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Cannabinoid receptor activation leads to massive mobilization of myeloid-derived suppressor cells with potent immunosuppressive properties

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Abstract

Summary—Cannabinoid receptor activation by agents such as Δ^9-tetrahydrocannabinol (THC) is known to trigger immune suppression. Here, we show that administration of THC in mice leads to rapid and massive expansion of $CD11b^+Gr\text{-}1^+$ myeloid-derived suppressor cells (MDSC) expressing functional arginase and exhibiting potent immunosuppressive properties both *in vitro* and *in vivo*. The induction of MDSC by THC was associated with a significant increase in granulocyte colony-stimulating factor (G-CSF). Moreover, administration of anti-G-CSF antibody inhibited the induction of MDSC by THC. THC was able to induce MDSC in TLR4 mutant C3H and C57BL10/ScN mice and hence acted independently of TLR4. Accumulation of MDSC in the periphery with a corresponding decrease in the proportion of $CD11b^+Gr\text{-}1^+$ cells in the bone marrow, as well as *in vivo* BrdU labeling and cell cycle analysis, showed that THC induced mobilization of these cells from bone marrow and their expansion in the periphery. Use of selective antagonists SR141716A and SR144528 against cannabinoid receptors, CB1 and CB2 respectively, as well as receptor-deficient mice showed that induction of MDSC was mediated through activation of both CB1 and CB2 receptors. These studies demonstrate that cannabinoid receptor signaling may play a crucial role in immune regulation via the induction of MDSC.

Keywords

arginase; cannabinoid receptors; G-CSF; immune suppression; myeloid-derived suppressor cells

Introduction

Recently, a suppressor cell population of myeloid lineage capable of reducing anti- tumor as well as inflammatory immune responses has been described [1-5]. These cells express CD11b and Gr-1, and have been named myeloid suppressor cells or myeloid-derived suppressor cells (MDSC) [2,3,6]. $CD11b^+Gr\text{-}1^+$ MDSC are a heterogeneous cell population including immature macrophages, granulocytes, dendritic cells and other myeloid cells, and possess potent immunosuppressive properties [2,6]. They have been shown to suppress T cell proliferation [7], inhibit the cytotoxicity of CD8 T cells or NK cells both *in vitro* and *in vivo* [3,6,8,9], down-regulate L-selectin expression on CD4/ CD8 T cells [10] and induce antigen-specific CD8 T cell tolerance in tumor bearing hosts [11]. L-Arginine metabolism is

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the major mechanism by which MDSC cause T cell suppression [1,2,12]. $CD11b^{+}Gr\text{-}1^{+}$ suppressive cells have also been identified during several inflammatory conditions [13-16].

Endogenous and exogenous cannabinoids signal through two major G-protein coupled cannabinoid receptors, CB1 and CB2 [17]. Delta-9-tetrahydrocannabinol (THC) is a natural cannabinoid compound from the plant *Cannabis sativa* [18]. THC and other cannabinoids have been extensively studied with respect to their immunomodulatory and anti-inflammatory properties [17]. Cannabinoids exert their immunomodulatory effects by various mechanisms. We and others have previously shown that that the immunosuppressive property of THC can be attributed in part to its ability to induce apoptosis in T lymphocytes, dendritic cells and macrophages [19-22]. THC has also been shown to trigger regulatory T cells (Treg) [23] as well induce anti-inflammatory cytokine production [18,23,24].

While both CB1 and CB2 receptors are expressed on cells of the immune system the precise nature of these receptors and the role of endogenous and exogenous cannabinoids in the regulation of the immune functions is not clear. Also, while many studies have demonstrated that cannabinoids exhibit anti-inflammatory properties, the precise mechanisms are unclear. In this study, we demonstrate for the first time that activation of cannabinoid (CB1 and CB2) receptors through administration of cannabinoids such as THC, into mice, triggers massive induction of arginase 1 expressing $CD11b^{+}Gr\text{-}1^{+}$ MDSC, with immunosuppressive properties.

Results

THC administration triggers massive induction of $CD11b^{+}Gr\text{-}1^{+}$ cells

C57BL/6 (B6) wild type (WT) mice were injected with vehicle or THC intraperitoneally (*i.p.*). The exudate cells in the peritoneal cavity were harvested after 16 h and analyzed. To our surprise, the administration of THC induced massive local accumulation of cells in the peritoneal cavity when compared to vehicle control (Fig 1A). Flow cytometric analysis using forward and side scatter revealed that majority of cells induced by THC were granular and larger in size (Fig 1B). We further phenotyped these cells using mAb to cell surface markers, CD3, Gr-1, CD11b, and F4/80 (Fig 1C). Cells from vehicle-injected mice showed phenotypic characteristics expected from a normal peritoneum. $CD11b^{high}$ cells in the peritoneum typically represent mature macrophages, which also express high F4/80. Interestingly, majority of cells (>90%) from THC-injected mice were positive for CD11b and Gr-1 markers. Close to one third proportion of the population also showed low or intermediate expression of F4/80. We performed a dose-response study by injecting (*i.p.*) wild-type mice with different doses of THC and analyzed the peritoneal exudate cells for the co-expression of CD11b and Gr-1 by flow cytometry. THC triggered a dose-dependent increase in the percentage (Fig 1D) as well as absolute numbers (Fig 1E) of $CD11b^{+}Gr\text{-}1^{+}$ cells in the peritoneum. In control mice, the $CD11b^{high}$ ($Gr\text{-}1^{-/-}$) cell population represents mature macrophages. This is typical of naïve peritoneum where mature macrophages form a significant proportion. Whereas, $CD11b^{+}Gr\text{-}1^{+}$ cells induced by THC expressed intermediate levels of CD11b. The $CD11b^{high}$ ($Gr\text{-}1^{-/-}$) macrophages disappear in the dot plots with increasing doses of THC (Fig 1D) likely because of the dilution effect resulting from several fold induction of $CD11b^{+}Gr\text{-}1^{+}$ cells or due to THC-induced killing of mature macrophages as reported previously [20]. The robust induction of $CD11b^{+}Gr\text{-}1^{+}$ cells seen here in response to THC also shows that unlike lymphocytes [19-21] or macrophages [20], these cells are resistant to THC-induced killing.

THC-induced CD11b$^+$Gr-1$^+$ cells are MDSC and mediate immunosuppression *in vitro* and *in vivo*

Next, we investigated if THC-induced CD11b$^+$Gr-1$^+$ were MDSC by studying their ability to mediate inhibition of T cell proliferation, which is one of the most important characteristics of MDSC. To this end, we performed a series of *in vitro* and *in vivo* studies. For *in vitro* assays, we determined the suppressive activity of purified peritoneal CD11b$^+$Gr-1$^+$ cells induced by THC on the proliferation of T cells stimulated with polyclonal as well as antigen-specific stimuli. T cell proliferation was assessed at by [3H]-thymidine uptake. THC-induced CD11b$^+$Gr-1$^+$ cells caused a dose-dependent decrease in proliferation of T cells stimulated with ConA with almost complete inhibition at a 1:2 ratio of CD11b$^+$Gr-1$^+$ cells to T cells (Fig 2A). THC-induced CD11b$^+$Gr-1$^+$ cells also significantly decreased proliferation of OT-II ova-transgenic T cells stimulated using agonist ova peptide (Fig 3B). As a control, we purified CD11b$^+$Gr-1$^+$ cells from peritoneal exudates induced by thioglycollate broth, which at early time points (4 h) triggers primarily neutrophils, and compared their ability to suppress T cell proliferation. Side-by-side comparison of suppressive activity showed that THC-induced CD11b$^+$Gr-1$^+$ cells were highly immunosuppressive compared to the CD11b$^+$Gr-1$^+$ neutrophils (Fig 3C, D).

Next, we used a well-established model of T cell-mediated liver inflammation induced by ConA to test the immunosuppressive properties of THC-induced CD11b$^+$Gr-1$^+$ cells *in vivo*. We adoptively transferred CD11b$^+$Gr-1$^+$ cells purified from the peritoneum of THC-injected mice into naïve mice before inducing hepatitis using ConA. The transferred CD11b$^+$Gr-1$^+$ cells significantly suppressed liver inflammation as indicated by decreased liver enzyme, alanine transaminase (ALT) levels, a commonly used marker for liver inflammation (Fig 2E). These data together suggested that the CD11b$^+$Gr-1$^+$ cells induced by THC exhibited the functional characteristics of MDSC.

Kinetics of THC- induced MDSC accumulation

The kinetics of MDSC induction by THC in peritoneum and spleen was determined by injecting mice with a single dose of THC, and analyzing recruitment of MDSC at different time points (0-24 h). A significant increase in frequency (Fig 3A) and absolute number of MDSC (Fig 3B) in peritoneum and spleen was detected as early as 8 h after THC injection, which peaked around 16 h and started to decline by 24 h.

Histological analysis of cellular infiltrates in peritoneum

We studied the morphology of THC-induced MDSC by Wright Giemsa staining. Cytospin preparations of peritoneal exudate cells from mice injected with vehicle or THC were stained and analyzed by light microscopy (Fig 3C). Vehicle-injected mice showed the characteristics mainly of lymphocytes and macrophages. In contrast, cells from THC-injected mice consisted of PMN-like cells with characteristic circular (or donut shaped) nucleus as well as monocyte-like cells. We also looked at cellular infiltrates in parietal peritoneum sections. H&E stained cross-sections of peritoneal tissue showed significant infiltration of cells in the THC group whereas, vehicle-injected mice showed no infiltration of cells (Fig 3D).

THC induces MDSC in TLR4-deficient mice

Previous studies have shown that toll-like receptor ligands can trigger induction MDSC [25-27]. The pure THC preparation used in the current study had undetectable levels of endotoxins. However, to further rule out the effect of endotoxins, as well as to investigate the possibility if THC acted via TLR4 in this case, we tested the same batch of THC in TLR4-deficient C57BL10/ScN mice and TLR4-mutant C3H mice. Both C57BL10/ScN and

C3H mice showed significant increase in frequency as well as absolute number of $CD11b^{+}Gr\text{-}1^{+}$ MDSC (Fig 4A, B) thereby suggesting, THC-induced recruitment of MDSC was independent of TLR4.

Cytokine/ chemokine response to THC

THC is known to suppress inflammatory cytokines induced by infectious agents or during inflammatory conditions *in vivo* [23,24]. However, we sought to determine if THC can induce any cytokine/chemokines *in vivo* resulting in the recruitment of MDSC. To this end, we injected WT mice with a single dose of THC or vehicle and collected sera at 6, 12 and 24 h. We analyzed sera for IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, IFN-γ, TNF-α, MIP-1α, MIP-1β, RANTES, MCP-1, GM-CSF, G-CSF, KC and eotaxin by Bioplex assay. THC had little or no effect on majority of these cytokines and chemokines at 6, 12 and 24 h as compared to basal levels induced by vehicle (data not shown), except for KC (CXCL1) and G-CSF. THC induced significant levels of chemokine, KC at 12 h and very high and persistent levels of G-CSF (~1500 pg/mL) at 12 and 24 h (Fig 5A). It is noteworthy that THC induced G-CSF levels significantly at an early time point, 6 h and to a very high level at 12-24 h. It is possible that the initial upregulation of G-CSF by THC leads to migration and activation of MDSC which in turn secrete more G-CSF causing increased mobilization and expansion of these cells.

GM-CSF has been shown to play an important role in induction MDSC associated with tumors [28]. However, we could not detect increase in GM-CSF in sera in the current model. To further investigate the role of GM-CSF and G-CSF in the induction of MDSC by THC, we determined the levels of these cytokines directly in the peritoneal exudates of vehicle and THC-injected mice. While there was a robust increase in the levels of G-CSF in the peritoneum of THC-injected mice as compared to vehicle controls, the GM-CSF levels were low and there was no significant difference between the two groups (Fig 5B).

Role of G-CSF in THC-induced accumulation of MDSC

To further confirm the role G-CSF the induction of MDSC by THC *in vivo*, we pretreated mice with anti-mouse G-CSF Ab before injecting THC. We observed that as low as 10 μg/ mouse of anti-G-CSF was able to significantly block the accumulation of MDSC in the peritoneum in response to THC (Fig 5C), indicating that G-CSF plays a crucial role in this process. These data suggest that THC may trigger migration and expansion of MDSC primarily by inducing G-CSF.

THC-induced MDSC express functional arginase

L-Arginine metabolism is an important pathway used by MDSC to blunt antitumor immunity [1] or reduce inflammatory responses [13-16]. Expression of arginase, the enzyme which metabolizes and depletes essential amino acid L-arginine, is the hallmark characteristic of MDSC. In order to test if THC-induced MDSC express functional arginase, we injected mice with vehicle or THC and determined arginase enzyme activity by spectrophotometric assay based on the principle of conversion of substrate L-arginine to L-ornithine. THC-induced cells from the peritoneum accounted for several fold higher levels of arginase activity when compared to vehicle controls thereby suggesting that THC-induced MDSC expressed functionally active arginase (Fig 6A).

THC induces migration and expansion of MDSC from bone marrow

In naïve mice, $CD11b^{+}Gr\text{-}1^{+}$ cells have been shown to be present in small numbers in peripheral tissues such as spleen and up to 18-50% in bone marrow depending on the mouse strain [6,29]. Because THC induces mobilization of MDSC massively and rapidly, we

speculated that the source of these cells could be bone marrow. To investigate this, we injected WT mice with THC and determined the percentage of $CD11b^{+}Gr\text{-}1^{+}$ cells at 0 and 16 h in bone marrow and peritoneum (Fig 6B). We observed a significant decrease in the percentage of $CD11b^{+}Gr\text{-}1^{+}$ cells in the bone marrow 16 h after THC administration with a corresponding increase in periphery (peritoneum). This indicated that $CD11b^{+}Gr\text{-}1^{+}$ cells were migrating from bone marrow in response to THC. Further, we wanted to know if the MDSC were also dividing in the periphery. Mice were given vehicle or THC, two hours after injecting with BrdU. Peritoneal cells harvested after 24 h were triple stained for CD11b, Gr-1 and intranuclear BrdU incorporation (Fig 6C). Close to 37% of the MDSCs in the peritoneum of the THC-injected mice were $BrdU^{+}$. Because cells incorporating BrdU in the bone marrow and then migrating to peritoneum in response THC would have contributed to this percentage, we determined active proliferation in the peritoneum by cell cycle analysis using propidium iodide (PI) staining. We found that a significant proportion (22.5%) of THC-induced MDSC and 23.3% of all cells induced by THC in the peritoneum were in S-phase indicative of active cycling when compared to 11.9% of peritoneal cells found in vehicle-treated mice. These data suggested that MDSC accumulating in the periphery in response to THC were undergoing active proliferation.

Analysis of MDSC subsets induced by THC

Although, MDSC are defined by their co-expression of CD11b and Gr-1, these cells have been known to contain heterogeneous mixture of myeloid cells with suppressive function. However, recently two major subsets of MDSC have been identified based on the expression of CD11b, Ly6-G and Ly6-C antigens. Granulocytic subsets (PMN-MDSC) express both Ly6-G and Ly6-C along with CD11b ($CD11b^{+}Ly6\text{-}G^{high}Ly6\text{-}C^{low/int}$) while monocytic subsets (MO-MDSC) express only Ly6-C and CD11b ($CD11b^{+}Ly6\text{-}G^{neg}Ly6\text{-}C^{high}$) [25,30,31]. We sought to identify these subsets among MDSC induced by THC. For this we used Ab specific to Ly6-G (clone: 1A8) and Ly6-C (clone: HK1.4) along with anti-CD11b. THC administration resulted in significant dose-dependent increase in the frequency (Fig 7A) as well as absolute numbers of $CD11b^{+}Ly6\text{-}G^{+}Ly6C^{+(int)}$ granulocytic (PMN) and $CD11b^{+}Ly6\text{-}G^{neg}Ly6C^{+(high)}$ monocytic MDSC in the peritoneum (Fig 7B, C) suggesting that THC significantly induces both major MDSC subsets.

Role of cannabinoid receptors

To investigate if induction of MDSC resulted from activation of cannabinoid receptors, we first used CB1 and CB2 antagonists, SR141716A (SR1) and SR144528 (SR2) respectively. WT mice were injected with SR1 or SR2 or both, two hours prior to administration of vehicle or THC. Mice pretreated with either SR1 or SR2 showed significantly less frequency (Fig 8A) and absolute number (Fig 8B) of MDSC when compared to mice injected with THC alone. This indicated that THC acts through both CB1 and CB2 receptors to induce the mobilization of MDSC. We did not observe any significant synergistic blocking with both the antagonists injected together.

To further establish the role of cannabinoid receptors, we used CB1 and CB2 receptor knockout mice. We injected WT, CB1KO ($CB1^{-/-}$) or CB2KO ($CB2^{-/-}$ or $Cnr2^{-/-}$) mice with vehicle or THC. In CB1 and CB2 KO mice, the percentages (Fig 8C) as well as the absolute numbers (Fig 8D) of MDSC in the peritoneum were significantly reduced after THC challenge when compared to WT mice, confirming the involvement of both the receptors. Furthermore, when we injected CB1 and CB2 KO mice with CB2 or CB1 antagonist (SR2 or SR1) respectively, and then administered THC, we observed that blocking CB2 receptors in CB1 KO mice and CB1 receptors in CB2KO mice had a synergistic effect and almost completely inhibited the induction of absolute numbers of MDSC in response to THC (Fig

8C, D). Cumulatively, these data suggested that THC induces MDSC by signaling primarily through both CB1 and CB2 receptors.

Discussion

Immunosuppressive and anti-inflammatory properties of cannabinoids, including THC, have been well established [17]. THC is known to cause immune suppression by various mechanisms including induction of apoptosis in effector T cells, suppressing the inflammatory cytokines production under certain activating conditions, as well as arresting cell proliferation [18]. However, the effect of cannabinoids on important suppressor cells populations, namely, MDSC is not known. MDSC were initially identified in large numbers in tumor-bearing mice and were found to effectively suppress anti-tumor immune responses [4-6]. There is strong evidence showing that these cells are immunosuppressive and inactivate T cells [1,2]. Several recent studies have shown their role in modulation of inflammatory diseases [14-16]. Induction of MDSC in cancer has also been attributed to be a consequence of chronic inflammation [32,33].

In the current study, we injected THC intraperitoneally in naïve mice in the absence of any activating conditions. Initially, we were perplexed to see the dramatic accumulation of cellular infiltrates in the peritoneal cavity in response to THC, which were found to be large and granular cells by preliminary assessment. Given the potent immunosuppressive properties of THC, we speculated that these may not be inflammatory infiltrates, but some type of suppressor cells. Further phenotypic and functional characterization revealed that THC-induced cells co-expressed CD11b and Gr-1 markers. We further observed that THC-induced $CD11b^{+}Gr\text{-}1^{+}$ cells expressed functional arginase and caused significant inhibition in the proliferation of T cells stimulated with polyclonal (ConA) as well as antigen-specific (ova) stimuli *in vitro*, and conA-induced T cell-mediated liver inflammation *in vivo*, all hallmark characteristics of MDSC. It should be noted that unlike THC-induced $CD11b^{+}Gr\text{-}1^{+}$ cells, purified $CD11b^{+}Gr\text{-}1^{+}$ neutrophils isolated 4 h after thioglycollate administration failed to exhibit similar immunosuppressive properties.

We and others have shown that THC suppresses inflammatory cytokines elevated during an inflammatory response [23,34]. However, the effect of THC on various cytokines in naïve mice has not been thoroughly investigated. Among an array of 23 different cytokines and chemokines tested, we made a striking observation that THC caused a dramatic induction in the levels of G-CSF, and to some extent KC, in sera of naïve mice. Very high induction of G-CSF was also observed directly in the peritoneum. We noted that blocking of G-CSF by anti-G-CSF Ab was able to significantly decrease THC-induced accumulation of MDSC *in vivo*. G-CSF is a crucial cytokine required for the differentiation of granulocytes such as neutrophils from bone marrow precursors, and which also regulates their migration, proliferation as well as function [35]. Anti-inflammatory properties of G-CSF have also been known [36]. A very recent study using a spontaneous metastatic memory carcinoma model showed that the number of MDSC in the spleen directly correlated with G-CSF transcript levels [37]. Differential accumulation of tumor infiltrating monocytic MDSC over granulocytic MDSC in tumor bearing hosts mediated by chemokines, particularly G-CSF has also been demonstrated [38].

We further showed that the effect of THC was independent of TLR4 and mediated directly through activation of both CB1 and CB2 cannabinoid receptors. This observation was supported by the findings that CB1 or CB2 select antagonists could block MDSC induction by THC partially but significantly and was confirmed using CB1 and CB2 receptor-deficient mice. It has been shown that bone marrow is the source of large numbers of $CD11b^{+}Gr\text{-}1^{+}$ precursors [6,29]. In the current study, THC seemed to cause the migration $CD11b^{+}Gr\text{-}1^{+}$

MDSC from bone marrow. THC also induced the proliferation of migrated MDSC in the periphery as evidenced by significant proportion of actively proliferating MDSC in the peritoneum of THC injected mice. Various cell types including macrophages, endothelial cells and fibroblasts are known to express CB1 and CB2 receptors [39]. THC may activate CB1/ CB2 receptors on these cells to induce the secretion of mediators such as G-CSF and KC (CXCL1). Inasmuch as, G-CSF and KC have been shown to induce the development, migration and expansion of granulocytes, particularly neutrophils, it is highly likely that these molecules may play a crucial role in MDSC induction. We have noted that unlike the *i.p.* administration of THC, *i.v.* injection does not result in robust induction of MDSC in spleen, lungs and peritoneum, although a significant induction in the liver was seen (data not shown). This may result from the fact that i.p. injection of THC may activate resident cells in the peritoneal cavity such as fibroblasts or immature macrophages, that are known to produce G-CSF [40]. This is also supported by our observation that high levels of G-CSF were found in the peritoneal cavity washings after THC injection by the *i.p.* route. Although, G-CSF is primarily known to promote neutrophil differentiation from bone marrow precursors, it is possible that G-CSF by itself or other factors such as KC induced by THC or a combination of G-CSF and THC, may promote mobilization of $CD11b^{+}Gr\text{-}1^{+}$ MDSC and arrest their differentiation into mature neutrophils or macrophages. Currently, we are further testing this possibility.

We observed that a single dose of THC, as low as 5-10 mg/kg, was able to induce significant levels of MDSC in mice, which is clinically relevant. Based on body surface area normalization (BSA) method [41], this translates to human equivalent dose (HED) of 15-30 mg/m^2 (0.40-0.81 mg/kg). In cancer patients, the dose of THC (dronabinol or Marinol®) recommended is usually 2.5-20 mg/m^2/day (0.068-0.54 mg/kg), as an antiemetic treatment during chemotherapy. Chan et al., demonstrated that rats injected with 50 mg/kg body weight of THC had a serum concentration of 10 μM THC within 10 h of administration [42]. Azorlosa et al., showed that levels as high as 1 μM could be attained in the plasma of humans after recreational use of marijuana [43], and in a separate report, it was shown that THC can get preferentially concentrated 15- to 20-fold in some tissues [44]. Such levels of THC may lead to significant suppression of the immune response leading to increased susceptibility to opportunistic infections and cancer. In fact, it was shown that treatment of mice with doses of 8 mg/kg significantly suppressed the responses of mice to infection with Legionella pneumophila [34]. In light of these, our results suggest that THC, at a pharmacological or recreational dose may induce MDSC and cause immunosuppression.

Studies from our laboratory suggest that cannabinoids are a double-edged sword. On one hand, cannabinoids can suppress malignancies of the immune system by inducing apoptosis of tumors that express cannabinoid receptors [45]. On the other hand, because they suppress anti-tumor immune response, they promote growth and metastasis of cannabinoid receptor-deficient tumors such as breast cancer that are resistant to cannabinoid-induced apoptosis [46]. Epidemiological studies have shown that marijuana smokers are more susceptible to certain types of cancer [47,48]. It is possible that induction of MDSC by cannabinoids may make an individual more susceptible to certain types of cancer, particularly those which don't express cannabinoid receptors. We previously showed that THC promotes the growth and metastasis of 4T1 mammary tumors that lack cannabinoid receptors by suppressing anti-tumor immunity [46]. Recent studies have suggested that 4T1 tumor growth is associated with MDSC induction [49]. Thus, it is tempting to speculate that THC further enhances induction of MDSC which may be responsible for the accelerated growth and metastasis of 4T1 tumors and studies are in progress to address this in detail.

The current study unravels a new mechanism by which cannabinoids trigger immune suppression. These findings are important in several ways: 1) THC is currently used

clinically to ameliorate nausea and vomiting as well as stimulate appetite during chemotherapy in cancer patients, and HIV/AIDS patients and to lower intraocular eye pressure to treat glaucoma [50]. Thus, it is important to know if such a treatment would impact the immune system and pose health hazards. Because MDSC are known to promote tumor growth, our observations indicate that THC treatment in cancer patients may potentiate adverse effects. 2) Because CB2 is expressed only on immune cells, CB2 select agonists may constitute better anti-inflammatory agents with less non-specific toxicity than the currently available anti-inflammatory agents. Thus, the demonstration that CB2 activation leads to MDSC which in turn may help suppress inflammation may provide novel avenues to develop anti-inflammatory agents. 3) Our studies shed light on the potential role of endocannabinoids that activate cannabinoid receptors on MDSC induction and consequent regulation of inflammation.

Materials and Methods

Reagents

THC, SR141716A (CB1 antagonist) and SR144528 (CB2 antagonist) were provided by National Institute on Drug Abuse, National Institutes of Health (Bethesda, MD). The mAb fluorescein isothiocyanate-conjugated anti-CD11b, anti-Ly-6C (clone: HK1.4), phycoerythrin-conjugated anti-CD3, anti-Gr-1 (clone: RB6-8C5), anti-F4/80, anti-Ly-6G, Alexa-647-labeled CD11b, unconjugated anti-BrdU and mouse Fc-block (anti-CD16/CD32) were purchased from eBioscience. Phycoerythrin-conjugated anti-Ly-6G mAb (clone: 1A8) was from BD Biosciences. Purified anti-mouse-G-CSF Ab was from R&D Systems. Anti-PE microbeads, magnetic sorting columns and equipment were from Miltenyi Biotech. Propidium iodide and RNAase were from Invitrogen. Concanavalin A, L-arginine, L-ornithine standard, Ninhydrin reagent, BrdU, Triton X-100, red blood cell lysis buffer and all other chemicals and reagents were from Sigma-Aldrich.

Mice

Female C57BL/6 (WT), C3H and OT-II mice (8-10 weeks) were purchased from National Cancer Institute, National Institutes of Health (Frederick, MD). CB2 knockout ($CB2^{-/-}$ or $Cnr2^{-/-}$) mice on B6 background and C57BL/10ScN mice were obtained from The Jackson labs (Bar Harbor, ME, USA). CB1 knockout ($CB1^{-/-}$) mice on B6 background were a gift from Dr. James Pickel, National Institute of Mental Health Transgenic Core Facility. Mice were housed under specific pathogen-free conditions in the Animal Resource Facility of University of South Carolina and all experiments were pre-approved by the Institutional Animal Care and Use Committee.

Administration of compounds and preparation of cells

Mice were injected with THC intraperitoneally. Exudate cells in the peritoneal cavity were harvested by performing lavage with sterile, ice cold PBS (5ml × 3). Cells were spun down and reconstituted in FACS buffer (PBS/ 2% FBS) for flow cytometry staining or in complete RPMI medium for *in vitro* experiments. Splenocytes were obtained after preparing the single cell suspension followed by RBC lysis. Bone marrow cells were obtained by flushing tibia with ice cold PBS followed by RBC lysis. For blocking experiments, SR1, SR2 compounds, or anti-G-CSF Ab were injected (i.p.) at the indicated doses 2 h prior to injecting THC.

Immunofluorescence staining and flow cytometry

For fluorescence-activated cell sorting (FACS) analysis, cells were blocked using mouse Fc-block (anti-CD16/CD32) and stained for various cell surface markers using fluorescently labeled mAb (10 μg/mL, in PBS containing 2% fetal bovine serum). After washing, stained

cells were analyzed in a flow cytometer (FC500; Beckman Coulter, Fullerton, CA). Only live cells were counted by setting gates on forward and side scatters to exclude debris and dead cells. Isotype antibody-treated cells served as staining controls. Intracellular staining was performed after fixing and permeabilizing the surface stained cells using Fix-perm reagents (eBioscience). Data obtained were analyzed in Cytomics CXP software (Beckman Coulter).

Histology

Harvested parietal peritoneum was fixed in 4% buffered paraformaldehyde and embedded in paraffin. Sections (5 μm) were stained with H&E for histological analysis under light microscope.

Wright-Giemsa staining

Cells were collected by cytospin onto glass slides and dried completely for 30 min. The slides were stained with Wright-Giemsa stain (Fisher Sci) according manufacturer's instructions and analyzed by light microscopy.

T cell proliferation assay

THC-induced MDSC were sorted by magnetic sorting using PE-conjugated Gr-1 Ab and anti-PE microbeads (Miltenyi Biotech). The sorted cells were >95% positive for CD11b and Gr-1 co-expression. For some experiments $CD11b^{+}Gr\text{-}1^{+}$ MDSC were sorted to >96% purity using FACS Aria (Becton and Dickinson) after labeling with fluorescently conjugated Ab. Purified MDSC were irradiated and cultured at different ratios with purified syngenic T cells (2×10^5) stimulated with Con A (4 μg/ml) in a 96-well round bottom plates, in complete RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 10 mM HEPES, 1 mM penicillin-streptomycin, and 50 μM β-mercaptoethanol. For antigen-specific stimulation, purified T cells (2×10^5) derived from OT-II mice were stimulated with agonist ova peptide ($OVA_{323–339}$, 1μg/mL) in the presence of irradiated splenocytes (5×10^5) as APC, and were co-cultured with different ratios of purified, irradiated MDSC. T cell proliferation was determined after 72 h culture by pulsing with [3H]thymidine (1 μCi/ well) during the final 12 h of culture. Cultures were harvested using a cell harvester and thymidine incorporation was measured in a beta counter (Perkin Elmer).

Peritoneal neutrophils

WT mice were injected with 1 mL of 3% thioglycollate broth (TGB) and peritoneal exudates were harvested after 4 h. This mixture of cells predominantly contain $CD11b^{+(high)}Gr\text{-}1^{+(high)}$ neutrophils (approximately 65-70%). The $CD11b^{+}Gr\text{-}1^{+}$ cells were enriched by sorting on FACS Aria to >96% purity and used as neutrophil controls.

Induction of hepatitis and adoptive transfer with MDSC

ConA was dissolved in pyrogen-free PBS at a concentration of 2.5 mg/ml and injected in to C57BL/6 mice intravenously at a dose of 12.5 mg/kg body weight to induce hepatitis. MDSC harvested from the peritoneum of THC-injected mice were purified by magnetic sorting using PE-conjugated Gr-1 Ab and anti-PE microbeads (Miltenyi Biotech). For adoptive transfer, mice received 5 million purified MDSC *i.v.*, 12 h before injecting ConA. Sixteen hours after ConA administration, blood was collected by retro-orbital bleeding. Hepatitis was assessed by measuring liver enzyme alanine aminotransferase (ALT) in sera.

Cytokine analysis

Sera from individual mice were obtained 6, 12, and 24 h after injecting with vehicle or THC and stored below -20°C until analysis. IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9,

IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, IFN-γ, TNF-α, MIP-1α, MIP-1β, RANTES, MCP-1, GM-CSF, G-CSF, KC and eotaxin concentrations in serum samples were determined using Bio-Plex chemiluminescence assay system (Bio-Rad).

Enzyme-linked immunosorbent assay (ELISA)

Peritoneal exudates were collected by lavage from vehicle or THC injected (*i.p.*) mice using 1 ml of sterile PBS. Cells were spun down to obtain supernatants. Sandwich ELISAs for GM-CSF (BioLegend) and G-CSF (Peprotetch) were performed, and the concentrations of cytokines in the samples were determined from standard curves after correcting for blank.

Arginase activity assay

Arginase activity in cell lysates was measured from the conversion of L-arginine to L-ornithine as described elsewhere [13]. Lysates of 2×10^6 peritoneal exudate cells from vehicle or THC-injected mice were prepared using cell lysis buffer containing protease inhibitor cocktail, and stored below -80°C until use. Arginase was activated by the addition of 10 mmol/L $MnCl_2$ (25 μL) to cell lysate (25 μL) and incubating at 55°C for 20 min. Carbonate buffer (150 μL, 100 mmol/L, pH 10) was then added along with 100 mmol/L L-arginine (50 μl) to initiate the reaction, and incubated at 37°C. Arg1 activity was stopped after 10 min by adding glacial acetic acid (750 μL). Ninhydrin reagent, 250 μL (2.5 gm of Ninhydrin + 40 mL of 6M phosphoric acid + 60 mL of glacial acetic acid) was added and tube were covered and boiled at 90-100°C for 1 h. Standards were created by using known amounts of L-ornithine and processed as above. Tubes were cooled and colored end point was measured using a spectrophotometer at 515 nm.

BrdU labeling *in vivo*

To study MDSC proliferation in vivo, mice were injected with BrdU (2 mg/mouse) 2 hours before injecting THC. Mice were maintained with drinking water containing 0.8 mg/mL BrdU After 24 h. Cells from the peritoneal cavity were harvested by gavage and stained for surface CD11b and Gr-1. After fixing and permeabilization, incorporated BrdU was stained by intranuclear staining protocol using DNAase I and FITC-conjugated anti-BrdU antibody (BD Biosci). Triple-stained cells were analyzed by flow cytometry on FC 500 (Beckman Coulter).

Propidium iodide (PI) staining

PI stating for cell cycle analysis was done by standard procedure. Briefly, cells were stained using anti-Gr-1 FITC to mark MDSC (typically >95% Gr-1^+ cells were $Gr1^+CD11b^+$ among vehicle and THC-induced cells in the peritoneum). Cells were fixed and resuspended in PI-reagent containing 0.1% Triton X-100, 0.2 mg/mL RNAase and 20μg/mL propidim iodide in PBS and incubated for 37°C for 15 min. Labeled cells were acquired on FC 500 flow cytometer (Beckman Coulter) using DNA analysis settings to gate out doublets and aggregates. DNA-PI signal on gated Gr-1^+ cells representing MDSC was analyzed using Multicycle software (Pheonix Systems).

Statistical analysis

In most experiments, we used groups of 3-6 mice, unless otherwise mentioned, that were analyzed individually and data depicted as mean ± SEM. The statistical comparisons between different treatment groups were done using Student's t test and differences with *P* value of <0.05 were considered to be significant. All experiments were repeated at least two times to test the reproducibility of results.

Acknowledgments

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Abbreviations

ALT alanine transaminase

B6	C57BL/6
BSA	body surface area normalization
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
HED	human equivalent dose
KC	keratinocyte chemoattractant
MDSC	myeloid-derived suppressor cell
MO-MDSC	monocyte like MDSC subset
PMN-MDSC	PMN-like MDSC subset
THC	Δ^9-tetrahydrocannabinol

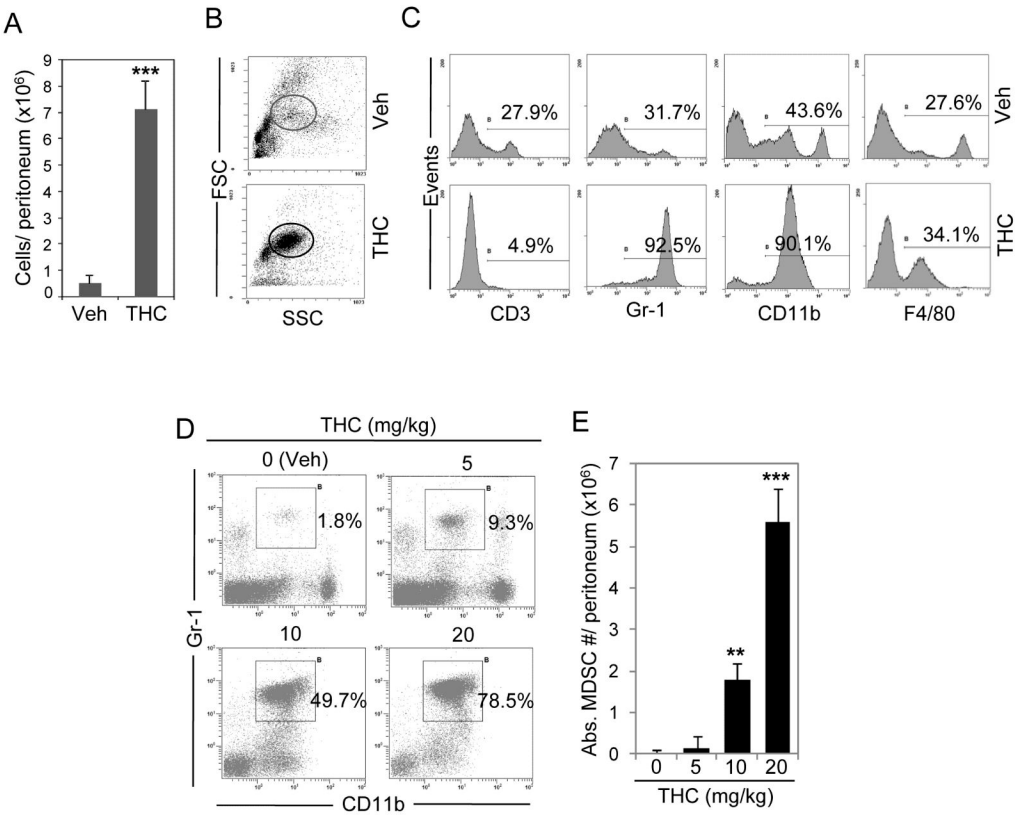

Figure 1.

THC induces CD11b^{+}Gr-1^{+} cells *in vivo*. (A) B6 WT mice were injected intraperitoneally with vehicle or THC (20 mg/kg). Total viable peritoneal exudate cells harvested after 16 h were quantified by trypan blue exclusion. ***P<0.001 versus vehicle control. Data are mean ± SEM (n = 6). Similar results were obtained in four independent experiments with 4-6 mice per group. (B) Cells were analyzed by flow cytometry. Representative dot plots showing forward scatter (FSC) and side scatter (SSC) analysis for granularity and size by flow cytometry. Oval gate represents larger and more granular cells. (C) Phenotypic characterization. Cells were stained for various cell surface markers using fluorescently labeled mAb and analyzed by flow cytometry. Representative histograms from 3 mice per group show percentage of positive cells for each antigen. Results represent similar observations from 2 independent experiments with 3-5 mice per group. (D) WT mice were injected with various doses of THC and after 12 h peritoneal exudate cells were stained using fluorescently labeled mAb to CD11b and Gr-1 antigens for MDSC and analyzed by flow cytometry. Representative dot plots show percentage of MDSC (gated double-positive cells). (E) Absolute numbers of MDSC calculated based on the percentages and total cell numbers. **P<0.01 versus vehicle control. Data are mean ± SEM (n = 5). (D & E) Similar results were obtained in three independent experiments with 3-5 mice per group.

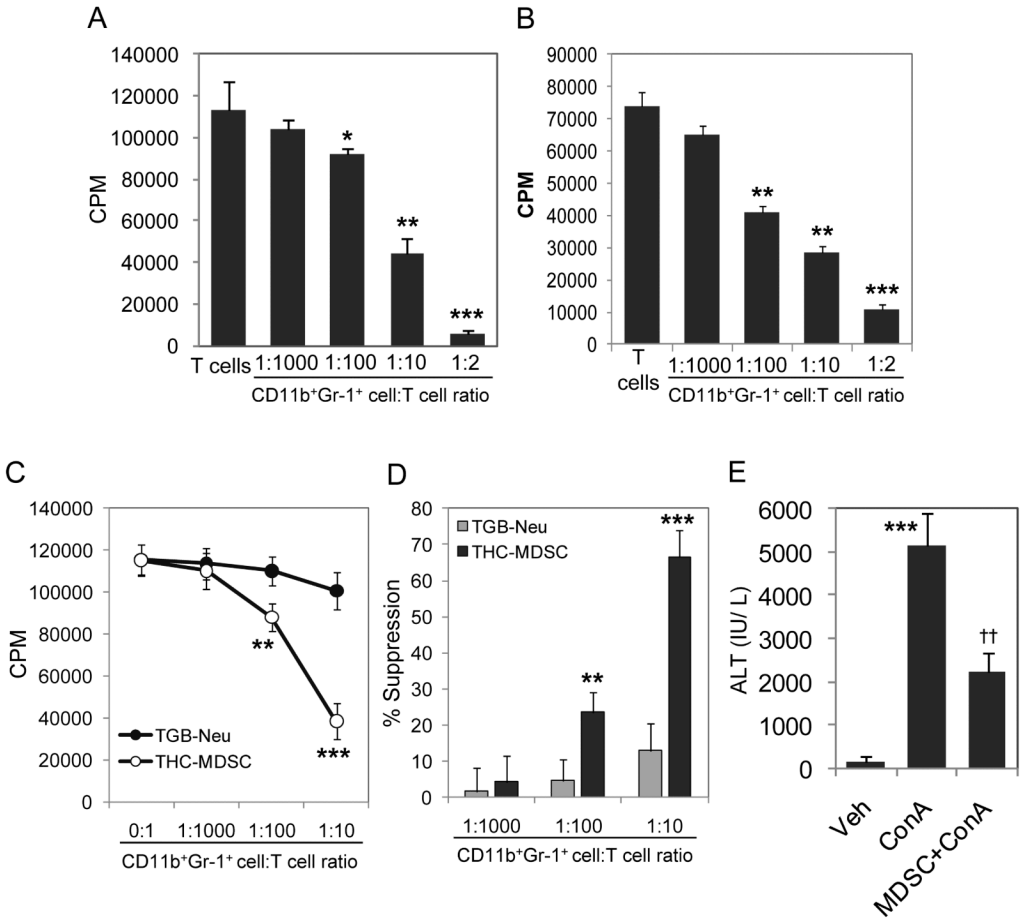

Figure 2.

THC-induced CD11b^{+}Gr-1^{+} cells are immunosuppressive *in vitro* and *in vivo*. *In vitro* T cell suppression determined by (A) co-culturing purified THC-induced MDSC with WT T cells stimulated using ConA or (B) OT-II T cells stimulated using ova-peptide in the presence of irradiated APC. T cell proliferation was measured by [3H]-thymidine incorporation (CPM, counts per minute) at 72 h. T cells stimulated in the absence of any MDSC served as positive control. (C) Sorted CD11b^{+}Gr-1^{+} cells from the peritoneum of THC or thioglycollate broth (TGB)-injected mice were used in T cell proliferation assay stimulated using ConA. (D) Percentage T cell suppression for TGB-neutrophils and THC-MDSC. **P*<0.05, ***P*<0.01, ****P*<0.001 versus control for all above. Data are mean ± SEM of triplicate determinations and representative of two experiments. (E) Serum ALT levels indicative of T cell-induced hepatitis measured 16 h after ConA challenge in mice with or without adoptively transferred purified THC-induced peritoneal MDSC (5 × 10^{6}/ mouse). ****P*<0.001 versus vehicle; ††*P*<0.01 versus ConA. Data are mean ± SEM (n = 4). Two independent experiments with 3-4 mice per group showed similar results.

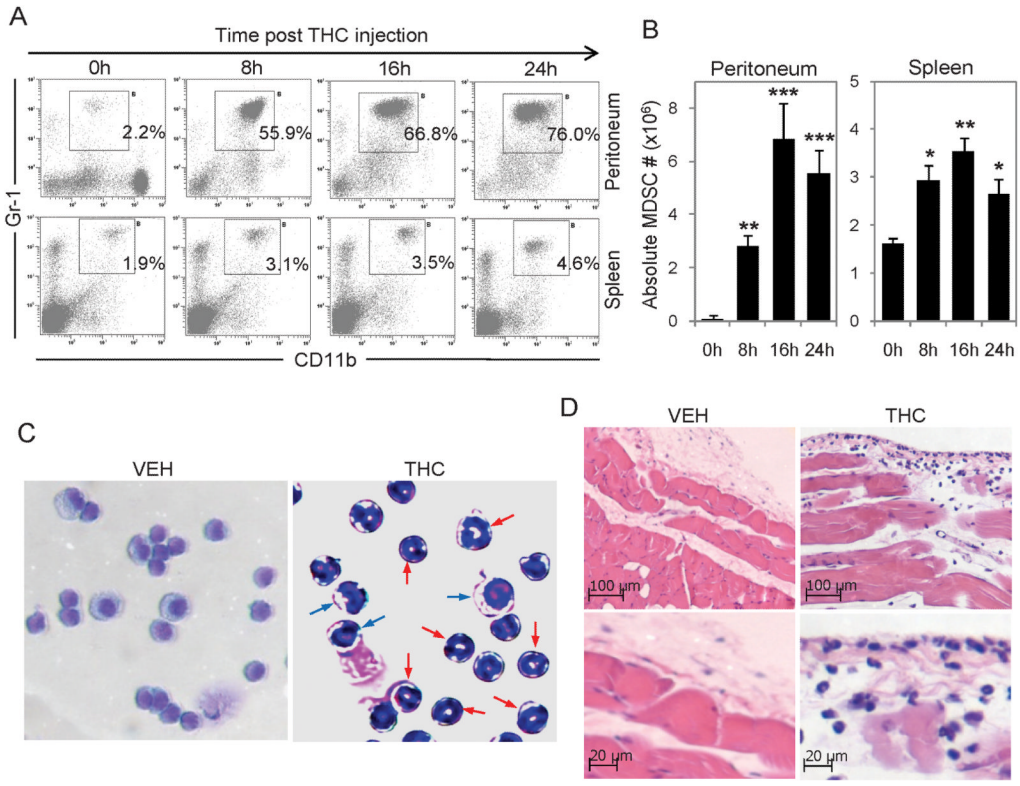

Figure 3.

Time course of THC-induced MDSC accumulation in peritoneum and spleen. Peritoneal exudates cells and splenocytes harvested at indicated time points after the administration of THC in WT mice (*i.p.*, 20 mg/kg) were analyzed for $CD11b^{+}Gr\text{-}1^{+}$ MDSC by flow cytometry. (A) Representative dot plots showing percentages of MDSC (gated). (B) Absolute MDSC cell numbers were calculated based on the percentages and total cell numbers. ***P<0.001, **P<0.01, *P<0.05 versus control (0 h). Data are mean ± SEM (n = 5) and representative of two separate experiments. (C) Morphological analysis. Representative photomicrographs (40× objective) of Wright-Giemsa stained cytospin preparations of peritoneal exudate cells harvested after 16 h from mice injected with vehicle or THC. MDSC induced by THC consist of PMN-like cells with characteristic circular nuclei (red arrows) and monocyte-type cells (blue arrows). (D) Representative H & E stained corss-sections of parietal peritoneum from mice injected with vehicle or THC showing significant cellular infiltrates in THC-injected mice. Similar results were obtained in two experiments with 3 mice per group (C & D).

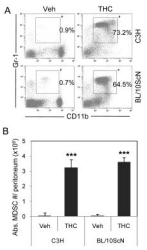

Figure 4.

THC induces MDSC in TLR4-deficient mice. To rule out the involvement of TLR4 ligands, we injected TLR4-deficient C3H and C57BL10/ScN mice with vehicle or THC (20 mg/kg, *i.p.*) and after 12 h, peritoneal exudate cells were harvested and stained and analysed for the co-expression of CD11b and Gr-1 by flow cytometry. (A) Representative dot plots showing percentage of gated MDSC. (B) Mean absolute number of MDSC ± SEM (n = 4) represent data from one out of two independent experiments. *** $P<0.001$ versus vehicle control.

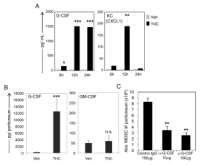

Figure 5.

Role of G-CSF in the induction of MDSC by THC *in vivo*. (A) WT mice were injected with vehicle or THC (20 mg/kg), blood was collected at 6, 12 and 24 h, and serum chemokines G-CSF and KC were determined by Bioplex assay. **P*<0.05, ***P*<0.01, ****P*<0.001 versus vehicle control. Data are mean ± SEM (n = 5) and represent one out of two independent experiments with similar results. (B) Detection of G-CSF and GM-CSF in the peritoneal exudates by sandwich ELISA. ****P*<0.001 and n.s. (not significant) versus vehicle control. Data are mean ± SEM (n=3) and representative of two separate experiments. (C) Antibody to G-CSF blocks the accumulation MDSC in response to THC. Absolute numbers of $CD11b^{+}Gr\text{-}1^{+}$ MDSC in the peritoneum 16 h after injecting THC in mice pretreated with control IgG or anti-G-CSF Ab. ***P*<0.01 versus IgG control. Data are mean ± SEM (n = 3). Two experiments with 3 mice per group showed similar results.

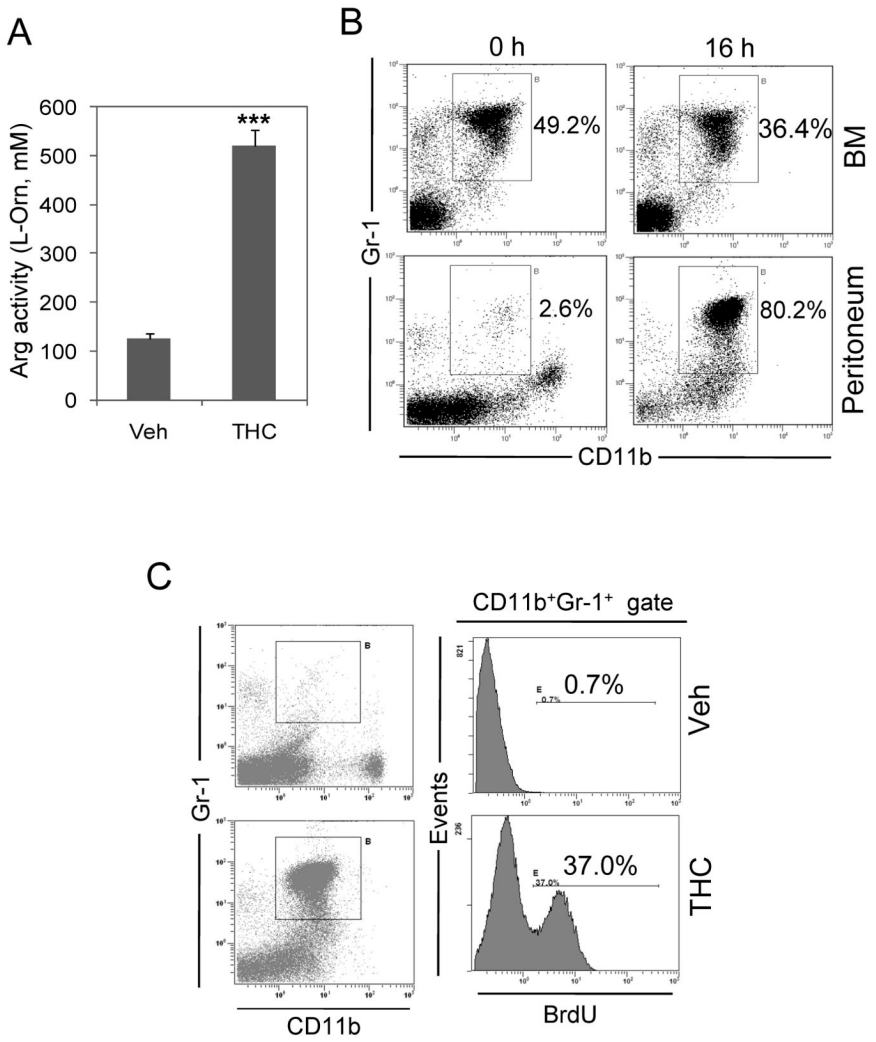

Figure 6.

(A) THC-induced MDSC express functional arginase. Arginase activity in the lysates of peritoneal exudate cells from vehicle or THC-injected WT mice determined on the basis of conversion of L-arginine to L-ornithine as described in the *Materials and methods* section. ***P<0.001 versus vehicle control. Data are mean ± SEM (n = 3) mice per group, and two experiments were performed. Each measurement was performed in triplicates. (B) $CD11b^+Gr\text{-}1^+$ cells migrate from bone marrow in response to THC. Representative dot plots of peritoneal and bone marrow cells harvested 0 or 16 h after THC treatment showing percentage of $CD11b^+Gr\text{-}1^+$ cells (gated), indicate possible migration of MDSC from bone marrow. Data are representative of two independent experiments with 3 mice per group. (C) THC-induced MDSC proliferate in periphery. *In vivo* BrdU labeling showing proliferation of THC-induced MDSC in peritoneum at 24 h. Representative histograms on right show percentage of $BrdU^+$ cells among gated $CD11b^+Gr\text{-}1^+$ MDSC shown on left. Two experiments with 3 mice per group showed comparable results.

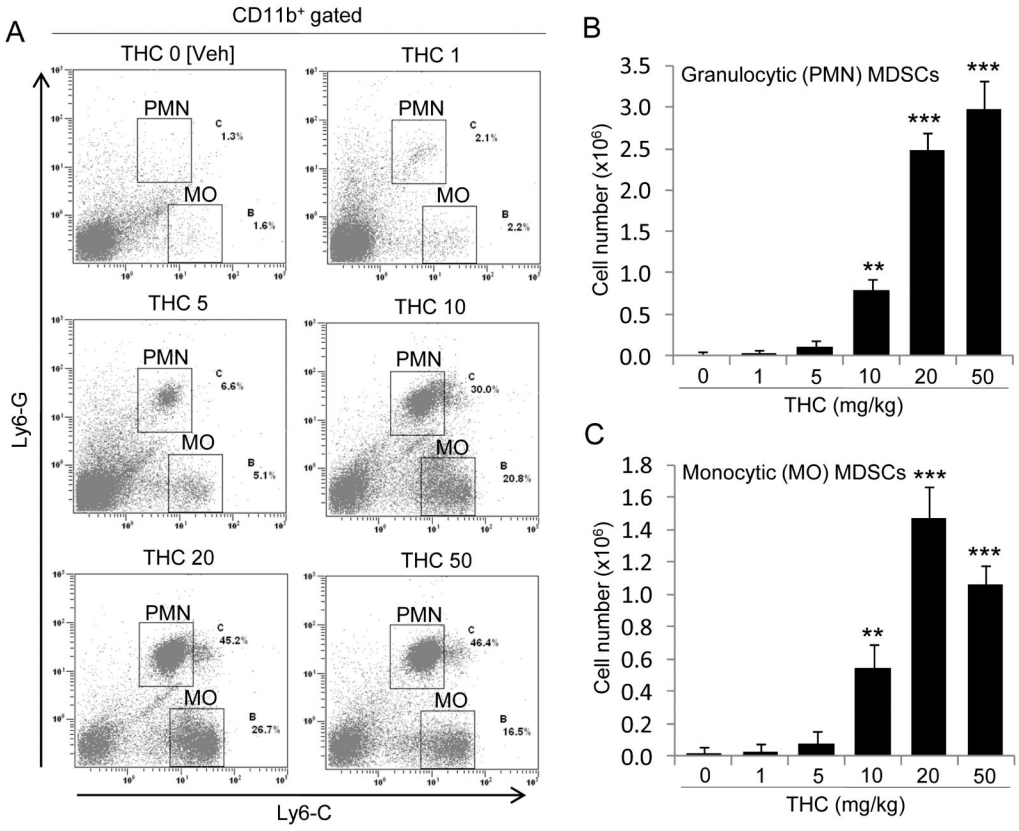

Figure 7.

Analysis of MDSC subsets induced by THC. To determine the granulocytic ($CD11b^{+}Ly6\text{-}G^{high}Ly6\text{-}C^{low}$) and monocytic ($CD11b^{+}Ly6\text{-}G^{-}Ly6\text{-}C^{high}$) subsets of MDSC, WT mice were injected with various doses of THC, peritoneal exudates cells were harvested after 12 h, triple stained for CD11b, Ly6-G and Ly6-C markers and analyzed by flow cytometry. (A) Ly6-G and Ly6-C expression on cells gated for $CD11b^{+}$ showing $CD11b^{+}Ly6\text{-}G^{+}Ly6C^{+(int)}$ granulocytic (PMN) and $CD11b^{+}Ly6\text{-}G^{neg}Ly6C^{+(high)}$ monocytic subsets. Representative dot plots show the percentages for granulocytic (PMN) and monocytic (MO) populations for various doses of THC. (B, C) Absolute cell numbers of each subset of MDSC or different doses of THC. $^{**}P<0.01$, $^{***}P<0.01$ versus control (THC, 0 mg/kg). Data are mean ± SEM (n = 5) and represent one out of two independent experiments.

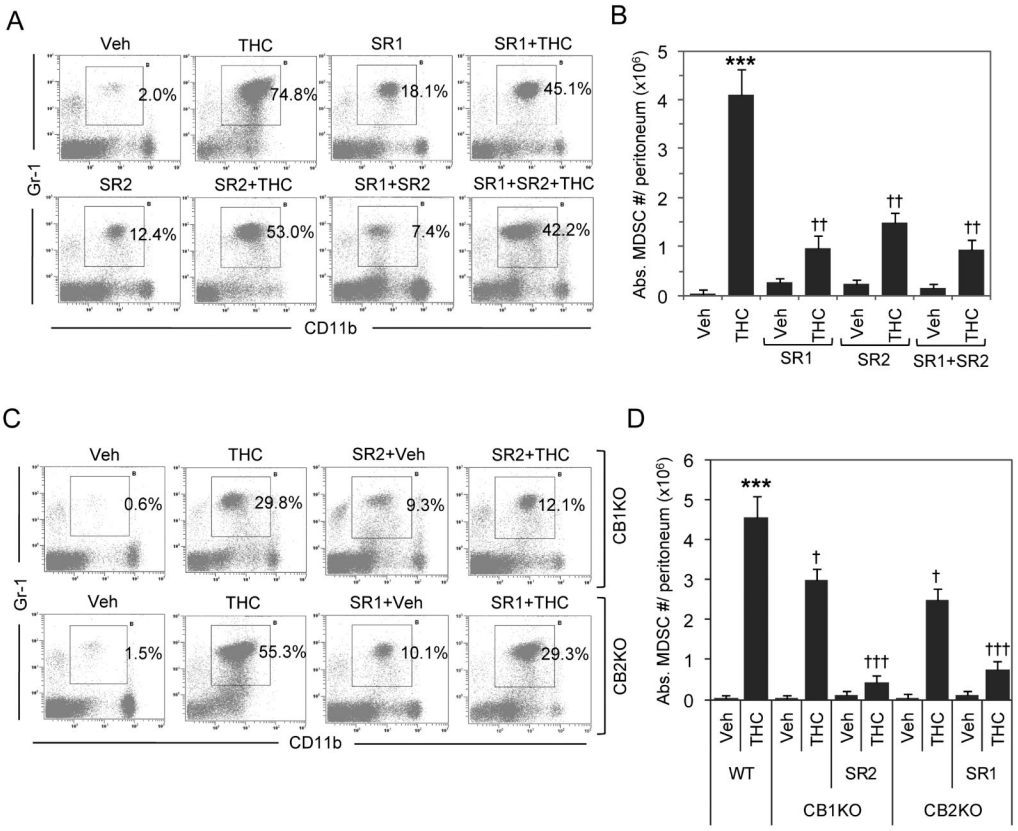

Figure 8.

Role of cannabinoid receptors in induction of MDSC by THC. WT mice were injected with CB1 (SR1) or CB2 antagonist (SR2) or both, two hours prior to injecting vehicle or THC (20 mg/kg). After 12 h, peritoneal exudates cells were harvested and stained for MDSC (CD11b and Gr-1) and analyzed by flow cytometry. (A) Representative dot plots showing percentage of MDSC. (B) Corresponding absolute MDSC numbers are shown. (C) WT, CB1KO or CB2KO mice were injected with vehicle or THC. Some CB1KO or CB2KO mice also received CB2 antagonist (SR2) or CB1 antagonist (SR1) respectively, two hours before THC injection. Representative dot plots showing percentage of MDSC. (D) Absolute MDSC cell numbers in the peritoneum at 12 h. ***$P<0.001$ versus WT-vehicle; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$, $^{\dagger\dagger\dagger}P<0.001$ versus WT-THC. Data are mean ± SEM (n = 3). Similar results were obtained in two experiments with 3 mice per group.