvested for analysis.

Purification and quantification of endocannabinoids

The extraction, purification and quantification of anandamide, 2-AG and PalEtn from immature and mature dendritic cells requires a set of different biochemical steps [22]. First, cells were Dounce-homogenized and extracted with chloroform/methanol/Tris/HCl 50 mM pH 7.5 (2 : 1 : 1, v/v) containing internal standards (5 pmol [$^2H_8$]anandamide, 100 pmol [$^2H_8$]2-AG, and 5 pmol [$^2H_4$]PalEtn). The lipid-containing organic phase was dried down, weighed, prepurified by open bed chromatography on silica gel. The resultant fractions were obtained by eluting the column with 9 : 1 and 1 : 1 (v/v) chloroform/methanol and then analyzed by liquid chromatography-atmospheric pressure, chemical ionization-mass spectrometry (LC-APCI-MS) by using a Shimadzu HPLC apparatus (LC-10ADVP) coupled to a Shimadzu (LCMS-2010) quadrupole MS via a Shimadzu APCI interface.

MS analyses were carried out in the selected ion monitoring (SIM) mode, as described previously [44]. The temperature of the APCI source was 400 °C, the HPLC column was a Phenomenex (5 μm, 150 × 4.5 mm) reverse-phase column, eluted as described [44]. Anandamide (retention time 14.5 min), PalEtn (retention time 19.0 min) and 2-AG quasi-molecular ions (m/z = 348.3, 379.3 and 300.3) were quantified by isotope dilution with the above-mentioned deuterated standards (same retention times and m/z = 356.3, 387.3 and 304.3) [44] and their amounts in pmoles normalized per 10^7 cells. Two LC-MS peaks for both deuterated and undeuterated mono-arachidonoylglycerol were found at retention times of 17.0 and 18.9 min, respectively, corresponding to 2-AG and 1(3)-AG, in agreement with the previous observation that 2-AG undergoes isomerization during the purification procedure [24]. Therefore, the amounts of 2-AG were calculated by adding the amounts of the two isomers. The amounts of endocannabinoids are expressed as pmols or nmols per 10^7 cells extracted. Data were statistically evaluated by ANOVA (Bonferroni-adjusted).

Total RNA isolation and RT-PCR analysis

Total RNA from immature and mature dendritic cells was extracted using Trizol reagent according to the manufacturer's recommendations (GibcoBRL). Following extraction, RNA was precipitated using ice-cold isopropanol, resuspended in diethyl pyrocarbonate (Sigma)-treated water and its integrity was verified following separation by electrophoresis into an 1% agarose gel containing ethidium bromide. RNA was further treated with RNAse-free DNAse I (Ambion DNA-free^{TM} kit) according the manufacturer's recommendations to digest contaminating genomic DNA and to subsequently remove the DNAse and divalent cations.

The expression of mRNAs for glyceraldehyde-3-phosphate dehydrogenase, FAAH, CB_1 and CB_2 receptors was examined by RT-PCR. Total RNA was reverse-transcribed using oligo dT primers. DNA amplifications were carried out in PCR buffer (Q-Biogen) containing 2 μL cDNA, 500 μM dNTP, 2 mM $MgCl_2$, 0.8 μM each primer and 0.5 U *Taq* polymerase (Q-Biogen). The thermal reaction profile consisted of a denaturation step at 94 °C for 1 min, annealing at 60 °C for 1 min and an extension step at 72 °C for 1 min. A final extension step of 10 min was carried out at 72 °C. The PCR cycles were 35 for CB_1, CB_2, FAAH and glyceraldehyde-3-phosphate dehydrogenase and were observed to be optimal and in the linear portion of the amplification curve (data not shown). Reactions were performed in a PE Gene Amp PCR System 9600 (Perkin-Emer). After PCR, the products were separated by electrophoresis on a 2% agarose gel containing ethidium bromide for UV visualization.

The specific human oligonucleotides were synthesized on the basis of cloned human cDNA sequences of glyceraldehyde-3-phosphate dehydrogenase, FAAH, CB_1 and CB_2. For glyceraldehyde-3-phosphate dehydrogenase, the primers sequences were 5′-CCCTTCATTGACCTCAACTA CATGGT-3′ (nucleotides 208–233; sense) and 5′-GAG GGCCATCCACAGTCTTCTG-3′ (nucleotides 655–677; antisense). The FAAH sense and antisense primers were 5′-GTGGTGCT(G/A)ACCCCCATGCTGG-3′ (nucleotides 469–475) and 5′-TCCACCTCCCGCATGAACCG CAGACA-3′ (nucleotides 561–569), respectively. The CB_1 sense and antisense primers were 5′-GATGTCTTTGGGA AGATGAACAAGC-3′ (nucleotides 365–373) and 5′-AG ACGTGTCTGTGGACACAGACATGG-3′ (nucleotides 460–468), respectively. For CB_2, the primers sequences were 5′-CCCATGCAGGA(G/T)TACATGATCCTGAG-3′ (nucleotides 20–29; sense) and 5′-CTCCGC(A/C)G(A/G) AAGCCCTC(A/G)TAC-3′ (nucleotides 64–70; antisense). The expected sizes of the amplicons were 470 bp for glyceraldehyde-3-phosphate dehydrogenase, 300 bp for FAAH, 309 bp for CB_1 and 150 bp for CB_2. The glyceraldehyde-3-phosphate dehydrogenase house-keeping gene expression was used in order to evaluate any variation in the RNA content and cDNA synthesis in the different preparations. Furthermore, the PCR primers for glyceraldehyde-3-phosphate dehydrogenase and FAAH were selected on the basis of the sequence of the FAAH gene (NCBI accession number AF098010) by including the introns 5476–6026 and 6173–6296, and of the sequence of the glyceraldehyde-3-phosphate dehydrogenase gene (NCBI accession number AH007340) by including the introns 3216–3305, 3413–3541, 3633–3722, 3839–3930 and 4013–4205, respectively. In the presence of contaminant genomic DNA, the expected size of the amplicons would be 1062 bp for glyceraldehyde-3-phosphate dehydrogenase and 1335 bp for FAAH, respectively. No PCR products were detected when the reverse transcriptase step was omitted (data not shown).

Western immunoblotting

Analytical SDS/PAGE (10%) was performed as described previously [45] on lysates from immature dendritic cells and from brain and spleen of rat used as positive control for CB_1, CB_2 and FAAH, respectively. Western blot analysis was then carried out with the CB_1, CB_2 and FAAH polyclonal antibody. Briefly, dendritic cells or rat organs were homogenized in lysis buffer (1 mM EDTA, 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1 mM Na-*ortho*vanadate, 1 mM Na-fluoronate, 1% NP-40, 0.1% SDS, 1% Triton,

0.25% Na-desoxycholate, 1 mM phenylmethanesulfonyl fluoride, 1 mg·mL^{-1} serine proteases inhibitors) using a Dounce homogenizer, incubated at 4 °C for 30 min and finally centrifuged at 10 000 ***g*** for 20 min The amount of proteins in each resulting supernatant was titrated by a Biorad assay. Supernatants were mixed 4 : 1 (v/v) with sample buffer (300 mM Tris/HCl pH 6.8, 50% glycerol, 500 mM dithiotreitol, 0.05% Bromophenol blue, 10% SDS) and boiled for 5 min prior to loading on a 0.75 mm-thick gel. Samples were subjected to electrophoresis (100 V) for 2.5 h under reducing conditions, and separated proteins were transferred onto Immobilon Protein Transfer at 30 mA overnight at 4 °C. The nitrocellulose membrane was preincubated with 5% nonfat dry milk in NaCl/Tris (10 mM Tris/HCl pH 8, 150 mM NaCl) for 30 min to block nonspecific binding. The membrane was incubated for 1 h in antibody at a dilution of 1 : 400 for CB_1 polyclonal antibody, 1 : 250 for CB_2 polyclonal antibody, and 1 : 200 for FAAH polyclonal antibody. A control was made in the same conditions using the CB_1 polyclonal antibody and the CB_2 polyclonal antibodies preabsorbed with the homologous antigens (4 μg·mL^{-1} antibody solution). Then, the membrane was washed 3 × 10 min in NaCl/Tris containing 0.05% Tween-20 (NaCl/Tris/Tween) and incubated with goat anti-(rabbit IgG) Ig conjugated with horseradish peroxidase (dilution 1 : 3000) for 1 h. The membrane was again washed 3 × 10 min in NaCl/Tris/Tween and rinsed in NaCl/Tris/Tween. Signals were detected with an ECL kit (Biorad). Control of specificities was performed by preadsorpbing the antibody by the homologous antigen at a concentration of 4 μg·mL^{-1} of antibody solution.

RESULTS

Endocannabinoids in dendritic cells

After a lipid extraction in chloroform/methanol, a separation was conducted using SiO_2 open bed chromatography. The separated lipids (9 : 1 fraction) were subjected to LC-APCI-MS analysis. The amounts in immature dendritic cells were 0.14 ± 0.02 pmol per 10^7 cells and 2.1 ± 1.0 pmol per 10^7 cells, for anandamide and 2-AG, respectively (means ± SD, $n = 4$). PalEtn was quantified at an amount of 8.2 ± 3.9 pmol per 10^7 cells (means ± SD, $n = 4$). Because the activation and the maturation of dendritic cells induce a series of events that lead to changes in dendritic cell phenotype and function, we have compared the amount of these compounds in immature dendritic cells that were used as control (100%) with those of dendritic cells made mature by stimulation with LPS and Der p 1 allergen (Fig. 1). We found that in mature dendritic cells the amounts of 2-AG were increased to 275.5 ± 59.1% and 189.8 ± 28.2% of control after LPS and Der p 1 stimulation, respectively (means ± SD, $n = 4$, $P < 0.05$ by ANOVA) (Fig. 1). By contrast, we observed no statistically significant effect on anandamide amounts (92.3 ± 22.1% and 91.5 ± 45.6% of control after LPS and Der p 1 stimulation, respectively, means ± SD, $n = 4$, $P > 0.05$) (Fig. 1). The amounts of PalEtn were also not significantly modified by cell maturation (110.8 ± 32.5% and 102.0 ± 38.9% of control for LPS and Der p 1 stimulation, respectively, means ± SD, $n = 4$) (Fig. 1).

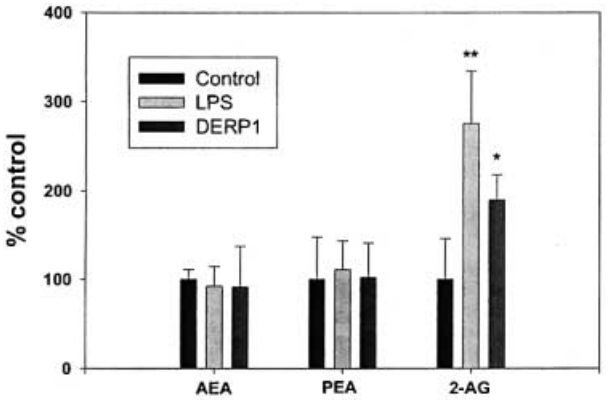

Fig. 1. Modulation of the levels of anandamide (AEA), PalEtn and 2-AG in dendritic cells treated with either vehicle (control), LPS or Der p 1. Data are expressed as per cent of controls and are means ± SD ($n = 4$). *$P < 0.05$ by ANOVA. Control levels were 0.14 ± 0.02, 8.2 ± 3.9 and 2.1 ± 1.0 pmol per 10^7 cells for AEA, PalEtn and 2-AG, respectively.

Analysis of cannabinoid receptors and fatty acid amide hydrolase

To determine the presence of the cannabinoid receptors (CB_1 and CB_2) and of the fatty acid amide hydrolase (FAAH), we used two independent methods. RT-PCR was used to determine the presence of the messenger RNAs, and Western immunoblot analysis was used to determine the presence of the corresponding proteins.

Using specific primers for human CB_1, amplification of immature and mature dendritic cell cDNA revealed the presence of mRNA transcripts of the expected length for CB_1 (Fig. 2A). Western immunoblotting of immature dendritic cells shows two bands at ≈ 83 and ≈ 64 kDa very similar to those detected in rat brain, used as positive control (Fig. 3A). The predicted size of the CB_1 protein based in its amino acid sequence following extrapolation from its corresponding cDNA is 53 kDa. However, previous studies demonstrated that the immunoreactive bands at 83 and 64 kDa most likely represent a receptor that has undergone post-translational modification such as glycosylation [46]. That the immunoreactive bands at 83 and 64 kDa were not due to nonspecific interactions is supported by the observation that preabsorbing of the CB_1 antibody with its corresponding blocking peptide eliminated almost all of the staining of these bands (Fig. 3A). The most abundant band in human immature dendritic cells was the one at ≈ 83 kDa which may be related to a predominant glycosylation form of the CB_1 receptor in these cells. Additionally, in the rat brain lysate we also observed a band at ≈ 41 kDa which could correspond to the truncated CB_1 receptor protein (data not shown) [46].

The expression of CB_2 mRNA in immature and mature dendritic cells was also demonstrated by using RT-PCR with specific human primers (Fig. 2A). Western blot analysis of proteins prepared from human immature dendritic cells shows the presence of three immunoreactive bands at ≈ 59, ≈ 45 and ≈ 39 kDa (Fig. 3B). The most abundant band was the one at ≈ 59 kDa, which was present in both human immature dendritic cells and in rat

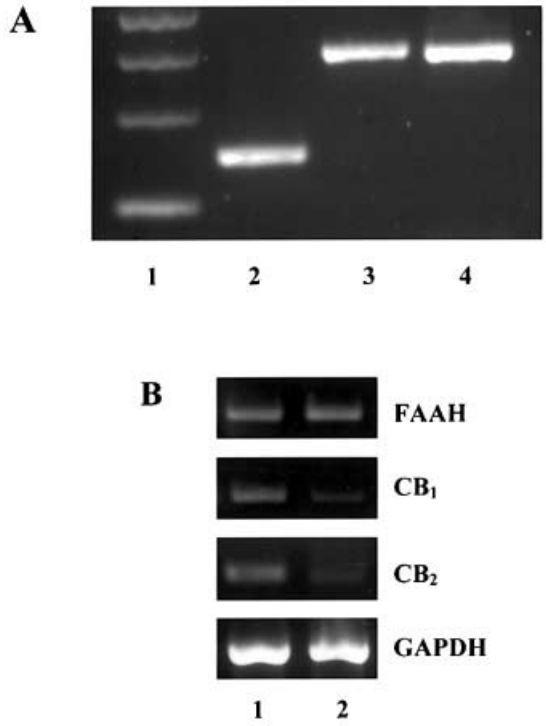

Fig. 2. FAAH, CB_1 and CB_2 mRNA expression in dendritic cells. (A) Expression in immature cells of mRNA transcripts with the expected sizes for CB_2 (lane 2), CB_1 (lane 3) and FAAH (lane 4). A 100 bp DNA ladder is shown starting from 100 bp (lane 1). (B) FAAH, CB_1 and CB_2 mRNA expression in immature dendritic cells (lane 1) or after stimulation with LPS (lane 2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in dendritic cells is shown as the housekeeping gene. The expected sizes of the amplicons were 300 bp for FAAH, 309 bp for CB_1, 150 bp for CB_2 and 470 bp for GAPDH. In (A) five times more PCR product than in (B) was loaded onto the agarose gel. In (B), data are not representative of all the samples analyzed, as in only three preparations out of the six analyzed was a decrease of mRNA transcripts observed.

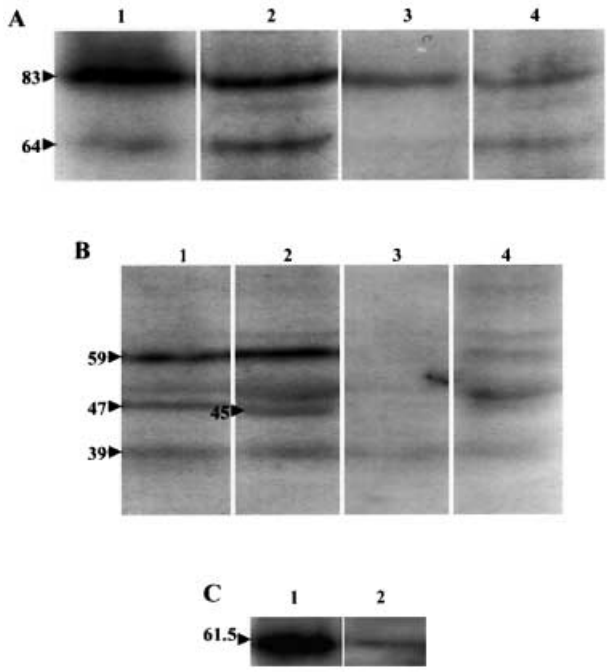

Fig. 3. Western immunoblotting of protein homogenates of human immature dendritic cells, rat brain and rat spleen. (A) Rat brain (lane 1) and dendritic cell (lane 2) lysates reacted with CB_1 antibody exhibit two immunoreactive bands at $\approx$83 kDa and $\approx$64 kDa. The immunostaining of these bands were reduced in rat brain (lane 3) and in dendritic cells (lane 4) lysates when the CB_1 antibody was preabsorbed with its corresponding homologous peptide. (B) Rat spleen (lane 1) and dendritic cells (lane 2) lysates reacted with CB_2 antibody show three immunoreactive bands: at $\approx$59, $\approx$47 and $\approx$39 kDa for the rat spleen lysate and at $\approx$59, $\approx$45 and $\approx$39 kDa for the dendritic cell lysate. Pre-adsorption of CB_2 antibody with the homologous antigen abolished the positive staining (lanes 3 and 4). (C) Rat brain (lane 1) and dendritic cell (lane 2) lysates reacted with FAAH antibody exhibit an intense immunoreactive band at $\approx$61.5 kDa.

spleen used as a positive control. This band, whose staining was totally abolished when the CB_2 antibody was preabsorbed with its corresponding antigen, might correspond to a glycosylated form of the CB_2 receptor protein. The dendritic cells band at $\approx$45 kDa and the rat spleen at $\approx$47 kDa were less intense and are consistent with the previous glycosylated forms of human and rat CB_2 receptors [47,48]. The $\approx$39 kDa band was very faint in both human immature dendritic cells and rat spleen and could correspond to the 39 kDa predicted size of the CB_2 protein based on its amino acid sequence extrapolated from the corresponding cDNA.

A FAAH mRNA transcript was also detected in human dendritic cells. RT-PCR amplification of cDNA of these cells shows a single band of the expected molecular size (Fig. 2A). We also determined the presence of the FAAH protein by Western blot analysis (Fig. 3C). An intense staining band at $\approx$61.5 kDa, corresponding to the predicted size of FAAH protein (62 kDa), based on its amino acid sequence extrapolated from its corresponding cDNA, was observed in immature dendritic cells as well as in rat brain lysates (Fig. 3C).

To examine the modulation of CB_1, CB_2 and FAAH mRNA expression in immature vs. mature dendritic cells, we compared the expression of these genes by RT-PCR in immature dendritic cells, used as controls, with that of dendritic cells after stimulation with LPS and Der p 1 allergen. Although in some cases a decrease of the expression of CB_1 and CB_2 receptor was observed with LPS (Fig. 2B), these findings could not be reproduced in all dendritic cell preparations examined.

DISCUSSION

The results presented here indicate for the first time that human dendritic cells contain anandamide, 2-AG and PalEtn. The amounts of anandamide and 2-AG in immature dendritic cells were similar to the ones detected in rat circulating macrophages [21,24], and also in this case 2-AG was the most abundant endocannabinoid. As compared to human lymphocytes [26], however, dendritic cells make 25 times less anandamide and much more 2-AG. PalEtn, which is not an endocannabinoid but exhibits cannabimimetic anti-inflammatory effects in immune cells [28,49], was more abundant than both anandamide and 2-AG, as

observed also in human blood [50]. Like rat circulating macrophages [21,24], and unlike human lymphocytes [26], LPS stimulation (and subsequent cell maturation) exerted no effect on dendritic cell anandamide levels. However, it must be pointed out that in the present study, where we investigated the effect of cell maturation on endocannabinoid levels, a much lower concentration of LPS (1 $\mu g \cdot mL^{-1}$) was used as compared to previous investigations [21,24,26]. Furthermore, the detection of increased levels of anandamide might have been prevented by degradation by FAAH, a factor less likely to affect 2-AG levels, which were 20-fold higher than those of anandamide. In fact, after LPS-induced maturation, the amounts of 2-AG were increased 2.8-fold, as in the mouse macrophage J774 cell line [24] and in rat circulating macrophages [20,24]. The fact that 2-AG levels are increased as a consequence of dendritic cell maturation was confirmed when this phenomenon was also induced by using the *D. pteronyssinus* mite allergen, Der p 1, which led to a 1.9-fold increase of 2-AG amounts. These data suggest that 2-AG originating from human dendritic cells might contribute to the important immune function played by these cells after activation and during bacterial infections and allergic responses. This hypothesis is supported by the previous observation that, unlike anandamide, which cannot activate efficaciously CB_2 receptors [51], 2-AG is the only endocannabinoid capable of functionally activating with the same efficacy not only CB_1 but also CB_2 receptors [51,52]. It is possible that 2-AG produced after Der p 1 stimulation is involved as a mediator in dendritic cell-induced polarization of the immune response during the allergic response [43]. However, it must be pointed out that the cells used in this study were obtained from healthy donors and that a different picture may have emerged if cells from allergic patients had been used instead. For example, though Der p 1 antigen is known to exhibit allergenic activity, when dendritic cells from healthy donors are incubated with Der p 1, dendritic cells preferentially acquire the capacity to favor a Th1 response, as if they had been stimulated with LPS [43]. Therefore, further experiments with dendritic cells from house dust mite allergic patients are required to fully understand the role of 2-AG in the development of the allergic response, which depends on several factors.

We did not assess whether the endocannabinoids and PalEtn produced from dendritic cells were released or not into the incubation medium as this would have required the use of a modified incubation medium containing either a blocker of endocannabinoid uptake or BSA [22]. Furthermore, detection of these compounds in the extracellular milieu usually requires the use of a number of blood cells much higher than that used in this study. However, it is worthwhile noting that 2-AG was recently shown to be released from macrophages following stimulation with the platelet activating factor [25].

Because endocannabinoids have been suggested to act as both autacoid and paracoid mediators [52], we next investigated whether dendritic cells also express cannabinoid receptors and FAAH, one of the enzymes mostly involved in endocannabinoid inactivation. The heterogeneous distribution of CB_2 receptors among cells of the immune system suggests that these receptors might exert their function on immune cells depending on their lineages and stage of differentiation [53]. Indeed, the expression of cannabinoid CB_1 and CB_2 receptors in immune cells appears to be regulated by LPS, cytokines and immunological stimuli. LPS downregulates CB_2 receptor mRNA in mouse splenocytes [54] and so does the immune-suppressive cytokine tumor growth factor-β in peripheral blood lymphocytes [55]. In contrast, anti-CD40 Ig upregulate both CB_1 and CB_2 receptor mRNA in mouse B splenocytes [12,54]. Finally, in a very recent study [56] it was found that the cannabinoid CB_2 receptor is expressed in macrophages differentially in relation to cell activation. CB_2 was undetectable in resident rat peritoneal macrophages, present at high levels in thioglycolate-elicited inflammatory and interferon γ-primed peritoneal macrophages, and detected at significantly diminished levels in LPS-activated peritoneal macrophages, whereas the CB_1 receptor was not detected regardless of cell activation. Our results show that both CB_2 mRNA and protein are expressed in immature human dendritic cells and that, although we could not observe in all preparations a differential expression between immature and mature dendritic cells, in some cases a reduction of CB_2 expression after LPS treatment was noticed, as previously reported for B cells [54]. We also found that both mRNA transcripts and protein for the cannabinoid CB_1 receptor are present in immature human dendritic cells, in amounts comparable to those of CB_2 receptors. Also the levels of CB_1 mRNA transcripts appeared to be slightly lower in mature than immature cells, unlike previously observed with LPS-stimulated human lymphocytes [26], but again this effect was not observed in all cell preparations.

Finally, we found that immature dendritic cells also express FAAH, whose mRNA levels do not appear to be modified by maturation induced by either LPS or Der p 1. This lack of effect is at variance with previous findings in human lymphocytes, where FAAH is downregulated by LPS, interleukin-12 and interferon-γ, and upregulated by interleukins-4 and -10 [26,13].

Taken together, these data show the presence of a complete endocannabinoid system in human dendritic cells. Our findings, together with previous reports in the literature regarding other immune cell types at different stages of maturation and activation, indicate that this signaling system might be regulated in dendritic cells in a similar way to macrophages and lymphocytes as far as the amounts of the endogenous ligands are concerned, but differently in terms of the levels of the two cannabinoid receptor subtypes and FAAH. Indeed, inflammatory, allergenic and septic stimuli always seem to stimulate the formation of either 2-AG or anandamide, or both, from macrophages, lymphocytes and dendritic cells. However, these increased endocannabinoid levels may not necessarily result in increased cannabinoid receptor stimulation, as CB_1 and in particular CB_2 receptors might be down-regulated by those same stimuli leading to enhanced amounts of 2-AG and anandamide.

The interactions of mature dendritic cells with naive T cells should be considered when speculating on the possible function of endocannabinoids in the immune response. It is possible, for example, that 2-AG produced by dendritic cells after LPS stimulation, i.e. during bacterial infection or septic shock, acts on T cell cannabinoid receptors to switch the immune response from a Th2 to a Th1 profile. Conversely, T cells, after interacting with dendritic cells, might produce anandamide and 2-AG, which then might feed back on CB_1

and CB_2 receptors detected here on dendritic cells. FAAH might then play a role in regulating this endocannabinoid-mediated T cell–dendritic cell communication. Finally, the presence of relatively high amounts of PalEtn in both immature and mature dendritic cells might indicate that the strong anti-inflammatory and anti-hyperreactivity actions of this compound, which occur via mechanisms yet to be elucidated [27], might be due in part to interaction with these cells, which are so deeply involved in allergy. In view of these considerations, our findings should warrant further studies on the pharmacological effects of THC, endocannabinoids and PalEtn on dendritic cells, and on the possible regulation of the endocannabinoid system following dendritic cell–T cell interactions.

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