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Δ^9-Tetrahydrocannabinol attenuates allogeneic host-versus-graft response and delays skin graft rejection through activation of cannabinoid receptor 1 and induction of myeloid-derived suppressor cells

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RECEIVED JANUARY 1, 2015; REVISED APRIL 30, 2015; ACCEPTED MAY 6, 2015. DOI: 10.1189/jlb.3A0115-030RR

ABSTRACT

Immune cells have been shown to express cannabinoid receptors and to produce endogenous ligands. Moreover, activation of cannabinoid receptors on immune cells has been shown to trigger potent immunosuppression. Despite such studies, the role of cannabinoids in transplantation, specifically to prevent allograft rejection, has not, to our knowledge, been investigated previously. In the current study, we tested the effect of THC on the suppression of HvGD as well as rejection of skin allografts. To this end, we studied HvGD by injecting H-2^k splenocytes into H-2^b mice and analyzing the immune response in the draining ingLNs. THC treatment significantly reduced T cell proliferation and activation in draining LNs of the recipient mice and decreased early stage rejection-indicator cytokines, including IL-2 and IFN-γ. THC treatment also increased the allogeneic skin graft survival. THC treatment in HvGD mice led to induction of MDSCs. Using MDSC depletion studies as well as adoptive transfer experiments, we found that THC-induced MDSCs were necessary for attenuation of HvGD. Additionally, using pharmacological inhibitors of CB1 and CB2 receptors and CB1 and CB2 knockout mice, we found that THC was working preferentially through CB1. Together, our research shows, for the first time to our knowledge, that targeting cannabinoid receptors may provide a novel treatment modality to attenuate HvGD and prevent allograft rejection.
***J. Leukoc. Biol.* 98: 435–447; 2015.**

Introduction

HvGD, perhaps better known as transplant rejection, is primarily a T cell-driven process involving recognition of alloantigens that leads to rejection of allograft cells or tissue. With immunosuppressive drugs, the success rate of allogeneic transplants has steadily increased over the years. However, the chronic use of these drugs often results in major complications for the graft recipient. The 4 major categories of immunosuppressive drugs used today include corticosteroids, calcineurin inhibitors, antiproliferatives, and mTOR inhibitors. These antirejection treatments focus largely on halting alloreactive T cell activation or proliferation in the host. By causing global inhibition of T cells, the host's innate immune system becomes severely impaired [1–5]. For instance, cyclosporine, a calcineurin inhibitor, can cause immunotoxicity through the destruction of liver tissue [1, 6]. In addition, antiproliferatives, such as MMF and mTOR inhibitors, cause leukopenia and can delay the functionality of the graft [2, 3]. Furthermore, use of any severe immunesuppressive drug is associated with an increased risk of infection, bacterial or viral, and cancer. Specifically, treatment of allograft rejection, with current drug options, can result in lymphomas as well as accelerated hepatitis infections [7, 8]. Such severe drug-related issues have necessitated the continued search for an HvGD treatment with a better cost-to-benefit ratio.

THC is one of many cannabinoids derived from the *Cannabis sativa* plant, which was first described in a 1964 paper by Gaoni and Mechoulam [9]. THC is a known ligand for CB1 and CB2, which were discovered in the 1990s [10, 11]. CB1 and CB2 are G-protein-coupled receptors that are expressed both in the CNS and in the periphery, including the immune system [12–15]. Upon activation, CB1 and CB2 receptors modulate adenylate cyclase and both calcium and potassium channels, reduce T cell proliferation, and have been associated with regulation of the cytokines leading to a shift from a proinflammatory Th1 to an anti-inflammatory Th2 response [16–19]. Although originally believed to be specific to the CNS, the CB1 receptor has since

Abbreviations: BL6 = C57BL/6 mice (wild-type), C_3H = C_3H/HeJ mice, CB = cannabinoid receptor, CD11b = cluster of differentiation molecule 11B, F1 = B6D2F1/J mice, FasL = Fas ligand, HvGD = host vs. graft disease (transplant rejection), ingLN = inguinal lymph node, KO = knockout, LN = lymph node, MDSC = myeloid-derived suppressor cell, MMF = mycophenolate, mTOR = mammalian target of rapamycin, THC = Δ^9-tetrahydrocannabinol

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been found in peripheral immune cells and is highly up-regulated upon T cell activation [14, 20].

The anti-inflammatory properties of THC have been very well characterized by our laboratory and others [21–23]. Recently, we made an exciting observation that administration of THC results in massive induction of MDSCs [24]. MDSCs are innate regulatory cells known to reduce T cell-driven inflammatory responses in cancer models [25]. In mice, MDSCs are defined as T cell-suppressive, immature cells of myeloid origin, positive for the cell-surface markers CD11b and Gr1 [25]. The heterogeneous population of progenitor and immature cells, which make up MDSCs, reduce inflammation by producing Arg-1, iNOS, or both [26]. Although the cell-surface markers of MDSCs are expressed in other immune cells, such as neutrophils, the method of T cell suppression differs in these 2 cell subsets. Unlike MDSCs, which primarily use L-arginine depletion, the small proportions of neutrophils which are suppressive preferentially use reactive oxygen species to suppress T cell proliferation [27, 28].

Currently, THC, under the brand name Marinol (dronabinol; Unimed Pharmaceuticals, Buffalo Gove, IL, USA), has been approved for medicinal use. Marinol has been used to alleviate pain and nausea associated with cancer treatments, to stimulate appetite in those with wasting diseases, such as HIV/AIDS, and to relieve spasticity in patients with multiple sclerosis patients [29]. Furthermore, our laboratory recently found that THC treatment significantly reduced symptoms associated with GvHD, where the immune cells from the allograft attack recipient tissue, in a CB-dependent manner [30]. Based on such studies, we hypothesized that cannabinoids may have the potential to be used in transplantation [31]. To our knowledge, THC has not been directly tested against allograft rejection in vivo. Because immune cells express CBs and produce endocannabinoids, studies focused on addressing the role of CB-ligand system may offer novel insights into their mechanism of action in enhancing the survival of an allograft.

In this study, we found that THC treatment reduced inflammation associated with HvGD and caused significant increase in the survival of allogeneic skin allografts. These effects of THC were primarily mediated by its ability to induce immunosuppressive MDSCs. The current study suggests a role for the cannabinoid system in the regulation of transplantation immunity and treatment.

MATERIALS AND METHODS

Mice

Female C57BL/6 (H-2^b wild-type, BL6) mice, aged 6–8 wk, with an average weight of 20 g, were obtained from the National Institutes of Health (NIH) National Cancer Institute (Frederick, MD, USA) and used as recipients. Female C_3H/HeJ (H-2^k, C_3H) mice from The Jackson Laboratory (Bar Harbor, ME, USA) were used as donors. CB1 $KO^{-/-}$ mice (H-2^b C57BL/6 background) were a gift from Dr. James Pickel (NIH National Institute of Mental Health Transgenic Core Facility, Bethesda, MD, USA). CB2 $KO^{-/-}$ mice (H-2^b C57BL/6 background) were obtained from The Jackson Laboratory. The CB1 and CB2 KO mice were used at 6 wk of age with an average weight of 20 g. B6D2F1/J (F1 H-$2^{d/b}$) mice, aged at least 8 wk with an average weight of 20 g, were obtained from The Jackson Laboratory and were also used as recipient mice. All mice were housed in pathogen-free conditions and allowed ad libitum access to filtered water and Teklad rodent diet 8604 (normal chow; Harlan Laboratories, Indianapolis, IN, USA) at the Association for Assessment and Accreditation of Laboratory Animal Care (Frederick, MD, USA)-accredited Animal Research Facility located at the University of South Carolina School of Medicine (Columbia, SC, USA). All experiments were conducted under an approved Institutional Animal Care and Use Committee animal protocol.

Treatment with THC and induction of acute HvGD

THC (procured from NIH National Institute on Drug Abuse-National Institutes of Health, Bethesda, MD, USA) dissolved in ethanol was diluted in 1× PBS to a concentration of 20 mg/kg. THC or a vehicle (ethanol diluted in 1× PBS) was administered intraperitoneally at a volume of 0.1 ml/mouse 2 h before allogeneic cell injection. To induce HvGD, we used a modified localized HvGD model developed by the Laboratory of Immunology at the NIH National Institutes of Allergy and Infectious Diseases (Bethesda, MD, USA) for footpad injection of allogenic (stimulatory) cells [32], which has also been used in other studies [33–35]. In brief, acute HvGD was induced by injecting 2.2×10^7 C_3H spleen cells subcutaneously. These cells were split, with one half injected into each of the right and left hind flanks, of BL6 recipient mice. The ingLNs were harvested daily for 5 d to study the localized HvGD, following MLR kinetics [36]. THC treatment was continued daily until the termination of the study (d 5). Likewise, CB antagonist SR141716 [5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide; CB1 antagonist], SR144528 [*N*-[(1*S*)-*endo*-1,3,3-trimethylbicyclo [2.2.1]heptan2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1*H*-pyrazole-3-carboxamide; CB2 antagonist], and AM-630 [6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methanone; CB2 antagonist] were dissolved in DMSO containing 20 μl Tween 80 (Sigma-Aldrich, St. Louis, MO, USA) and then diluted in 1× PBS to a concentration of 20 mg/kg. The CB antagonist vehicle consisted of DMSO with 20 μl Tween 80 diluted in 1× PBS. Receptor antagonists were administered via intraperitoneal injection at a volume of 0.1 ml/mouse 1 h before each THC treatment. Vehicle control groups received ethanol or DMSO diluted in 1× PBS to a similar concentration as that of the treatment groups.

Monoclonal antibodies, reagents, and flow cytometer

Antibodies used for flow cytometric analysis (BioLegend, San Diego, CA, USA) included Fc block (clone 93), FITC conjugated anti-H-2^b (clone KH95), PE-conjugated anti-H-2^k (clone 15-5-5), Alexa Fluor 488-conjugated anti-CD3e (clone 145-2C11), PE/Cy7-conjugated anti-CD4 (clone GK1.5), PE-conjugated anti-CD8a (clone 53-6.7), PE/Cy7-conjugated anti-CD25 (clone PC61), PE/Cy5-conjugated anti-CD28 (clone 37.51), PE/Cy5-conjugated anti-CD44 (clone IM7), PE-conjugated anti-CD69 (clone H1.2F3), PE-conjugated anti-Gr1 (clone RB6-8C5), and FITC conjugated anti-CD11b (clone M1/70). HvGD ingLN and peritoneal exudates were assessed by flow cytometric analysis. Briefly, ingLN cells (10^6 cells in 25 μl) from BL6-recipient mice were incubated with Fc receptor antibodies (5–10 min) and incubated with conjugated antibodies (20–30 min at 4°C). After incubation with conjugated antibodies, cells were washed twice with 1× PBS/2% FBS buffer. The stained cells were then assessed by flow cytometer (FC500; Beckman Coulter, Brea, CA, USA), and the resulting data were analyzed by Cytomics CXP software (Beckman Coulter). All flow-cytometer plots were gated on live cells only. Two-color flow-cytometric analysis was performed for assessing the persistence of donor H-2^k cells in the recipient ingLN, spleen, and peritoneal exudates. Three-color flow-cytometric analysis was used to profile the ingLN response to disease and treatment. Two-color flow-cytometric analysis was used to look for MDSC populations in the peritoneal exudate.

Cytokine and chemokine analysis

The Sandwich ELISA MAX kits (BioLegend) were used to assess the level of IFN-γ, TNF-α, MCP-1, IL-2, and IL-6 cytokines. The Sandwich ELISA kit for G-CSF (Abcam, Cambridge, United Kingdom) was also used. Cells from the ingLNs (5×10^6 cells/ml) from HvGD mice were cultured in 0.2 ml aliquots in 96-well, round-bottom, tissue-culture plates for 16–20 h. To assess MCP-1 and G-CSF levels in the peritoneal exudate, a 1 ml 1× PBS lavage was collected, as described previously [37]. Cytokine production was quantified from cell supernatants (stored at −20°C). Absorbance was measured at 450 nm using

a Wallac 1420 Victor2 Multilabel Counter (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA).

Thymidine analysis of ex vivo proliferation from allograft restimulation

LN cells from HvGD BL6 mice, collected 10 d after treatment with an THC injection, were cultured in triplicate (0.2 mL/well in a round-bottom, 96-well plate) with mitomycin C-inactivated C_3H spleen cells, for 4 d. Sixteen hours before collection and analysis, [^{3}H]thymidine (2 μCi/well) was added to the cell cultures. The radioactivity was measured using a liquid-scintillation counter (MicroBeta Trilux; PerkinElmer Life and Analytical Sciences).

MDSC isolation

We have referred to $Gr1^+CD11b^+$ cells as *MDSCs*, although such a phenotype is also seen on other cells, including neutrophils. The confirmation that this phenotype ($Gr1^+CD11b^+$) represents MDSCs comes from their ability to suppress T cell proliferation as shown in the "Results" section. We have indicated the use of purified $Gr1^+CD11b^+$ MDSCs in such instances in which the cells have been enriched via positive selection.

Sixteen hours after THC injection, mice were euthanized, and the peritoneal exudate was collected [24]. In brief, the peritoneal cavity was washed 3 times with ice-cold 1× PBS (5 ml/wash) for 5 min with agitation to recover cells. The cells were resuspended in 1 ml, treated with Fc block for 10 min, and labeled with PE-conjugated anti-Gr1. The EasySep-positive PE selection kit (STEMCALL Technologies, Vancouver, BC, Canada) procedure was followed to isolate $Gr1^+$ cells, as described previously [38]. After isolation, cells were labeled with FITC-conjugated anti-CD11b and assessed for purity using flow-cytometric analysis.

MDSC depletion assay

MDSCs were depleted by the intraperitoneal injection of Ly-6G/Ly-6C-purified Ab (clone RB6-8C5), as described [39]. Depletion Ab was given 3 h after every other THC treatment in doses of 0.1 and 0.05 mg/mouse. Control mice received isotype-matched control antibodies. Mice were euthanized at the end of the 5 d model as outlined previously. Peritoneal exudates were assessed for the presence of MDSCs and H-2^k cells, and ingLN were assessed for cellularity and T cell activation.

MDSC adoptive transfer

Isolated THC induced MDSCs (5×10^6), >95% pure, as described earlier [40], were transferred into BL6 HvGD mice via an intraperitoneal injection 30 min after subcutaneous injection of graft cells. Mice were euthanized 18 or 90 h after graft injection. Peritoneal exudates were assessed for the presence of MDSCs and MDSC chemokines, MCP-1 and G-CSF, and ingLNs were assessed for cellularity and T cell populations.

Dual skin allograft transplant

We performed dual skin grafts on each recipient mouse; one allograft (right graft bed) and one autograft (left graft bed). First, graft skin was obtained from the tail of a C_3H donor mouse and transferred into the recipient mice, either BL6 or F1. Second, tail-skin grafts from autologous donor mice were transplanted on to the recipient mice, either BL6 donor to BL6 recipient or F1 donor to F1 recipient. Recipient mice were treated with THC, or the vehicle, 2 h before the skin-graft procedure. Mice were kept in protective casts for 9 d following the graft procedure [41]. Grafts were assessed for rejection starting on d 9 after graft placement. Treatment of either THC or the vehicle was given intraperitoneally every other day for 14 d, and after that point, all treatments were stopped. For assessment of CB1 involvement, the pharmacological antagonist SR141716A (Tocris Bioscience, Bristol, United Kingdom) was given (20 mg/kg, i.p.) 1 h before each THC treatment. Mice were scored as +/+, accepted the graft; +/−, partially rejected the graft (<50% scabbed over or less than a halving in graft size); or −/−, fully rejected the graft. For depicting graft survival +/+ and +/− skin grafts were considered viable, and −/− skin grafts were nonviable.

Statistical analysis

HvGD experiments ($n \geq 4$ mice per treatment group) were repeated 2 or more times to ensure repeatability. Skin-graft experiments were run in triplicate ($n > 7$ mice was used for statistical analysis). Data are shown as means ± SEM. Student's t test was used to compare data between 2 groups. One-way ANOVA with a Tukey post hoc test was used to compare 3 or more groups. A log-rank (Mantel-Cox) test was used to determine the significance of survival curves. Experimental groups were compared to the vehicle, $P < 0.05$ was considered significant.

RESULTS

THC treatment reduces lymphocyte proliferation in ingLNs

To determine whether THC could have a therapeutic role in allograft rejection, we modified a mouse model of acute HvGD with a strong and rapid, host immune response to the graft [32]. To ensure that our model of HvGD was optimized, we looked at cellularity in the draining ingLN at various time points (data not shown). We found that, by d 4, maximum absolute T cell counts had been reached; therefore, additional experiments were ended 4 d after allogenic cell injection. As such, the recipient mice (BL6 haplotype H-2^b) were given a bolus (2.2×10^7 cells) of graft (C_3H haplotype H-2^k) splenocytes subcutaneously 2 h after THC (20 mg/kg body weight) or vehicle treatment (ethanol diluted in 1× PBS). Treatment was administered intraperitoneally daily (**Fig. 1A**). Mice were assessed for symptoms of HvGD 4 d after graft injection, with T cell proliferation and Th1-associated cytokine secretion used as markers of disease induction. The draining ingLNs were collected and assessed for T cell proliferation. The data showed that total cellularity in the ingLN in HvGD mice was significantly decreased with THC treatment from the >5-fold increase observed in vehicle-treated HvGD mice (Fig. 1B). Flow-cytometric analysis revealed that T cell subset ($CD3^+CD4^+$ and $CD3^+CD8^+$) proportions were increased in vehicle-treated HvGD mice compared with naive mice (Fig. 1C and D). However, HvGD mice given the THC treatment showed a significant reduction in both T cell populations, bringing these T cell numbers back to naïve levels (Fig. 1C and D).

THC treatment suppresses secretion of proinflammatory cytokines

Previously, our laboratory group showed that THC treatment can reduce cytokines and chemokines associated with inflammatory disease models [42, 43]. Because HvGD is known to be accompanied by increases in Th1-associated cytokines, the effect of THC treatment on the proinflammatory environment in the ingLN of HvGD mice was assessed. Cytokines well known for their involvement in transplant rejection include IFN-γ and IL-2, which are typically expressed at high levels early after receipt of the graft; TNF-α, which increases as HvGD progresses; and IL-6, which has a role in graft tolerance by skewing the Th1/Th2 balance [44]. To this end, the ingLNs from THC- or vehicle-treated (ethanol diluted in 1× PBS) HvGD mice were collected 4 d after the injection of graft cells and cultured for 24 h to assess spontaneous cytokine secretion in the draining LN microenvironment. IL-2, a cytokine known

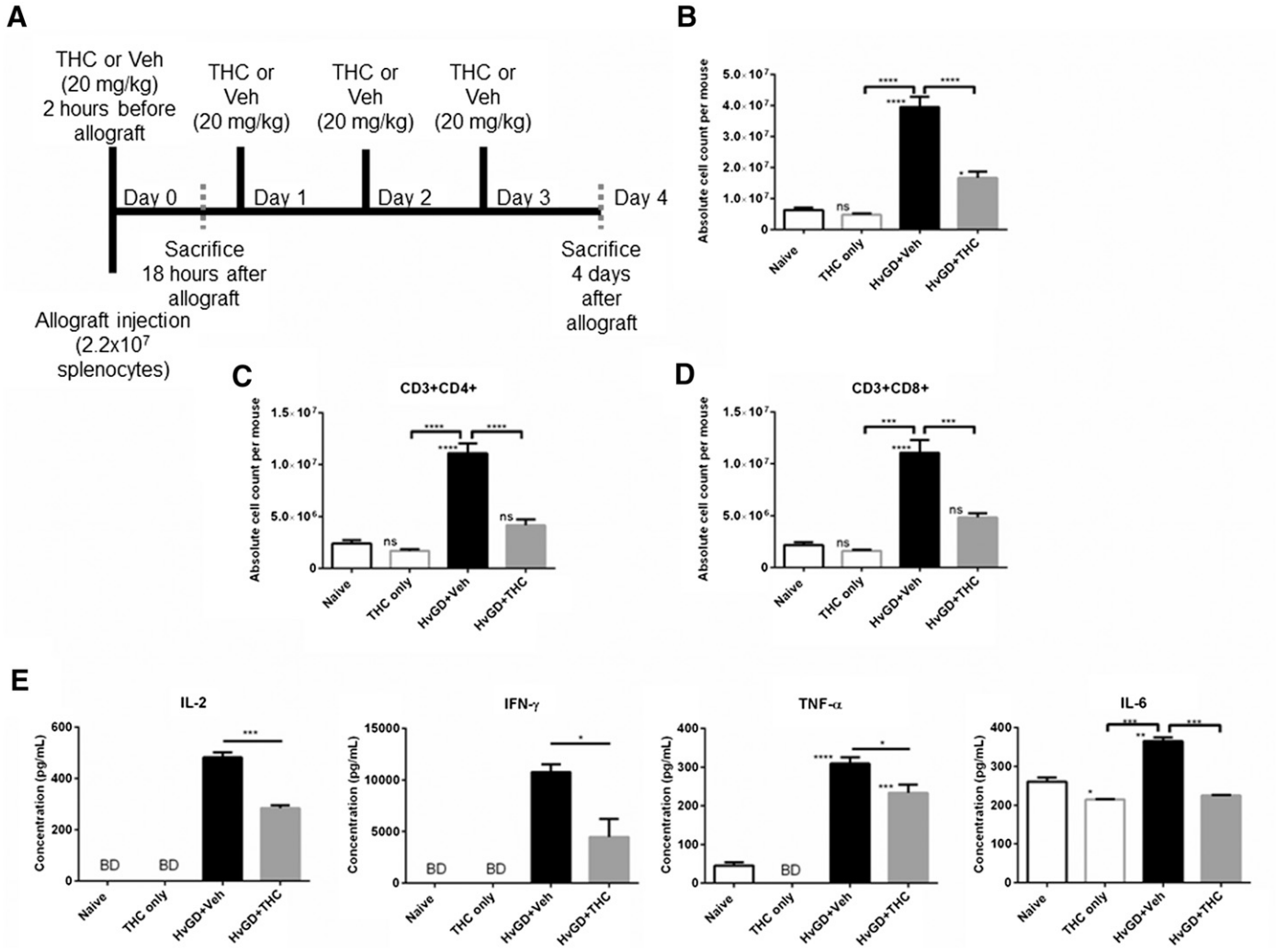

Figure 1. THC treatment ameliorates HvGD. C57BL/6 (BL6) mice were given THC or the vehicle (ethanol diluted in 1× PBS, $n = 5$ mice per group) 2 h before graft cells (C_3H 2.2×10^7 s.c.) and daily after graft injection until they are killed. On d 4 after allograft injection, T cell populations were assessed by harvesting draining ingLN and then triple-staining the cells with fluorochrome-labeled antibodies against CD3, CD4, and CD8 antigens. IngLN cells, collected on d 4 after allograft injection, were also cultured for 24 h, and supernatants harvested to assay the spontaneously produced Th1 cytokines. (A) Schematic of procedure and treatment regimen. (B) Absolute cell count in the ingLN. (C–D) Absolute number of $CD3^+CD4^+$ (C) and $CD3^+CD8^+$ (D) cells in the ingLN. (E) Spontaneous secretion of IL-2, IFN-γ, TNF-α, and IL-6. Representative data of 3 experiments are shown (means ± SEM). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0001$ Student's t test.

to increase T cell proliferation, and IFN-γ, which is associated with CTL killing, were elevated in vehicle-treated mice with HvGD but were reduced by nearly 50% upon THC treatment (Fig. 1E, left). Additionally, TNF-α and IL-6 (Fig. 1E) were overexpressed in vehicle-treated HvGD mice; however, a significant reduction was seen in THC-treated mice. To ensure that THC treatment was in fact targeting the HvGD T cell response, rather than ablating naïve T cell function, mice were treated with THC alone, as a control. Cell counts in the ingLN (Fig. 1B), absolute numbers of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells in the ingLN (Fig. 1C–D), and cytokines (Fig. 1 E) in mice treated with THC alone were not changed significantly compared with naïve mice. These data are consistent with the observation that even long-term administration of THC, over 12 mo, does not alter lymphocyte subsets proportions, proliferation, or T cell apoptosis in naïve male rhesus monkey [45]. Thus, in subsequent experiments, we primarily compared $HvGD^+$Veh versus $HvGD^+$THC groups.

IngLN activation is decreased by THC administration

One hallmark of HvGD is that a second transplant with the same haplotype disparity will be rejected by the host more rapidly than the initial graft. To determine whether THC could favorably affect this outcome of allograft rejection, HvGD mouse ingLNs were restimulated ex vivo. HvGD mice treated with either vehicle (ethanol diluted in 1× PBS) or THC were rested for 10 d, from the time of the last treatment injection, and then restimulated with C_3H splenocytes. Proliferation from restimulation was assessed using [^{3}H]thymidine incorporation. Mice given the vehicle treatment showed significantly increased rates of proliferation compared with THC-treated mice (**Fig. 2A**). Having found that ex vivo restimulation was decreased in THC-treated

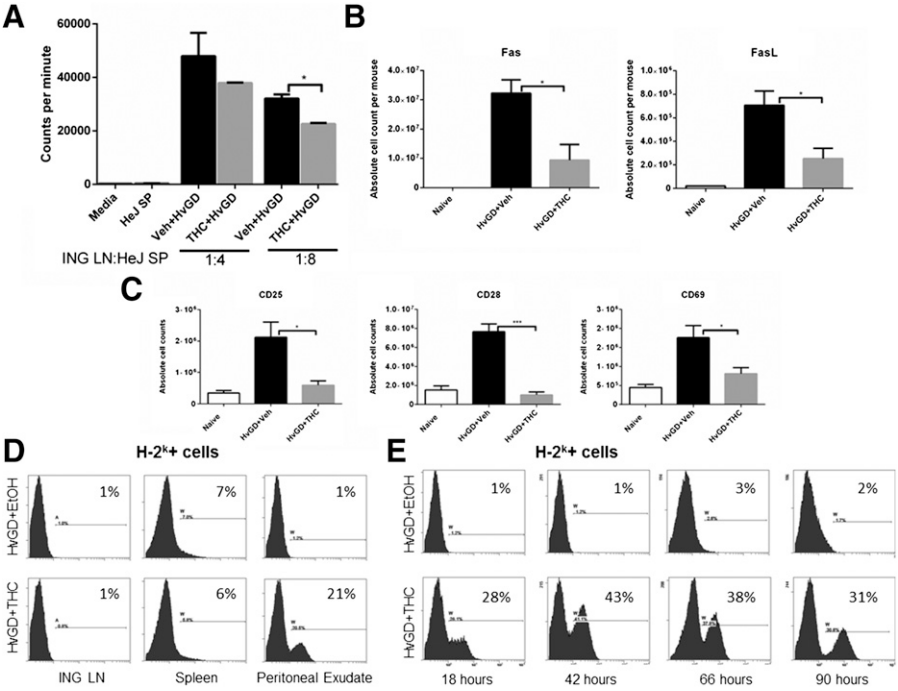

Figure 2. THC treatment reduces lymphocyte activation in the recipient mice and allows for allograft persistence. IngLNs were harvested from BL6 HvGD mice ($n = 5$ mice per group) 10 d after final THC treatment, d 14 after allograft injection. Next, ingLNs were harvested and restimulated in vitro with donor C_3H spleen cells (mitomycin C inactivated). Proliferation of ingLN cells was assessed 4 d after in vitro restimulation using [^{3}H] thymidine. IngLN activation status was determined 4 d after allograft injection by individually staining the cells for antibodies against CD25, CD28, and CD69 antigens. (A) C_3H restimulation in vehicle (ethanol diluted in 1× PBS) and THC-treated HvGD mice (data shown as means ± SEM). (B) Expression of Fas receptor and FasL on ingLN cells (data shown as means ± SEM). (C) T cell activation marker expression, CD25, CD28, and CD69 on ingLN cells (similar results seen in 2 replicate experiments). (D) H-2^k cell persistence in recipient mice. (E) H-2^k cell persistence in the peritoneal cavity throughout the 5 d (similar results seen in replicate experiments). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0001$ Student's t test.

HvGD mice, we next looked at the number of FasL-expressing cells in the ingLNs. We found that significantly fewer cells expressed FasL (Fig. 2B, upper) and that the number of active T cells expressing the Fas receptor was also significantly reduced (Fig. 2B lower) in THC-treated HvGD mice. Furthermore, T cell activation markers were analyzed to ascertain how THC affects proliferation rates after treatment. To this end, ingLNs were collected 4 d after graft-cell injection from both the vehicle- and THC-treated mice and were evaluated for cells positively expressing activation markers. Expression of CD25, which is found on memory T cells and is part of the IL-2 receptor; CD28, a necessary costimulatory molecule that interacts with CD80/86 on APCs; and CD69, an early activation marker, were assessed in HvGD ingLNs by flow cytometry. HvGD mice treated with THC had significantly fewer CD25$^+$, CD28$^+$, and CD69$^+$ cells in the ingLNs than did the vehicle-treated mice (Fig. 2C).

THC decreases the inflammatory response allowing for H-2^k graft cell persistence

In HvGD, the final step in graft rejection is apoptosis of the graft cells caused by CTLs and NK cells [46]. Because THC treatment caused a reduction in the number and proportion of CD3$^+$CD8$^+$ cells and FasL expression, H-2^{k+} cells were evaluated to determine whether treatment extended the life of the graft cells. The draining ingLNs, spleen, and peritoneal cavity were selected as possible sites of graft-cell persistence. Although no cells positive for H-2^k were found in the vehicle-treated (ethanol diluted in 1× PBS) HvGD mice (Fig. 2D), allograft cells were detected in the peritoneal cavity of THC-treated HvGD mice (Fig. 2D). The H-2^{k+} cells were measurable in the peritoneal cavity as early as 16 h after injection of the graft (Fig. 2D). To analyze whether daily THC treatments were sufficiently immunosuppressive to allow for continued persistence of the graft cells, the peritoneal exudate was monitored at 24 h intervals. Interestingly, H-2^{k+} cells persisted for the entire 90 h duration of THC treatment (Fig. 2E).

THC treatment induces suppressive MDSCs in HvGD mice

Our laboratory has shown that high levels of Gr1$^+$CD11b$^+$ MDSCs are induced by a single intraperitoneal injection of THC into naïve mice and that such cells are highly immunosuppressive [24]. THC-induced Gr1$^+$CD11b$^+$ MDSCs found in the peritoneal cavity have been shown to suppress T cell proliferation via the depletion of the amino acid L-arginine [24, 47, 48]. To test whether a THC injection in the current HvGD model also triggers Gr1$^+$CD11b$^+$ MDSCs, we harvested the cells from the peritoneal cavity, 18 h after graft injection and stained them for Gr1$^+$CD11b$^+$ expression. Although HvGD$^+$Veh (ethanol diluted in 1× PBS)-treated mice showed <3% MDSCs, equivalent to an absolute count around 0.04×10^6 cells and similar to naïve mice, HvGD$^+$THC-treated mice showed a marked increase in Gr1$^+$CD11b$^+$ MDSC, the proportion of which increased to 75%, or 3.2×10^6 cells, at 18 h and dropped to 46%, or 2.4×10^6 cells, at 90 h (**Fig. 3A**). As many immature myeloid cells express the cell surface markers Gr1 and CD11b, we looked at the T cell suppressive property of these double-positive cells to ensure that they were indeed MDSCs [49, 50]. To that end, we next looked at the Gr1$^+$CD11b$^+$ MDSCs from the peritoneum of THC-treated HvGD mice, isolated at 18 h, which were purified (>95% purity Gr1$^+$CD11b$^+$ cells) using positive selection based on expression of Gr1, as described earlier [40]. Isolated MDSCs were cocultured, at various concentrations, in an MLR with BL6 ingLNs as responders and C_3H spleen cells treated with mitomycin C, as stimulators (1:8 ratio). Proliferation of the BL6 ingLN cells was measured using thymidine incorporation.

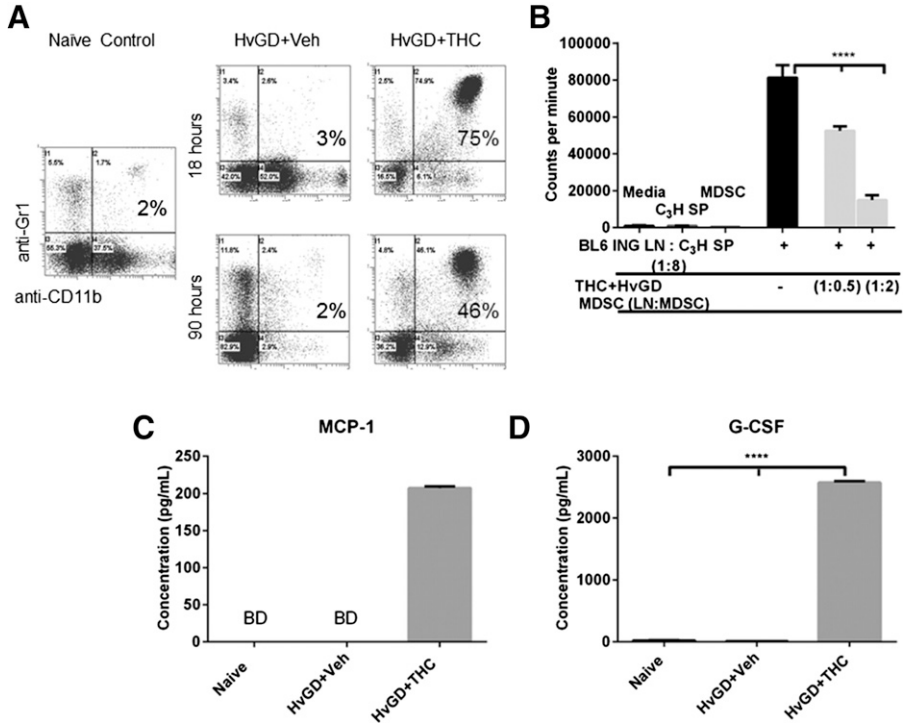

Figure 3. THC treatment induces immunosuppressive MDSCs. Cells were isolated from peritoneal exudates ($n = 5$ mice per group) either 18 or 90 h after graft injection, and stained for Gr-1 and CD11b stained to identify MDSCs, MDSCs were also isolated using positive selection PE-magnetic beads for Gr1. C_3H cells and MDSCs were mitomycin C-inactivated before plating with BL6 ingLN cells. Proliferation of ingLN cells was assessed 4 d after MLR stimulation using thymidine. (A) $Gr1^+CD11b^+$ MDSCs in the peritoneal cavity, at 18 and 90 h after graft cell injection (similar results seen in replicate experiments). (B) MDSC suppression of MLR against C_3H cells ($P < 0.01$, 1-way ANOVA and Tukey honestly significant difference post hoc test). (C–D) Peritoneal exudates were collected 18 h after graft injection (1 ml ice-cold 1× PBS) and assessed for MDSC-homing cytokines. (C) MCP-1 (CCL2) and (D) G-CSF. Representative data of replicate experiments are shown (means ± SEM). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0001$ ANOVA/Tukey.

$Gr1^+CD11b^+$ MDSCs (from here on denoted as *MDSCs*) significantly reduced T cell proliferation in a dose-dependent manner (Fig. 3B).

Because MDSC induction has been associated with inflammatory disease, we next set out to show that THC treatment, and not the disease model, was driving MDSC recruitment [51–53]. Previous studies from our laboratory demonstrated that CB ligand treatment increased the level of MCP-1 (CCL2), which was also associated with an increase in MDSCs at the tumor site [54, 55]. Additionally, we looked at G-CSF, which in our laboratory was previously shown to be elevated in THC-mediated MDSC induction [24]. To that end, the peritoneum exudate was collected, with a single lavage of 1 ml 1× PBS, and assessed for cytokine and chemokine levels. We found MCP-1 and G-CSF were elevated only in THC-treated HvGD mice (Fig. 3C and D, respectively).

THC induced MDSCs have an integral role in the amelioration of HvGD

To determine the importance of MDSCs in reducing overall HvGD symptoms, we used the anti-Ly-6G/Ly-6C purified Ab (RB6-8C5) to deplete $Gr1^+$ MDSCs. The depletion Ab was given 3 h after every other THC treatment in decreasing doses of 0.1 and 0.05 mg/mouse. The ingLN cellularity for $CD3^+CD4^+$ (**Fig. 4A**) and $CD3^+CD8^+$ (Fig. 4B) T cells was significantly increased in HvGD mice, which was decreased following treatment with THC and control Ab. Interestingly, $HvGD^+THC$ mice that received anti-Ly-6G/Ly-6C Ab showed reversal of the THC effect, resulting in increased numbers of T cells (Fig. 4A and B). Furthermore, the effect of MDSC depletion on T cell activation markers, including CD25, CD28, and CD44, was assessed. Upon treatment with the anti-Ly-6G/Ly-6C Ab, we saw a significant increase in cells positive for the CD25, CD28, and CD44 activation markers (Fig. 4C). To ensure that RB6-8C5 depleted the MDSCs, $Gr1^+CD11b^+$ cells in the peritoneal cavity were analyzed. RB6-8C5 caused a stark decrease in the MDSC proportion in THC-treated HvGD mice from 67% to about 11% (Fig. 4D). Finally, RB6-8C5 administration, along with THC treatment, resulted in a significant decrease in the number of $H\text{-}2^{k+}$ cells in the RB6-8C5 group, consistent with a decrease in allograft persistence (Fig. 4E).

Because MDSC depletion decreased the efficacy of the THC treatment in HvGD mice, we tested whether adoptive transfer of THC-induced MDSCs into allograft-recipient mice would suppress HvGD. MDSCs were isolated from THC-treated mice 18 h after treatment, as outlined previously. Isolated THC-induced MDSCs (5×10^6), at >95% purity, were then transferred into BL6 HvGD mice via an intraperitoneal injection 30 min after subcutaneous injection of graft cells. Efficacy of adoptive cell transfer in HvGD mice was assessed by ingLN cellularity and T cell populations 4 d after the injection of graft cells. We noted that transfer of MDSCs suppressed the HvGD, as indicated by the total number of cells (**Fig. 5A**) as well as the $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subsets (Fig. 5B), although to a lesser extent than for THC. Additionally, we looked at the peritoneal exudate for MDSC persistence as well as MDSC homing cytokine levels (18 h after graft cell injection). When the peritoneal exudate was enumerated for $Gr1^+CD11b^+$ cells in MDSC-transferred HvGD mice, we found that there were significantly reduced numbers of MDSCs in the peritoneal cavity compared with the THC-treated group (Fig. 5C). In corroboration with reduced overall cellularity within the peritoneum, the proportion of persisting $H\text{-}2^{k+}$ cells was reduced by 50% in MDSC-treated, compared with THC-treated, HvGD mice (data not shown). In addition, MCP-1 and G-CSF were undetectable in mice adoptively transferred with MDSCs when

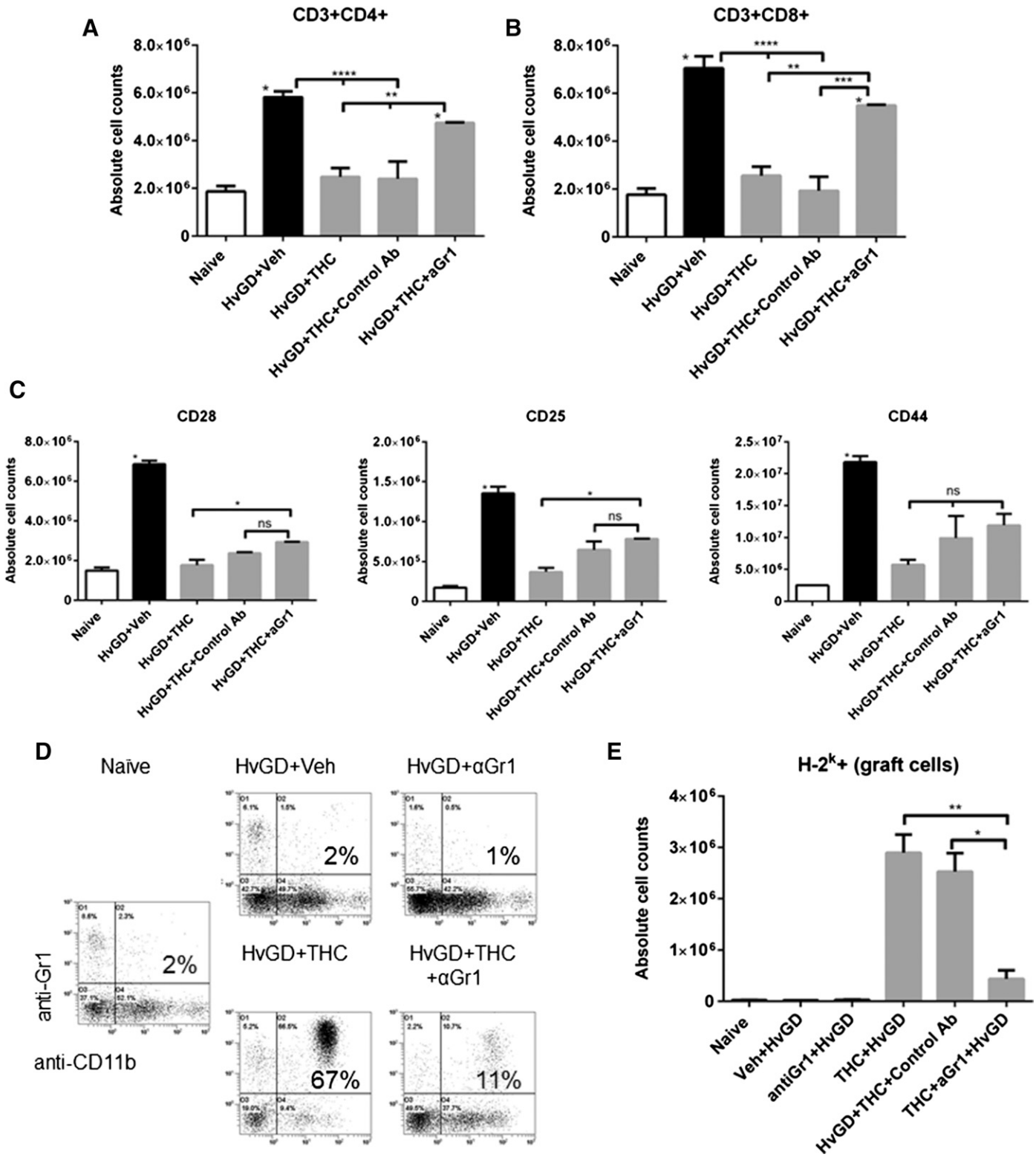

Figure 4. Role of MDSCs in HvGD modulation as determined via MDSC depletion. MDSCs were depleted by intraperitoneal injection of Ly-6G/Ly-6C-purified Abs (RB6-8C5) or isotype matched control Abs (n = 5 mice per group). Depletion Ab was given 3 h after every other THC treatment in decreasing doses of 0.1 and 0.05 mg/mouse. Peritoneal exudates were assessed for the presence of MDSCs and donor H-2^k cells, and ingLN were assessed for cellularity and T cell activation, d 4 after graft injection. (A) Absolute cell count of $CD3^+CD4^+$ T cells in ingLN. (B) Absolute cell count of $CD3^+CD8^+$ T cells in ingLN. (C) T cell activation marker expression, CD25, CD28, and CD44 (right), on ingLN cells. (D) Proportion of MDSCs in the peritoneal cavity. (E) Allograft cell persistence at the conclusion of the 5 d experiment. Representative data of 3 experiments are shown (means ± SEM). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0001$ Student's t test or ANOVA/Tukey.

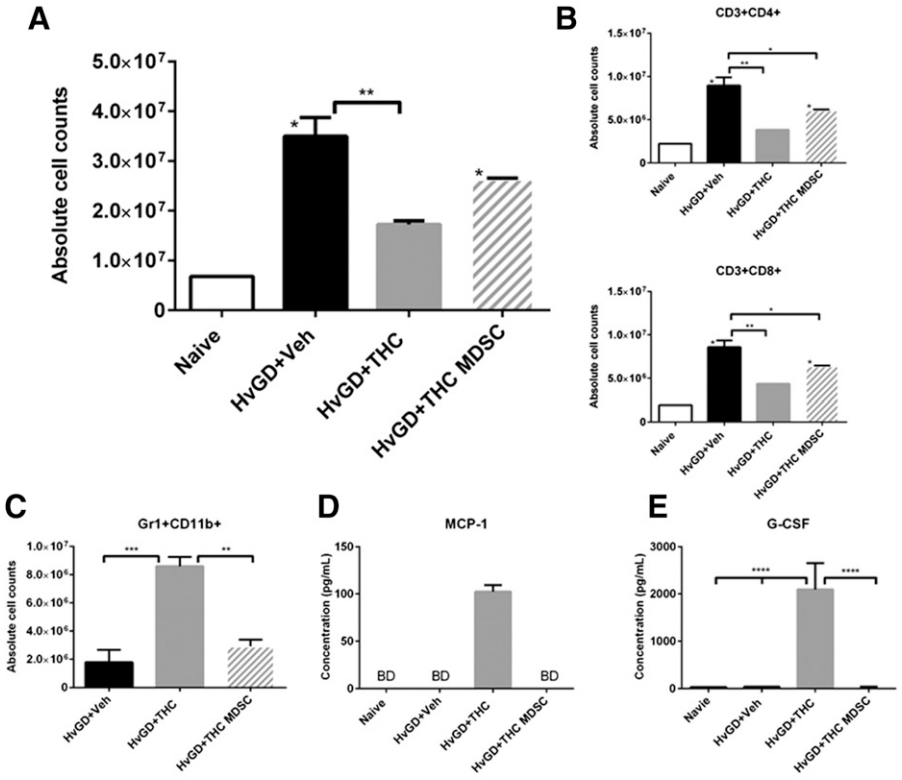

Figure 5. Role of MDSCs in HvGD modulation as determined via adoptive transfer. Isolated THC induced MDSCs (5×10^6), >95% pure, were transferred into BL6 HvGD mice ($n = 5$ per group) via an intraperitoneal injection 30 min after subcutaneous injection of graft cells. Mice were euthanized at 2 time points: day 4 for ingLN analysis and 18 h for peritoneal lavage analysis after graft injection. Peritoneal exudates were assessed for the presence of MDSCs and the MDSC-homing chemokines. (A) Cellularity in the ingLN. (B) T cell populations, $CD3^+CD4^+$ (top) and $CD3^+CD8^+$ (bottom). (C) Overall MDSC cellularity of the peritoneal cavity. (D) MCP-1 levels in the peritoneal exudate. (E) G-CSF levels in the peritoneal exudate. Similar results seen in replicate experiments (means ± SEM). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0001$ ANOVA/Tukey.

compared with those exposed to THC, which showed very high levels (Fig. 5D and E). These data showed that adoptively transferred MDSC may be less effective because they may not be able to survive or home.

THC-mediated reduction of HvGD occurs in a CB1-dependent manner

Immune cells can express both CB1 and CB2 receptors; as such, specific antagonists were used in vivo to ascertain whether THC was working preferentially through activation of CB1, CB2, or both receptors [14, 30, 55, 56]. We found that although both CBs were expressed in the ingLNs, the expression of CB1 was stronger than that of CB2 (data not shown). To address the relative role of CB1 vs. CB2, BL6 mice were treated with either CB1 or CB2 antagonist (SR141716 or SR144528, respectively, at 20 mg/kg, i.p.) 1 h before each THC treatment. As seen earlier, $HvGD^+$THC mice showed decreased absolute cellularity of the ingLNs compared with $HvGD^+$Veh ($DMSO^+$ 20 μl Tween-80 diluted in 1× PBS) mice. It is noteworthy that $HvGD^+THC^+$ CB1 antagonist-treated mice, but not $HvGD^+THC^+$ CB2 antagonist-treated mice, showed a reversal in ingLN cellularity compared with $HvGD^+$THC mice, thereby suggesting that THC was acting through CB1 but not CB2 (**Fig. 6A**). To further corroborate that the CB2 receptor was not the main CB involved, we used another CB2 antagonist (AM630) [15] and found that this antagonist was also not able to reverse the effect of THC on HvGD (Fig. 6B). As confirmation that THC was working preferentially through the CB1 receptor, CB1 KO mice were incorporated into the HvGD model as recipients. Thus, we induced HvGD in BL6 wild-type and CB1 KO mice and treated them with THC. The data indicated that THC treatment did not reduce ingLN cellularity in HvGD mice with a nonfunctional CB1 receptor compared to THC-treated wild-type HvGD mice (Fig. 6C). Next, we studied MDSC induction in wild-type, CB1 KO, or CB2 KO allograft-recipient mice and found that the proportion of MDSCs in the peritoneal exudate was 84% in wild-type BL6 mice, whereas CB1 KO mice had 25% and the CB2 KO mice had 63% (Fig. 6D). Looking not only at MDSC proportion but also at absolute cell counts, we noted a significant reduction from CB1 but not CB2 KO mice, 8.6×10^6 and 17.1×10^6 cells, respectively, when compared with wild-type mice, which averaged 16.2×10^6 peritoneal-exudate MDSCs (Fig. 6E).

THC significantly extends life of allogenic skin grafts

Although all the data shown above indicated that THC can suppress HvGD, we next tested directly whether THC treatment could reduce rejection of complete haplotype mismatched skin allografts. To that end, tail skin from donor C_3H mice, kept in place on the recipient mouse using a compression cast in lieu of sutures, acted as a complete mismatch allograft [41]. The cast was removed after 9 d. Allograft rejection was assessed daily starting on d 9 after transplant, 1 h after cast removal. Mice were treated with either THC or vehicle (ethanol diluted in 1× PBS) every other day for 14 d via intraperitoneal injection as described earlier. The data showed that the skin allograft survival curve for THC-treated BL6-recipient mice, mean survival >12 d was significantly extended compared with vehicle-treated mice (mean survival < 10 d) (**Fig. 7A**). Axillary and brachial LNs, from the skin-transplant mice, were restimulated with mitomycin C-treated C_3H splenocytes ex vivo, and proliferation was analyzed using thymidine incorporation. THC-treated skin graft-recipient

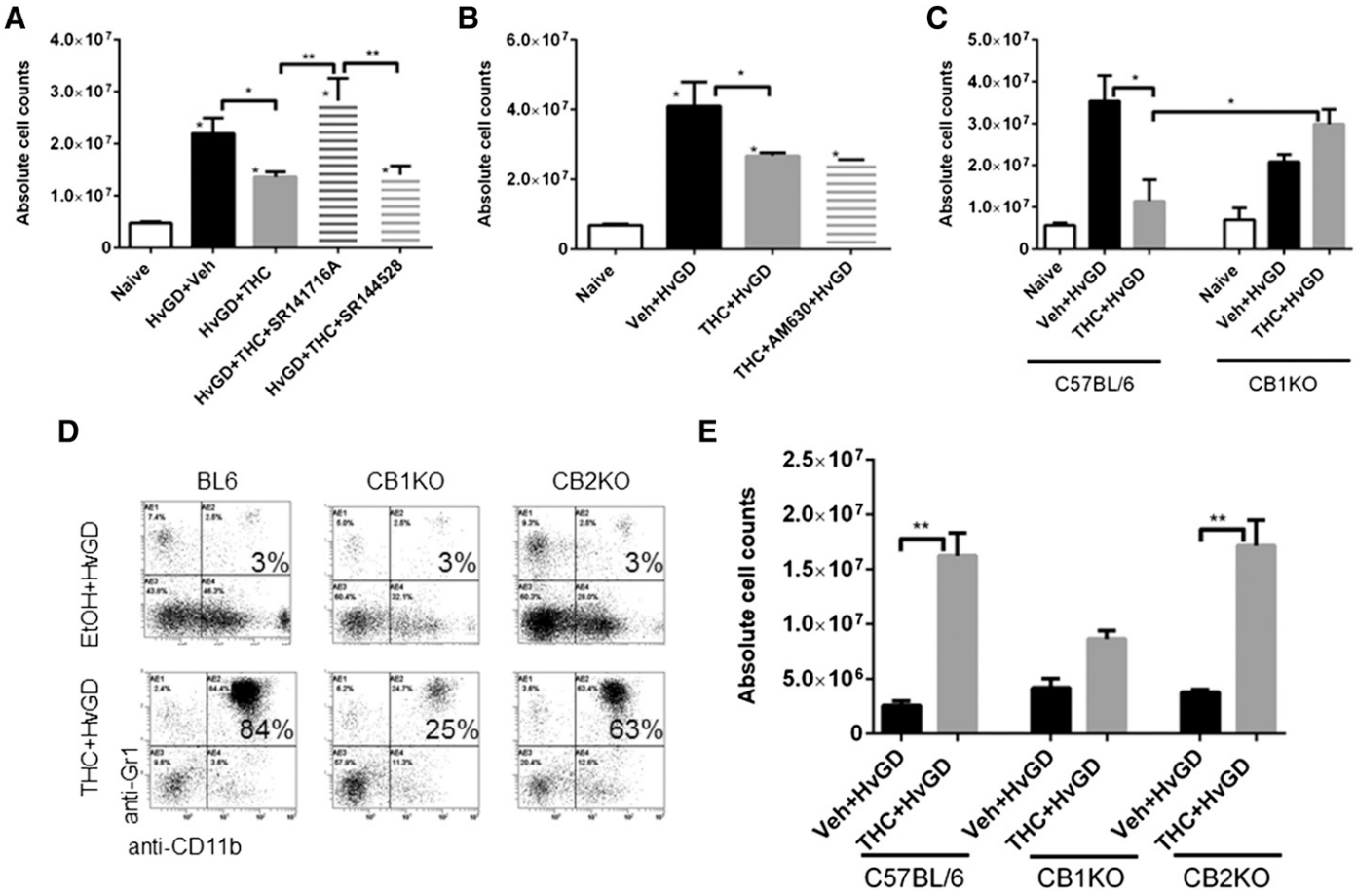

Figure 6. THC works through CB1 to ameliorate HvGD. BL6 mice ($n = 5$ per group) were given either a CB1 or a CB2 antagonist 1 h before treatment with THC or the vehicle (DMSO$^+$ 20 μl Tween-80 diluted in 1× PBS). (A) Absolute cellularity in ingLN, d 4 after graft injection, using CB2 and CB1 antagonists (SR144528 and SR141716A, respectively) (data shown as means ± SEM). (B) Absolute cellularity in ingLNs, d 4 after graft injection, using CB2 antagonist AM630 (data shown as means ± SEM). (C) Cellularity on ingLN in BL6 and CB1 KO HvGD mice, d 4 after graft injection. (D) THC induction of peritoneal MDSCs in BL6, CB1 KO, and CB2 KO HvGD mice, 18 h after graft injection (means ± SEM). (E) Absolute cell counts of peritoneal MDSCs in BL6, CB1 KO, and CB2 KO HvGD mice. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0001$ Student's t test or ANOVA/Tukey.

mice showed significantly less proliferation than did vehicle-treated graft mice (Fig. 7B). To show that the protective effects of THC on the skin allograft were not specific toward 1 MHC haplotype, we moved our dual-graft model into B6D2F1/J mice, which have a different haplotype disparity H-2$^{d/b}$ than do BL6 mice compared with the H-2^k allograft. We again found that THC treatment was able to significantly extend the skin allograft survival curve (mean survival = 12 d) compared with vehicle treatment (mean survival < 10 d) (Fig. 7C). Furthermore, having shown that CB1 function was integral in reducing the inflammatory response to allograft cells, we used the antagonist (SR141716A) along with the THC treatment in the BL6 skin-graft model. A significant reduction in the life of the skin graft was observed upon pharmacologically blocking the CB1 (mean survival < 11 d) compared with the THC treatment without the CB1 antagonist (mean survival = 14 d) (Fig. 7D).

DISCUSSION

Although the immunosuppressive properties of THC have been well elucidated, whether THC could be used to prevent allograft rejection has not, to our knowledge, been previously studied [57]. Recently, we suggested that activation of CBs may provide a novel approach to preventing transplant rejection [31]. THC has been found to affect nearly every aspect of cellular immunity, including T cells, NK cells, macrophages, neutrophils, mast cells, and B cells (as reviewed throughout the years [12, 21, 22, 58–60]. THC can also affect the biasing of T cell differentiation from Th1 to Th2 [19, 61–63]. Moreover, THC can also induce immunosuppressive cells, such as MDSCs and regulatory T cells [24, 30, 63]. Thus, it was worth investigating whether THC could suppress allograft rejection.

In the current study, we found that THC-induced MDSCs may have a critical role in suppressing allograft rejection. This was evident from experiments using the Ab RB6-8C5, which depleted the MDSC in vivo and caused a reversal of the ability of THC to ameliorate HvGD. THC-induced MDSCs suppressed the T cell proliferation against allogeneic cells in vitro. Furthermore, adoptive transfer of THC-induced MDSCs into HvGD mice caused significant suppression of the HvGD response. Together, these data provided strong support for THC suppressing allograft rejection through induction of MDSCs. Our laboratory has

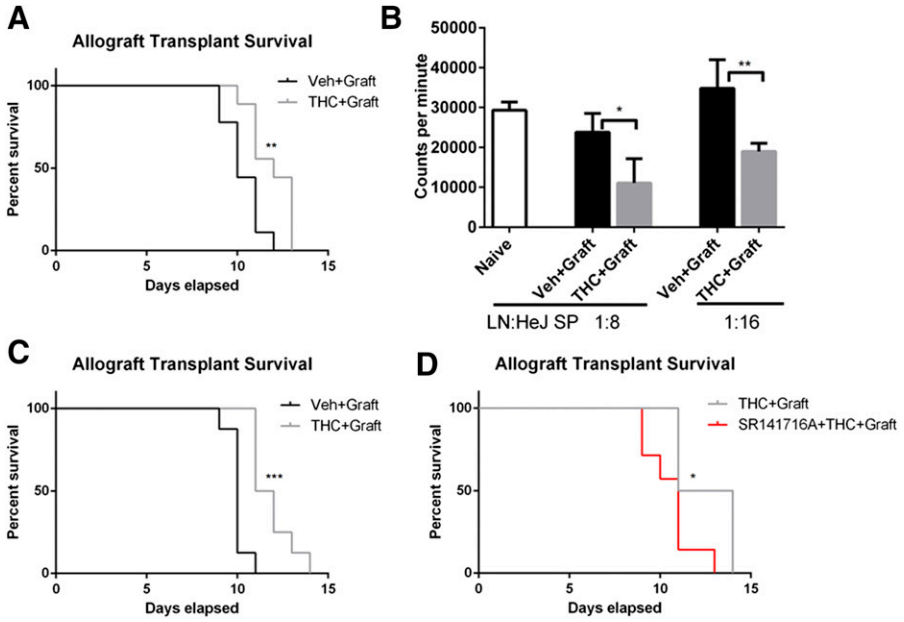

Figure 7. THC treatment significantly extends survival of allogenic skin grafts. The dual skin-graft method was used to include a technical control (all autograft skin transplants survived). THC treatment was given every other d for 2 wk. (A) C_3H allograft on BL6 recipient mice: survival of allograft in THC and vehicle (ethanol diluted in 1× PBS)-treated mice (means ± SD, $n = 9$ vehicle-treated mice and $n = 9$ THC-treated mice). (B) Skin-graft rejection mice from panel A were rested 10 d (from the last treatment) and then the axillary and brachial LNs were restimulated ex vivo with C_3H splenocytes. Thymidine assay was used to determine proliferation. (C) C_3H allograft on B6DF1/J (F1) recipient mice: survival of allograft in THC- and vehicle-treated mice (means ± SD, $n = 8$ vehicle-treated mice and $n = 9$ THC-treated mice). (D) C_3H allograft on BL6 recipient mice survival of allograft in THC or SR141716A$^+$THC-treated mice (means ± SD, $n = 6$ SR141716A$^+$ THC-treated mice and $n = 4$ THC-treated mice). Log-rank test (survival curves) and ANVOA/Tukey $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.0001$.

shown previously that THC treatment triggers rapid and massive induction of MDSCs in naïve mice after a single dose [24]. Although MDSCs were first associated with cancer as part of the tumor microenvironment, which decreased T cell-based antitumor immunity and increased tumor progression, recent studies have indicated that MDSCs may also be induced at sites of inflammation [25, 64, 65]. The suppressive functionality of MDSCs is most commonly attributed to the release of Arg-1 or iNOS [26]. THC-induced MDSCs have been shown to be suppressive to T cell proliferation in response to both polyclonal and antigen-specific stimuli [24]. The importance of MDSCs in creating tolerance in grafts was shown recently through the adoptive transfer of LPS-induced MDSCs intravenously [66]. The transfer of MDSCs delayed the rejection of minor antigen disparity in H-Y skin grafts [66]. MDSCs were also present in tolerated grafts, including MHC-mismatched kidney transplants in rats [50]. Furthermore, the suppressive function of MDSCs was deemed vital because MDSCs that were unable to produce Arg-1 via IL-13 treatment were less effective in ameliorating GvHD in lethally irradiated BALB/c mice [67]. However, adoptive transfer of MDSCs was not sufficient to cause graft persistence in fully mismatched antigen disparity [66]. In the current study, we found that the adoptive transfer of purified THC-induced MDSCs, suppressed the immune response in the LNs of mice with HvGD, and reduced the total numbers of cells, including $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells, although this was not as effective as administration of THC alone. These data suggested that MDSCs, at least in part, have a role in THC-mediated suppression of allogeneic response in the recipient. THC may be more effective in vivo because immunosuppression can be induced through additional mechanisms, such as induction of regulatory T cells, switching from Th1 to Th2, apoptosis in activated T cells, and suppression of cytokines [19, 30, 42, 61, 63, 68, 69]. Alternatively, treatment with MDSCs may not be as effective because the cells may not be able to maintain an immature, and, therefore, suppressive, phenotype after adoptive transfer or may not migrate to the grafted site to mediate suppression, unlike those MDSCs directly induced by THC. We concluded this was due to a lack of MDSC-homing cytokines, such as MCP-1 and G-CSF, which are necessary for THC-mediated MDSC migration and retention of suppressive capabilities [70]. The findings of De Wilde et al. [66], which show that MDSCs are directly involved in tolerance, lend credence to our findings that THC, a known MDSC inducer, reduces HvGD symptomology, attenuating acute transplant rejection.

In the current study, the THC-mediated effect on allograft response was regulated through CB1 rather than CB2. Recently, a study looking at MLR in vitro found that CB2 was necessary for THC-induced T cell suppression [71]. Although the findings of Robinson et al. [71] showed a CB2-dependent decrease in IL-2 production in vitro, the role of MDSCs was not assessed in that study. In contrast, the current research suggests that CB1 is involved in the resolution of HvGD. For instance, we found that THC was able to significantly reduce the in vivo proinflammatory response, including T cell activation and proliferation, to allograft in wild-type BL6 mice with functional CB1 and CB2 receptors. However, when the functionality of CB1 was compromised, either by genetic KO or pharmacological blockade, THC was less effective in inhibiting the proinflammatory response, including T cell proliferation. These findings are supported by Gaffal et al. [72] who showed that CB1 does have a role in the regulation of chemokines associated with T cell-dependent inflammation. In contrast, a loss of CB2 function from pharmacological blockade had little to no impact on THC-mediated immunosuppression. The observation that CB1 activation was necessary for HvGD amelioration was explained in part when the role of CBs in MDSC induction was observed. We showed that THC-mediated induction of MDSCs in this model was dependent on a functional CB1. Moreover, the use of another CB ligand, the endogenous cannabinoid anandamide, has been found to induce

MDSCs in a CB1-dependent manner [54]. Additionally, the THC-mediated shift in T cell differentiation from Th1 to Th2 has recently been found to involve both CB1 and CB2 [19]. Newton et al. [19] found that the shift was due to the CB1-dependent decrease in IL-12Rβ1 binding and a concurrent CB2-dependent increase in GATA-3 expression. As such, CB1 and CB2 activation creates a simultaneous drop in IFN-γ and a rise in IL-4. The role of CB1 in T cell differentiation brings to light the often-overlooked involvement of this receptor with peripheral immune cells. Although CB2 is expressed at higher levels in naïve immune cells, CB1 expression has been found to be elevated upon activation, specifically in T cells, and is known to have a crucial role in the LPS-induced immune response [20, 73, 74]. Furthermore, THC treatment of T cell lines, in lieu of activation, resulted in elevated CB1 levels [75]. Together, these findings support THC-mediated immune modulation being involved in both CB1 and CB2, and furthermore, CB preference may depend on the nature of the immune response as well as the activation state.

In the current study, we found that THC treatment in HvGD mice led to significant suppression of proinflammatory cytokines. In particular, THC significantly reduced the secretion of IL-2, IL-6, and IFN-γ, which are associated with the loss of graft function and, ultimately, lead to allograft destruction. Our laboratory has reported that THC treatment in an autoimmune hepatitis model can reduce both TNF-α and IL-6 levels in serum [43]. Furthermore, THC treatment led to reduced IL-2 and IFN-γ secretion in ConA-activated splenic T cells [76]. In addition to reducing proinflammatory cytokines, necessary for T cell proliferation, THC can also reduce the expression of receptors necessary for T cell activation. We found that THC was able to significantly reduce the number of cells expressing CD25, CD28, CD69, and CD44 costimulatory and activation markers in HvGD mice. Additionally, THC treatment brought the expression of these T cell-activation receptors to levels not significantly different from naïve mice. Costimulator ICOS expression and transcription can be down-regulated via THC treatment of anti-CD3/CD28 stimulated $CD4^+$ splenic T cells [77]. CB binding has also been found to inhibit T cell activation through costimulator interference. Focusing on the role of APCs in T cell activation, a recent study found that heat-stable antigen expression was decreased on macrophages upon THC treatment, resulting in extended T cell unresponsiveness [78]. Furthermore, CD28 blockade is known to have a major role in tolerance, although not sufficient to prevent graft rejection. Although there has been no direct evidence that ties THC to the depletion of the costimulatory molecule CD28, it is known that THC treatment inhibits pathways associated with CD28 binding [79]. CD28 activation is involved in PI3K/Akt and NF-κB proliferative pathways. Binding of CD28 to PI3K, protein kinase C-θ, or growth factor receptor-bound protein 2 have all been suggested as NF-κB activation-regulating pathways [80]. NF-κB activation is important in graft rejection because recent literature shows that impaired NF-κB activation can be correlated to skin-graft tolerance [81]. Because PI3K subunit p110δ is up-regulated in transplanted H-Y-mismatched heart-graft tissue, CD28/PI_3K/NF-κB pathway could be integral in rejection [82]. THC treatment inhibits NF-κB DNA binding, and high levels of IL-10 can suppress PI3K binding of CD28 [79, 83]. The research reported here acts as a bridge to those concepts. In addition, reduced CD28 costimulation, as well as THC treatment, has been implicated in the development of anergy in mouse lymphocytes [84, 85]. As such, we suggest that THC treatment induces an increase in Th2 cytokines, which inhibit CD28 binding, thereby reducing NF-κB and allowing for graft tolerance.

Together, the current study shows, for the first time to our knowledge, that the CB-ligand system may have a critical role in allograft rejection. THC treatment reduced the T cell response in the host by dampening the secretion of proinflammatory cytokines and expression of T cell activation markers. Additionally, THC treatment resulted in delayed graft destruction, even in a MHC disparity model of allogenic skin transplant. Induction of highly immunosuppressive MDSCs following THC treatment proved to be necessary, at least in part, for THC-mediated attenuation of allograft rejection. We also noted that this effect of THC was dependent on activation of CB1 rather than CB2. The current study sets the stage for additional studies on the cannabinoid system in regulating transplant rejection involving potential manipulation of endocannabinoids, receptors, and the use of CB-select agonists that are not psychoactive.

AUTHORSHIP

P.S.N., M.N., and J.M.S. performed experimental design. J.M.S. performed experiments and analysis. P.S.N., M.N., and J.M.S interpreted the data. J.M.S. wrote the manuscript. P.S.N., M.N., and J.M.S. evaluated the manuscript evaluation.

ACKNOWLEDGMENTS

This work was supported in part by the U.S. National Institute of Health (NIH) National Institute of Mental Health Grant R01-MH094755, NIH National Center for Complementary and Integrative Health (NCCIH) Grant P01-AT003961, and NIH National Institute of General Medical Sciences Grant P20-GM103641 (to P.S.N.), NIH National Institute of Environmental Health Sciences Grant R01-ES019313 (to P.S.N. and M.N.), NIH NCCIH Grant R01-AT006888, and the U.S. Veterans Administration Merit Award BX001357 (to M.N.). We thank Dr. Phillip Chandler (Georgia Regents University, Augusta, GA, USA) for his help in establishing the skin-graft procedure.

DISCLOSURES

The authors declare no competing financial interests.

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KEY WORDS:
T cell suppression · skin allograft · allograft tolerance · THC