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ORIGINAL ARTICLE

Cannabinoids Inhibit T-cells via Cannabinoid Receptor 2 in an In Vitro Assay for Graft Rejection, the Mixed Lymphocyte Reaction

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Abstract Cannabinoids are known to have anti-inflammatory and immunomodulatory properties. Cannabinoid receptor 2 (CB2) is expressed mainly on leukocytes and is the receptor implicated in mediating many of the effects of cannabinoids on immune processes. This study tested the capacity of Δ^9-tetrahydrocannabinol (Δ^9-THC) and of two CB2-selective agonists to inhibit the murine Mixed Lymphocyte Reaction (MLR), an in vitro correlate of graft rejection following skin and organ transplantation. Both CB2-selective agonists and Δ^9-THC significantly suppressed the MLR in a dose dependent fashion. The inhibition was via CB2, as suppression could be blocked by pretreatment with a CB2-selective antagonist, but not by a CB1 antagonist, and none of the compounds suppressed the MLR when splenocytes from CB2 deficient mice were used. The CB2 agonists were shown to act directly on T-cells, as exposure of $CD3^+$ cells to these compounds completely inhibited their action in a reconstituted MLR. Further, the CB2-selective agonists completely inhibited proliferation of purified T-cells activated by anti-CD3 and anti-CD28 antibodies. T-cell function was decreased by the CB2 agonists, as an ELISA of MLR culture supernatants revealed IL-2 release was significantly decreased in the cannabinoid treated cells. Together, these data support the potential of this class of compounds as useful therapies to prolong graft survival in transplant patients.

Keywords Cannabinoids · Cannabinoid receptor 2 · Mixed lymphocyte reaction · T-cells · Immunosuppression

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Introduction

Cannabinoids were reported to have effects on immune responses as early as the 1970s (Gupta et al. 1974; Johnson and Wiersema 1974; Nahas et al. 1974; Neu et al. 1970), but the basis for this activity was not understood until the cannabinoid receptors were cloned. To date, two cannabinoid receptors have been identified, designated CB1 and CB2. The CB1 receptor was found at the highest levels on neurons in the central nervous system (Galiegue et al. 1995; Herkenham et al. 1991; Matsuda et al. 1990) and to a lesser extent on cells of the immune system and testes (Daaka et al. 1996; Galiegue et al. 1995; Waksman et al. 1999). However, the CB2 receptor was found to be expressed primarily on cells of the immune system, including B-cells, natural killer cells (NK cells), monocytes, polymorphonuclear cells, T-cells, and activated microglia (Galiegue et al. 1995; Munro et al. 1993; Murikinati et al. 2010). Discovery that these receptors were expressed on leukocytes provided a rationale for the functional effects of Δ^9-THC on the immune system, which have been reported to

suppress nearly every type of immune cell. There are numerous reports on the suppression of macrophages by Δ^9-THC, primarily by decreasing their antigen-presenting abilities. Macrophages exposed to Δ^9-THC resulted in the inhibition of phagocytosis, antigen processing of certain proteins, capacity for co-stimulation, nitric oxide production, pro-inflammatory cytokine release and production in macrophages and microglia (reviewed by Klein and Cabral 2006), as well as the migration of activated microglia (Fraga et al. 2011). Treatment with Δ^9-THC was found to suppress lymphocyte recruitment, proliferation, and function following inflammatory stimuli and to modulate cytokine and antibody levels and types (reviewed by Roth 2002 and Croxford and Yamamura 2005). In addition, Δ^9-THC was found to induce a shift from T helper 1 (Th1) to T helper 2 (Th2) cells following *Legionella pneumophila* infection (reviewed by Klein et al. 2003).

In much of the preceding literature on Δ^9-THC, it was not determined whether the cannabinoid was altering immune function through the CB1 or the CB2 receptor, although a few studies have shown effects to be exclusively through CB2 (Eisenstein et al. 2007; McCoy et al. 1999; Yuan et al. 2002). Until recently, this question could only be approached using selective antagonists for the two receptors. The development of synthetic cannabinoids that are selective for CB2 (Huffman et al. 1996, 1999, 2005; Marriott et al. 2006) has allowed direct testing of the hypothesis that agonist activation of this receptor down-regulates immune responses. CB2-selective agonists have been shown to be anti-inflammatory and immunosuppressive in mouse models of a wide variety of conditions where immune responses are detrimental, including Experimental Autoimmune Encephalitis (EAE), which is a mouse model of multiple sclerosis (Maresz et al. 2007; Zhang et al. 2009b), ischemic/reperfusion injury following an induced stroke (Ni et al. 2004; Zhang et al. 2007, 2009a), rheumatoid arthritis (Sumariwalla et al. 2004), inflammatory bowel disease (Storr et al. 2009), spinal cord injury (Adhikary et al. 2011; Baty et al. 2008), sepsis (Tschöp et al. 2009), autoimmune uveoretinitis (Xu et al. 2007), osteoporosis (Ofek et al. 2006) and systemic sclerosis (Servettaz et al. 2010a).

Organ transplantation and skin grafts are conditions in which activated immune responses greatly hinder the success of the transplant. Specifically, alloreactive T-cells, which recognize histoincompatible antigens on transplanted tissue, mediate tissue and organ rejection (reviewed by Heeger 2003). Δ^9-THC, given in vivo to mice, has been reported to inhibit ex vivo reactivity of spleen cells from treated animals when exposed to histoincompatible spleen cells in vitro in the Mixed Lymphocyte Reaction (MLR), an in vitro correlate of graft rejection (Zhu et al. 2000). Whether the effect was via CB1 or CB2 receptors was not explored. As CB2-selective cannabinoids have been shown to inhibit T-cells in several experimental conditions, as evidenced by decreasing production of the cytokines IL-2, IL-6, IFN-γ, and TNF-α, inhibiting migration of T-cells to inflammatory stimuli, and inhibiting proliferation of T-cells (Borner et al. 2009; Cencioni et al. 2010; Maresz et al. 2007; Xu et al. 2007; Ghosh et al. 2006; Coopman et al. 2007), it was hypothesized that CB2-selective agonists would block graft rejection.

The current study explored the potential of Δ^9-THC and two CB2-selective agonists, JWH-015 and O-1966, for their capacity to inhibit the MLR in vitro, which is a correlate of in vivo graft rejection. It was found that these cannabinoids directly suppressed T-cells in a dose-dependent manner, through activation of the CB2 receptor. The results suggest that CB2-selective cannabinoids are a candidate class of compounds as novel therapeutic agents to prevent graft rejection following transplantation.

Materials and methods

Mice

Six week-old, specific pathogen-free C3HeB/FeJ and C57BL/6 J female mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Founder CB2 receptor deficient (CB2R k/o) mice, on a C57BL/6 J background were obtained from the National Institutes of Health (Bethesda, MD) and bred in the Animal Core of the Center for Substance Abuse Research, P30 Center for Excellence, at Temple University School of Medicine Central Animal Facility.

Compounds

Δ^9-tetrahydrocannabinol (Δ^9-THC) was provided by The National Institute on Drug Abuse (NIDA, Rockville, MD). Δ^9-THC was supplied as a solution of 50 mg/ml in absolute ethanol and stored at 4 °C. JWH-015 (CB2-selective agonist) was purchased from Tocris Biosciences (Bristol, UK). O-1966 (CB2-selective agonist) was a generous gift from Anu Mahadevan (Organix, Woburn, MA). SR141716A (CB1-selective antagonist) and SR144528 (CB2-selective antagonist) were obtained from Research Triangle Institute (Research Triangle Park, NC). Each of these compounds was supplied as crystals and stored at −20 °C. Before each use, JWH-015, SR141716A, and SR144528 were dissolved in absolute ethanol and O-1966 was dissolved in DMSO.

The solutions were added drop-wise to the medium used for the assay (RPMI-1640) to obtain the desired concentration.

One-way mixed lymphocyte reaction (MLR)

Mice were sacrificed and their spleens aseptically removed. Single cell suspensions were obtained by passing spleens through nylon mesh bags (Sefar Inc., Depew, NY) in RPMI-

1640 with 5 % fetal bovine serum (FBS) containing 50 μM 2-mercaptoethanol (2-Me), and 100 U/ml penicillin and streptomycin sulfate. All reagents were purchased from Gibco Life Technologies (Carlsbad, CA), with the exception of FBS, which was purchased from HyClone Laboratories (Logan, UT). Red blood cells were lysed by hypotonic shock for 10 s with sterile water. Responder spleen cells from C57BL/6 mice were resuspended in RPMI with 10 % FBS, 50 μM 2-Me, and 100 U/ml penicillin and streptomycin sulfate. Splenocytes from C3HeB/FeJ were similarly prepared to serve as the stimulator cells, but they were inactivated by treatment with 50 μg/ml of mitomycin C for 20 min at 37 °C. The cells were washed 3 times to remove mitomycin C from the medium and resuspended to the desired concentration using a Beckman Coulter Z1 Dual Cell and Particle Counter (Beckman Coulter Inc., Indianapolis, IN). Responder cells (8×10^5) and stimulator cells (8×10^5) were co-cultured in 200 μl in 96 well plates for 48 h at 37 °C in 5 % CO_2. In wells where it was desired, 50 μl of cannabinoid was added to 100 μl responder cells 3 h prior to mixing with 50 μl stimulator cells. If antagonists were used, 50 μl were added to 50 μl responder cells for 2 h prior to adding the agonist, followed by a 3 h incubation with 50 μl agonist, before mixing with 50 μl stimulator cells. After a 48 h incubation period, cultures were pulsed with 1 μCi/well [^{3}H]-thymidine and harvested 18 h later onto glass fiber filters (Packard, Downers Grove, IL) using a Packard multichannel harvester, and placed in vials in liquid scintillation solution (Cytoscint, MP-Biomedical, Irvine, CA). [^{3}H]-thymidine incorporation on the filters was measured using a Packard 1900 TR liquid scintillation counter. Data were corrected for background by subtraction of [^{3}H]-thymidine incorporation in the absence of stimulator cells. Results are expressed as a Suppression Index (SI), where untreated spleen cells are given a value of 1.00 (100 %), and responses of cultures receiving treatment with cannabinoids are calculated as:

$$\mathrm{SI} = \frac{\text{Mean counts per minute} \big/ \text{cannabinoid treated cultures}}{\text{Mean counts per minute} \big/ \text{untreated cultures}}$$

Fluorescence activated cell sorting (FACS)

Splenocytes were resuspended in staining buffer: PBS containing 1 % BSA (Sigma, St. Louis, MO). Cells were incubated with 1 μg/10^6 cells of 2.4G2 antibody specific for Fcγ III/II receptor at 4 °C for 5 min to prevent nonspecific binding. Cells were then incubated with 0.5 μg/10^6 cells of PE-conjugated rat anti-mouse CD11b and PerCP-conjugated rat anti-mouse CD3ε (BioLegend, San Diego, CA), for 30 min on ice. Cells were then washed twice with sorting buffer: PBS containing 0.1 % BSA (Sigma). Cells were resuspended in sorting buffer (PBS containing 0.1 % BSA) to a concentration of 40×10^6 cells/ml, and then sorted using the FACSAria™ system (BD Biosciences, San Jose, CA). Purity of sorted cells was checked by analyzing a sample from each sorted population ($CD3^+$ and $CD11b^+$) on the flow cytometer at the completion of sorting. Cell purity was 99 % for all experiments.

mRNA expression analysis

Splenocytes were harvested and either immediately sorted or cultured in the MLR for 24 h before sorting by flow cytometry into $CD3^+$ and $CD11b^+$ populations as described above. Total RNA was extracted using an Rneasy® Mini Kit (Qiagen, Valencia, CA) according to the provided protocol. RNA concentration and purity was checked with a NanoDrop2000 (Thermo Fisher Scientific, Waltham, MA). 1 μg of RNA was then reverse transcribed to cDNA using the RT^2 First Strand Kit (Qiagen) following the provided protocol. For quantitative PCR (qPCR), cDNA was diluted 10-fold in DEPC water and 4 μl was added to 16 μl of Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) containing 200 nM of forward and reverse primers specific for mouse CB2 or mouse β-Actin (Invitrogen, Grand Island, NY). The qPCR was performed using a Mastercycler ep Realplex2 (Eppendorf, Hamburg, Germany) starting with 1 cycle at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 75 °C for 30 s, 57 °C for 30 s, and a melting curve analysis. The relative quantification of CB2 was calculated based on the number of cycles required for the fluorescence emission to reach the threshold level (CT) of CB2, normalized to the CT of the reference gene β-Actin. To ensure the amplification was of CB2 message and not contaminating genomic DNA, RNA samples that were not reverse transcribed were run with each reaction. The following primers were used: CB2 Forward 5′- GTGATCTTCGCC TGCAACTTT -3′, CB2 Reverse 5′-GGAGTCGACCC CGTGGA -3′, β-Actin Forward 5′-AGCTTCTTTGCAGCT CCTTCGTTGC-3′, and β-Actin Reverse 5′-ACCAGCG CAGCGATATCGTCA-3′.

ELISA

IL-2 levels in the MLR culture supernatant were determined using the Quantikine® Mouse IL-2 Immunoassay (R&D Systems, Inc., Minneapolis, MN). 96 well microplates were obtained pre-coated with a polyclonal antibody specific for mouse IL-2. The supernatant was incubated for 2 h at room temperature, after which any unbound antigen was removed by five washes. Enzyme-linked polyclonal antibody for mouse IL-2 was added and incubated at room temperature for 2 h. Following five washes to remove unbound antibody, a stabilized hydrogen peroxide and chromogen substrate

solution was added and incubated for 30 min at room temperature protected from light, followed by addition of dilute hydrochloric acid stop solution. The optical density was determined using a POLARstar Omega microplate reader (BMG LABTECH, Offenburg, Germany).

Cell viability

Cell viability was assessed using cell cultures that were run in parallel with each experimental MLR. Viability from experiments was measured by flow cytometry using the LIVE/DEAD® Fixable Dead Cell Stain Kit from Molecular Probes, Inc. (Eugene, OR). 1×10^6 cells from cultures were resuspended in 1 ml FCM Staining Buffer and incubated for 30 min at room temperature with 1 μl Dead Cell Stain. Cells were washed twice and resuspended in FCM staining buffer and analyzed using LSRII (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences). In addition, cell viability in several experiments was also checked with Trypan blue exclusion test. Cultures run in parallel with the experimental MLR were diluted to 1.6×10^6 cells/ml and 0.2 % Trypan Blue was added. The cells were scored for viability using a hemocytometer.

Apoptosis

The presence of apoptotic cells was examined using the APO-BrdU™ TUNEL Assay Kit from Molecular Probes, Inc (Eugene, OR) and Vybrant® FAM Poly Caspases Assay Kit from Molecular Probes, Inc. For TUNEL assays, 2×10^6 cells per sample in MLR culture were collected 0, 24, and 48 h after stimulator cells were added. The cells were fixed with 1 % (w/v) paraformaldehyde in PBS for 15 min on ice and then permeabilized by adding 3 mL ice-cold 70 % ethanol in PBS. The cells were stored in this solution at −20 °C until day 3 of the experiment. The TUNEL assay was then performed by following the protocol provided by the manufacturer. For caspase assays, 1×10^6 cells per sample in MLR culture were collected at 0, 24, and 48 h after stimulator cells were added, and the assay performed by following the protocol provided by the manufacturer.

Statistics

Data were transformed to normalized ratios, to accommodate non-normality of the data. Comparisons between groups were tested using ANOVA with vertical group comparisons at each dose. Least square means were used for horizontal and vertical comparisons between groups and doses. No adjustment was made for multiple comparisons. Statistical significance was defined as p values<0.01 or 0.001.

Results

Cannabinoids inhibit the MLR in a dose-dependent manner via the CB2 receptor

To determine the effect of Δ^9-THC, JWH-015 and O-1966 on the MLR, 8×10^5 C57BL/6 responder splenocytes were pretreated for 3 h with cannabinoid or vehicle before addition of 8×10^5 mitomycin C inactivated C3HeB/FeJ splenocytes. Figure 1 shows that pretreatment with all three cannabinoids inhibited the MLR in a dose-dependent manner, with suppression observed between 8 and 32 μM compared to vehicle controls. For the CB2-selective agonists, significant suppression was observed at 4 uM. Using a Live/Dead stain, cell viability was assessed and no difference observed in the number of dead cells between control and cannabinoid treated groups. A representative group from data collected for Fig. 1, showed cells from MLR cultures that received no treatment were 88.7 % live, cells that were treated with ethanol vehicle were 86.9 % live, and cells treated with 32 μM Δ^9-THC were 87.9 % live. Similarly, cells from other cultures that were treated with 32 μM JWH-015 or O-1966 were 88.6 % and 88.5 % live, respectively. Viability was checked in each experiment hereafter, and cells were 85–90 % live in all experiments.

To verify whether the cannabinoids were inducing suppression of the MLR via CB1 or CB2 receptors, CB1- and CB2-selective antagonists were used. C57BL/6 responder splenocytes were pretreated for 2 h with the CB1-selective antagonist SR141716A, the CB2-selective antagonist SR144528, or ethanol vehicle. The cells were then treated for 3 h with Δ^9-THC, JWH-015, O-1966, or vehicle controls, before mitomycin C inactivated C3HeB/FeJ splenocytes were added to each well. As shown in Fig. 2, pretreatment with the CB2-selective antagonist significantly blocked suppression by Δ^9-THC, JWH-015 and O-1966, while pretreatment with the CB1-selective antagonist had no effect on the suppression induced by any of the three cannabinoids.

To corroborate the pharmacological evidence that Δ^9-THC, JWH-015, and O-1966 act via the CB2 receptor, splenocytes from CB2 receptor knockout (CB2R k/o) mice were treated with these compounds and tested in the MLR. As shown in Fig. 3, pretreatment with Δ^9-THC, JWH-015 or O-1966 inhibited the MLR when cells from wild-type mice were used, but not in cultures containing splenocytes from CB2R k/o mice. No difference in viability was observed between cultures from wild-type or CB2R k/o mice, with all treatments yielding viability between 85 % and 90 % viable cells.

Together, these results support the conclusion that Δ^9-THC, JWH-015, and O-1966 are suppressing the MLR via the CB2 receptor.

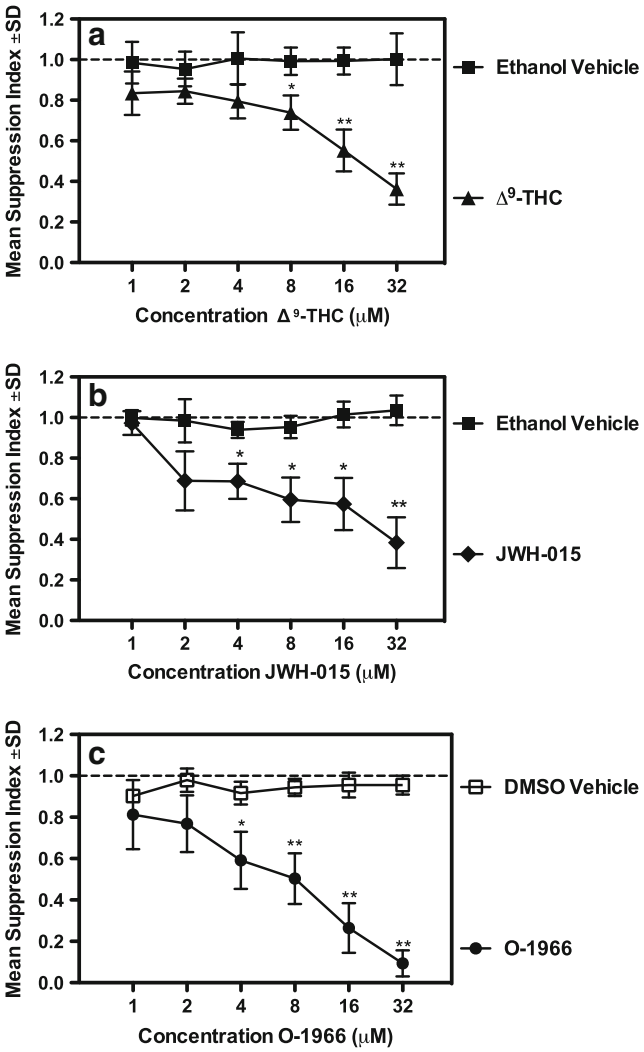

Fig. 1 The cannabinoids Δ^9-THC, JWH-015 and O-1966 inhibit the MLR in a dose-dependent manner. C57BL/6 responder splenocytes were pretreated for 3 h with: Panel **a**: Δ^9-THC (▲) or ethanol vehicle (■), Panel **b**: JWH-015 (♦) or ethanol vehicle (■) or Panel **c**: O-1966 (●) or DMSO vehicle (□). Concentrations of ethanol (a, b) or DMSO (c) vehicle correspond to the amount needed to dissolve each concentration of cannabinoid from 1 μM to 32 μM (1.25×10^{-3} % to 0.4 % v/v). The Δ^9-THC experiment was repeated 3 times, and the JWH-015 and O-1966 experiments were repeated 4 times each, with quadruplicate wells for each treatment in all experiments. Data are the mean ± S.D. *$p<0.01$, **$p<0.001$ (ANOVA, versus vehicle) Values for vehicle are not significantly different from 1.0 (no treatment) at any concentration

JWH-015 and O-1966 inhibit the release of IL-2 in the MLR

To examine the effect of cannabinoids on one aspect of T-cell function, the release of IL-2 in the MLR was examined. Culture supernatants from unfractionated spleen cells were collected 24 h after the start of the MLR incubation. Figure 4 shows both JWH-015 and O-1966 inhibited IL-2 release in a dose-dependent manner, indicating that the cannabinoids inhibit this parameter of T-cell function. Furthermore, when splenocytes from CB2R k/o mice were used, O-1966 treatment did not inhibit IL-2 release, indicating this effect is CB2 receptor dependent.

JWH-015 and O-1966 inhibit T-cells

The question was addressed as to whether the cannabinoids act directly on the T-cells, on accessory cells, or on both types of cells. Splenocytes from wild-type C57BL/6 mice were sorted into highly purified subpopulations using flow cytometry. Specifically, $CD3^+$ (T-cells) and $CD11b^+$ (myeloid derived cells) populations were selected and individually treated with JWH-015 or ethanol vehicle, or O-1966 or DMSO vehicle, before being added back to the remainder of the untreated spleen cells, which were either CD3 or CD11b depleted, to restore the normal spleen population. The reconstituted cells were then incubated with mitomycin C inactivated C3HeB/FeJ stimulator splenocytes. Figure 5 shows that complete inhibition of the MLR was observed only in cultures containing $CD3^+$ cells that had been treated with a cannabinoid. In cultures that received cannabinoid treated $CD11b^+$ cells, significant inhibition was only observed for $CD11b^+$ cells that were treated with 32 μM JWH-015 ($p<0.01$), while treatment of $CD11b^+$ cells with any dose of O-1966 did not reach statistical significance. Further, $CD11b^+$ cells treated with 8 μM, 16 μM, or 32 μM of JWH-015 or O-1966 were significantly less inhibited than unsorted cells treated with the same dose of cannabinoid, indicating that the observed effect of CB2 agonists can be attributed primarily to a direct effect on the T-cells.

To rule out the possibility that cannabinoids were inhibiting $CD3^+$ cells to a greater extent than $CD11b^+$ cells from disproportionate expression of CB2 receptors, quantitative PCR was performed to measure CB2 receptor (CB2R) RNA expression levels in purified $CD3^+$ cells and $CD11b^+$ cells immediately after spleens were removed (T_0) and after 24 h in the MLR (T_{24}). CB2R expression was not significantly different between these cell populations at T_0. By T_{24}, both populations had significantly increased CB2R expression, with a 26.6-fold increase in $CD11b^+$ cells and a 6.9-fold in $CD3^+$ cells (Fig. 6). Thus, the data do not support the hypothesis that the reason for the increased inhibition by $CD3^+$ cells was due to a greater expression of CB2R.

To further verify that the cannabinoids can directly suppress T-cells, splenocytes were sorted into a $CD3^+$ population. Purified T-cells were treated for 3 h with O-1966 or DMSO vehicle and then activated with the anti-CD3 and anti-CD28 antibodies. In the presence of O-1966 (Fig. 7), there was a dose-dependent, marked decrease in proliferation. This experiment shows conclusively that the cannabinoids can act directly on T-cells, as proliferation could be inhibited by the cannabinoid in the absence of accessory cells.

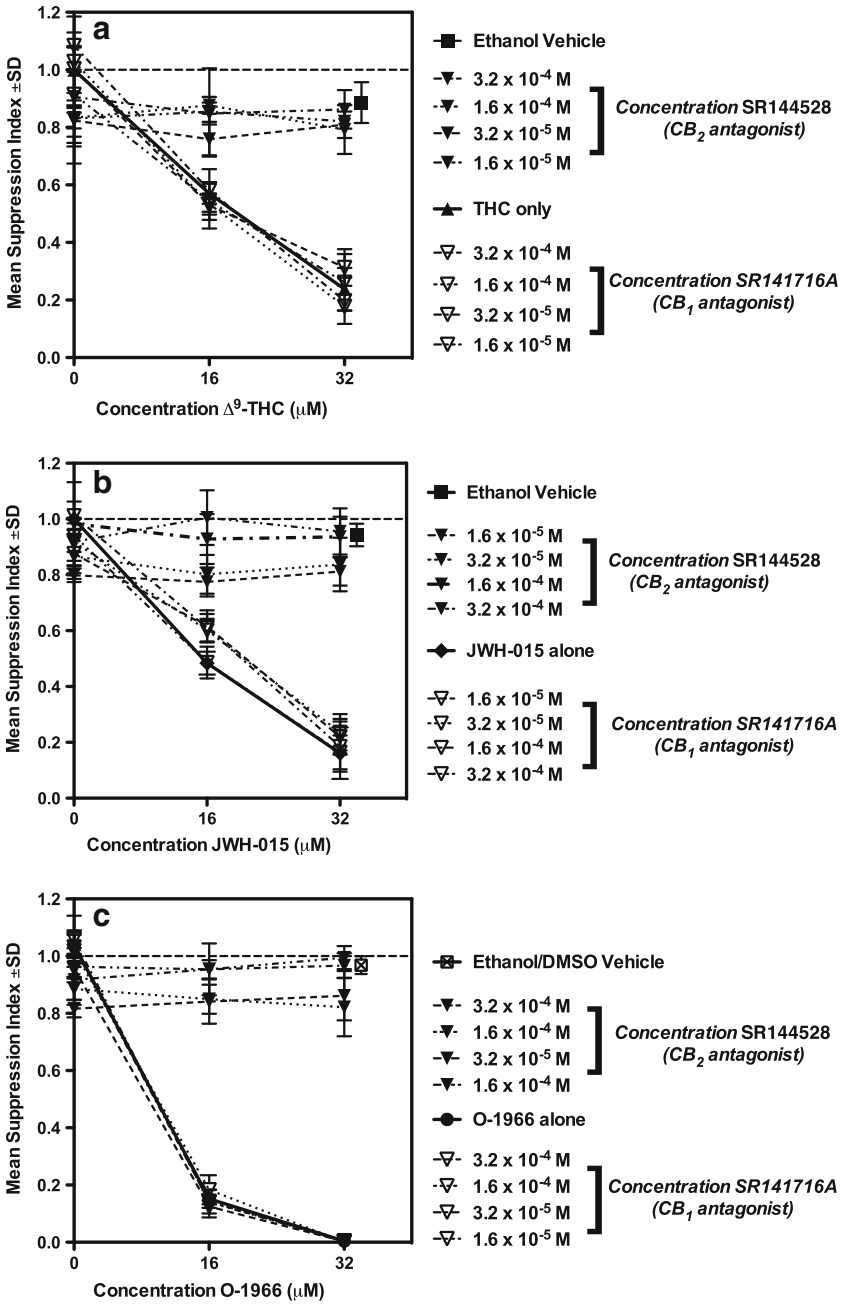

Fig. 2 Δ^9-THC and the CB2-selective agonists, JWH-015 and O-1966, inhibit the MLR via the CB2 receptor. C57BL/6 responder splenocytes were pretreated with varying concentrations of SR141716A, a CB1 antagonist, (▽), SR144528, a CB2 antagonist (▼), or ethanol vehicle (■) for 2 h. The cultures were then treated with one of three cannabinoids. Panel **a**: Δ^9-THC (▲) or ethanol vehicle (■). Panel **b**: JWH-015 (◆) or ethanol vehicle (■). Panel **c**: O-1966 (●) or DMSO vehicle (□). Vehicle controls were the amount of ethanol needed to dissolve the antagonist (0.05 % v/v) plus 0.4 % v/v of ethanol (**a**,**b**) (■) or the amount of DMSO (**c**) (⊠), needed to dissolve 32 μM Δ^9-THC, JWH-015 or O-1966. Data are mean ± S.D. of 3 separate experiments, with quadruplicate wells for each treatment. $p<0.001$ (ANOVA, agonist + CB1 antagonist versus agonist alone, agonist + CB2 antagonist versus vehicle)

JWH-015 and O-1966 do not induce apoptosis

A possible mechanism that has been proposed for cannabinoid mediated immunosuppression is through the induction of apoptosis of activated immune cells (Lombard et al. 2007; McKallip et al. 2002). While the present paper reports membrane integrity of the cells in the experiments was unchanged by cannabinoid treatment, as measured by LIVE/DEAD staining in each experimental condition, more precise measurements were used to detect and measure apoptotic cells. To detect cells in the early stages of apoptosis, MLR cultures treated with JWH-015, O-1966, or vehicle were harvested at the start of the culture, and 24 and 48 h into the assay. Levels of caspases 1, 3, 4, 5, 7, 8, and 9 were measured by flow cytometry using a caspase assay kit (Vybrant® FAM Poly Caspases Assay Kit, Molecular Probes, Inc., Eugene, OR). Figure 8a shows that, while the number of caspase positive cells increased as time in culture increased, there were no differences between cells that received no treatment or treatment with vehicle, as compared with treatment with a cannabinoid. Additionally, DNA fragmentation was measured using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Fig. 8b), to test cells from MLR cultures that were harvested at the start of the assay (T_0), and 24 or 48 h after culture initiation. At all time points tested, there was no difference between treatment groups,

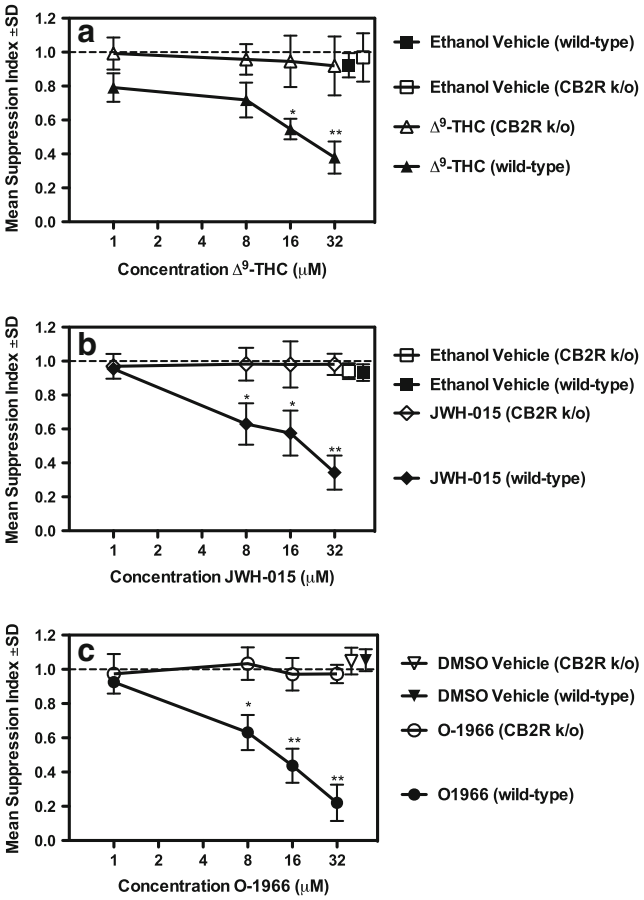

Fig. 3 Δ^9-THC, JWH-015, and O-1966 do not suppress the MLR when splenocytes from CB2R k/o are used. Splenocytes from CB2-deficient mice (open symbols) or wild-type mice (closed symbols) were treated for 3 h. Panel **a**: Δ^9-THC (WT: ▲, k/o: △) or ethanol vehicle (WT: ■, k/o: □). Panel **b**: JWH-015 (WT: ◆, k/o: ◇) or ethanol vehicle (WT: ■, k/o: □). Panel **c**: O-1966 (WT: ●, k/o: ○) or DMSO vehicle (WT: ▼, k/o: ▽). Concentrations of ethanol (**a**,**b**) or DMSO (**c**) vehicle correspond to the amount needed to dissolve the highest concentration of cannabinoid (0.4 % v/v). Each experiment was repeated 3 times, with quadruplicate wells for each treatment. *p<0.01, **p<0.001 (ANOVA, WT versus CB2R k/o). Values for vehicle are not significantly different from 1.0 (no treatment)

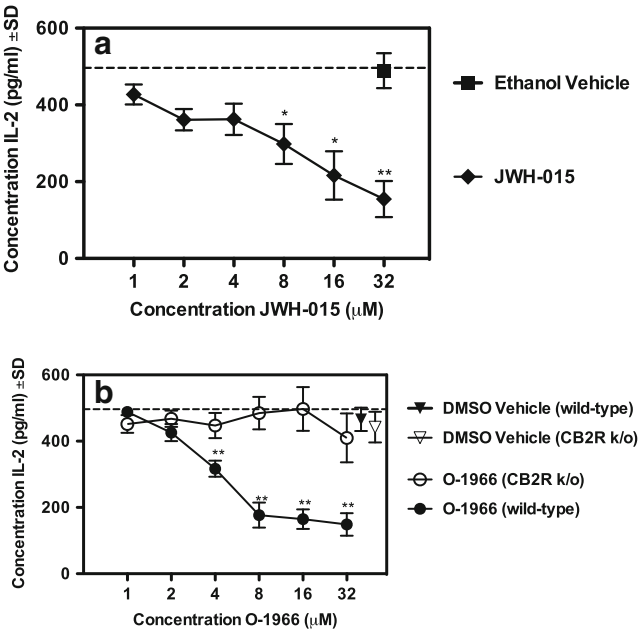

Fig. 4 JWH-015 and O-1966 inhibit the release of IL-2 in the MLR. To determine the effect of CB2-selective cannabinoids on the release of IL-2, CB2R k/o responder splenocytes (*open symbols*) or wild-type littermates (*closed symbols*) were pretreated for 3 h with: Panel **a**: JWH-015 (◆) or ethanol vehicle (■). Panel **b**: O-1966 (WT: ●, k/o: ○) or DMSO vehicle (WT: ▼, k/o: ▽). The cultures were incubated for 24 h; supernatants were collected; and concentrations of IL-2 were assessed by ELISA. Concentrations of ethanol or DMSO vehicle correspond to the concentration needed to dissolve the highest concentration of cannabinoid. JWH-015 data are the mean of 3 separate experiments with triplicate wells for each treatment, and O-1966 are the mean of 2 separate experiments with triplicate wells for each treatment. *p<0.01, **p<0.001 (ANOVA, A: JWH-015 versus vehicle; B: WT versus k/o). Values for vehicle are not significantly different from 1.0 (no treatment)

showing that apoptosis is not the mechanism by which JWH-015 and O-1966 are suppressing the MLR.

Discussion

The results presented in this paper show that Δ^9-THC, a mixed CB1/CB2 agonist, and two CB2-selective agonists can inhibit the Mixed Lymphocyte Reaction (MLR), an in vitro correlate of organ and skin graft rejection. The inhibition by all three compounds in the MLR was shown to be CB2 dependent, as pretreatment with the CB2-selective antagonist, SR144528, completely reversed the suppression, while pretreatment with the CB1-selective antagonist, SR141716A, had no effect. Further, suppression did not occur in CB2 receptor knockout (CB2R k/o) mice. In addition, the CB2-selective cannabinoids, JWH-015 and O-1966, decreased the release of IL-2 in the MLR, which did not occur when splenocytes from CB2R k/o mice were used. Evidence is also presented showing that inhibition of the MLR occurred predominantly when the $CD3^+$ population, but not the $CD11b^+$ population, was treated with the CB2-selective agonists. This difference was not due to differential expression of the CB2 receptor because before activation, $CD3^+$ cells and $CD11b^+$ cells expressed comparable levels of CB2 mRNA and after activation, $CD11b^+$ cells expressed CB2 mRNA levels many fold higher than $CD3^+$ cells. The CB2 agonists were also shown to inhibit proliferation of purified T-cell populations stimulated with antibodies.

Suppression was observed in the MLR by Δ^9-THC, JWH-015, and O-1966, in the range of concentrations from 4 μM to 32 μM. At 32 μM, Δ^9-THC suppressed the MLR by 64 %±0.08, JWH-015 by 62 %±0.06, and O-1966 by

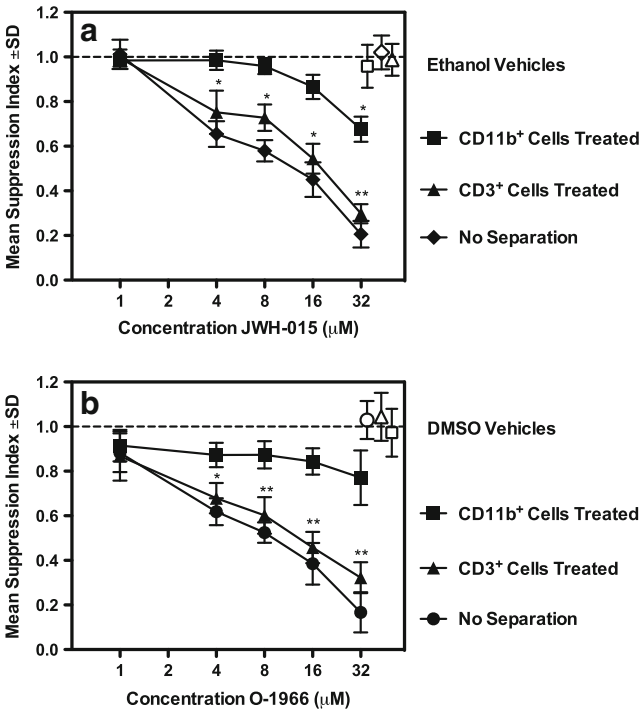

Fig. 5 JWH-015 and O-1966 directly inhibit T-cells. C57BL/6 splenocytes were sorted by flow cytometry into $CD3^+$, $CD11b^+$, or $CD3^-CD11b^-$ fractions. $CD3^+$ fractions (▲) or $CD11b^+$ fractions (■) were treated for 3 h with the desired cannabinoid. Panel **a**: JWH-015 or ethanol vehicle. Panel **b**: O-1966 or DMSO. $CD3^+$ treated populations were combined with untreated $CD11b^+$ and $CD3^-CD11b^-$ FACS sorted cell subsets to reconstitute the normal splenocyte population for carrying out the MLR. Likewise, $CD11b^+$ treated populations were combined with untreated $CD3^+$ and $CD3^-CD11b^-$ populations. Data are the mean of 3 separate experiments, with quadruplicate wells for each treatment. Treated fraction versus vehicle: *p<0.01, **p<0.001 (ANOVA)

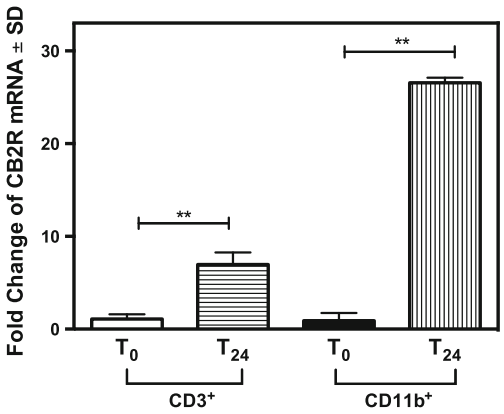

Fig. 6 CB2 receptor (CB2R) expression increases in the MLR. C57BL/6 splenocytes were sorted by flow cytometry immediately after harvest (T_0) or after 24 h in the MLR (T_{24}). $CD3^+$ and $CD11b^+$ populations were collected, RNA was extracted, reverse transcribed, and analyzed by quantitative PCR. The fold change of CB2R expression in $CD3^+$ cells from T_0 (▭) to T_{24} (▤) and in $CD11b^+$ cells from T_0 (■) to T_{24} (▥) is shown. Levels of CB2R were normalized to the reference gene β-Actin. Data are the mean of 2 experiments. **p<0.001 (ANOVA, versus T_0)

90.4 %±0.07. Based on the affinity of these compounds for the CB2 receptor (Δ^9-THC: 3.9–75.2 nM (Howlett et al. 2002), JWH-015: 13.8 nM (Showalter et al. 1996), O-1966: 23 nM (Wiley et al. 2002), these doses would seem to be high by several orders of magnitude. However, other investigators have also reported effects on immune function by cannabinoids in this dosage range (Adhikary et al. 2012; Borner et al. 2009; Ngaotepprutaram et al. 2012; Zhu et al. 1993). Previous research has shown that the concentration of Δ^9-THC needed to suppress B- and T-lymphocytes in vitro must be increased proportionally to increases of serum levels in the culture media, (Nahas et al. 1977; Klein et al. 1985). MLR cultures were incubated in medium containing 10 % fetal bovine serum, an amount that necessitated micromolar concentrations of cannabinoids in previous studies. It should be noted that in the present experiments, extensive controls were included to address any concerns about the possibility of cell toxicity, non-receptor mediated or off target effects, or conditions that are not physiological due to the concentrations of cannabinoids used. In every experiment, parallel cultures were stained with Invitrogen's LIVE/DEAD dead cell stain that was analyzed by flow cytometry. In preliminary experiments trypan blue exclusion was also used as a test of cell viability. In no experiment did any cannabinoid agonist, antagonist, vehicle control, or any combination of agonists, antagonists, and vehicle control increase the number of dead cells compared to cultures that received no treatment. These results show that exposure of the spleen cells to the doses of cannabinoids that resulted in suppression was not toxic, and thus, cell death can be excluded as the cause of the suppression of the MLR. As a further control, apoptosis was also measured in cannabinoid treated MLR cultures by testing levels of several caspases, which are important mediators in the induction of apoptosis. Neither JWH-015 nor O-1966 were found to increase levels of apoptosis in the MLR. In addition, the TUNEL assay, which measures DNA fragmentation seen in apoptotic cells was also

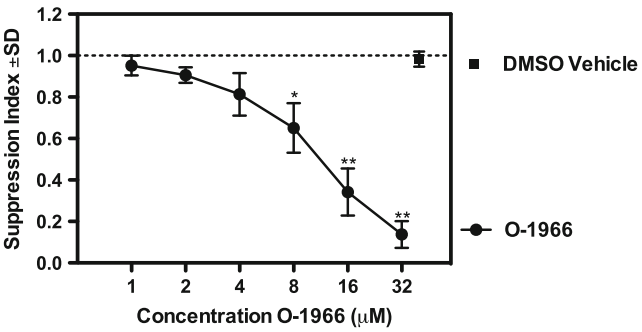

Fig. 7 O-1966 inhibits T-cell proliferation in response to activation by anti-CD3 and anti-CD28 antibodies. Purified C57BL/6 T-cells ($CD3^+$) were treated for 3 h with O-1966 (●) or DMSO vehicle (■). The T-cells were added to a plate coated with 25 μg anti-CD3 antibody/well, and soluble anti-CD28 antibody (0.4 μg/well) was added to each well. Data are the average of 3 separate experiments with quadruplicate wells for each treatment. *p<0.01, **p<0.001. (ANOVA, versus vehicle)

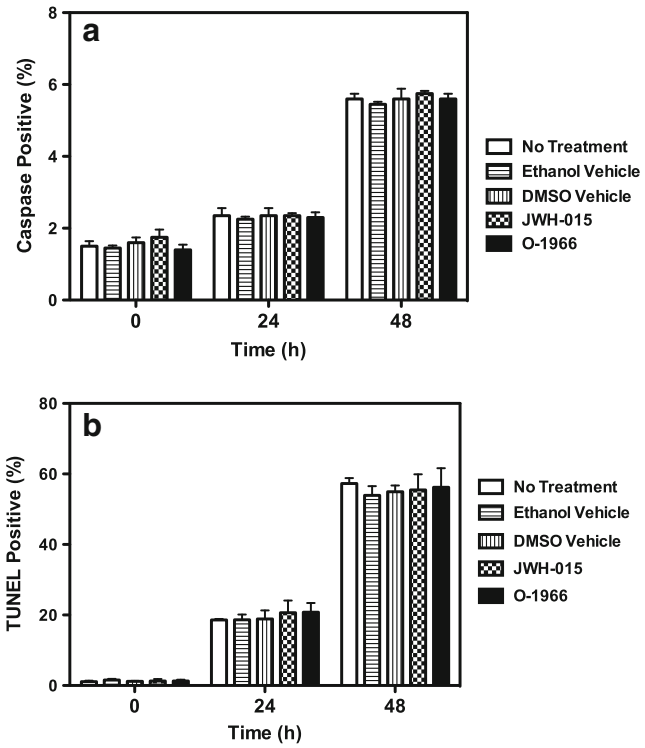

Fig. 8 JWH-015 and O-1966 do not induce apoptosis in the MLR. To determine if the CB2-selective cannabinoids induce apoptosis in the MLR, cells were harvested at time zero, or after 24 or 48 h in culture. Panel **a**: cells were stained for activation of caspases and analyzed by flow cytometry. Panel **b**: TUNEL assay. Cells received no treatment (▭), 32 μM JWH-015 (▩), ethanol vehicle (▤), 32 μM O-1966 (■), or DMSO vehicle (▥). Concentrations of ethanol or DMSO vehicle correspond to the concentration needed to dissolve the respective cannabinoid. Data are mean of 3 separate experiments, with duplicate wells for each treatment. There was no significant difference in numbers of cells positive for activated caspases or TUNEL positive cells at any time points tested

employed. Interestingly, all cultures showed marked increases in TUNEL positive cells by 48 h in culture, but there was no differential increase in the wells receiving the cannabinoids. The experiments presented in this paper also show that the cannabinoid doses that resulted in immunosuppression, even though seemingly high, were exerting their effect through the CB2 receptor, as suppression was 100 % blocked by the CB2-selective antagonist. Further, cells taken from mice lacking the CB2 receptor (CB2R k/o mice) were not suppressed when exposed to the cannabinoids, and the cannabinoids did not inhibit their IL-2 production, showing that these cannabinoids did not have generalized toxicity. Another potential concern is whether the effect of the cannabinoids at these micromolar concentrations is physiologically relevant. Several studies have shown that Δ^9-THC and CB2-selective agonists have in vivo immunosuppressive effects. For example, in mice, Δ^9-THC inhibited antitumor immunity (5 mg/kg) (Zhu et al. 2000), and increased the Th2 phenotype following *Legionella pneumophila* infection (8 mg/kg) (Newton et al. 2009). O-1966 has been shown to be effective in treating spinal cord injury (Baty et al. 2008; Adhikary et al. 2011) and stroke (Zhang et al. 2007, 2009a) at doses from 1 mg/kg to 10 mg/kg. JWH-133, another CB2-selective agonist, was shown to improve outcomes in models of atherosclerosis (Hoyer et al. 2011), systemic sclerosis (Servettaz et al. 2010b), colitis (Storr et al. 2009), stroke (Murikinati et al. 2010), autoimmune uveoretinitis (Xu et al. 2007), and inflammation following LPS challenge (Rajesh et al. 2007; Ramirez et al. 2012) at doses from 1 mg/kg to 20 mg/kg. We have performed preliminary studies using O-1966 to block skin graft rejection in vivo and found it to be effective at a similar dose range (data not shown). Thus, there seems to be a poor correlation between the doses needed in vitro to demonstrate efficacy of these cannabinoids in the immune system, and the in vivo doses that demonstrate anti-inflammatory and immunosuppressive effects. At present there is no explanation for why such high doses are needed in vitro, and it is not uncommon in pharmacology to see similar situations.

The suppressive effect of Δ^9-THC on the MLR and T-cells, the cells that proliferate in the MLR, was previously shown after in vivo administration of Δ^9-THC (5 mg/kg), which decreased ex vivo proliferation in the MLR (Zhu et al. 2000). Another group showed that treatment of splenocytes with Δ^9-THC in vitro could inhibit proliferation in the MLR, but using CB1/CB2 k/o mice, this group concluded that the inhibition was CB1- and CB2-independent (Springs et al. 2008). Our data show very clearly that Δ^9-THC added in vitro inhibits proliferation in the MLR in a CB2-dependent manner, based on both use of receptor specific antagonists and use of cells from CB2 receptor k/o mice. In accordance with our results, several studies have shown that the inhibition of T-cells by cannabinoids to be CB2 mediated. Yuan et al. reported that Δ^9-THC treatment decreased mRNA levels for IL-2 and IFN-γ in T-cells activated with anti-CD3 and anti-CD28 antibodies, and that this suppression could be reversed by treatment with the CB2 antagonist SR144528 (Yuan et al. 2002). Other groups used CB2 selective agonists, including JWH-015, and found that they inhibited molecules involved in T-cell receptor signaling in primary human T-cells and Jurkat T-cells following antibody activation (Borner et al. 2009). Other CB2-selective cannabinoids significantly decreased proliferation of T-cells and IL-2 release in response to various methods of stimulation (Cencioni et al. 2010; Ihenetu et al. 2003; Maresz et al. 2007). Our data clearly demonstrate a direct effect of CB2-selective cannabinoids on T-cells in the context of graft rejection.

Ideally, the anatomically disparate expression of CB1 and CB2 would allow for the use of compounds selective for CB2, and thus eliminate the unwanted psychoactive effects from CB1 activation, while maintaining the anti-inflammatory and immunosuppressive properties. While CB2 receptors have been found to have limited neuronal expression (Gong et al. 2006; Van Sickle et al. 2005), recent reports show that CB2

agonists modulate cocaine's rewarding and locomotor stimulating effects (Xi et al. 2011) and affect symptoms of depression (Onaivi et al. 2008), suggesting a function in the central nervous system for this class of compounds. However, intravenous administration of O-1966 to mice in doses up to 30 mg/kg did not produce any effect in the tetrad test, a common series of behavioral analyses used to assess the psychoactive effects of cannabinoids (Zhang et al. 2007). More work should be done to evaluate the neuronal effects of CB2-selective agonists in order to fully understand the prospect of this class of compounds as potential immunomodulatory therapeutics.

CB2-selective cannabinoids have been proposed as possible candidates to block graft rejection (Nagarkatti et al. 2010), but to date there are no published studies prior to the present one showing that this class of compounds is effective in this regard. The ability to transplant organs has been a major advance in medical science, saving thousands of lives. Previously, the obstacle to achieving graft survival was the inability to prevent immunologic organ rejection between allogeneic donors and recipients. This obstacle was overcome by two critical breakthroughs. First, the development of tissue typing permitted assessment of the relatedness of the MHC antigens of the prospective donor and recipient to determine compatibility. Second was the discovery of immunosuppressive compounds that could be administered chronically to the recipient to prevent rejection. Use of these compounds is required in almost all cases of organ transplantation because an exact tissue match cannot be found, only a close one. Unless immunosuppressive therapy is given, the immune system perceives even the small differences, and eventually mounts an immune response that results in destruction of the graft. The graft is rejected primarily via expansion of activated antigen-specific T-cells that recognize antigens on the incompatible tissue as foreign, and replicate (reviewed by Heeger 2003).

Most current protocols for immunosuppressive therapy use compounds that inhibit T-cells, and a vast majority include calcineurin inhibitors (tacrolimus or cyclosporine) and T-cell depleting antibodies (Colvin-Adams et al. 2013; Kandaswamy et al. 2013; Kim et al. 2013; Smith et al. 2013; Valapour et al. 2013). However, the current strategies employing these therapeutic agents have several problems. Even with continuous anti-rejection therapy, eventually many grafts are rejected after several years. Further, the standard anti-rejection drugs tacrolimus and cyclosporine, have been implicated in the development of diabetes, hypertension, and nephrotoxcity (Merville 2005). New therapeutic approaches are needed and CB2-selective agonists could potentially be given alone or in combination with current standard treatments, in order to lower the required doses tacrolimus and cyclosporine.

Further studies to investigate the potential use of CB2-selective cannabinoids as a treatment to prevent graft rejection are desirable. Studies to elucidate the mechanism by which the cannabinoids inhibit T-cells in graft rejection are in progress. Also, the CB2-selective agonists need to be further tested for their capacity to suppress graft rejection in animal transplantation models.

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Disclosure The authors declare that they have no conflict of interest.

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