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Differential migratory properties of monocytes isolated from human subjects naïve and non-naïve to *Cannabis*

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Abstract

This study evaluates the migratory potential of monocytes isolated from two groups of human subjects: naïve and non-naïve to *Cannabis*. Phytocannabinoids (pCB), the bioactive agents produced by the plant *Cannabis*, regulate the phenotype and function of immune cells by interacting with CB_1 and CB_2 receptors. It has been shown that agents influencing the phenotype of circulating monocytes influence the phenotype of macrophages and the outcome of immune responses. To date, nothing is known about the acute and long-term effects of pCB on human circulating monocytes. Healthy subjects were recruited for a single blood draw. Monocytes were isolated, fluorescently labeled and their migration quantified using a validated assay that employs near infrared fluorescence and modified Boyden chambers. CB_1 and CB_2 receptor mRNA expression was quantified by qPCR. Monocytes from all subjects (n = 10) responded to chemokine (c–c motif) ligand 2 (CCL2) and human serum stimuli. Acute application of pCB significantly inhibited both the basal and CCL2-stimulated migration of monocytes, but only in subjects non-naïve to *Cannabis*. qPCR analysis indicates that monocytes from subjects non-naïve to *Cannabis* express significantly more CB_1 mRNA. The phenotype of monocytes isolated from subjects non-naïve to *Cannabis* is significantly different from monocytes isolated from subjects naïve to *Cannabis*. Only monocytes from subjects non-naïve to *Cannabis* respond to acute

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exposure to pCB by reducing their overall migratory capacity. Our study suggests that chronic exposure to *Cannabis* affects the phenotype of circulating monocytes and accordingly could influence outcome of inflammatory responses occurring in injured tissues.

Keywords

Monocyte; *Cannabis*; Cannabinoid; CCL2; Transmigration; Neuroinflammation

Introduction

Circulating monocytes migrate into damaged tissues where they differentiate into macrophages of two major phenotypes, M1 and M2 (Garden and Moller 2006; Colton 2009; van de Veerdonk and Netea 2010). M1 and M2 macrophages release distinct cytokines, chemokines and other mediators, and thus play a critical role in the outcome of immune responses. Cannabinoids regulate many aspects of monocyte biology, including their differentiation into M1 and M2 phenotypes, as well as their ability to produce immune modulators, indicating that these compounds represent promising therapeutics for regulating immune responses (Montecucco et al. 2008; Roth et al. 2002; Klein and Cabral 2006; Raborn and Cabral 2010; Patinkin et al. 2008; Raborn et al. 2008; Miller and Stella 2008; Walter et al. 2003). Accordingly, regimented cannabinoid treatments alleviate the pathogenesis and symptoms measured in animal models of various human immune diseases, both in the periphery and CNS, with a portion of these therapeutic effects due to cannabinoids regulating monocyte biology. Based on this evidence, significant effort was invested in better understanding how cannabinoids regulate monocyte biology with the aim of developing novel therapeutic approaches designed to treat immune diseases (Baker et al. 2000; Maresz et al. 2007; Ni et al. 2004; Zhang et al. 2009; Castillo et al. 2010; Landucci et al. 2011). Despite this effort, little is known about how cannabinoids regulate monocyte migration, an early and fundamental step of immune response implementation.

Endogenous cannabinoids (endocannabinoids, eCB) are lipids that activate cannabinoid receptors expressed by many cell types (Mach and Steffens 2008). The two primary eCBs, arachidonoylethanolamine (AEA or anandamide) and 2-arachidonoyl glycerol (2-AG), are produced by many cell types, including endothelial cells and T cells (Rossi et al. 2010; Zhang et al. 2011; Huang et al. 2010). pCB encompass ~70 terpenophenolic compounds isolated from the *Cannabis* plant and include Δ^9-tetrahydrocannabinol (THC), cannabinol (CBN) and cannabidiol (CBD). Both eCBs and some pCBs activate CB_1 and CB_2 receptors (both are G protein-coupled receptors, GPCRs) (Pertwee et al. 2010). THC and CBD are the two most abundant cannabinoids produced by many *Cannabis* varieties, while CBN is a degradation product of THC (Izzo et al. 2009).

Several laboratories reported that activation of CB_1 and CB_2 receptors regulates both the basal and chemokine-stimulated migration of cells belonging to the monocytic lineage. However, it is difficult to draw a conclusion on whether cannabinoids truly regulate human monocyte migration because there are striking discrepancies in the results reported dependent upon the cell lines (Kishimoto et al. 2003; Franklin and Stella 2003; Walter et al.

2003; Patinkin et al. 2008; Montecucco et al. 2008; Sacerdote et al. 2000). Here, we sought to revisit this question using freshly isolated human monocytes since they have a more direct relevance to human health. In the first set of experiments, we validated both the use of freshly isolated $CD14^+$ monocytes and the quantification of their migratory response using an unbiased fluorescent technique (Miller and Stella 2009). Using this approach, we then asked whether monocytes isolated from *Cannabis* naïve and non-naïve subjects exhibit differential sensitivity to various modulators of cell migration, including cannabinoids.

Materials and methods

Patients and study design

Subjects were recruited for this pilot study according to the rules prescribed by the Human Subjects Committee at the University of Washington. Blood was obtained by vein-puncture from the ante-cubital vein of healthy human subjects under a protocol approved by the Human Subjects Committee at the University of Washington. Donors provided prior written informed consent to the procedure and use of the sample. (Table 1) No *Cannabis* was administered to subjects in this study; these data are based on the report of the subjects in an intake questionnaire.

Materials

Gibco® RPMI1640 (Invitrogen, Carlsbad, CA). DRAQ-5™ (Axxora, San Diego, CA). CP55940, JWH-015, SR144528 (SR2), THC, CBD and CBN (NIDA). AEA and 2-AG (Cayman Chemical, Ann Arbor, MI, USA). CCL2 and CCL2 neutralizing antibody (Calbiochem®/EMD Chemicals, Gibbstown, NJ, USA). NucleoSpin® RNA XS kit (Macherey–Nagel, Düren, Germany/Chlontech, Mountainview, CA, USA); Human spleen and human brain total RNA (Zyagen, San Diego, CA, USA). Sprint RT Complete-Double PrePri-med (Clontech, Mountain View, CA, USA). LNA-based FAM-labeled Universal Probe Library Human set (Roche Applied Science (Indianapolis, IN, USA). Miltenyi LD Midi Macs® separation unit and monocyte isolation kit II (Miltenyi Biotech Inc, Auburn, CA, USA).

Monocyte isolation

The buffy coat was collected after centrifugation of whole blood using BD Vacutainer® CPT Cell Preparation Tubes with sodium citrate (VWR Scientific, San Francisco, CA). The buffy coat was rinsed with PBS (30 ml, centrifuged at 3,000 rpm × 15 min). The resulting pellet was resuspended in buffer and monocytes isolated using an ex vivo deletion strategy developed by Miltenyi Biotech (Midi Macs™ cocktail kit). This strategy uses negative selection (indirect cell labeling) according to the manufacturer's instructions. The cells retained on a magnetized column are positive for CD3, CD7, CD16, CD19, CD56, CD123 and CD235a, while the unlabeled cells which pass through the magnet are purportedly $CD14^+$ and negative for the other markers.

Migration quantification

To quantify cell migration, we used a method that we recently developed and is based on the modified Boyden chamber (Miller and Stella 2009). Briefly, filters (pore diameter = 5 μm)

were coated with human fibronectin (10 μg/ml in PBS for 30 min). Isolated CD14 + monocytes were fluorescently labeled with DRAQ-5 (700 nM, 10 min at 37 °C in RPMI 1640 supplemented with 0.1 % BSA). Cells were then rinsed in RPMI (0.1 % BSA) and re-suspended for a final density of 10^4 cells per 390 μl per upper well. Lower wells were loaded with media (82 μl) containing vehicle (0.1 % DMSO for basal migration) or the chemo-attractant tested. When cannabinoids were tested for their effect on a chemo-attractant, these compounds were added to both the upper and lower wells. Fluorescence emitted by the cells that had migrated toward the bottom filter area was detected with an Odyssey® Imaging system (Li-COR Biosciences, Lincoln, NE) (Miller and Stella 2009). To validate this method, we performed manual counting by imaging the bottom of the filter with a Zeiss Axiovert microscope (Carl Zeiss, Thornwood, NY) in three high-powered fields (320×). Here, cells that were counted were defined as identifiable by DRAQ-5-stained nuclei and phalloydin-stained actin filaments. Cells were counted in three fields (area 0.1 mm^2) from each condition, these numbers were averaged and multiplied by 320 (area of one entire well was 32 mm^2).

Cannabinoid mixture

Phytocannabinoids were incubated both individually and in a mixture (Mix) with human monocytes. The ratio for this Mix was calculated as THC + CBN/CBD = 5.2. Final concentrations were: THC = 1 μM, CBN = 300 nM and CBD = 250 nM, which is based on a phenotype system in which any plant analysis sample with a value >1 is considered a ‘drug type’ plant as opposed to a ‘fiber type’ plant (Doorenbos 1971; Fetterman et al. 1971).

qPCR

Total RNA was isolated and purified with the NucleoSpin® RNA XS kit. The RNA quality and concentration was assessed spectrophotometrically (A260/A280 >1.9) by a NanoDrop instrument (Thermo Scientific, Rockland, DE, USA). Total RNA from human spleen and brain were used as reference (Zyagen, San Diego, CA, USA). Specifically, 250 ng of total RNA were reverse-transcribed into cDNA with Sprint RT Complete-Double Pre-Primed (according to the manufacturer’s instructions). Probe based qPCR was performed in triplicate using the LNA-based FAM-labeled Universal Probe Library Human set combined with target-specific primers suggested by the freeware tool Universal Probe Library Design Center and FastStart Universal Probe Master (ROX) Mix (Roche) using a StepOnePlus Real-Time PCR System (ABI, Foster City, CA, USA). The best primers/probe set tested for each target was selected by comparing amplification consistency in human brain and spleen samples. Primers and probes for CNR1 and CNR2 are in Online Resource 1A. To compare samples from different origins (i.e., monocytes, brain and spleen), we quantified several housekeeping genes and assessed the consistency of their relative Ct values for normalization. We determined the Ct values of the following mRNAs: 1-hypoxanthine phosphoribosyl-transferase (HPRT), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YW-HAZ), beta-actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and TATA box binding protein (TBP) (Online Resource 1B). From this panel we selected TBP because its amplification levels in the samples that we were comparing were within the same range (Online Resource 2). Thus, the target/TBP ratio was used to compare the relative target expression in different

samples using the modified $\Delta\Delta C_T$ method (Pfaffl 2001). In all samples, CNR1 expression was compared to its level in brain and CNR2 compared to its level in spleen. Data are displayed as direct fold change (RQ) and represent the mean of triplicate qPCR per target.

Data analysis

Data were analyzed with Prism 4.0 (GraphPad, San Diego, CA) and results are expressed as mean ± SEM. For migration, the effect of each compound on basal migration was calculated (after systematically subtracting the background fluorescent signal) as: Migration = (migration with conditioned media/basal migration) × 100. In the case of CCL2 stimulation: migration = (migration with CCL2 + CB/migration stimulated by CCL2) × 100.

Results

Validation: CCL2 increases the migration of freshly isolated human monocytes

To validate the method used in this study, we used freshly isolated monocytes from a healthy subject and confirmed a correlation between the fluorescence signal (near-infrared) emitted by cells that had migrated through the filter and actual number of cells by counting them (Fig. 1a). This result extends our previous study in which we also found a correlation between the fluorescent signal and actual cell number in mouse microglial cells in culture (Miller and Stella 2009). To stimulate the migration of human monocytes, we tested both CCL2 (monocyte chemoattractant protein 1) and serum freshly isolated from this subject (Table 2), and found that both stimuli enhanced cell migration (419 ± 38 % and 861 ± 253 %, respectively; $n = 17$). To determine whether the stimulatory response induced by freshly isolated serum is due to CCL2, we incubated serum with a CCL2 neutralizing antibody (10 μg/ml) and found that indeed this antibody blocked the stimulatory response induced by serum (Fig. 1b). This result indicates that the majority of the migratory stimulatory response induced by freshly isolated serum on monocyte migration requires CCL2, confirming and extending previous reports (Weiss et al. 1998; Conductier et al. 2010; Prinz and Priller 2010; Mahad and Ransohoff 2003). Together these results validate our approach to study ex vivo migratory response of human monocytes.

Differential migratory responses of monocytes: *Cannabis* naïve and non-naïve subjects

We isolated monocytes from ten healthy human subjects: five subjects were naïve to *Cannabis* (i.e., either never used this plant or had been naïve for more than 3 years), and five subjects were non-naïve (current use of *Cannabis* >twice a week) (Table 2). We then tested how CCL2, serum and pCB regulate their cell migration. We found that monocytes isolated from *Cannabis* non-naïve subjects exhibit a reduced response to both CCL2 and serum compared to monocytes isolated from naïve subjects (Fig. 2). We also found that both basal and stimulated (CCL2 and serum) migration of monocytes isolated from *Cannabis* non-naïve subjects were inhibited by pCB, whereas these responses measured in monocytes isolated from *Cannabis* naïve subjects were not affected (Fig. 3a, b). Remarkably, synthetic compounds and eCBs (other than PEA) did not affect the migration of monocytes isolated from both *Cannabis* naïve and non-naïve subjects (Online Resource 3a–d). Together these results suggest that monocytes isolated from *Cannabis* naïve and non-naïve subjects exhibit different migratory responses and that only monocytes isolated from *Cannabis* non-naïve

subjects are sensitive to pCB. This selective sensitivity to pCB is specific, because, both synthetic cannabinoids and eCB do not affect the migratory response of monocytes isolated from both groups of subjects.

Differential cannabinoid receptor expression

Is this differential sensitivity of monocytes to pCB due to changes in CB receptor expression? Because of the small number of monocytes isolated from each subject, as well as the absence of antibodies that specifically label cannabinoid receptors (Atwood and Mackie 2010), we measured changes in CB receptor mRNA expression by qPCR using TBP mRNA as the house-keeping gene (Online Resource 1). We found that monocytes isolated from *Cannabis* non-naïve subjects express fourfold higher amounts of CB_1 receptor mRNA as compared to cells isolated from *Cannabis* naïve subjects, whereas CB_2 receptor mRNA remained unchanged between the two cohorts (Fig. 4a, b). These results show that the sensitivity of monocytes isolated from non-naïve subjects to pCB correlates with enhanced expression of CB_1 receptors.

Discussion

Circulating monocytes play a fundamental role in the initiation of inflammatory and immune responses (Geissmann et al. 2003). The molecular components controlling the ability of circulating monocytes to infiltrate diseased tissue, including crossing the blood brain barrier, represent promising targets for drugs designed to treat diseases involving chronic inflammatory and immune responses. Here we report that monocytes isolated from human subjects naïve and non-naïve to *Cannabis* respond differentially to specific modulators of cell migration.

CCL2, a potent chemo-attractant, is produced by various cell types (including endothelial cells, fibroblasts, astrocytes and microglia) in response to growth factors, cytokines and oxidative stress (Deshmane et al. 2009). Thus, CCL-2 gradients participate in directing circulating monocytes toward injured tissue, such as the CNS in the case of multiple sclerosis (MS), cerebral ischemia and brain tumors (Conductier et al. 2010; Gonzalez-Navarro et al. 2008; Kulkarni and Anders 2008; Fujita et al. 2011). Indeed, CCL2 production increases in diseased tissue and many immune cells express CCL2 receptors (Dawson et al. 2003). We found that the CCL2-stimulated migration of monocytes is attenuated in non-naïve subjects. While we still do not understand the molecular details of this reduced response (e.g., whether it is due to decreases in CCL2 receptor expression and/or coupling), this result suggests that chronic use of *Cannabis* affects the ability of circulating monocytes to respond to a potent chemo-attractant known to recruit monocytes toward damaged and inflamed tissues. This result is remarkable when considering that numerous patients regularly use *Cannabis* as medicine (an estimated 20 % of patients with MS) (Chong et al. 2006).

Most subtypes of immune cells express cannabinoid receptors (Miller and Stella 2008). We found that monocytes isolated from subjects non-naïve to *Cannabis* express more CB_1 receptors than monocytes isolated from subjects naïve to *Cannabis*, a result that corroborated a study reporting a similar response in PBMCs (Nong et al. 2002). To our

knowledge, only one study tested whether cannabinoids affect the migration of freshly isolated human monocytes (Montecucco et al. 2008) (Online Resource 3). Note that the cannabinoid compound tested was JWH-015 at 20 μM, a concentration that leads to both CB_1, CB_2 and GPR55 cross-activation (Lauckner et al. 2008) and to receptor-independent effects (Stella 2010). Here we tested JWH-015 at 3 μM, a concentration that still cross-activates CB_1, CB_2 and GPR55 receptors, while minimizing receptor-independent effects, and found that this aminoalkylindole compound does not regulate the migration of freshly isolated monocytes. In fact, none of the synthetic cannabinoids that we tested affected monocyte migration. Considering this lack of response to synthetic cannabinoids, the inhibitory response induced by phyto-CB that we measured only in monocytes isolated from non-naïve subjects suggests a mechanism involving the selective sensitization to this subfamily of compounds acting on CB receptors. To our knowledge, this is the first example of *Cannabis* use leading to this selective sensitizing of immune cells to pCBs.

In conclusion, our study suggests that chronic *Cannabis* use affects key aspects of monocyte migration; both their basal migration and response to potent chemo-attractant stimulants. Accordingly, the ability of circulating monocytes to invade tissues under healthy and diseased conditions, and how therapeutic compounds control this step, may differ when considering subjects naïve or not to *Cannabis*, as in the case in patients with MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CB	Cannabinoid
eCB	Endocannabinoid
pCB	Phytocannabinoid
AEA	Arachidonoylethanolamine
PEA	Palmioylethanolamine
THC	Tetrahydrocannabinol
CBN	Cannabinol
CBD	Cannabidiol
PBMC	Peripheral blood mononuclear cell
CCL2	C–C motif ligand 2 or monocyte chemo-attractant protein 1

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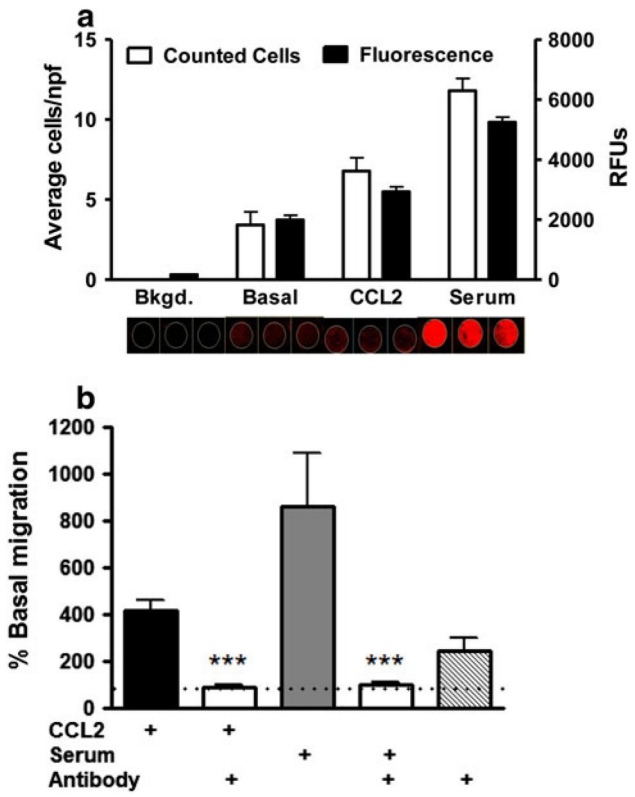

Fig. 1.

CCL2 increases the migration of freshly isolated human monocytes. **a** Correlation between cell quantity and fluorescence units: freshly isolated monocytes were labelled with DRAQ5 and phalloydin. After incubation and migration, non-migrated cells were wiped from the top side of the filter, rinsed with PBS and fluorescence values for migrated cells (bottom of the filter) were quantified on the Odyssey® imaging system. After fluorescence scanning, membranes were manually counted on a Zeiss Axiovert microscope. Average number of cells in three representative fields per condition correlated well with fluorescence values. **b** CCL2 is necessary for serum-induced migration. Using the potent chemo-attractant, CCL2, and human serum, we established positive controls with CCL2 and serum. Applying a CCL2 neutralizing antibody to the conditioned media in the lower chambers inhibited this migratory induction to basal levels. As a control, neutralizing antibody on its own showed no inhibitory activity on migration

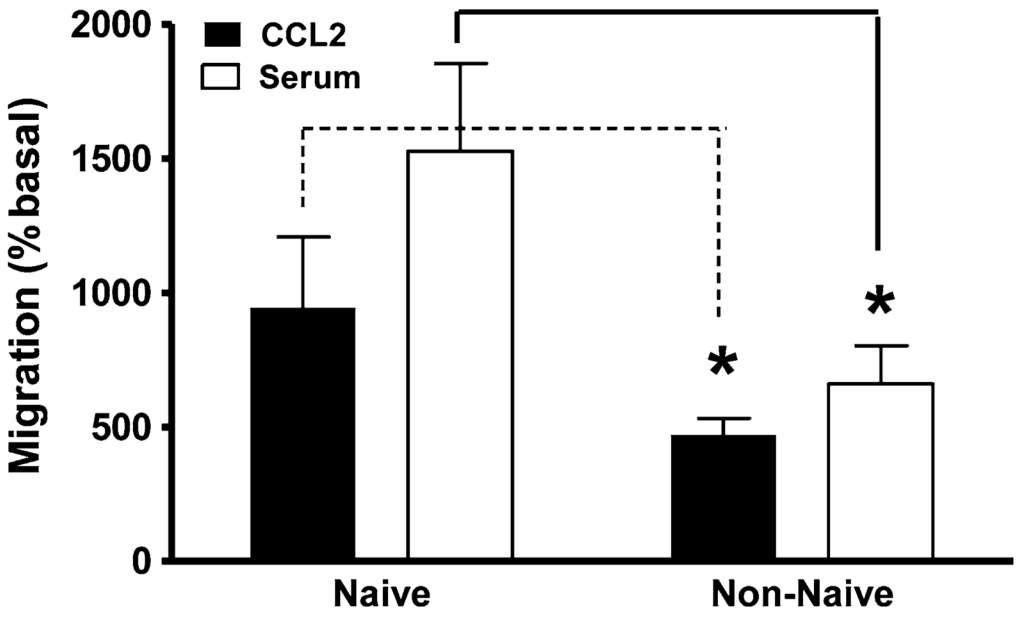

Fig. 2.

Differential migratory responses of monocytes isolated from *Cannabis* naïve and non-naïve subjects. Monocytes isolated from five subjects ($n = 3$ for each condition), who were naïve to *Cannabis* use, have a significantly increased response to CCL2 and serum compared to monocytes isolated from subjects who were non-naïve to *Cannabis* use. Significance $p < 0.05$ (students' *t* test,)

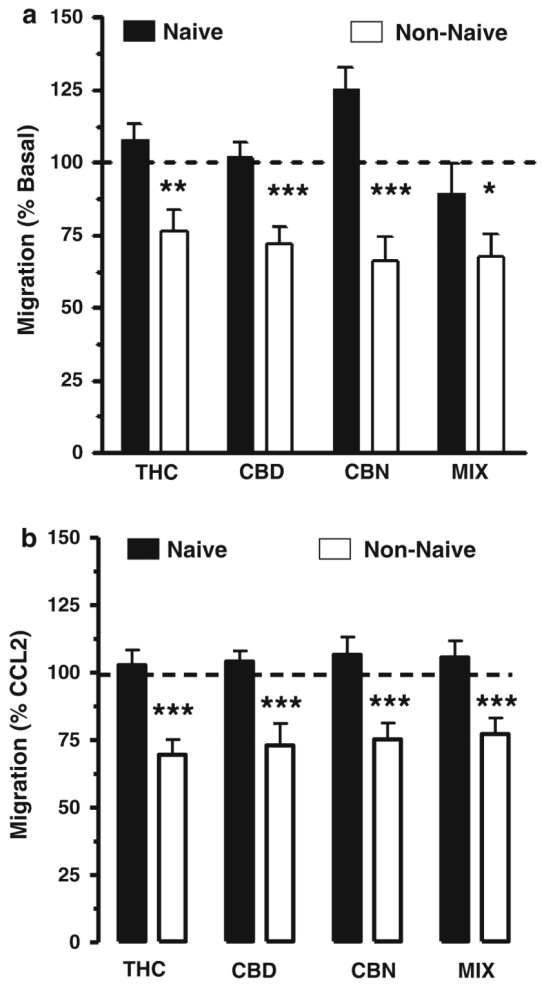

Fig. 3.

Differential sensitivity to pCB between *Cannabis* naïve and non-naïve subjects. (**a**) Basal migration of freshly isolated human monocytes was significantly reduced by addition of phytocannabinoids (pCB) to the lower wells of the migration chamber: THC, cannabidiol (CBD), cannabinol (CBN) and a set *ratio* of these compounds (MIX). Basal migration was significantly inhibited in the non-naïve cohort. (**b**) Additionally, the migratory response toward CCL2 was also reduced by ~25 % when the pCBs were added to CCL2 in the lower chambers. In control experiments (not shown) pCBs alone in the upper chamber with cell loading did not affect basal migration. Statistical significance ***$p < 0.001$; **$p < 0.001$; *$p < 0.05$ (unpaired, two-tailed *t* test calculated by GraphPad)

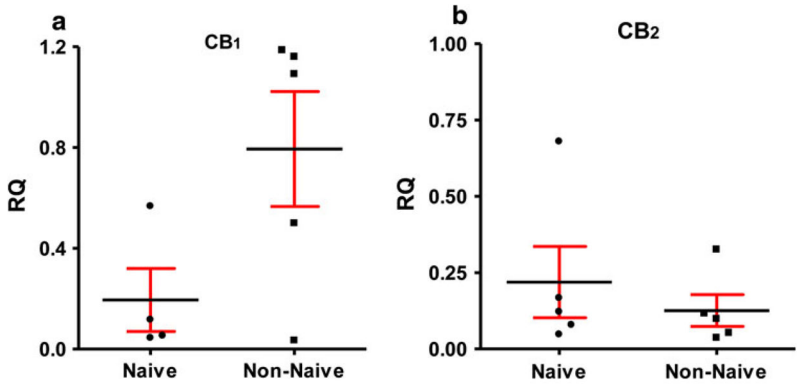

Fig. 4.

Cannabinoid receptor message in human monocytes: **a** subjects non-naïve to *Cannabis* have fourfold increase in CB_1 receptor mRNA compared to naïve subjects. Total RNA was amplified using qPCR and $\Delta\Delta C_T$ calculations using TBS as a control housekeeping gene was used for quantification. Human brain RNA was used as a positive control and calibration sample. The difference did not reach statistical significance. **b** No difference was found in mRNA for CB_2 receptor between the cohorts. Human spleen RNA was used as a positive control (*RQ* relative quantification)

Table 1

Human subjects data

Gender	Female	Male
Number of subjects	11	14
Average age (±SD)	36 (±12.2)	31.3 (± 4.4)

Table 2

Human subjects data 2

Gender	Female	Male
Number of subjects	5	5
Average age (±SD)	28.6 (±3.5)	32.8 (±3.4)
Naïve to Cannabis	3	2
Non-naïve to Cannabis	2	3