Stimulation of peripheral cannabinoid receptor CB2 induces MCP-1 and IL-8 gene expression in human promyelocytic cell line HL60

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Abstract Using the recently developed methodology of nucleic acid microarrays spotted with specific cDNAs probes belonging to different gene families, we showed for the first time that nanomolar concentrations of the cannabinoid ligand CP-55940 upregulated the expression of two different members of the chemokine gene family: the α-chemokine interleukin-8 (IL-8) and the β-chemokine monocyte chemotactic protein-1 (MCP-1), in the promyelocytic cell line HL60 transfected with peripheral cannabinoid receptors (CB2). These genomic modulations observed on large-scale cDNA arrays were first confirmed by Northern blot studies. Furthermore, ELISA evaluations in culture supernatants indicated that the cannabinoid-induced activation of these two chemokine genes was followed by enhanced expression and secretion of the corresponding proteins. These upregulations initially observed in transfected HL60 cells overexpressing CB2 receptors, also occurred in normal non-transfected HL60 cells. The enhancement of IL-8 and MCP-1 gene transcription and protein production was shown to be pertussis toxin sensitive attesting that this phenomenon was a G_i protein-coupled receptor-mediated process as expected for cannabinoid receptors. More specifically, the abolition of the cannabinoid-induced effect by the specific CB2 antagonist SR 144528 indicated a strict peripheral cannabinoid-mediated process. Altogether, our data highlight a possible new function of peripheral cannabinoid receptors in the modulation of immune and inflammatory responses.

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Key words: Monocyte chemotactic protein; Interleukin 8; Cannabinoid receptor

1. Introduction

Cannabinoid receptors belong to the G protein-coupled receptor (GPCR) super family. To date, two receptor types have been identified and are referred to as CB1 and CB2 [1–3]. CB1 receptors are expressed in the central nervous system and some peripheral tissues whereas CB2 is only present at the periphery and especially on cells of immune origin [4–6]. However, despite the characterization and the localization of these receptors on immune cells [5,6], the identification of second messenger systems [7], the finding of putative endogenous ligands [8,9] and the availability of synthetic agonists and specific antagonist ligands [10,11], the function of cannabinoid receptors in the immune system still remains an enigma. So far, with the exception of few examples [12–14], most of the studies showed immunosuppressive effects of cannabinoids in very different areas of immunity, for instance inhibition of lymphocyte proliferative response [15,16], antibody production [17], cytotoxic activity or cytokine production [18]. These widespread suppressive effects interfering with many different functions were observed in most cases at cannabinoid ligand concentrations much higher (>1 μM) than those required for binding to the receptor and raised the question of the relevance of the observed effects in terms of the receptor specificity.

In an attempt to elucidate the role of CB2 receptors in the immune system we used an original approach. Our analysis was carried out using a cell line overexpressing CB2: in this system, we compared the patterns of gene expression before and after the stimulation of cells with physiological concentrations of cannabinoid ligands, using large-scale cDNAs microarrays spotted with hundreds of cDNAs probes grouped into different gene families involved in different biological processes. We then investigated whether the proteins under the control of the modulated genes similarly varied in both transfected and wild-type cells, and finally we made sure that the observed events were mediated via CB2 receptors by the use of specific antagonist ligands.

2. Materials and methods

2.1. Reagents

CP-55940 was obtained from Pfizer. SR 144528 (*N*-((1*S*)-endo-1,3,3-trimethylbicyclo(2,2,1)heptan-2-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide) and SR 141716A (*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride) were synthesized at the chemistry department, Sanofi Recherche (Montpellier, France). Pertussis toxin (PTX) was purchased from Sigma chemicals.

2.2. Cell culture and treatment

Cell lines were grown at 37°C in humidified 5% CO_2 in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 25 mM HEPES, 2.5 mM sodium pyruvate and 20 μg/ml gentamicin. The human promyelocytic cell line HL60 was purchased from ATCC (American tissue culture collection). Stable transfection of the HL60 cell line with CB2 (HL60-CB2) was performed as follows: cells were transfected with the recombinant plasmid pCDNA3-CB2 by electroporation and then maintained in the presence of 600 μg/ml Geneticin. Cells were screened 4 weeks later for CB2 receptor expression by flow cytometry using an anti-CB2 receptor polyclonal antibody [19]. For RNA extraction, cells were grown in a culture medium with 0.5% FCS 24 h prior treatment with 10 nM CP-55940 or 200 nM SR 144528 for different time periods.

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Abbreviations: MCP-1, monocyte chemotactic protein 1; IL-8, Interleukin 8; MIP-1α, macrophage inhibitory protein 1α; GRO-α, growth regulatory oncogene α; PTX, pertussis toxin

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2.3. RNA isolation and northern blot analysis

Total RNA was isolated from cells by lysis in guanidinium isothiocyanate and purified by cesium chloride gradient ultracentrifugation. The integrity of RNAs was assessed by analysing 18S and 28S ribosomal RNA. Poly(A)$^+$ RNA was then extracted with a fast track mRNA isolation Kit (Invitrogen NV, Leek, The Netherlands). For Northern blot analysis, 1 μg poly(A)$^+$ RNA or 20 μg total RNA per lane was electrophoresed in 1% agarose-formaldehyde gels. RNA was transferred to Nytran (Schleicher and Schuell, France), cross-linked and hybridized to ^{32}P-labeled cDNA in a Church buffer [20]. The human MCP-1 and IL-8 radiolabeled probes were prepared from PCR products obtained with the following primers: 5′-TCCAGCATGAAAGTCTCTGC-3′ and 5′-TGGAATCCTGAACCCACTTC-3′ (human MCP-1) or 5′-TTGGCAGCCTTCCTGATTTC-3′ and 5′-AACTTCTCCACAACCCTCTG-3′ (human IL-8).

2.4. ^{32}P-Labeled cDNAs synthesis and Atlas human cDNA expression array I hybridization

The Atlas human cDNA expression array I was purchased from Clontech (Palo Alto, CA). ^{32}P-Labeled cDNA probes were performed using 1 μg poly(A)$^+$ RNA from HL60-CB2 cells (control) or HL60-CB2 treated with 10 nM CP-55940 for 1 h. Briefly, poly(A)$^+$ were reverse-transcribed using gene-specific primers and MMLV reverse transcriptase in the presence of [α-^{32}P]dATP. Complex [^{32}P]cDNA from each sample was purified on a chroma-spin column and counted. About 3×10^6 cpm from each probe were hybridized to a separate Atlas human cDNA array membrane in Church buffer at 68°C overnight. After a high-stringency wash, blots were exposed to Amersham Hyperfilm MP with an intensifying screen.

2.5. Measurements of chemokine production

CB2-transfected and wt HL60 cells were incubated at 37°C in a 5% CO_2 atmosphere at 10^6 cells/ml with the indicated concentrations of CP-55940 in RPMI medium supplemented with 0.5% FCS. After a 24 h incubation culture supernatants were collected and levels of IL-8, MCP-1, MIP-1α or GRO-α were determined using ELISA kits from R&D Systems (Abingdon, UK). In some experiments, cells were treated overnight with 100 ng/ml PTX and extensively washed before exposure to CP-55940. When needed the CB1 and CB2 receptor antagonists SR 141716 and SR 144528, respectively, were added to the cells 30 min before the addition of CP-55940. The data shown are means of duplicate wells and experiments were done at least twice. In some experiments results were expressed as % increase in chemokine production as follows:

$$\%\ \text{increase} = \frac{\text{cytokine in treated sample} - \text{cytokine in control}}{\text{cytokine in control}} \times 100$$

3. Results

3.1. Induction of MCP-1 and IL-8 genes expression through CB2 receptor

We have previously demonstrated by RT-PCR that the human promyelocytic cell line (HL60) expresses mRNAs for the peripheral cannabinoid receptor CB2 whereas mRNAs for the central cannabinoid receptor CB1 are undetectable [6], making these cells very suitable to monitor the gene expression profile associated with a stimulation of the peripheral cannabinoid receptor. In order to amplify the genomic changes associated with a functional coupling of the peripheral receptor, transfected HL60 cells stably overexpressing the CB2 receptor (HL60-CB2) were used in a first approach.

The gene expression profile of HL60-CB2 cells, stimulated for 1 h with 10 nM CP-55940, was thus compared with that of their non-stimulated counterparts. The hybridization of their respective poly(A)$^+$ RNAs to separated Atlas human cDNA expression membranes made it possible to simultaneously assess in one single hybridization the expression of 588 cDNAs reported to play key roles in many different biological processes. The patterns shown in Fig. 1 clearly reveal for the first time that stimulation of the CB2 receptors led to an increased expression of two chemokine genes: MCP-1 and IL-8.

To confirm that changes in hybridization signals corresponded to real changes in mRNA abundance between the two samples, Northern blots were performed using specific MCP-1 and IL-8 probes. As shown in Fig. 2A, incubation of HL60-CB2 with the CB agonist for 1 h induced a significant increase in both MCP-1 and IL-8 mRNA levels. These inductions were not artefactually restricted to CB2 overexpressing cells but were also observed at a lower level in normal non-transfected cells and especially in MCP-1 mRNAs. Kinetic studies (Fig. 2B) showed a differential time-dependent increase in MCP-1 and IL-8 mRNAs. Whereas MCP-1 mRNA expression reached a peak after 1 h of treatment with the CB agonist, 3 h were required for a maximum IL-8 mRNA expression. In both cases however, the levels of transcripts similarly declined after a 6 h treatment.

The receptor-specificity of MCP-1 and IL-8 mRNAs upregulation was evaluated at the respective optimal time with the

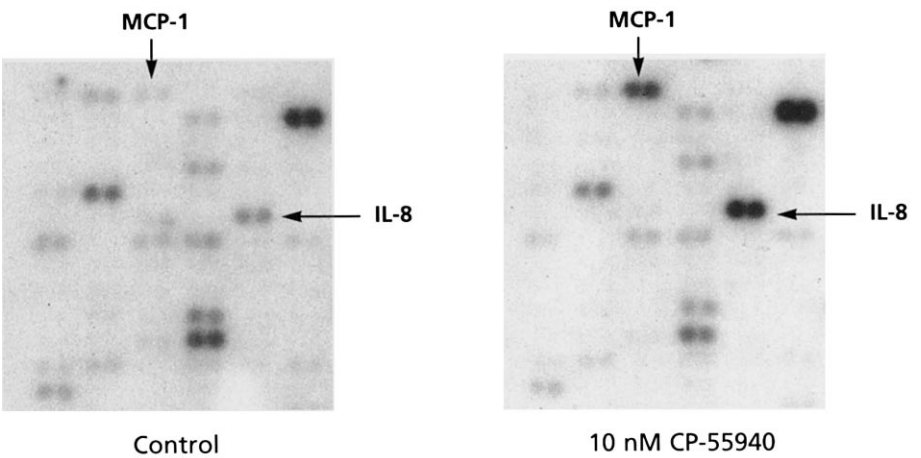

Fig. 1. Differential gene expression in control and CP-55940 treated human promyelomonocytic cell line HL60 stably transfected with CB2. Poly(A)$^+$ RNA was isolated from the indicated samples. ^{32}P-labeled cDNA probes were generated from each poly(A)$^+$ sample and hybridized to separate Atlas human cDNA arrays I membranes as described in Section 2. Autoradiography was performed overnight with an intensifying screen.

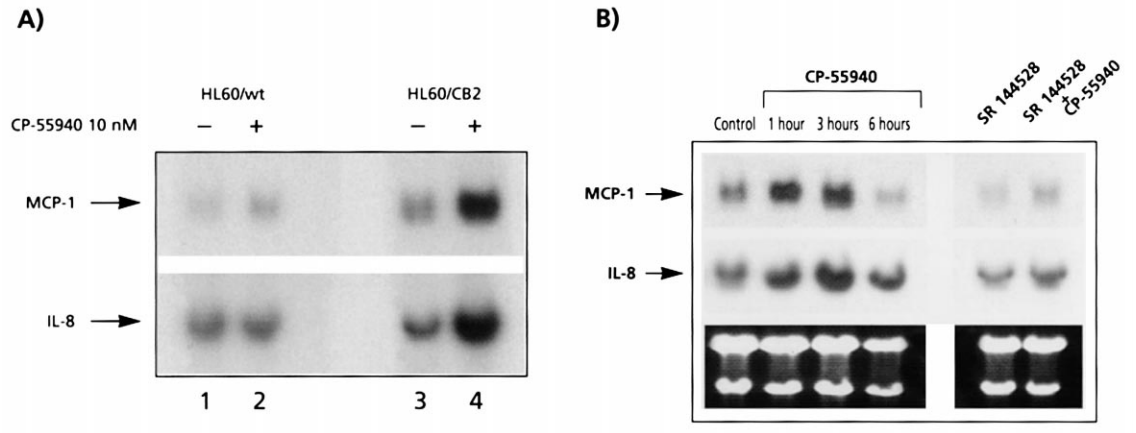

Fig. 2. MCP-1 and Il-8 are specifically inducible in HL60-CB2 cells by the cannabinoid agonist CP-55940. Total RNA was isolated from approximately 2×10^7 cells. Northern blots were performed using 20 μg of total RNA per lane. Filters were sequentially hybridized to the [^{32}P]cDNA probes MCP-1 and IL-8. Ethidium bromide-stained gels indicate the integrity and the relative amount of RNA loaded in individual lanes. A: Effect of CP-55940 in upregulating MCP-1 and IL-8 mRNA expression in HL60-CB2 and HL60 wild type. Cells were maintained in culture medium containing 0.5% fetal calf serum for 24 h and then incubated for 1 h at 37°C with (lanes 2 and 4) or without (lanes 1 and 3) 10 nM of CP-55940. B: Time course of CP-55940 on MCP-1 and IL-8 mRNA expression in HL60-CB2 cells, and effect of the CB2 antagonist SR 144528. Cells were incubated with or without 10 nM CP-55940 for up to 6 h. For SR 144528 effects, HL60-CB2 cells were treated with 200 nM of SR 144528 alone or in association with 10 nM CP-55940 for 3 h.

CB2 specific antagonist SR 144528 tested at 200 nM alone or in combination with 10 nM CP-55940. Fig. 2B shows that the CP-induced effects on MCP-1 and IL-8 mRNAs were completely blocked by SR 144528, clearly indicating a CB2 receptor-mediated process. The decrease in the basal level of both mRNA populations when cells were treated with SR 144528 alone confirmed the inverse agonist profile of this compound [21].

3.2. *Effect of CP-55940 on MCP-1 and IL-8 protein production*

To test whether the upregulation of MCP-1 and IL-8 gene transcription was followed by the production of the corresponding proteins, we evaluated the levels of these chemokines in the culture supernatants of HL60 cells stimulated for 24 h with the cannabinoid agonist CP-55940. First, the range of increase in IL-8 and MCP-1 protein level in wild-type HL60 was compared with that in CB2-transfected HL60 after stimulation with 100 nM CP. In both cell subtypes CP increased IL-8 production by 52 and 70% respectively whereas in the same conditions MCP-1 production was enhanced by 80 and 42%, respectively (Fig. 3). The two proteins being very significantly upregulated in CB2 transfected and normal HL60 cells as well, these latter were used in most of the following experiments unless otherwise stated.

Since cannabinoid receptors are linked to G_i protein, in a first approach to demonstrate the receptor specificity in this phenomenon, we evaluated the effect of the G_o-G_i protein inhibitor PTX on the CP-induced increase in these two chemokines expression level. As shown in Fig. 4a,b, PTX totally abolished the stimulating activity of the cannabinoid ligand on IL-8 and MCP-1 production whatever the CP-55940 concentrations tested, clearly indicating a G_o-G_i protein-coupled receptor-mediated process. In the absence of PTX, the EC_{50} of CP-55940 for both IL-8 and MCP-1 enhancements were evaluated at about 2 nM. This low nanomolar concentration was in line with the affinity of this ligand for the cannabinoid receptors [22], strengthening the notion of a cannabinoid receptor-induced effect.

However, CP-55940 displays high but equivalent affinity for both CB1 and CB2 receptors. Therefore, to ascertain that the CB2 was the receptor subtype involved in the observed effects, as for the studies on gene expression profile, the CB2 antagonist SR 144528 [11] was used in combination with the agonist. Fig. 4c,d shows that the stimulating effect induced by 30 nM CP-55940 on IL-8 and MCP-1 was abolished by the specific CB2 antagonist. A complete inhibition was observed at

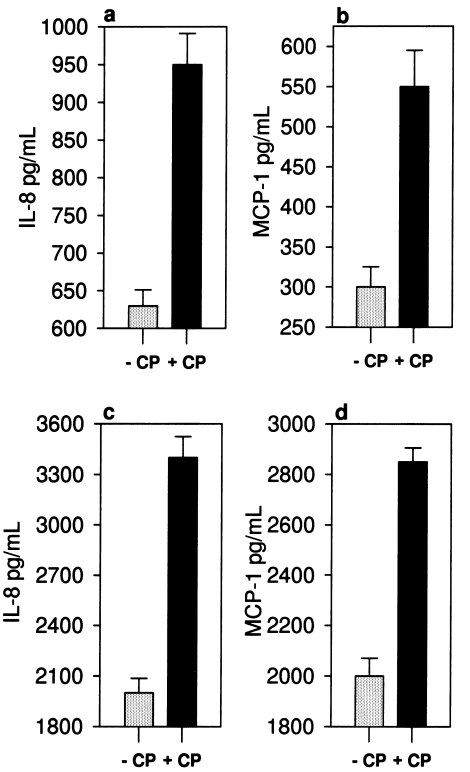

Fig. 3. Effect of CP-55940 on MCP-1 and IL-8 protein production. The levels of MCP-1 and IL-8 proteins were evaluated by ELISA in the culture supernatants of wt (a and b) or CB2 (c and d) HL60 cells stimulated for 24 h with 100 nM CP-55940.

200 nM antagonist with an IC50 around 30 nM in both cases. A similar result was obtained in CB2-transfected HL60 cells where, in addition to the CB2 antagonist, the CB1 antagonist SR141716 [10] was also tested and shown to be unable to block the stimulating effect of CP-55940 on MCP-1 production (Fig. 4e). These data confirmed a specific CB2-receptor-mediated effect as already observed at the transcriptional levels. In contrast to what was clearly observed with the potent synthetic ligand CP-55940, neither the putative endogenous ligand anandamide, nor the psychoactive component of marijuana Δ^9-THC were able to modulate the production of these two chemokines (not shown).

Finally, we tested the effect of CP-55940 on two other chemokines, GRO-α which belongs to the same α-chemokine family as IL-8 and MIP-1α, a β-chemokine family member as MCP-1. The experiment depicted in Fig. 5 shows that unlike IL-8 or MCP-1, neither GRO-α nor MIP-1α production was affected by the CP-55940 treatment.

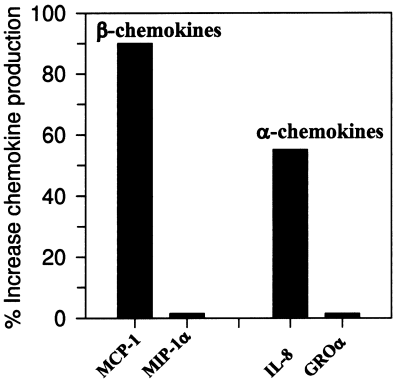

Fig. 5. Effect of CP-55940 on different α- and β-chemokine family members. The levels of MCP-1, IL-8, GRO-α and MIP-1α was measured by ELISA in the culture supernatants of wt-HL60 stimulated for 24 h with 100 nM CP-55940.

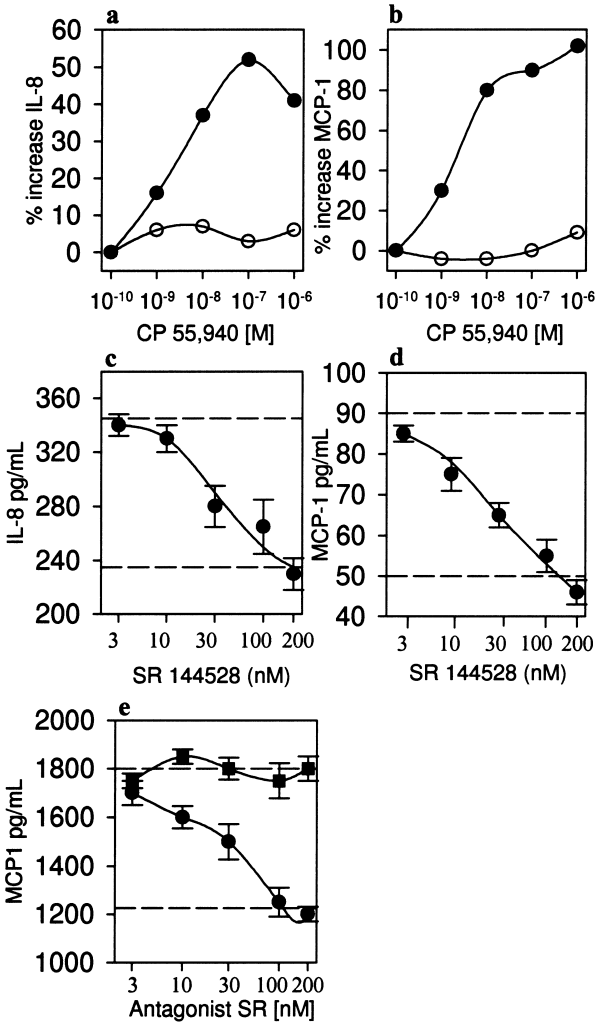

Fig. 4. Receptor specificity of the CP-induced effect. The CP-induced increase in IL-8 (a) and MCP-1 (b) in wt-HL60 was evaluated in the presence (○) or the absence (●) of 100 ng/ml PTX after a 24 h incubation. The antagonistic effect of SR 144528 on IL-8 (c) and MCP-1 (d) upregulation was tested in wt HL60 stimulated with 30 nM CP for 24 h. Comparison between the CB1 antagonist SR 141716 (■) and the CB2 antagonist SR 144528 (●) on MCP-1 production was similarly done in CB2-HL60 (e). Lower and upper dashed lines indicate basal and CP induced chemokine production respectively.

4. Discussion

With regard to the impact on the immune system, cannabinoids have been mainly shown to impair immunological functions in vitro and in vivo, including deficiency of lymphocyte blastogenesis, suppression of antibody formation, deficiency of cytokines production, etc. [23]. However, these reports have little meaning since most of them were observed using drug concentrations outside the physiological range and probably via receptor-independent pathways. An additional intricacy is the fact that the two receptor subtypes so far characterized are often coexpressed in the same cells, leading to a difficult interpretation of the respective commitment of both receptors in the immune system. Therefore, the exact function of the CB receptor in the immune system is still poorly understood although some work has clearly suggested that the CB2 receptor seems to be involved in the cannabinoid immunomodulating effects [11,12].

Recently, gene modulation studies have generated a great interest due to the fact that all changes in cell type or state, whatever the causes, are correlated with changes in gene expression at both magnitude and timing levels. The ability to detect and identify the differentially expressed genes in these processes is a powerful tool for studying any biological event.

In this report, we investigated the effect of a cannabinoid ligand on gene transcriptional regulation in an attempt to decipher the function of CB2 in the immune system. Here, we took the advantage of technological advances to simultaneously analyze the expression pattern of hundreds of genes at a single time. To this end, we used an approach based on hybridization to large scale arrays containing 588 human cDNAs fixed onto nylon membranes. These hundreds of genes are organized into groups of functional classes playing key roles in different cellular events. As a cellular system, we used the promyelocytic cell line HL60 that only expresses the CB2 receptor and lacks CB1 receptor mRNA. Moreover, since CB2 receptor density is generally low, in order to amplify an eventual cannabinoid-induced effect on gene modulation, we artificially overexpressed the receptor of interest by transfection.

Using this approach, we demonstrated that nanomolar concentrations of CB ligands CP-55940 were able to potently upregulate the gene expression of two different chemokines IL-8 and MCP-1. These enhancements at the genomic level were correlated with an increase in the production of the corresponding proteins. Furthermore, this observation was extended to the non-transfected wild-type cells whose IL-8 and MCP-1 gene expression levels were lower and whose modulation might have been unnoticed without the help of the corresponding transfected counterparts.

To our knowledge, this is the first time that a cannabinoid ligand is shown to induce a modulation of these chemokines. These effects were specifically mediated by the peripheral cannabinoid receptor, since (i) the CB2 specific receptor antagonist SR 144528 prevented IL-8 and MCP-1 induction upon CP-55940 stimulation, (ii) PTX, a G_o-G_i protein inhibitor, abolished the CP-induced effects.

Over 40 chemokines have been identified to date and subdivided into two main families, the α- (or CXC-) chemokines and the β- (or CC-) chemokines whose IL-8 and MCP-1 are respective members [24]. We therefore wondered whether stimulation of IL-8 and MCP-1 was the sign of a general rise in many chemotactic factor expressions. This issue was assessed by evaluating the effect of CP-55940 on GRO-α, a member of the CXC-chemokines as is IL-8, and MIP-1α which, like MCP-1, belongs to the CC-chemokine family. In contrast to what was observed with IL-8 and MCP-1, neither GRO-α nor MIP-1α protein expression was affected, indicating a possible specific molecular targeting of the CB agonist, leading to attraction of only restricted leukocyte populations. Indeed, IL-8 is an important chemotactic factor for neutrophils only [25] whereas MCP-1 is mainly involved in the attraction of monocytes [26]. These two chemokines have been detected in a wide variety of inflammatory diseases such as asthma, glomerulonephritis, rheumatoid arthritis, atherosclerosis and inflammatory bowel disease [24]. Their potential role in these disease processes make an inhibitor of their activities of great clinical interest.

Therefore, the very surprising observation that CB2 receptors mediated the modulation of these two major inflammatory mediators brought new insights on their function and might provide a novel target for therapeutic interventions. Also intriguing was the absence of effect of the putative endogenous ligand anandamide, meaning that perhaps another endogenous ligand mimicking the effect of the agonist CP-55940 likely remains to be identified. In this matter, the availability of the highly specific CB2 receptor antagonist SR 144528 represents a valuable tool not only to help the characterization of possibly new candidate ligands but also to go further into the understanding of the role of CB2 receptors in the modulation of leukocyte recruitment and finally to evaluate their commitment in inflammatory models where these two chemokines are involved.

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