Targeting Cannabinoid Receptors as a Novel Approach in the Treatment of Graft-versus-Host Disease: Evidence from an Experimental Murine Model

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ABSTRACT

Allogeneic hematopoietic cell transplantation (HCT) is widely used to treat patients with life-threatening malignant and non-malignant hematological diseases. However, allogeneic HCT often is accompanied by severe and lethal complications from graft-versus-host disease (GVHD), in which activated donor T cells recognize histocompatibility antigenic mismatches and cause significant toxicity in the recipient. In the current study, we tested the hypothesis that activation of cannabinoid receptors on donor-derived T cells may prevent GVHD. We tested the effect of Δ^9 -tetrahydrocannabinol (THC) in an acute model of GVHD that was induced by transferring parental C57BL/6 (B6) spleen cells into (C57BL/6 $\times$ DBA/2) F_1 (BDF1) mice. Transfer of B6 cells into BDF1 mice produced severe acute GVHD in the recipient, characterized by lymphoid hyperplasia, weight loss, T

helper I cytokine production and mortality. THC administration led to early recovery from body weight loss, reduced tissue injury in the liver and intestine, as well as complete survival. THC treatment reduced the expansion of donor-derived effector T cells and blocked the killing of host-derived immune cells while promoting Foxp3⁺ regulatory T cells. Impaired hematopoiesis seen during GVHD was rescued by treatment with THC. The ability of THC to reduce the clinical GVHD was reversed, at least in part, by administration of cannabinoid receptor (CB) 1 and CB2 antagonists, thereby demonstrating that THC-mediated amelioration of GVHD was cannabinoid receptor-dependent. Our results demonstrate for the first time that targeting cannabinoid receptors may constitute a novel treatment modality against acute GVHD.

Introduction

Allogeneic hematopoietic cell transplantation is a proven and standard clinical treatment option used for patients with life-threatening malignant and nonmalignant hematological diseases (Ferrara and Deeg, 1991; Bortin et al., 1992). However, one of the severe complications that develops after allogeneic hematopoietic cell transplantation is graft-versus-host disease (GVHD) (Korngold and Sprent, 1978), in which activated host-reactive effector donor T cells recognize the histocompatibility antigen mismatches, thereby attacking the genetically disparate recipient. Bone marrow transplanta-

tion is one of the most commonly used approaches to provide the source of allogeneic hematopoietic cells. Development of GVHD leads to general and profound immunosuppression, anemia, weight loss, inflammatory processes targeting spleen, liver, gastrointestinal tract, and skin, and ultimately the death of the recipient (Ferrara and Deeg, 1991; Welniak et al., 2007).

The median survival rate of patients with moderate to severe acute GVHD is reported to be less than 6 months (Ferrara and Deeg, 1991; Welniak et al., 2007). Donor T cells play a crucial role in development of GVHD (Korngold and Sprent, 1978; Ferrara and Deeg, 1991). In both murine and clinical settings, depletion of donor T cells has been shown to reduce the risk of GVHD. However, such an approach decreases the chances of engraftment and increases the recurrence of malignancy (Martin et al., 1988; Poynton, 1988). Moreover, the current immunosuppressive drugs available to treat GVHD show positive response in only a small proportion of patients and are often associated with development of

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ABBREVIATIONS: GVHD, graft-versus-host disease; CB, cannabinoid receptor; PE, phycoerythrin; THC, Δ^9 -tetrahydrocannabinol; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; IFN, interferon; IL, interleukin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; Th, T helper; Treg, regulatory T cell; CFU-GM, colony-forming unit-granulocyte macrophage; CTL, cytotoxic T lymphocyte; VEH, vehicle; AM251, *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; SR144528, 5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-*N*-[(1*S*,2*S*,4*R*)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1*H*-pyrazole-3-carboxamide.

serious side effects, including nephrotoxicity and cardiotoxicity, thereby reducing the quality of life in recipients of bone marrow transplantation (Storb et al., 1986; Buckner and Clift, 1989; Ferrara and Deeg, 1991; Welniak et al., 2007). Thus, there is an emerging need to regulate GVHD to promote graft-versus-tumor effect, without causing severe toxicity resulting from the expansion of donor-derived T cells.

Cannabinoids, the active ingredients found in *Cannabis sativa*, have been shown to exhibit a wide range of pharmacological properties (Klein, 2005; Mackie, 2006; Pertwee, 2009). Cannabinoids mediate their effects primarily through the G protein-coupled cannabinoid receptors CB1 and CB2, which are negatively coupled to adenylyl cyclase (Pertwee and Ross, 2002; Mackie, 2006). Studies from our laboratory and elsewhere have suggested that cannabinoids exhibit potent anti-inflammatory properties and therefore can be used to treat autoimmune and inflammatory diseases (Klein, 2005; Nagarkatti et al., 2009). Cannabinoids have been shown to inhibit tumor cell growth and angiogenesis, suggesting their potential use in the treatment of gliomas, prostate and breast cancers, and malignancies of immune origin (McKallip et al., 2002a; Hall et al., 2005; Lombard et al., 2005; Ramer et al., 2010). Δ^9 -Tetrahydrocannabinol (THC) is one of the most extensively investigated ingredients found in cannabis. THC activates both CB1 and CB2, thereby mediating both psychotropic and anti-inflammatory properties. THC is used clinically in patients with cancer and HIV/AIDS to increase appetite and decrease nausea (Hall et al., 2005; Pertwee, 2009). It is also used to help patients with glaucoma by reducing pressure within the eye (Mackie, 2006; Pertwee, 2009).

Inasmuch as our previous studies suggested that THC exhibits anti-inflammatory and immunosuppressive properties (Hegde et al., 2008; Nagarkatti et al., 2009, 2010; Pandey et al., 2009), we tested the possibility of its use in treating GVHD in a parent $\rightarrow$ F₁ model. We hereby demonstrate for the first time that administration of THC during allogeneic transplantation can significantly suppress GVHD.

Materials and Methods

Mice. Female C57BL/6 (B6, H-2^b, CD45.2⁺) and (C57BL/6 $\times$ DBA/2) F₁ (BDF1, H-2^{b/d}, CD45.2⁺) mice (6–8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in specific pathogen-free conditions and received filtered water and normal chow. All experiments were approved by our institutional Animal Care and Use Committee.

Induction of Acute GVHD and Treatment with THC. Six- to 8-week-old normal female B6 mice (H-2^b) were used as donors. Acute GVHD was induced by injecting 5×10^7 B6 spleen cells intravenously into normal female BDF1 recipient mice. THC at a concentration of 20 mg/kg body weight was dissolved in ethanol and diluted in PBS. THC or vehicle was then administered intraperitoneally (0.1 ml/mouse) into recipient BDF1 mice 1 h after B6 donor cell injection. THC treatment was continued every alternate day thereafter until the termination of the study. Likewise, cannabinoid receptor antagonists AM251 (*N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; CB1 antagonist) and SR144528 (5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-*N*-[(1*S*,2*S*,4*R*)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1*H*-pyrazole-3-carboxamide; CB2 antagonist) were dissolved in dimethyl sulfoxide containing one drop of Tween 80 (Sigma-Aldrich, St. Louis, MO) and were further diluted in PBS. Antagonists were injected intraperitoneally (0.1 ml/

mouse) 1 h before THC injection. All control groups received vehicles such as ethanol or dimethyl sulfoxide similarly diluted in PBS.

Monoclonal Antibodies, Reagents, Flow Cytometry. The following antibodies used for flow cytometric analysis were purchased from BD Biosciences (San Jose, CA): antimurine Fc γ receptor, fluorescein isothiocyanate-conjugated anti-H-2D^d, and phycoerythrin-conjugated anti-H-2D^b. Isotype-matched control antibodies were used for background staining. GVHD spleens were analyzed for the persistence of donor lymphocytes by flow cytometry. Donor cell splenic chimerism was analyzed by two-colored flow cytometric analysis. In brief, spleen cells (10^6 cells in 100 μ l) from BDF1 recipients, on day 21, were incubated with Fc γ receptor antibodies for 10 min and then washed three times in PBS/1% fetal bovine serum buffer after incubation with each antibody on ice for 30 min. Stained cells were then analyzed using a flow cytometer (FC500; Beckman Coulter, Fullerton, CA). Data were analyzed using Cytomics CXP software (Beckman Coulter). Triple staining analyses was carried out to measure apoptosis in the donor cell population. Splenocytes were incubated with antibodies against H-2D^b and H-2D^d for 30 min on ice followed by wash. The percentage of apoptotic cells was determined on either gated donor or host cells by performing TUNEL assay (Roche Diagnostics, Indianapolis, IN). In addition, three-color analysis was performed on gated cells by using fluorescein isothiocyanate-, phycoerythrin-, or biotin-conjugated monoclonal antibodies to analyze the CD4⁺ and Treg cell population.

Cytokine Analysis. The levels of cytokines IFN- γ and IL-2 in samples were measured by sandwich ELISA using ELISA kits purchased from Peprotech (Rocky Hill, NJ). To measure cytokine production in vitro with or without concanavalin A, spleen cells (4.0×10^6 cells/ml) from GVHD mice were cultured in 1-ml aliquots in 24-well tissue culture plates. The supernatants were harvested after 24-h culture, and cytokine production was quantified using an ELISA kit.

Spleen Index. The intensity of systemic GVHD was assessed by measuring the spleen index by using the following formula: spleen index = (spleen weight/body weight of each test F₁ mouse)/(mean (spleen weight/body weight) of age- and sex-matched normal F₁).

In Vitro Colony Assay. Bone marrow cells were flushed with RPMI medium 1640 from the femurs. To perform colony-forming unit-granulocyte macrophage (CFU-GM) assay, 10^5 bone marrow cells were cultured in 1% methylcellulose culture medium (Methocult M 3430 kit; Stem Cell Technology, Vancouver, Canada). This medium was supplemented with 1% bovine serum albumin, 30% fetal bovine serum, 3 U erythropoietin, 2 mM L-glutamine, and 2% pokeweed mitogen-stimulated spleen cell-conditioned medium. Cells were cultured in 1 ml of medium in a 35-mm Petri dish at 37°C in a 5% CO₂ atmosphere. The numbers of colonies were counted on day 7 using an inverted microscope.

Ex Vivo Spontaneous Proliferation Assay. To measure the spontaneous proliferation of splenocytes from BDF1 mice, cells were cultured in triplicate in 200- μ l aliquots in 96-well plates for 24 h with the addition of 1 μ Ci/well [³H]thymidine, during the last 8 h. Radioactivity incorporated into DNA was measured using a liquid scintillation counter.

⁵¹Cr Release Assay to Detect Antihost CTL Activity In Vitro. Specific antihost cytotoxic T lymphocyte (CTL) activity was measured in the spleens of GVHD mice using P815 (H-2^d) or EL-4 (H-2^b) target tumor cells. The target cells were labeled for 1 h at 37°C with 50 μ Ci ⁵¹Cr/2 $\times 10^7$ cells, and 100- μ l aliquots were then added to the wells of microtiter plates. Responder cells (4.0×10^6 cells) were cocultured with 1.0×10^6 irradiated (30 Gy) splenocytes of normal BDF1 (stimulator cells) in the presence of recombinant IL-2 (100 U) in a 96-well tissue culture plate. After 5 days of culture, effectors were harvested and tested for their ability to lyse target cells. Effector cells were tested in triplicates at four effector to target ratios of 100:1, 50:1, 25:1, and 12.5:1. The plates were incubated at 37°C for 4 h, and percentage of cytotoxicity was calculated by the following formula: percentage of cytotoxicity = $100 \times (\text{counts/min}$

samples – counts/min background)/(counts/min maximum – counts/min background).

Histopathology. On day 21 after GVHD induction, tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections (5 μ m) were cut and stained with hematoxylin and eosin and examined under a light microscope to assess the inflammation associated with GVHD.

Statistical Analysis. Statistical analysis was performed with Prism 4.03 software (GraphPad Software, Inc., San Diego, CA). Data are presented as the mean $\pm$ S.E.M. Student's *t* test was used to compare data between two groups. Results from body weight were analyzed by using the nonparametric Mann-Whitney test. Experimental groups were compared with controls, and *p* < 0.05 was considered significant.

Results

THC Administration Ameliorates Weight Loss and Splenomegaly Associated with GVHD. To investigate whether cannabinoids can be used in the treatment of GVHD, we developed an acute parent $\rightarrow$ F₁ GVHD model in which the activated donor cells recognize the recipient's cells as foreign and destroy them, whereas the recipient's cells recognize the donor as self. To this end, C57BL/6 splenocytes were injected intravenously into BDF1 recipient mice on day 0. Beginning day 1, THC (20 mg/kg body weight) or vehicle

was administered intraperitoneally every alternate day. We observed progressive weight loss in vehicle-treated GVHD-induced mice until the termination of the experiment on day 20 (Fig. 1A). In addition, three of six mice (50%) from this group died by day 20 in two independent experiments. In contrast, THC-treated BDF1 mice, in which GVHD had been induced, showed no significant weight loss and 100% of the mice survived (Fig. 1A). In parallel, vehicle-treated mice with acute GVHD also developed significant splenomegaly with marked increase in total cellularity that was dramatically reduced after THC treatment (Fig. 1, B–D). In these experiments, administration of THC alone into C57BL/6 mice did not cause any significant effect on body weight (Fig. 1A), splenic index (Fig. 1C), or total spleen cellularity (Fig. 1D).

Prevention of GVHD by THC Is Associated with Decreased Donor T Cell Function. Next, we used four groups of mice to make in-depth analysis of the effect of THC on GVHD: normal mice treated with vehicle or THC and GVHD mice treated with vehicle or THC (designated as VEH, THC, GVHD+VEH, and GVHD+THC, respectively). We first cultured the spleen cells from these mice in vitro with [³H]thymidine, to measure cell proliferation (Fig. 2A). The development of splenomegaly in vehicle-treated GVHD mice was accompanied by marked increase in spontaneous ex vivo

Fig. 1. Effect of THC on clinical indicators during acute GVHD. Acute parent $\rightarrow$ F₁ GVHD was induced by intravenous injection of C57BL/6 splenocytes into groups of five to six BDF1 recipient mice on day 0. THC (20 mg/kg body weight) or the vehicle was administered intraperitoneally every alternate day beginning day 1. As controls normal mice injected with vehicle or THC alone were also included as indicated. A, the mean $\pm$ S.E.M. of body weight in GVHD-induced recipient BDF1 mice. B, splenomegaly in various groups: normal mice+vehicle (a), normal mice+THC (b), GVHD mice+vehicle (c), and GVHD mice+THC (d). C, spleen index calculated as described under *Materials and Methods* on days 7 and 21 after donor cell transfer. D, total spleen cellularity for each treatment on day 21 is shown. Data are expressed as means $\pm$ S.E.M. **, *P* < 0.005; *, *P* < 0.01.

Fig. 2. Effect of THC administration on spontaneous ex vivo proliferation and cytokine production by splenocytes from recipient BDF1 mice. Parent $\rightarrow$ F₁ GVHD was induced as described in Fig. 1. A, splenocytes were harvested from GVHD-induced recipient mice, and the degree of spontaneous proliferation was studied by culturing cells for 4 h with [^3H]thymidine. Data are expressed as mean $\pm$ S.E.M. of triplicate cultures. B and C, splenocytes were cultured in vitro, and IL-2 and IFN- γ production was measured, respectively, by ELISA. Vertical bars represent mean $\pm$ S.E.M. of triplicate cultures. *, $P < 0.01$; #, undetectable. D, effect of THC on antihost CTL activity during GVHD. Spleen cells from the recipient mice were harvested on day 21 and cultured in vitro with irradiated splenocytes of normal BDF1 mice. After 5 days of culture, effectors were harvested and tested for their ability to lyse P815 (H-2^d) target cells using ^{51}Cr -release assay. E:T, effector to target. Results shown are mean $\pm$ S.E.M. of percentage of cytotoxicity from quadruplicate cultures. Similar results were obtained in two replicate experiments.

proliferation of transferred spleen cells (Fig. 2A). Treatment of GVHD mice with THC significantly reduced spontaneous proliferation, thereby suggesting that THC was suppressing the donor cell proliferation. To investigate the role of THC treatment on inflammatory cytokines, spleen cells from GVHD mice were cultured in medium alone for 24 h to measure the spontaneous production of Th1 cytokines by activated T cells. The data presented in Fig. 2, B and C showed that splenocytes obtained from vehicle-treated GVHD mice expressed significant levels of IL-2 and IFN- γ , whereas THC-treated GVHD mice had significantly decreased levels of these cytokines. We next evaluated the CTL activity of spleen cells derived from vehicle- or THC-treated GVHD mice on day 14. CTL activity was significantly decreased against P815 (H-2^d) tumor targets in GVHD+THC groups compared with GVHD+VEH-treated mice (Fig. 2D).

THC Administration Inhibits Donor-Derived Proliferating T Cell Expansion in Acute GVHD. To further investigate the specific effects of THC on donor and recipient cell populations, we stained the splenocytes with antibodies against H-2^d and H-2^b so that we could distinguish donor-derived (H-2^b) versus recipient-derived (H-2^{b/d}) cells. Data from a representative experiment (Fig. 3A) indicated that in vehicle-treated GVHD mice the percentage of donor-derived cells (61.8%) was significantly higher than those derived from the recipient (12.6%). This proportion was dramatically reversed in THC-treated GVHD mice, thereby suggesting that THC was inhibiting GVHD. Calculation of absolute cell numbers (Fig. 3B) further corroborated these observations.

We have previously shown that THC induces apoptosis in T cells upon administration in vivo (McKallip et al., 2002b, 2005). To investigate whether THC-mediated suppression of GVHD resulted from induction of apoptosis in donor cells, we gated these cells and analyzed them for apoptosis using TUNEL assay (Fig. 3C). We noted that in the GVHD+THC groups a significant proportion of cells was undergoing apoptosis (49.14%) compared with the GVHD+VEH groups (8.77%). In addition, when the cells from the recipient mice were similarly analyzed, they did not show significant levels of apoptosis (data not shown). Together, these data suggested that THC may mediate apoptosis in activated donor T cells, which may account for the inhibition of GVHD.

THC Treatment Prevents Impaired Hematopoiesis during Acute GVHD. Developing acute GVHD is associated with suppression of hematopoietic activity by bone marrow cells of the recipient (Xenocostas et al., 1987; Mori et al., 1998). In the current study, we investigated whether THC treatment would reverse this toxicity. As shown in Fig. 4, A and B, vehicle-treated GVHD mice exhibited impaired hematopoiesis associated with marked reduction in the number of granulocyte-macrophage progenitor cells (CFU-GM) on day 21 after B6 cell transfer compared with vehicle- or THC-treated mice in which GVHD had not been induced. In contrast, bone marrow cells isolated from THC-treated GVHD mice formed a significantly larger number of CFU-GM, proving that THC treatment reversed the suppression of hematopoiesis seen during acute GVHD.

Fig. 3. Effect of THC on donor cell chimerism in acute GVHD. GVHD was induced followed by treatment with vehicle or THC as described in Fig. 1. Vehicle-treated normal mice were used as a control. On day 21 after GVHD induction, donor chimerism was determined by harvesting splenocytes and double-staining the cells with antibodies against H-2^d and H-2^b antigens. A, donor cells were identified by expression of H-2^b only, whereas host-derived F₁ cells were identified by combined expression of H-2^d and H-2^b (H-2^{b/d}). B, absolute numbers of donor- and host-derived cells in the GVHD+VEH and GVHD+THC groups. **, $p < 0.01$ Student's *t* test. Data are mean of five mice per group. Splenocytes from normal F₁ mice were used as a control for double-staining. C, donor cells were gated and analyzed for apoptosis using TUNEL assay.

THC Treatment Reverses the Toxicity Induced during GVHD to Target Organs. We evaluated the effect of THC on GVHD-related tissue injury in liver and colon. As shown in Fig. 4C, the hepatic portal vein areas showed marked infiltration of mononuclear cells in vehicle-treated GVHD mice, which was significantly reduced after THC treatment. Likewise, the colon from vehicle-treated GVHD mice was highly infiltrated with lymphocytic cells, and the intestinal crypts were depleted. In contrast, THC treatment showed significantly less or almost no infiltration and well defined crypts, similar to normal control mice. Thus, THC treatment significantly reduced the GVHD-associated tissue injury in allogeneic recipients.

THC Effect in Acute GVHD Is Mediated through Cannabinoid Receptors. THC is known to mediate its effects in vivo through the cannabinoid receptors, CB1 and CB2. To investigate whether the THC-mediated suppression of GVHD resulted from the activation of CB1 and/or CB2, we analyzed the effect of THC in GVHD mice treated with CB1 or CB2 select antagonists. We observed that suppression of GVHD, as indicated by reduced splenomegaly, mediated by THC, was significantly reversed by blocking either CB1 or CB2 (Fig. 5A). In addition, a combination of CB1 and CB2 select antagonists could completely reverse the THC-mediated decrease in spleen index in GVHD mice (Fig. 5B). These results suggested that THC was mediating its effect through both CB1 and CB2.

Fig. 4. Effect of THC on CFU-GM during acute GVHD. Parent $\rightarrow$ F₁ GVHD was induced by intravenous injection of C57BL/6 splenocytes into BDF1 recipient mice that were treated with vehicle or THC as described in Fig. 1. On day 21, bone marrow cells were harvested, and CFU-GM assay was performed as described under *Materials and Methods*. A, photographs depict the formation of colonies on methylcellulose: vehicle (a); THC (b); GVHD+VEH (c); and GVHD+THC (d). B, the numbers of colonies were counted on day 7 of culture. Vertical bars represent mean $\pm$ S.E.M. of CFU-GM per 10⁵ cells from four individual mice. **, $p < 0.001$. C, histological examination of tissues obtained from liver and colon 21 days after induction of GVHD. On day 21, three to four mice from each group were sacrificed and examined for histopathology in normal control mice (a and d), vehicle-treated GVHD mice (b and e), and THC-treated GVHD mice (c and f). The sections were stained with hematoxylin and eosin and examined under light microscopy (magnification: 20 $\times$). Representative sections of liver (a–c) and colon (d–f) are depicted.

Fig. 5. Effect of select CB1 and CB2 antagonists on GVHD-associated splenomegaly after THC treatment. GVHD was induced as described in Fig. 1 followed by treatment with vehicle, THC, and CB1/CB2 antagonists as described under *Materials and Methods*. A, photographs of representative spleens from each group. B, spleen index was determined on day 21. Vertical bars represent mean $\pm$ S.E.M. of five mice per group. *, $p < 0.001$; #, $P < 0.01$ versus THC-treated group; ##, $P < 0.001$ versus THC-treated group.

THC Treatment Increases Foxp3⁺ T Regulatory Cells during GVHD. Previous studies from our laboratory demonstrated that THC treatment increases the number of Foxp3⁺ T regulatory cells in a hepatitis model, and, unlike other T cells, Tregs may be resistant to apoptosis induced by THC (Hegde et al., 2008). Moreover, there is increasing evidence to suggest that Tregs afford protection against GVHD after allogeneic bone marrow transplantation (Rezvani et al., 2006). Thus, in the current study, we investigated the induction of Tregs during GVHD and the impact of THC treatment. We noted that in GVHD mice, whereas the percentage of CD4⁺Foxp3⁺ Tregs did not change significantly compared with naive mice treated with vehicle, there was significant increase in the absolute numbers of Tregs, which reflects the regulatory mechanism triggered during an inflammatory response (Fig. 6, A and B). It is noteworthy that in the GVHD+THC groups, there was an additional increase in both the percentage and numbers of Tregs compared with the GVHD+VEH groups, thereby suggesting that THC may induce Tregs during GVHD (Fig. 6, A and B). To further determine whether the Tregs were derived from the donor or the recipient, we analyzed the spleen cells from GVHD+VEH- and GVHD+THC-treated mice and triple-stained them for donor (H-2^b)- and recipient (H-2^d)-derived cells as well as Foxp3 (Fig. 6C). The results indicated that in GVHD+VEH mice, the proportion of Tregs from the donor and the recipient were similar, whereas in GVHD+THC mice, the proportion of donor-derived Tregs was 2-fold higher than those derived from the recipient (Fig. 6C). These data indicated that most of the increase in Tregs found in GVHD+THC mice compared with the GVHD+VEH mice were indeed derived from the donor. Together, our data indicated an interesting

Fig. 6. THC treatment increases Foxp3⁺ T regulatory cells during GVHD. Induction of GVHD and treatment with vehicle or THC was performed as described in Fig. 1. Normal mice injected with vehicle were used as a control. On day 21, splenocytes from recipient mice were harvested and analyzed by flow cytometry for CD4⁺Foxp3⁺ T cells. A, recipient spleen cells stained for CD4 and Foxp3. B, absolute number of CD4⁺Foxp3⁺ Tregs is shown. Data represent mean $\pm$ S.E.M. of five mice per group. *, $p < 0.05$, Student's t test.

finding that THC suppressed the donor-derived proliferation of effector T cells while promoting the donor-derived Tregs.

Discussion

Allogeneic hematopoietic stem cell transplantation is a useful therapy that has saved many lives from malignant and nonmalignant hematological diseases. Despite the availability and use of immunosuppressive drugs, 60 to 75% of patients do not respond or respond poorly to this treatment, leading to deadly complications of GVHD, including lethal runting disease, which is defined by wasting, diarrhea, and skin lesions (Welnia et al., 2007; Broady and Levings, 2008). Thus, there is an urgent need to develop more effective treatments with fewer side effects (Broady and Levings, 2008). In the current study, we demonstrate for the first time that cannabinoids, such as THC, are highly effective in the treatment of GVHD. We tested the efficacy of THC using an acute

model of GVHD, which consisted of transferring parental B6 spleen cells into BDF1 mice. The GVHD was accompanied by lymphoid hyperplasia, weight loss, Th1 cytokine production, severe inflammation in various organs and tissues, and mortality. THC treatment suppressed the expansion of donor-derived T cells and prevented the loss of host-derived immune cells. THC administration led to early recovery from loss of body weight, reduced tissue injury, and increased survival. Impaired hematopoiesis seen during GVHD was rescued by treatment with THC. Moreover, the donor-derived T cells from THC-treated GVHD mice failed to proliferate and mediate cytotoxicity against the recipient's cells. It is noteworthy that THC-mediated protection from GVHD was regulated in vivo through activation of CB1 and CB2. These data together suggested that targeting cannabinoid receptors may constitute a novel treatment modality against acute GVHD.

Mouse models of GVHD have been invaluable in under-

Fig. 6. *Continued.* C, spleen cells were triple-stained for H-2^b, H-2^d, and Foxp3. Next, the donor (H-2^b-) and host-derived (H-2^{b/d}) cells were gated and analyzed for Foxp3⁺ cell population. Representative flow profiles are depicted.

standing the biology and pathogenesis of GVHD and developing better conditioning regimens, prophylaxis, and treatment. There is a diverse array of mouse strain combinations used to induce GVHD, which can influence the role of Th1/Th2 as well as Tregs in triggering GVHD (Welniak et al., 2007). For example, infusion of parental donor spleen cells from the DBA/2 strain into an unirradiated (C57BL/6 × DBA/2) F₁ mouse induces chronic GVHD, whereas infusion of spleen cells from the other parent, C57BL/6, induces acute GVHD (Welniak et al., 2007), as seen in the current study. Acute GVHD is initiated in this model by activation and attack primarily by donor CD4⁺ T cells against host tissue bearing the alloantigens (Via and Shearer, 1988). The engraftment of cytotoxic donor T cells and production of Th1 cytokine are also critical in the progression of GVHD (Via et al., 1987; Hakim et al., 1991). Acute GVHD targets many organs such as the skin, intestine, liver, lung, and lymphoid tissues and is most often characterized by a Th1-type cellular response (Welniak et al., 2007). In the current study, histopathological examination indicated that GVHD caused significant inflammation and necrosis in the liver and colon and marked splenomegaly, all of which were reversed after THC treatment. Moreover, the GVHD was accompanied by a significant induction of Th1 cytokines, which were also down-regulated by THC.

Cannabinoids exert a plethora of pharmacological actions that are mediated through activation of CB1 and CB2 (Pertwee and Ross, 2002; Pertwee 2009). CB1 and CB2 are heptahelical receptors that belong to the large super family of receptors that are coupled to G proteins. In a recent study, it

was shown that bone marrow stromal cells express endocannabinoids, whereas hematopoietic stem and progenitor cells express CB2 (Jiang et al., 2011). Furthermore, it was shown that the CB₂/CB₂ agonist axis mediates the repopulation of hematopoiesis and mobilization of hematopoietic stem and progenitor cells, suggesting CB₂ agonists may be therapeutically useful during clinical conditions such as bone marrow transplantation. In the current study, using CB1 and CB2 antagonists, we found that THC-mediated its effect through activation of both CB1 and CB2 in vivo to suppress GVHD. At the molecular level, although CB1 is predominantly expressed in the central nervous system, it is also expressed at significant levels on immune cells (Galiègue et al., 1995; Pertwee and Ross, 2002). The immune cells also express high levels of CB2 (Galiègue et al., 1995; Pertwee and Ross, 2002). THC along with other endogenous ligands targets both of these receptors as an agonist (Pertwee and Ross, 2002). Therefore, activation of CB1/CB2 on immune cells by agents such as THC has a synergistic effect. Furthermore, blocking of either receptor alone partially reverses the THC effect, whereas combined blocking of both receptors can completely reverse the THC effect. Data presented in Fig. 5 support this phenomenon of dual mediation of responses to THC via CB1/CB2 on immune cells. Our previous work on autoimmune hepatitis also supports this phenomenon (Hegde et al., 2010). This is also the reason we have seen that agents such as THC that activate both CB1 and CB2 are more effective than CB1 or CB2 select agonists for causing immunosuppression. We have previously shown that activation of T cells with THC triggers molecular signaling involving down-regulation of

Raf-1/mitogen-activated protein kinase/extracellular signal-regulated kinase/extracellular signal-regulated kinase/ribosomal kinase pathway, leading to translocation of Bad to mitochondria and this signaling can be inhibited by both CB1 or CB2 antagonists (Jia et al., 2006). Thus, we believe that the downstream signaling events after cannabinoid receptor activation are similar.

It should be noted that the dose of THC used in this study has relevance to the pharmacological doses of THC used in humans. In our study, THC was administered every alternate day and was used at 20 mg/kg body weight, which converts to 60 mg/m² based on area body surface normalization according to the Center for Drug Evaluation and Research guidelines (Center for Drug Evaluation and Research, 2005). The maximum recommended human dose of Marinol, when used as an antiemetic in cancer patients, is up to 90 mg/m²/day. Moreover, a long-term study carried out in rodents with higher doses of THC up to 150 mg/kg/day did not report major adverse effects or fatalities (Chan et al., 1996).

Cannabinoids are being evaluated for their therapeutic potential on the basis of their anti-inflammatory actions. Preclinical and clinical studies indicate anti-inflammatory properties of cannabinoids in nervous-tissue inflammation, inflammatory bowel disease, hepatic inflammation, arthritis, and vascular inflammation (Mackie 2006; Nagarkatti et al., 2009; Pertwee 2009). Studies from our laboratory and elsewhere have indicated that THC may mediate its anti-inflammatory properties through multiple mechanisms (Nagarkatti et al., 2009; Pandey et al., 2009; Rieder et al., 2010). Our laboratory has demonstrated the apoptotic effects of THC in T cells, B cells, and macrophages as one of the major mechanisms of immunosuppression (McKallip et al., 2002b; Do et al., 2004). Furthermore, THC has been indicated to promote a shift in Th cell differentiation from Th1 to Th2. THC treatment was shown to decrease the production of IFN- γ and IL-2 and increase IL-4, IL-10, and transforming growth factor β (Klein et al., 2000). Additional studies from our laboratory have shown that THC can activate Foxp3⁺ regulatory T cells in a murine hepatitis model (Hegde et al., 2008) as well as potent myeloid-derived suppressor cells in vivo (Hegde et al., 2010). In the current study, we observed an increase in regulatory T cells in donor cell population, indicating that the suppression of effector T cell function in THC-injected mice can be in part caused by the up-regulation of regulatory T cells. The precise mechanisms through which THC may induce apoptosis in donor-derived effector T cells while sparing and promoting the expansion of Tregs is unclear. It is not known whether this results from differential regulation of cannabinoid receptor expression, which needs further evaluation. Studies from our laboratory and others have established that THC and other cannabinoids can effectively suppress inflammatory response (Karsak et al., 2007; Michalski et al., 2007; Hegde et al., 2008; Jamontt et al., 2010). Moreover, we have also previously shown that THC can inhibit malignancies of the immune system (McKallip et al., 2002a; Lombard et al., 2005; Jia et al., 2006). Thus, cannabinoid receptors may be an excellent target candidate for use in graft-versus-leukemia treatment as well.

In summary, the data presented here demonstrate that THC may be an effective drug in the prevention and treatment of GVHD. Our studies open new avenues to explore cannabinoid receptor targeting as a novel therapeutic ap-

proach for treating GVHD. Of significant interest is the potential use of CB2-selective agonists that are nonpsychoactive or manipulation of endocannabinoids, to treat GVHD or promote graft-versus-leukemia.

Authorship Contributions

Participated in research design: M. Nagarkatti and P. S. Nagarkatti.

Conducted experiments: Pandey.

Performed data analysis: Pandey, Hegde, M. Nagarkatti, and P. S. Nagarkatti.

Wrote or contributed to the writing of the manuscript: Pandey, Hegde, M. Nagarkatti, and P. S. Nagarkatti.

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