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Low dose oral cannabinoid therapy reduces progression of atherosclerosis in mice

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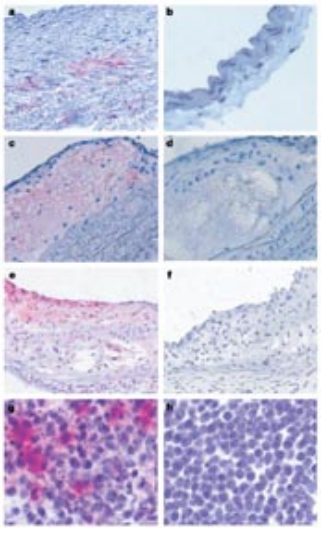
Atherosclerosis is a chronic inflammatory disease, and is the primary cause of heart disease and stroke in Western countries[1]. Derivatives of cannabinoids such as delta-9-tetrahydrocannabinol (THC) modulate immune functions[2] and therefore have potential for the treatment of inflammatory diseases. We investigated the effects of THC in a murine model of established atherosclerosis. Oral administration of THC (1 mg kg^{-1} per day) resulted in significant inhibition of disease progression. This effective dose is lower than the dose usually associated with psychotropic effects of THC. Furthermore, we detected the CB2 receptor (the main cannabinoid receptor expressed on immune cells[2,3]) in both human and mouse atherosclerotic plaques. Lymphoid cells isolated from THC-treated mice showed diminished proliferation capacity and decreased interferon-γ secretion. Macrophage chemotaxis, which is a crucial step for the development of atherosclerosis[1], was also inhibited *in vitro* by THC. All these effects were completely blocked by a specific CB2 receptor antagonist[4]. Our data demonstrate that oral treatment with a low dose of THC inhibits atherosclerosis progression in the apolipoprotein E knockout mouse model, through pleiotropic immunomodulatory effects on lymphoid and myeloid cells. Thus, THC or cannabinoids with activity at the CB2 receptor may be valuable targets for treating atherosclerosis.

It is now generally recognized that atherosclerosis is a chronic inflammatory disease that can lead to acute clinical events following plaque rupture and thrombosis[1,5]. Current treatments for atherosclerosis are mainly based on drugs that lower plasma cholesterol concentration and blood pressure. In particular, statins have proven to reduce cardiovascular events significantly, not only as a consequence of their cholesterol-lowering properties but also through their more recently identified anti-inflammatory and immunomodulatory effects[6]. Nevertheless, atherosclerosis remains the primary cause of heart disease and stroke in Western countries, accounting for up to 50% of deaths. The identification and development of

promising new anti-inflammatory therapies is therefore of great medical interest. Immunosuppressive and anti-inflammatory effects of cannabinoids have been reported[7, 8, 9], and pre-clinical studies have provided therapeutic rationale for their use in treating autoimmune diseases such as multiple sclerosis[10] and rheumatoid arthritis[11]. In murine collagen-induced arthritis (a mouse model of rheumatoid arthritis), cannabidiol, a major cannabinoid derivative, ameliorates chronic inflammation by inhibiting T-helper type 1 (T_H1) responses, as shown by reduced proliferation and interferon-γ (IFN-γ) production by lymph node cells from treated mice[11]. In support of the immunomodulatory role of cannabinoids, receptors for THC have been identified on several types of immune cells. The CB1 receptor is expressed predominantly in the brain, but CB2 receptor expression is found primarily on cells of the immune system, including B cells, T cells and monocytes[2]. It has been suggested that the immunomodulatory effects of cannabinoids are mediated by the CB2 receptor expressed on immune cells[3]. The fact that THC-mediated inhibition of T-helper cell activation is not observed in CB2 receptor knockout mice strongly supports this hypothesis[3]. These immunomodulatory properties suggest that cannabinoid derivatives might be beneficial in the treatment of atherosclerosis.

We hypothesized that cannabinoid treatment would alter inflammatory processes pivotal for the development of atherosclerosis, thus limiting disease progression. We first analysed the expression of CB2 receptors in atherosclerotic plaques of human and mouse diseased arteries. Immunohistochemistry revealed the presence of the CB2 receptor within human coronary atheroma as well as in atherosclerotic lesions of mouse aortic arch and root, but no CB2 staining was observed in non-diseased arteries (**Fig. 1**). CB1 receptors were not detected in any vascular tissue (data not shown). Double immunofluorescence staining confirmed that CB2 receptors are expressed by macrophages and T lymphocytes within atherosclerotic lesions (**Fig. 2**).

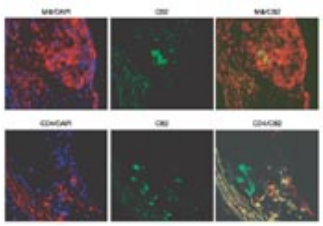
Figure 1: The cannabinoid receptor CB2 is expressed in human and mouse atherosclerotic plaques.



a–**h**, Representative cryosections showing CB2 receptor expression (pink staining) in human coronary atherosclerotic lesion (**a**), normal carotid artery from wild-type mouse (**b**), aortic arch atherosclerotic lesion from $ApoE^{-/-}$ mouse (**c**, **d**), aortic root atherosclerotic lesion from $ApoE^{-/-}$ mouse (**e**, **f**), and spleen (follicular area) from wild-type (**g**) or $CB2^{-/-}$ mouse (**h**). Sections were immunolabelled with an anti-CB2 receptor antibody (**a**–**c**, **e**, **g**, **h**), or with secondary antibody only (**d**, **f**).

High resolution image and legend (139K)

Figure 2: The cannabinoid receptor CB2 is expressed on macrophages and T lymphocytes within atherosclerotic plaques of $ApoE^{-/-}$ mice.



Shown are representative photomicrographs of ApoE$^{-/-}$ mouse aortic root cryosections, with the lumen of the artery at the right side of each photo. Immunofluorescence double-labelling was performed for CB2 receptors (green) and a cell-specific marker (red) for macrophages (MΦ) or T lymphocytes (CD4). Nuclei were stained in blue with DAPI. Analysis of atheroma from 4 different mice showed similar results.

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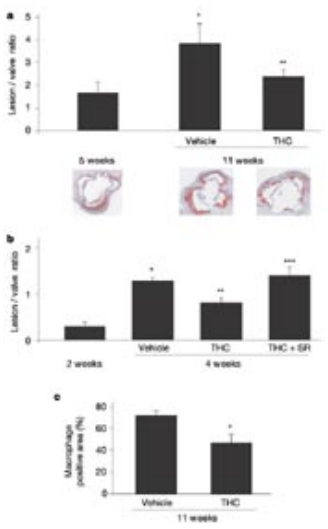
The anti-atherosclerotic potential of THC was tested in the apolipoprotein E knockout (ApoE$^{-/-}$) mouse model of atherosclerosis. We chose THC, the major constituent of marijuana, because its immune modulating effects have been well documented[7, 9, 10]. THC is already commercially available, for example as an anti-vomiting drug or for use in treating anorexia. Given that any new anti-inflammatory therapy should be well tolerated and preferably devoid of psychotropic effects, we first performed dose–response experiments of THC on early atherosclerotic lesion development. The maximal effect of THC on reduction of atherosclerotic lesions was observed at a dose of 1 mg kg^{-1} per day, with lower effects at higher doses (data not shown). Similar effects of cannabinoid treatment have been observed in previous *in vivo* studies[11, 12]. However, the underlying mechanisms for these observations remain unclear. Analysis of THC levels in blood serum of THC-treated mice (1 mg kg^{-1}) showed a concentration of 0.6 ng ml^{-1}, which is lower than the dose usually associated with psychotropic effects in either humans or mice[13, 14, 15, 16]. THC is known to be very lipid-soluble and is stored in fat tissue, which might explain the low THC levels observed in the serum of hypercholesterolemic mice. As the ApoE$^{-/-}$ mouse model used in our experiments shows significant accumulation of fat tissue, especially within the vessel wall, we might speculate that active THC is stored in the atherosclerotic plaque. In addition, circulating THC could be bound to the vast numbers of circulating lipid particles. Given the unique lipid metabolism of ApoE$^{-/-}$ mice and the high liposolubility of THC, ApoE$^{-/-}$ mice might be uniquely sensitive to very low doses of THC.

On the basis of these results, we chose a dose of 1 mg kg^{-1} THC for daily oral administration in order to test its therapeutic efficiency on advanced, established atherosclerosis. ApoE$^{-/-}$ mice were fed with a high-cholesterol diet for 5 weeks, and THC was then administered daily for the next 6 weeks, while maintaining the cholesterol diet. After the initial 5 weeks on a high-cholesterol diet, atherosclerotic lesions were clearly detectable within the aortic roots of ApoE$^{-/-}$ mice (**Fig. 3a**). More advanced vascular lesion development occurred in the aortic roots compared with the abdominal aorta, as demonstrated in previous studies[17, 18, 19]. After 11 weeks on a high-cholesterol diet, there was marked progression of atherosclerotic lesions within the aortic roots of control mice, but THC-treated mice showed significantly reduced progression of atherosclerotic lesions (**Fig. 3a**). Similar results were observed in the abdominal aorta (data not shown).

Figure 3: Reduced atherosclerotic plaque development and macrophage content in THC-treated ApoE$^{-/-}$ mice.

a, Representative cryosections of mouse aortic roots, stained with Sudan IV for lipid deposition, and quantification of atherosclerotic lesions. After 5 weeks on a high-cholesterol diet, ApoE$^{-/-}$ mice developed atherosclerotic lesions (n = 5). THC



(1 mg kg^{-1}) was orally administered during the last 6 weeks of the 11-week diet (n = 6 for THC-treated and n = 8 for control mice). Data represent mean values ± s.e.m. Single asterisk, $P < 0.05$ (compared with ApoE$^{-/-}$ at 5 weeks); double asterisk, $P < 0.05$ (compared with ApoE$^{-/-}$ at 11 weeks without THC). **b**, The CB2 receptor antagonist SR144528 abolishes the anti-atherosclerotic effect of THC. Atherosclerotic lesion development was detectable after 2 weeks on a high-cholesterol diet (n = 4). THC (1 mg kg^{-1}) alone or together with SR144528 was orally administered during the last 2 weeks of the 4-week diet, with control mice receiving corresponding dilutions of the SR144528 vehicle (n = 6 per group). Data represent mean values ± s.e.m. Single asterisk, $P < 0.05$ (compared with ApoE$^{-/-}$ at 2 weeks); double asterisk, $P < 0.05$ (compared with ApoE$^{-/-}$ at 4 weeks without THC); triple asterisk, $P < 0.05$ (compared with THC-treated ApoE$^{-/-}$ mice). **c**, Reduced macrophage infiltration in atherosclerotic plaques from THC-treated mice after 11 weeks on a high-cholesterol diet. Quantification of labelling with an anti-macrophage antibody (macrophage-positive cells divided by the area of atherosclerotic lesion, n = 6 for both groups). Values represent mean values ± s.e.m.; single asterisk, $P < 0.05$.

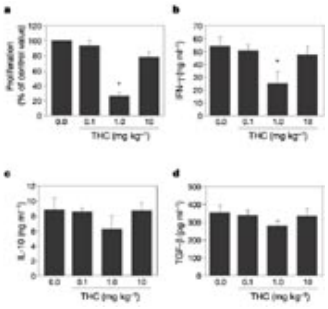
High resolution image and legend (60K)

To determine whether this anti-atherosclerotic effect of THC was mediated through the CB2 receptor, we performed experiments using THC in the presence of the CB2 receptor-specific antagonist SR144528 (ref. 4). The inhibitory effect of THC on early atherosclerotic lesion progression was completely abolished in the presence of the CB2 receptor antagonist (**Fig. 3b**). There were no differences in serum cholesterol, triglyceride levels or body weights between the treatment groups. None of the THC-treated mice died during treatment or showed unhealthy behaviour. Quantitative immunostaining analysis of atherosclerotic lesions showed significantly reduced macrophage infiltration per lesion in THC-treated mice (**Fig. 3c**). The initial stages of cell recruitment into atherosclerotic lesions involve rolling and adhesion of leukocytes to the vessel wall. Intravital microscopy was used to visualize the effect of THC on leukocyte recruitment within the mesenteric microvasculature[20]. These experiments revealed significantly reduced leukocyte adhesion in THC-treated animals (1.82 ± 0.65 cells per 100 μm) compared with controls (4.03 ± 0.44 cells per 100 μm). The effect of THC on leukocyte adhesion was reversed when animals were also treated with the CB2 receptor antagonist SR144528 (4.28 ± 0.98 cells per 100 μm; $P < 0.05$ for control versus THC and for THC versus THC + SR144528).

The progression of atherosclerosis may result from an imbalance between pro- and anti-inflammatory mediators in response to endothelial injury[21]. Several reports demonstrate that T cells play an important role during early atherosclerosis development[22,23]. It has been shown that T_H1 cells represent the predominant population of activated T cells within atherosclerotic lesions[24,25], and that THC treatment affects the T_H1/T_H2 balance of activated T cells[8,9]. We hypothesized that the observed anti-atherosclerotic effects of THC might result from a modified cytokine expression pattern in the THC-treated mice. We therefore investigated the influence of THC treatment on inflammatory responses at the beginning of atherosclerosis development. We analysed proliferative responses and cytokine profiles of lymphoid cells isolated from mice on a high-cholesterol diet with and without administration of different doses of THC. Compared with untreated mice, treatment with 1 mg kg^{-1} THC significantly reduced proliferative responses of *in vitro* stimulated splenocytes, whereas both low (0.1 mg kg^{-1}) and high (10 mg kg^{-1}) doses had no significant effect (**Fig. 4a**). Similar results were obtained with lymph node cells (data not shown). We examined culture

supernatants for T_H1 cytokines (IFN-γ, interleukin (IL)-12), T_H2 cytokines (IL-4, IL-10) and transforming growth factor (TGF)-β. Similar to the proliferative response, only the 1 mg kg^{-1} THC dose significantly reduced IFN-γ compared with controls. However, this dose resulted in only a modest, non-significant downregulation of IL-10 and TGF-β (**Fig. 4b–d**). In both THC-treated and untreated groups, expression of IL-4 and IL-12 was not detectable. Thus, during atherosclerosis, an oral dose of THC at 1 mg kg^{-1} seems to exert anti-inflammatory activity through suppression of the T_H1 response, resulting in a shift in the T_H1/T_H2 balance. The inhibitory effect of THC was bell-shaped, with neither the low nor the high doses having an anti-inflammatory effect.

Figure 4: THC reduces proliferative responses and inhibits T_H1 polarization.



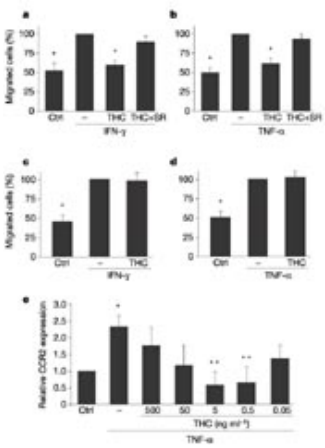
a–**d**, Isolated splenocytes from THC-treated (0.1 mg kg^{-1}; 1 mg kg^{-1}; 10 mg kg^{-1}) or control ApoE$^{-/-}$ mice fed the high-cholesterol diet were stimulated with Con A for 72 h (**a**) and the proliferation rate determined using the non-radioactive MTS colorimetric assay; or were stimulated for 48–72 h and concentrations of IFN-γ (**b**), IL-10 (**c**) and TGF-β (**d**) in the cell culture supernatants were determined by ELISA. Values represent mean values ± s.e.m.; $n = 6$ mice per group; single asterisk, $P < 0.05$ (compared with control).

High resolution image and legend (62K)

Early in atherosclerosis development, endothelial dysfunction in response to cardiovascular risk factors triggers recruitment of leukocytes (monocytes/macrophages and T lymphocytes) into the vessel wall. We addressed the possibility of whether THC treatment inhibits leukocyte migration using a functional *in vitro* experiment. Thioglycollate-elicited peritoneal macrophages were isolated from ApoE$^{-/-}$ or wild-type mice and assayed for their migration capacity in response to monocyte chemoattractant protein-1 (MCP-1). Treatment with THC at a concentration corresponding to the serum levels observed in THC-treated mice (0.6 ng ml^{-1}) significantly inhibited ApoE$^{-/-}$ macrophage migration (**Fig. 5a**, **b**). Similar results were obtained with cells obtained from wild-type mice (data not shown). These effects were completely blocked by the CB2 antagonist SR144528 (**Fig. 5a**, **b**), and were also absent when peritoneal macrophages isolated from CB2$^{-/-}$ mice were used (**Fig. 5c**, **d**), demonstrating that effects of THC on chemoattraction are in fact CB2 receptor-dependent. We analysed the expression levels of one of the MCP-1 receptors, CCR2, on splenocytes stimulated *in vitro* with different concentrations of THC. As shown in **Fig. 5e**, THC markedly reduced CCR2 expression in splenocytes stimulated with tumour-necrosis factor-α (TNF-α). The efficacy of THC at reducing CCR2 levels was bell-shaped, with significant reduction at 0.5 and 5 ng ml^{-1} THC, but less reduction at lower and higher doses.

Figure 5: THC reduces migration capacity and CCR2 expression *in vitro*.

a–**d**, Thioglycollate-elicited peritoneal cavity macrophages obtained from ApoE$^{-/-}$ (**a**, **b**) or CB2$^{-/-}$ (**c**, **d**) mice ($n = 4$ for both groups) were analysed for their *in vitro* migration response to the chemoattractant MCP-1. Cells were stimulated in duplicate with 1 ng ml^{-1} IFN-γ (**a**, **c**) or 10 ng ml^{-1} TNF-α (**b**, **d**) in the presence or absence of 0.6 ng ml^{-1} THC and 1 μM of the CB2 antagonist SR144528. Data represent mean values ± s.e.m. Single asterisk, $P < 0.05$ (compared with IFN-γ or



TNF-α-stimulated cells). **e**, Isolated splenocytes obtained from ApoE$^{-/-}$ mice (n = 4) were stimulated with 10 ng ml^{-1} TNF-α in the presence or absence of different doses of THC (0.05–500 ng ml^{-1}). Relative expression levels of CCR2 messenger RNA were determined by quantitative real-time PCR. Data represent mean values ± s.e.m. Single asterisk, $P < 0.05$ (compared with control, unstimulated cells); double asterisk, $P < 0.05$ (compared with TNF-α-stimulated cells).

High resolution image and legend (66K)

We have shown that relatively low oral doses of THC (1 mg kg^{-1}), initiated after manifestation of clinically detectable artery lesions, significantly inhibit atherosclerosis progression in mice. This anti-atherosclerotic effect is probably mediated by the CB2 receptor, as it is strongly expressed by macrophages and T lymphocytes within atherosclerotic lesions; furthermore, the inhibitory effects of THC on atherosclerosis progression are abolished in the presence of a CB2 receptor antagonist. We also provide evidence that the anti-atherosclerotic properties of THC are associated with a reduction of the T_H1 response and an inhibition of monocyte/macrophage migration to the site of inflammation. Our findings *in vitro* that THC affects leukocyte migration and CCR2 expression levels at biologically relevant concentrations lend further support to our *in vivo* findings. This key influence of THC on leukocyte recruitment reinforces the potential anti-inflammatory properties of THC during atherogenesis. Our results suggest that cannabinoid derivatives with activity at the CB2 receptor may be valuable clinical targets for treating atherosclerosis.

Methods

Reagents

Synthetic delta-9-THC Marinol (Dronabinol; Unimed Pharmaceuticals, Inc.) was dissolved at 0.1 mg ml^{-1} in 5.5% fat milk (w/v) in water. CB2 receptor antagonist SR144528 was provided by Sanofi-Synthélabo. THC (0.1 mg kg^{-1}; 1 mg kg^{-1} or 10 mg kg^{-1} per day) and SR144528 (0.7 mg kg^{-1} per day)[8] in 1.5% fat milk (w/v) were administrated orally in the drinking water. For *in vitro* experiments, delta-9-THC was purchased as a stock solution of 1 mg ml^{-1} in methanol (Cambridge Isotope Laboratories, Inc.) and further diluted in warm medium immediately before use. All *in vitro* experiments were performed by adding corresponding dilutions of the THC vehicle (methanol) to the non-THC-treated controls.

Animals

As a model of *in vivo* atherosclerosis, we used 10-week-old male ApoE$^{-/-}$ C57Bl/6 mice fed with a high-cholesterol diet (1.25% cholesterol, 0% cholate; Research Diets). For histological and atherosclerotic plaque development analysis, littermate ApoE$^{-/-}$ mice were fed with a high-cholesterol diet for 4 weeks (early atherosclerotic lesion development), or 11 weeks (advanced established atherosclerosis). THC (0.1 mg kg^{-1}; 1 mg kg^{-1} or 10 mg kg^{-1}) was administered during the last 2 weeks of the 4 week diet group (n = 6 per group), and during the last 6 weeks of the 11 week diet group (1 mg kg^{-1}; n = 6). In parallel, control ApoE$^{-/-}$ mice

(littermates) received milk without THC (n = 6 or 8 for the 4 and 11 week treatment groups, respectively). For CB2 receptor antagonist studies on early atherosclerotic lesion development, SR144528 was administrated in parallel with THC, and control mice received corresponding dilutions of the SR vehicle (methanol; n = 6 per group). For cell proliferation and cytokine secretion assays, littermate ApoE$^{-/-}$ mice were divided into four groups (control, THC 0.1 mg kg^{-1}; 1 mg kg^{-1}; 10 mg kg^{-1}; n = 6 per group) and fed a high-cholesterol diet for 4 weeks. THC or milk only was administered during the last 2 weeks of diet. CB2$^{-/-}$ mice were generated as previously described[3]. All animal studies were approved by the local Ethical Committee.

Immunostaining

Surgical specimens of human coronary atheroma were obtained using protocols approved by the Review Committee at the University Hospital of Geneva. Immunostaining of acetone-fixed cryosections of human and mouse arteries or spleens was performed as previously described[26, 27], using monoclonal antibodies for mouse CD4 and Mac-3 (PharMingen), and rabbit polyclonal antibody specific for both human and mouse CB2 receptor (Cayman Chemical).

Atherosclerotic lesion size and histological quantification

Atherosclerotic lesions within the thoraco-abdominal aorta and aortic roots were analysed by Sudan IV staining for lipid deposition. Quantification of lipid deposition and macrophage content (immunostaining) were performed by computer image analysis using MetaMorph6 software (Zeiss) as previously described[27].

Blood analysis

For measurements of cholesterol and triglyceride content, blood samples were collected at the beginning and the end of the diet treatment period. HDL and VLDL cholesterol fractions of sera were measured by fast protein liquid chromatography. THC levels in blood (after 2 and 6 weeks of treatment) were measured by gas chromatography/mass spectrometry[28] with a detection limit of 0.1 ng ml^{-1}.

Intravital microscopy

Intravital microscopy of the microvasculature of the mesentery was performed as previously described[20]. To visualize leukocytes, animals were injected intravenously with 50 µl of 0.05% rhodamine 6G (Sigma Aldrich) immediately before microscopy[29]. ApoE$^{-/-}$ mice were divided into three groups (control, THC; THC + SR144528; n = 4 per group) and fed with a high-cholesterol diet for 4 weeks. THC (1 mg kg^{-1}) and SR144528 were administered during the last 2 weeks of diet.

Proliferation assay

Splenocytes or lymph node cells were isolated from THC- or milk-treated mice (n = 6 per group) and cultured in 96-well plates at a concentration of 5×10^6 cells ml^{-1}. Culture medium consisted of RPMI 1640 supplemented with 25 mM HEPES buffer, 2 mM L-glutamine,

100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin and 10% heat-inactivated FBS. Cells were stimulated in triplicates with varying concentrations of concanavalin A (Con A; Sigma). After 72 h, cell proliferation was determined using a non-radioactive MTS cell proliferation assay (Promega) according to the manufacturer's guidelines.

Real-time PCR analysis

Total RNA from mouse splenocytes was isolated with Tri-reagent (MRC, Inc), and real-time polymerase chain reaction (PCR) analysis (TaqMan, Applied Bio-systems) for CCR2 expression was performed as previously described[30].

Cytokine analysis

For cytokine analysis, splenocytes and lymph node cells were cultured under the same conditions as described for the proliferation assay and stimulated with 2 μg ml^{-1} Con A. Supernatants were recovered after 48 h (for IFN-γ, IL-12 and TGF-β measurement) and 72 h (for IL-4 and IL-10 measurement). Murine IFN-γ, IL-12 (p70), IL-4, IL-10 and TGF-β were assayed by enzyme-linked immunosorbent assay (ELISA) using paired antibodies according to the manufacturer's instructions (R&D Systems).

Transmigration assay

Macrophages from the peritoneal cavity of thioglycollate-injected wild-type, $ApoE^{-/-}$ or $CB2^{-/-}$ mice (n = 4 mice per group) were isolated 4 days post-injection and stimulated (in triplicate) for 4 h with 1 ng ml^{-1} mIFN-γ or 10 ng ml^{-1} mTNF-α in the presence of THC. Inhibition experiments were performed by 10 min pre-incubation with 1 μM of the specific CB2 antagonist SR144528. Stimulated and unstimulated cells were transferred into the upper compartment of transwell filter migration chambers. Migration was triggered by the addition of 1 nM MCP-1 (R&D Systems) to the lower transwell chamber compartment. After 90 min incubation, the number of migrated cells was determined by counting (blinded observers) 10 microscopic fields per well.

Statistical analysis

All results are expressed as mean ± s.e.m. Differences between the values were considered significant at $P < 0.05$ using the two-tailed Student's t-test.

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Competing interests statement

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