

Title Page

**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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Running Title Page

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Cannabinoids suppress graft-versus-host disease

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CB, cannabinoid receptor; BMT, bone marrow transplantation; ConA, concanavalin A; FITC, fluorescein isothiocyanate; GVHD, graft-versus-host disease; GVT, graft-versus-tumor; HCT, hematopoietic cell transplantation; mAb, monoclonal antibody; PE, phycoerythrin; THC, delta-9-tetrahydrocannabinol; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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ABSTRACT

Allogeneic hematopoietic cell transplantation (HCT) is widely used to treat patients with life threatening malignant and nonmalignant hematological diseases. However, allogeneic HCT is often 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 delta-9-tetrahydrocannabinol (THC) in an acute model of GVHD that was induced by transferring parental C57Bl/6 (B6) spleen cells into (C57Bl/6 X DBA/2) F1(BDF1) mice. Transfer of B6 cells into BDF1 mice produced severe acute GVHD in the recipient, characterized by lymphoid hyperplasia, weight loss, Th1 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⁺ Tregs. 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 CB1 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 (HCT) 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 following allogeneic HCT 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 transplantation (BMT) 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 death of 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 associated often with development of serious side effects including nephrotoxicity and cardiotoxicity, thereby reducing the quality of life in BMT recipients (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 so as to promote graft-versus-tumor (GVT) effect, without causing severe toxicity resulting from the expansion of donor-derived T cells.

Cannabinoids, the active ingredients found in *Cannabis sativa* are 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). Recent 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 as well as malignancies of immune origin (McKalip et al., 2002a; Hall et al., 2005; Lombard et al., 2005; Ramer et al., 2009). Δ -9-Tetrahydrocannabinol (THC) is one of the most extensively investigated ingredients found in cannabis. THC activates both CB1 and CB2 receptors thereby mediating both psychotropic and anti-inflammatory properties. THC is used clinically in cancer and HIV/AIDS patients to increase appetite and decrease nausea (Hall et al., 2005; Pertwee, 2009). It is also used to help glaucoma patients 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; Pandey et al., 2009a; Nagarkatti et al. 2010), 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.

Methods

Mice

Female C57BL/6 (B6, H-2^b, CD45.2⁺) and (C57BL6XDBA/2) F1 (BDF1, H-2^{b/d}, CD45.2⁺) mice (6-8 weeks old) were purchased from The Jackson laboratory. Animals were housed in specific pathogen-free conditions and received filtered water and normal chow. All experiments were approved by institutional Animal Care and Use committee.

Induction of acute GVHD and treatment with THC

Six to 8 weeks old normal female B6 mice (H-2^b) were used as donors. Acute GVHD was induced by injecting 5×10^7 B6 spleen cells *i.v.* into normal female BDF1 recipient mice. THC (Δ^9 -tetrahydrocannabinol) at a concentration of 20 mg/kg body weight was dissolved in ethanol and diluted in PBS. THC or vehicle was then administered *i.p.* (0.1 mL/ mouse) into recipient BDF1 mice, 1 h after B6 donor cell injection. THC treatment was continued every alternate day thereafter till the termination of the study. Similarly, cannabinoid receptor antagonists AM251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide, CB1 antagonist) and SR144528 (5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]-1H-pyrazole-3-carboxamide, CB2 antagonist) were dissolved in dimethyl sulfoxide (DMSO) containing one drop of Tween 80 (Sigma-Aldrich) and were further diluted in PBS. Antagonists were injected *i.p.* (0.1 mL/ mouse) 1 h before THC injection. All control groups received respective vehicles such as ethanol or DMSO similarly diluted in PBS.

Monoclonal antibodies, reagents, Flow cytometry

The following antibodies used for flow cytometric analysis were purchased from BD Biosciences (San Diego): anti-murine Fc γ receptor, FITC-conjugated anti-H-2D^d and PE-conjugated H-2D^b. Isotype-matched control Abs 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. Briefly, 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%FBS 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 in Cytomics CXP software (Beckman Coulter). Triple staining analyses was carried out to measure apoptosis in 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 apoptotic cells were determined on either gated donor or host cells by performing TUNEL assay (Roche). In addition, three color analysis was performed on gated cells by using FITC, phycoerythrin or biotin conjugated mAbs to analyze CD4⁺ and Treg cell population.

Cytokine analysis

The levels of cytokines IFN- γ and IL-2 in samples were measured by sandwich ELISA using respective ELISA kits purchased from Peprotech (Rocky Hill, NJ). To measure cytokine production in vitro with or without Con 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 ELISA kit.

Spleen index

Intensity of systemic GVHD was assessed by measuring spleen index using 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 RPMI1640 from the femurs. To perform colony-forming unit-granulocyte macrophage (CFU-GM) assay, 1.0×10^5 BM 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, 3U erythropoietin, 2 mmol/L L-glutamine and 2% pokeweed mitogen-stimulated spleen cell-conditioned medium. Cells were cultured in 1 ml 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 inverted microscope.

Ex vivo spontaneous proliferation assay

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

⁵¹Cr release assay to detect anti-host CTL activity in vitro

Specific anti-host 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 µCi ⁵¹Cr/ 2×10^7 cells and 100 µl aliquots were then added to the wells of microtiter plates. Responder

cells (4.0×10^6 cells) were co-cultured with 1.0×10^6 irradiated (30 Gy) splenocytes of normal BDF1 (stimulator cells) in presence of rIL-2 (100U) 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 E:T ratios of 100:1, 50:1, 25:1 and 12.5:1. The plates were incubated at 37°C for 4 h and percent cytotoxicity was calculated by the following formula: % cytotoxicity = $100 \times (\text{counts/min samples} - \text{counts/min background}) / (\text{counts/min maximum} - \text{counts/min background})$.

Histopathology

On day 21 after GVHD induction, tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections cut were 5- μm thick and stained with hematoxylin and eosin and examined under light microscope to assess the inflammation associated with GVHD.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 4.03 software (GraphPad Software, Inc.). Data are presented as the mean \pm SEM. Student's t-test was used to compare data between two groups. Results from body weight were analyzed by using non-parametric 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 if cannabinoids can be used in the treatment of GVHD, we developed an acute parent→F1 GVHD model in which the activated donor cells recognize the recipient's cells as foreign and destroy it while the recipient's cells recognize the donor as self. To this end, C57BL/6 splenocytes were injected *i.v.* into BDF1 recipient mice on day 0. Beginning day 1, THC (20 mg/kg body wt) or vehicle was administered *i.p.* 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). Additionally, 3 out of 6 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 following THC treatment (Fig 1B, C, 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 4 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 in Figs 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* proliferation of transferred spleen cells (Fig 2A). Treatment of GVHD mice with THC significantly reduced the 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 2B and C showed that splenocytes obtained from vehicle-treated GVHD mice expressed significant levels IL-2 and IFN- γ while THC-treated GVHD mice had significantly decreased levels of these cytokines. We next evaluated the cytotoxic T lymphocyte (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 when compared to GVHD+vehicle 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 can distinguish donor-derived (H-2^b) versus recipient-derived (H-2^{b/d}) cells. Data from a representative experiment is shown in Fig 3A, which 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; McKallip et al., 2005). To investigate if 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 GVHD+THC groups, a significant proportion of cells were undergoing apoptosis(49.14%) when compared to GVHD +vehicle groups (8.77%). Also, 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 haematopoiesis 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 if THC treatment would reverse this toxicity. As shown in Fig 4A and B, vehicle-treated GVHD mice exhibited impaired haematopoiesis associated with marked reduction in number of granulocyte-macrophage progenitor cells (CFU-GM) on day 21 after B6 cell transfer when compared to vehicle or THC-treated mice in which GVHD had not been induced. In contrast, bone marrow cells isolated from THC-treated GVHD mice formed significantly greater number of CFU-GM proving that THC treatment reversed the suppression of haematopoiesis seen during acute GVHD.

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 following THC treatment.

Similarly, 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 cannabinoid receptors, CB1 and CB2. To investigate if 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 splenomegaly, mediated by THC, was significantly reversed by blocking either CB1 or CB2 receptors (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 receptors.

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, while the percentage of CD4⁺Foxp3⁺ Tregs did not change significantly when compared to naïve 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 6A, B). Interestingly, in GVHD+THC groups, there was an additional increase in both the percentage and numbers of Tregs when compared to GVHD+Veh groups thereby suggesting that THC may induce Tregs during GVHD (Fig 6A, B). To further determine if 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^{b/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 were two 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 when compared to the GVHD+Veh mice, were indeed derived from the donor. Together, our data indicated an interesting 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, 60–75% of patients do not respond or respond poorly to this treatment leading to deadly complication of GVHD, including lethal ruting disease, defined by wasting, diarrhea, and skin lesions (Welniak 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 haematopoiesis 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. Importantly, THC-mediated protection from GVHD was regulated in vivo through activation of CB1 and CB2 receptors. 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 understanding of 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 DBA/2 strain into an un-irradiated (C57BL6 x 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. The 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 which were all reversed following THC treatment. Moreover, the GVHD was accompanied by significant induction of Th1 cytokines which were also down regulated by THC.

Cannabinoids exert plethora of pharmacological actions that are mediated through activation of CB1 and CB2 receptors (Pertwee and Ross, 2002; Pertwee 2009). CB1 and CB2 are heptahelical receptors which belong to the large super family of receptors that are coupled to G-proteins. In a very recent study, it was shown that bone marrow stromal cells express endocannabinoids, whereas hematopoietic stem and progenitor cells (HSPCs) express CB2 receptors (Jiang et al., 2011). Furthermore, it was shown that the CB₂/CB₂ agonist axis mediates repopulation of hematopoiesis and mobilization of HSPCs thus 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 CNS, it is also expressed at significant levels on immune cells (Galiegue et al., 1995; Pertwee and Ross, 2002). The immune cells also express high levels of CB2 (Galiegue et al., 1995; Pertwee and Ross, 2002). THC along with other endogenous ligands targets both these receptors as an agonist (Pertwee and Ross, 2002). Therefore, activation of CB1/ CB2 receptors 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 the receptors can completely reverse the THC effect. Data presented in Fig 5 supports this phenomenon of dual mediation of responses to THC via CB1/CB2 receptors on immune cells. Our previous work on autoimmune hepatitis also supports this phenomenon (Hegde et al., 2010). This is also the reason why 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/ERK kinase (MEK)/ERK/RSK pathway leading to translocation of Bad to mitochondria and that this signaling can be inhibited by both CB1 or CB2 antagonists (Jia et al., 2006). Thus, we believe that the downstream signaling events following 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 body area surface normalization as per the Center for Drug Evaluation and Research guidelines. 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; Rieder et al.; Pandey et al., 2009). Our laboratory has earlier 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 also shown to decrease the production of IFN- γ and IL-2, and increase IL-4, IL-10 and TGF- 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 suppression of effector T cell function in THC-injected mice can be in part due to upregulation of regulatory T cells. The precise mechanisms through which THC may induce apoptosis in donor-derived effector T cells while sparing as well as promoting the expansion of Tregs is unclear. It is not known if this results from differential regulation of cannabinoid receptor expression, which needs further evaluation. Studies from our laboratory and by 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 (GVL) 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 up new avenues to explore cannabinoid receptor targeting as a novel therapeutic approach to treat GVHD. Of significant interest is the potential use of CB2 select agonists that are non-psychoactive or manipulation of endocannabinoids, to treat GVHD or promote GVL.

Authorship Contributions

Participated in research design: Nagarkatti M, Nagarkatti PS

Conducted experiments: Pandey R

Performed data analysis: Pandey R, Hegde VL, Nagarkatti M, Nagarkatti PS

Wrote or contributed to the writing of the manuscript: Pandey R, Hegde VL, Nagarkatti M,
Nagarkatti PS

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Footnotes

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Legends for Figures

Figure 1. Effect of THC on clinical indicators during acute GVHD. Acute parent→F1 GVHD was induced by *i.v.* injection of C57BL/6 splenocytes into groups of 5-6 BDF1 recipient mice on day 0. THC (20 mg/kg body wt) or the vehicle was administered *i.p.* every alternate day beginning day 1. As controls, normal mice injected with vehicle or THC alone were also included as indicated. Panel A shows the mean \pm SEM of body weight in GVHD-induced recipient BDF1 mice. Panel B shows splenomegaly in various groups: a) normal mice+vehicle, b) normal mice+THC, c) GVHD mice+ vehicle, d) GVHD mice+THC. Panel C shows spleen index calculated as described in Methods on days 7 and 21 after donor cell transfer. In panel D, total spleen cellularity for each treatment on day 21 is shown. Data expressed as means \pm SEM. ** P<0.005; * P<0.01.

Figure 2. Effect of THC administration on spontaneous *ex vivo* proliferation and cytokine production by splenocytes from recipient BDF1 mice. Parent→F1 GVHD was induced as described in Fig 1. In Panel A, splenocytes were harvested from GVHD-induced recipient mice and degree of spontaneous proliferation was studied by culturing cells for 4 h with ³thymidine. Data were expressed as mean \pm SEM of triplicate cultures. In panel 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 SEM of triplicate cultures.* P<0.01. # Undetectable. Panel D: Effect of THC on anti-host cytotoxic T lymphocyte (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 ⁵¹Cr-release assay. Results shown are mean \pm SEM of

percent cytotoxicity from quadruplicate cultures. Similar results were obtained in two replicate experiments.

Figure 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. Panel A: Donor cells were identified by expression of H-2^b only while host-derived F1 cells by combined expression of H-2^d and H-2^b (H-2^{b/d}). Panel B shows absolute numbers of donor derived and host derived cells in GVHD+Veh and GVHD+THC groups. **p<0.01 Student's t test. Data are mean of 5 mice/ group. Splenocytes from normal F1 mice were used as a control for double-staining. In panel C, donor cells were gated and analyzed for apoptosis using TUNEL assay.

Figure 4. Effect of THC on colony-forming unit-granulocyte macrophage (CFU-GM) during acute GVHD. Parent→F1 GVHD was induced by *i.v.* 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 in Methods. Panel A: The photograph depicts the formation of colonies on methylcellulose: a) Vehicle; b) THC; c) GVHD+Vehicle; d) GVHD+THC. Panel B: The numbers of colonies were counted on day 7 of culture. Vertical bars represent mean ± SEM of CFU-GM per 10⁵ cells from 4 individual mice. **indicates p<0.001. Panel C: Histological examination of tissues obtained from liver and colon 21 days after induction of GVHD. On day 21, 3-4 mice for 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 20X). A representative section of liver (a, b, c) and colon (d, e, f) has been depicted.

Figure 5. Effect of select CB1 and CB2 antagonists on GVHD associated splenomegaly following THC treatment. GVHD was induced as described in Fig 1 followed by treatment with vehicle, THC and CB1/CB2 antagonists as described in Methods. Panel A shows photographs of representative spleen from each group. Spleen index was determined on day 21 (panel B). Vertical bars represent mean \pm SEM of 5 mice/ group. * $p < 0.001$; # $P < 0.01$ vs THC treated group; ## $P < 0.001$ vs THC treated group.

Figure 6. THC treatment increases Foxp3⁺ T regulatory cells during GVHD. Induction of GVHD and treatment with vehicle or THC was performed as described in Fig1. 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. Panel A shows recipient spleen cells stained for CD4 and Foxp3. In panel B, absolute number of CD4⁺Foxp3⁺ Tregs is shown. Data represent mean \pm SEM of 5 mice/ group. * $p < 0.05$, Student's t test. In panel C, spleen cells were triple stained for H-2^b, H-2^d and Foxp3. Next, the donor (H-2^b)-derived and host-derived (H-2^{b/d}) cells were gated and analyzed for Foxp3⁺ cell population. Representative flow profiles (Panel C) are depicted.

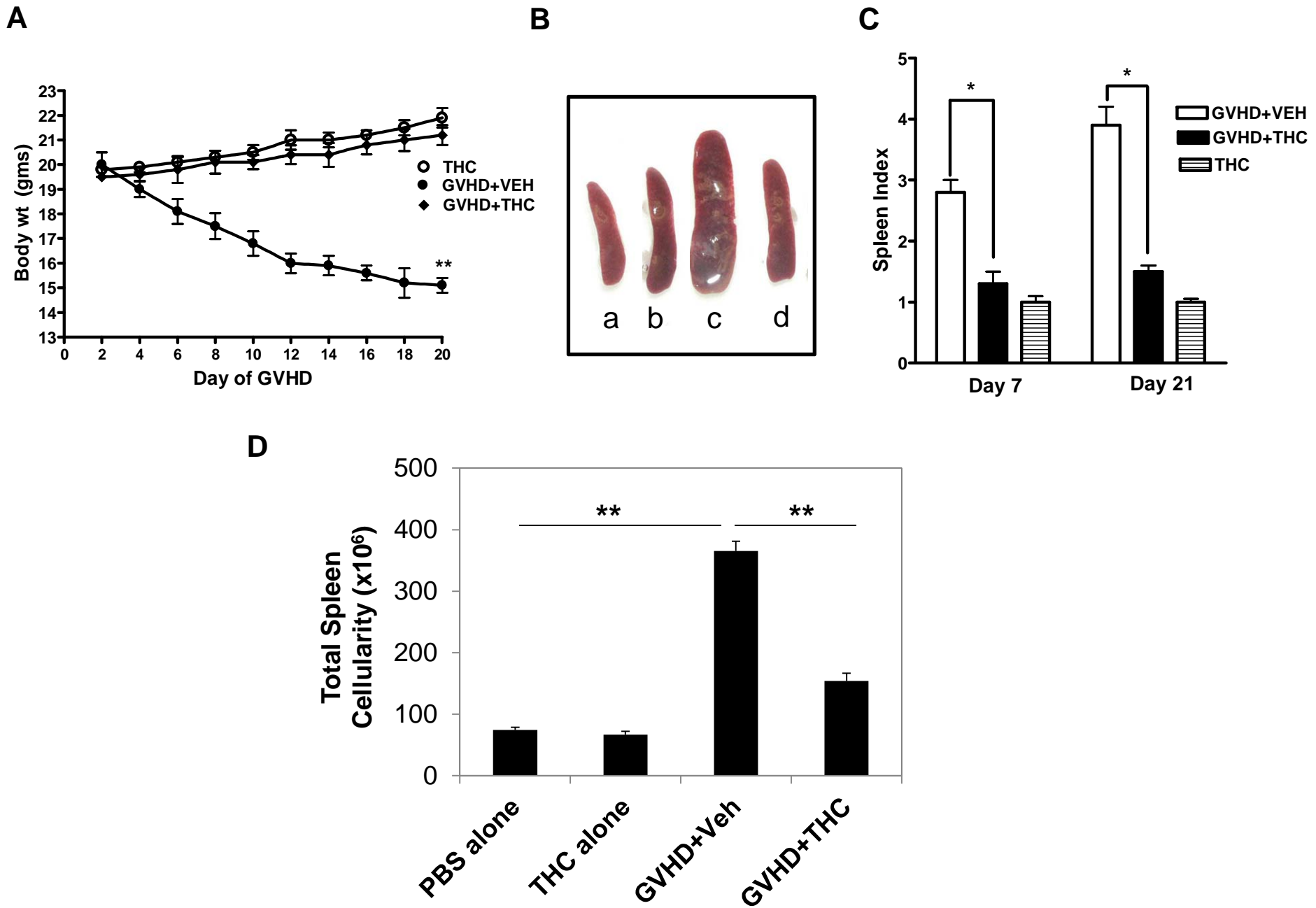


Figure 1

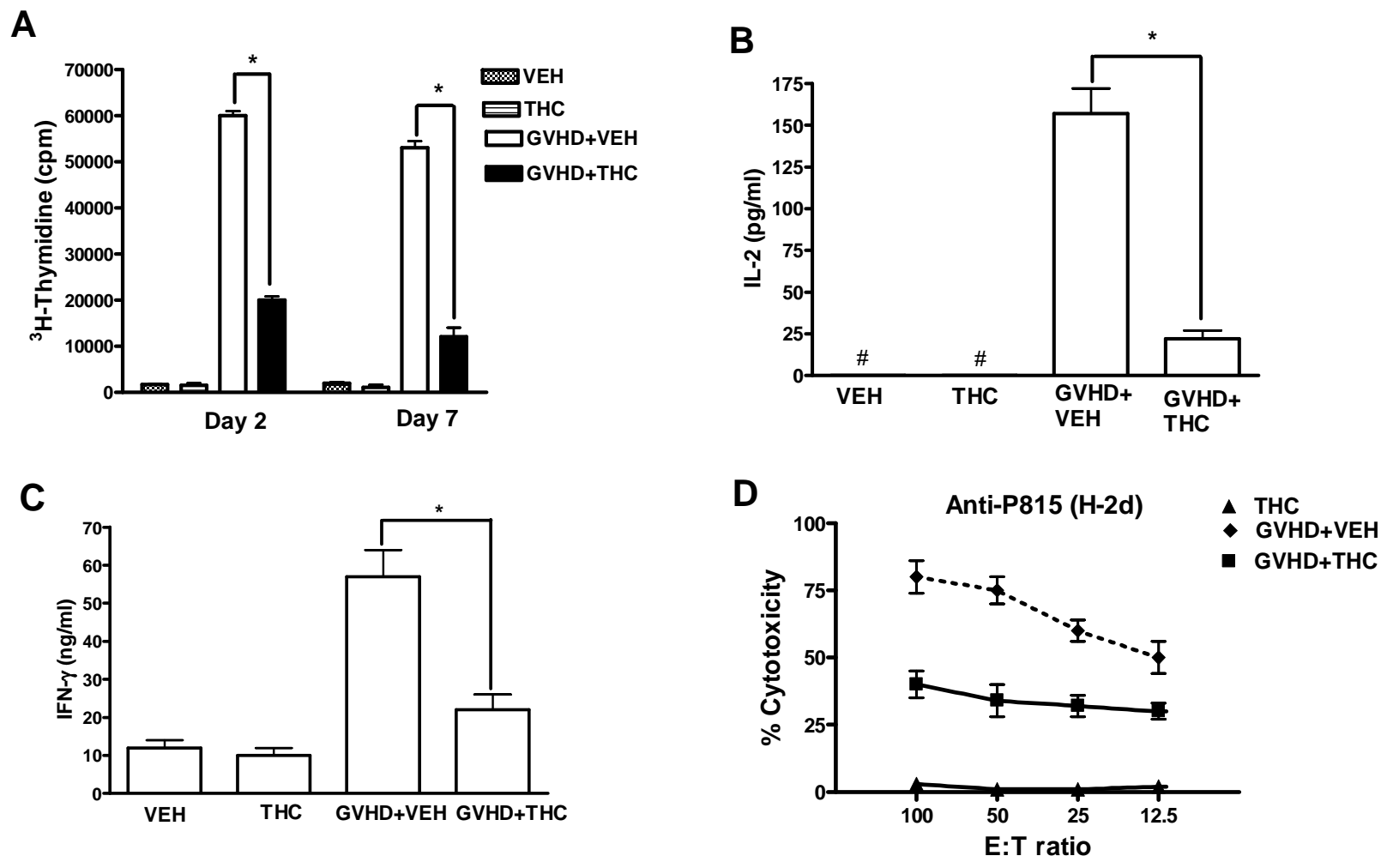


Figure 2

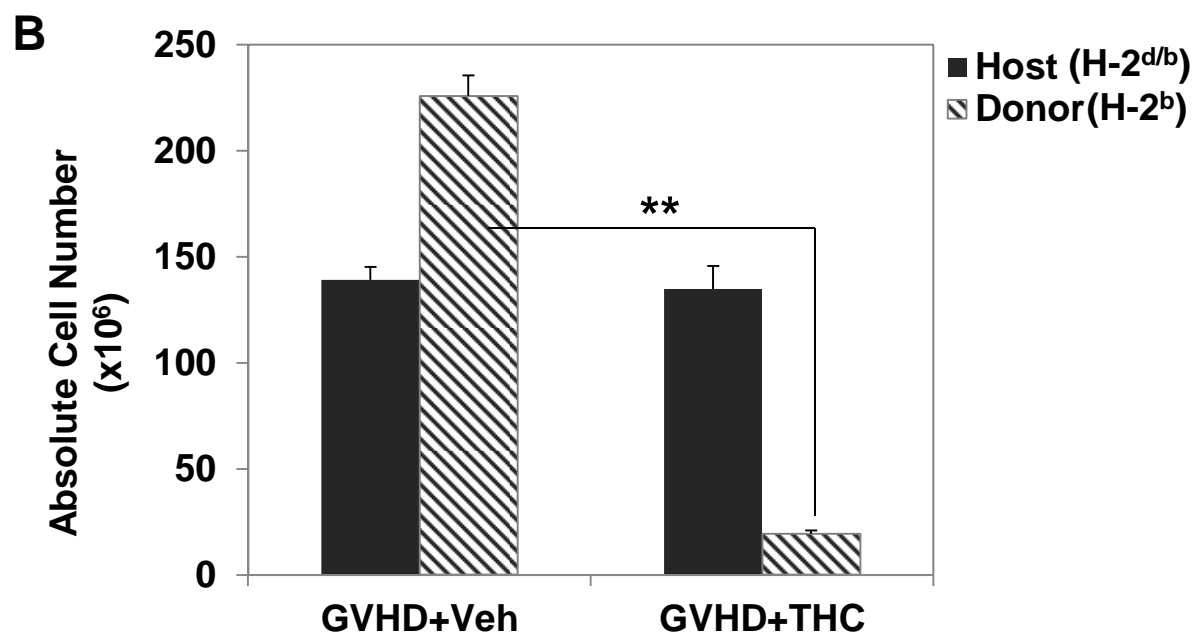
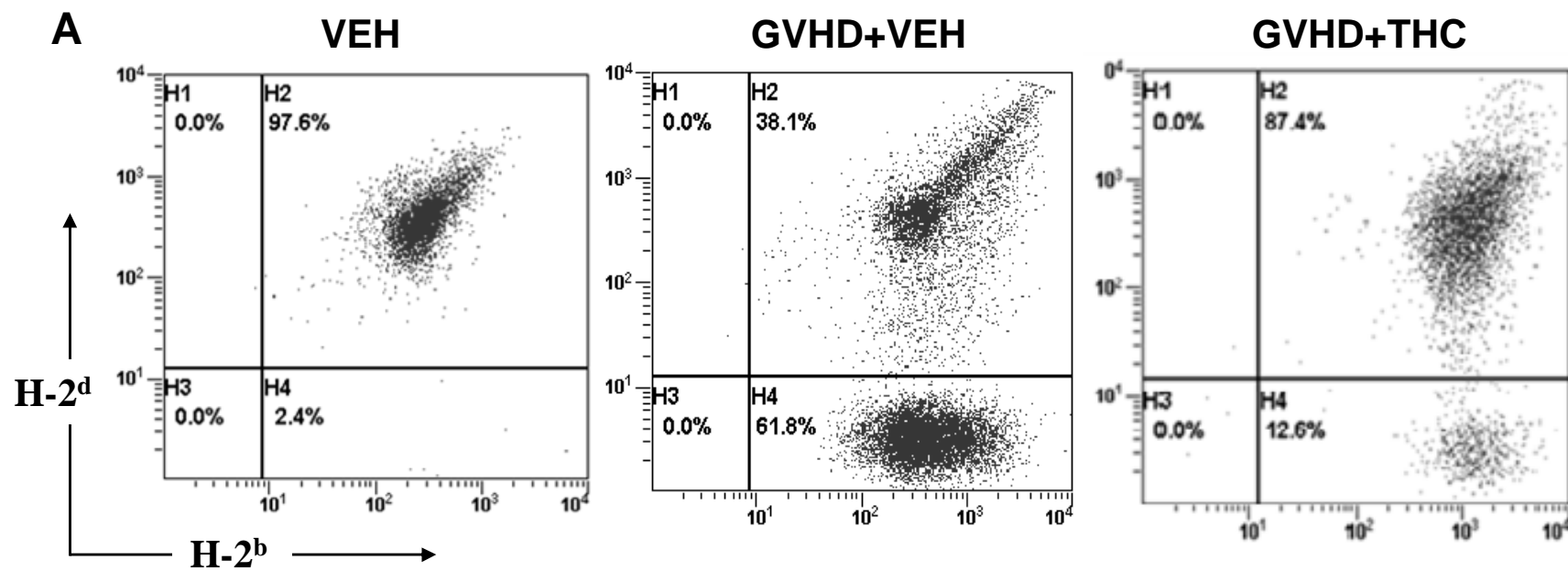
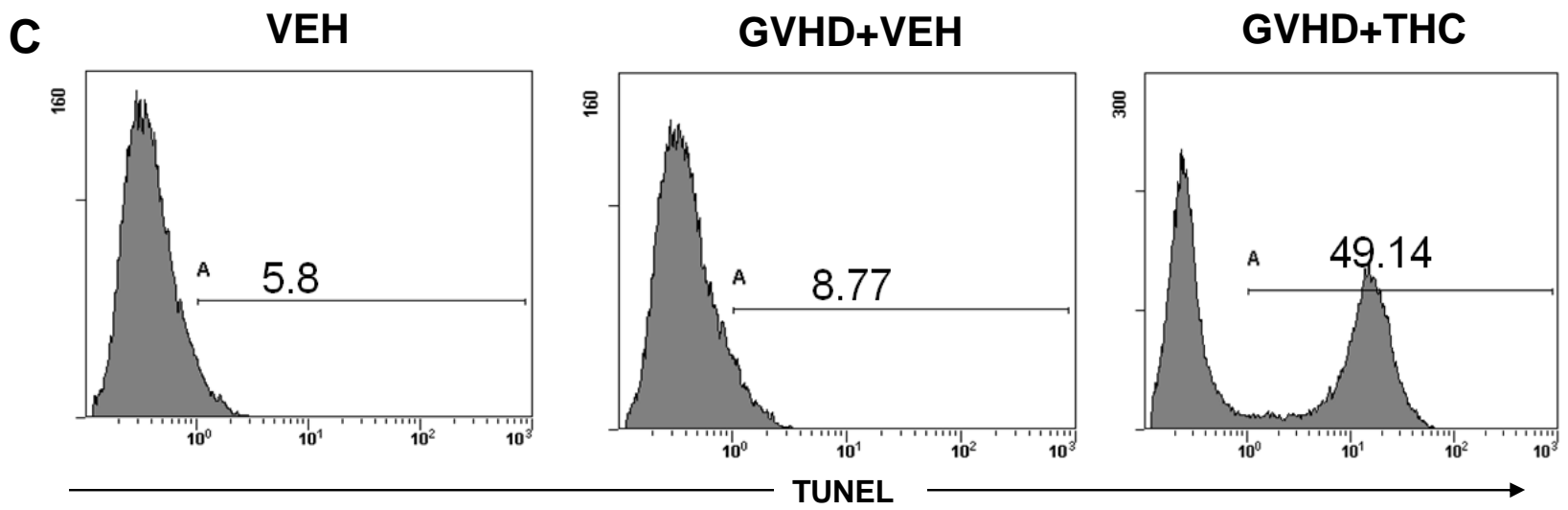
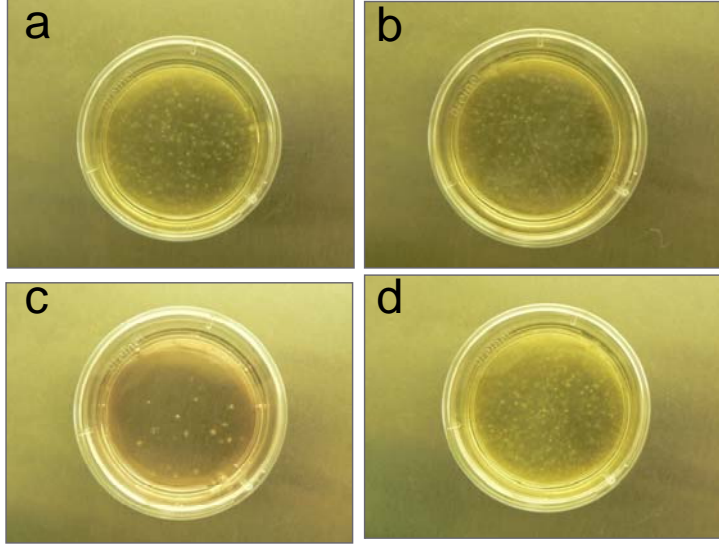
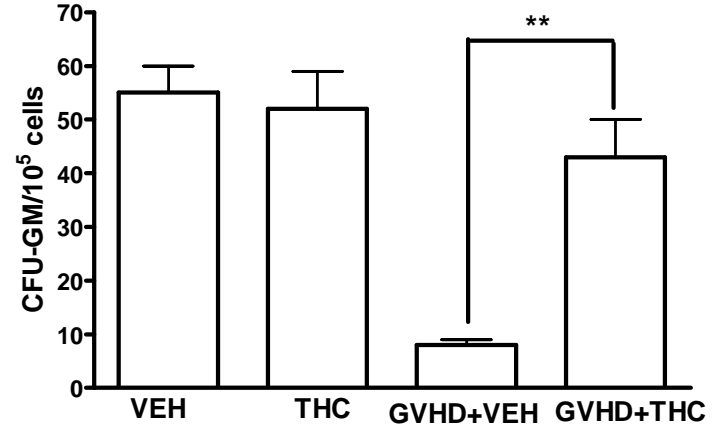
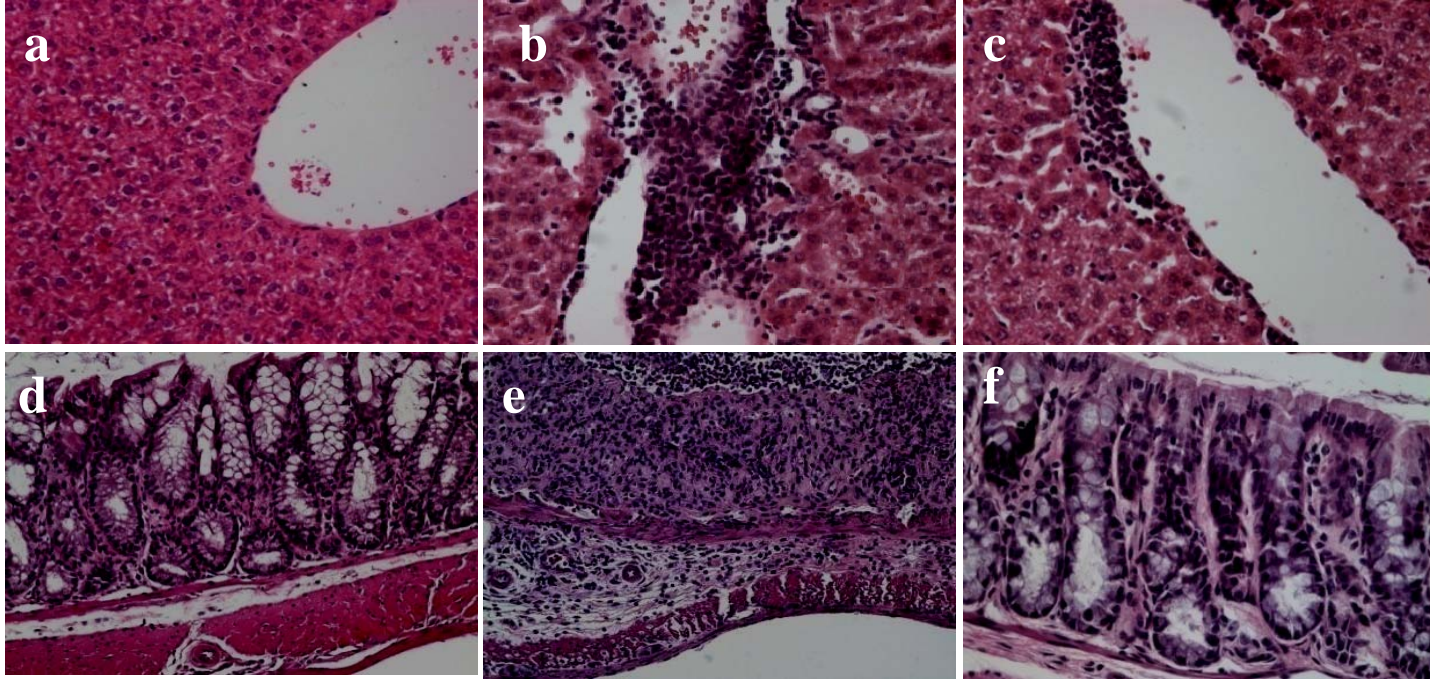


Figure 3



A**B****C****Figure 4**

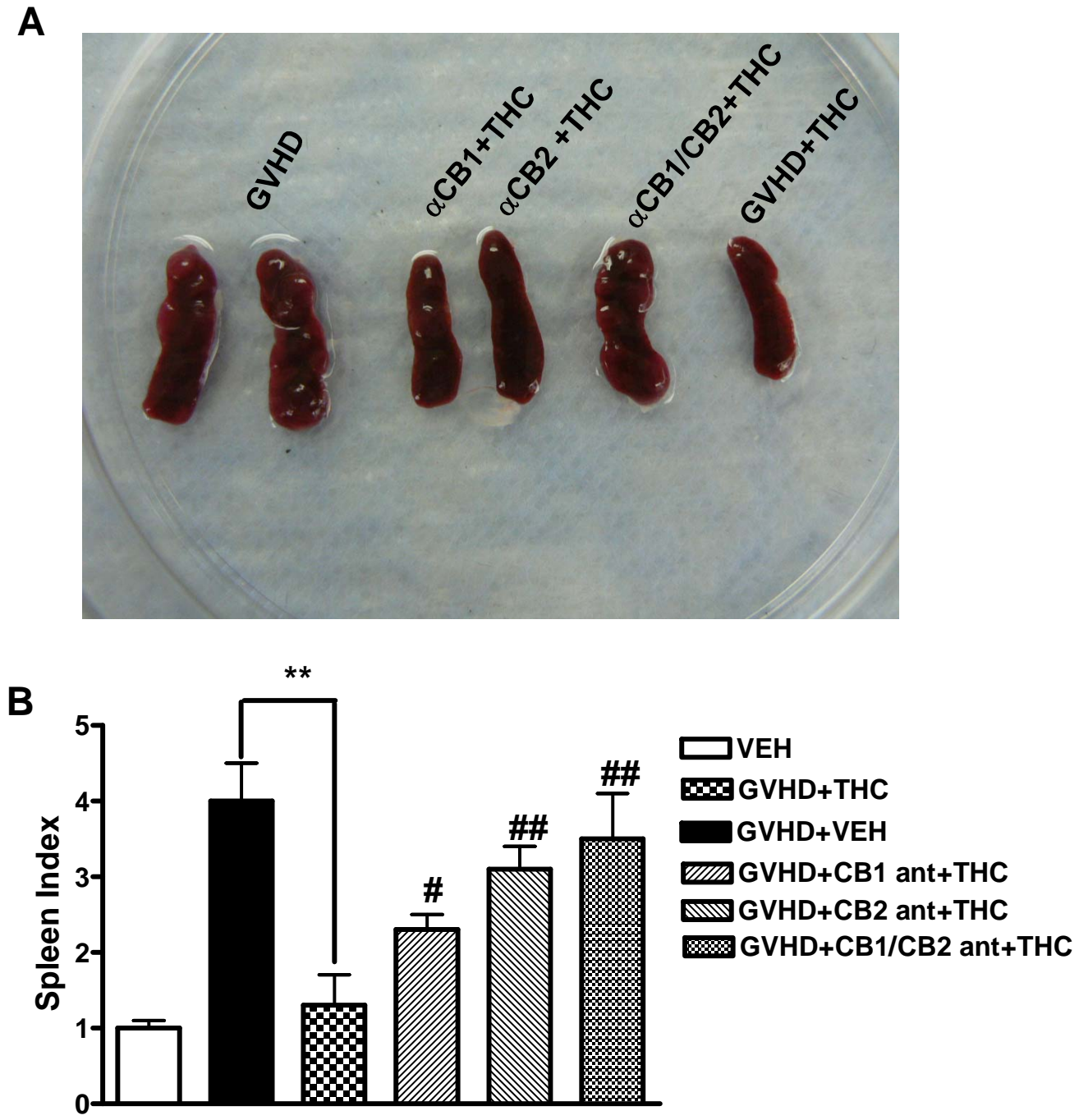


Figure 5

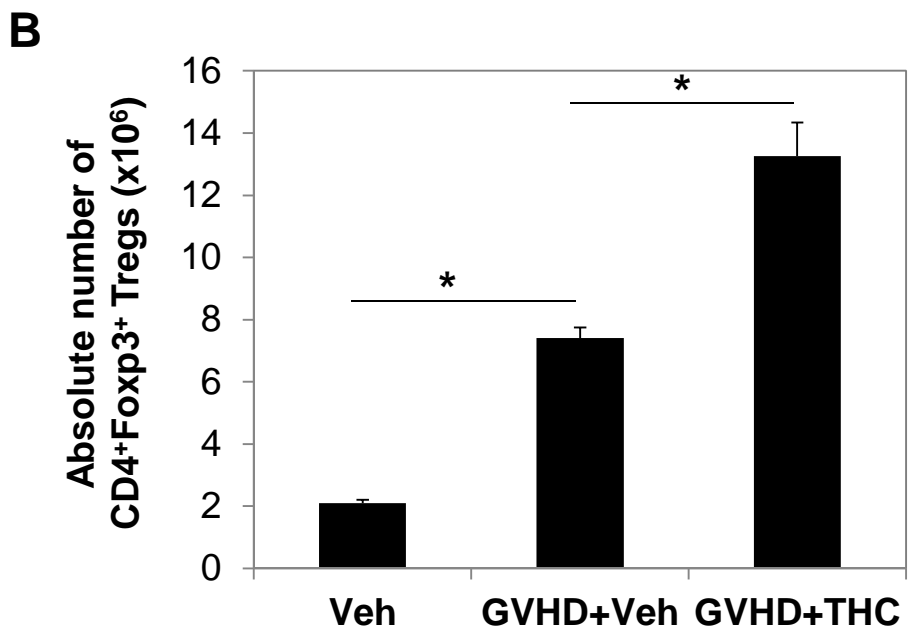
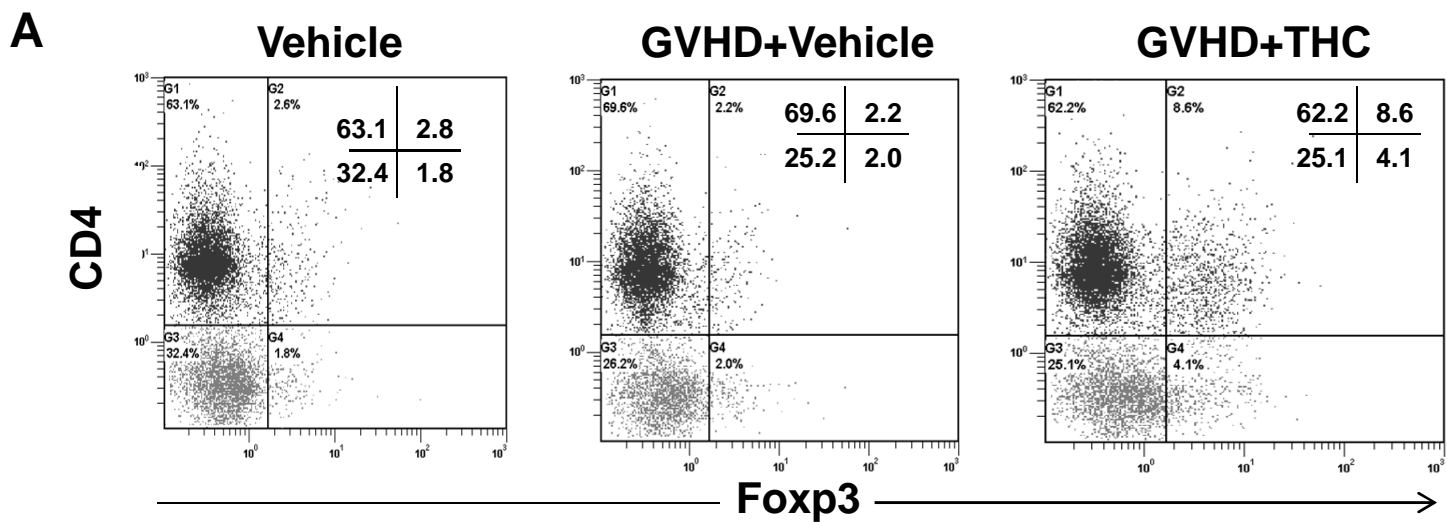
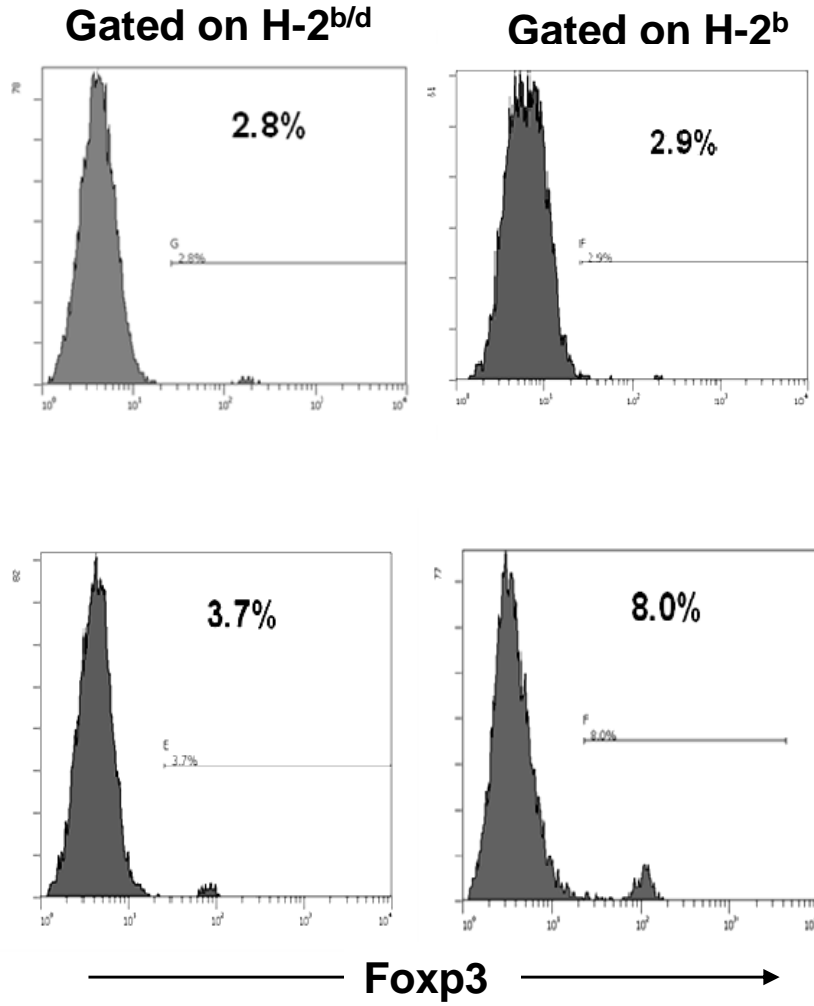
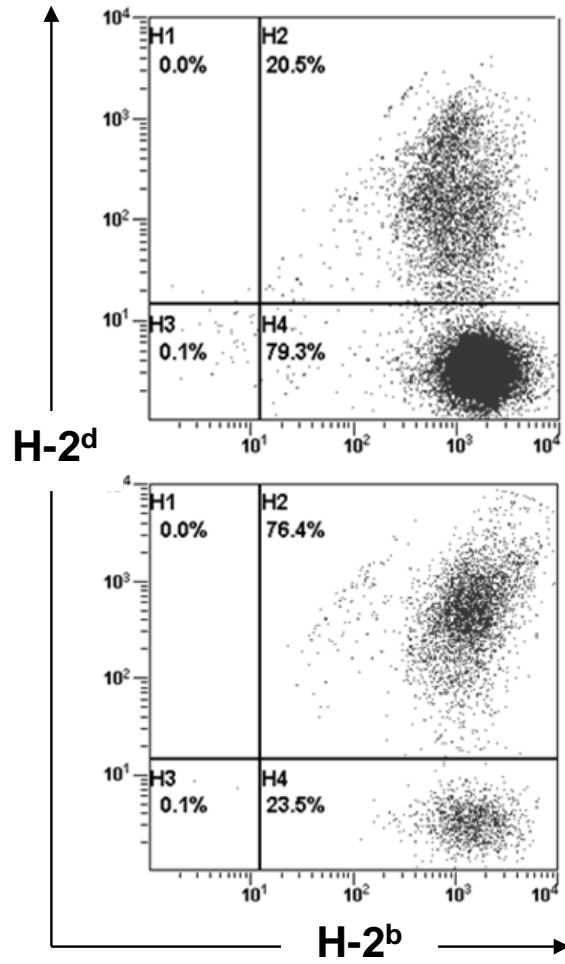


Figure 6

C



GVHD+Veh

GVHD+THC

FcγR3

Figure 6