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Title Page

Attenuation of experimental autoimmune hepatitis by exogenous and endogenous cannabinoids: Involvement of regulatory T cells

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Running Title Page

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Anandamide or AEA, arachidonoylethanolamide; AST, aspartate transaminase; AIH, autoimmune hepatitis; THC, delta-9-tetrahydrocannabinol; foxp3, Forkhead helix transcription factor p3; MAFP, methylarachidonyl fluorophosphate; MNCs, mononuclear cells; Tregs, regulatory T cells.

Abstract

Immune-mediated liver diseases including autoimmune and viral hepatitis are a major health problem worldwide. Natural cannabinoids such as Δ^9-tetrahydrocannabinol (THC) effectively modulate immune cell function and have shown therapeutic potential in treating inflammatory diseases. We investigated the effects of THC in a murine model of Concanavalin A-induced hepatitis. Intraperitoneal administration of THC after ConA challenge inhibited hepatitis as shown by significant decrease in liver enzymes and reduced liver tissue injury. Furthermore, THC treatment resulted in significant suppression of crucial inflammatory cytokines in ConA-hepatitis. Interestingly, THC treatment in ConA-injected mice led to significant increase in absolute number of $Foxp3^+$ T regulatory cells in liver. Surprisingly, select cannabinoid receptor (CB1 or CB2) agonists were not able to block hepatitis either independently or in combination. However, CB1/CB2 mixed agonists were able to efficiently attenuate hepatitis similar to THC. The modulatory effect of THC in ConA-induced hepatitis was reversed by both CB1 and CB2 antagonists. We also observed that endogenous cannabinoid anandamide was able to reduce hepatitis by suppressing cytokine levels. Also, deficiency or inhibition of endocannabinoid hydrolyzing enzyme, fatty acid amide hydrolase (FAAH), which leads to increased levels of endogenous cannabinoids, resulted in decreased liver injury upon ConA challenge. Our data demonstrate that targeting cannabinoid receptors using exogenous or endogenous cannabinoids and use of FAAH inhibitors may constitute novel therapeutic modalities to treat immune-mediated liver inflammation.

Liver diseases are a serious human health problem worldwide. The pathogenesis of immune-mediated liver diseases is very complex and involves several inflammatory cells and cytokines. Growing evidence suggests that major human liver diseases such as autoimmune and viral hepatitis are caused in general by activated T cell-mediated immune responses (Chisari and Ferrari, 1995; Kita et al., 2001). Autoimmune hepatitis (AIH) is a severe form of liver disease characterized by progressive destruction of the hepatic parenchyma, cellular infiltration, hypergammaglobulinemia, and autoantibodies. The prevalence of AIH is estimated to range between 50 and 200 cases per million population and accounts for 5.9% of all liver transplantations in the United States. AIH affects women more than men (3.6:1), and all ages and ethnic groups are susceptible to it (Czaja and Freese, 2002).

T cell-mediated hepatitis can be induced in mice by intravenous injection of mitogenic plant lectin, concanavalin A (ConA), which leads to polyclonal activation of T cells resulting in clinical and histological symptoms of acute hepatitis, including elevation of transaminase activities within 8-24h (Tiegs et al., 1992). It is a well-established mouse model for AIH. Activated $CD4^+$ T cells, natural killer T (NKT) cells, and Kupffer cells are the most prominent effector cells in this model which, together with macrophages and eosinophils, induce hepatocyte killing by contact or indirectly by producing large amounts of inflammatory cytokines (Gantner et al., 1995; Schumann et al., 2000; Takeda et al., 2000; Tiegs et al., 1992). Among several pro-inflammatory cytokines involved, TNF-α and IFN-γ play a critical role in direct induction of liver cytotoxicity, because anti-TNF-α and anti-IFN-γ mAbs protect mice from ConA-induced liver injury (Gantner et al., 1995; Kusters et al., 1996).

Delta-9-tetrahydrocannabinol (THC), the major psychoactive component of marijuana (*Cannabis sativa*) has wide ranging pharmacological properties (Mackie, 2006). The cannabinoid

compounds possess significant immunosuppressive and anti-inflammatory properties (Croxford and Yamamura, 2005). THC and cannabinoid receptor agonists have shown promise in several models of inflammation (Pertwee, 2002; Quartilho et al., 2003; Steffens et al., 2005). Cannabinoid receptors CB1 and CB2 are hetero-trimeric G protein-coupled receptors, which are activated by THC, as well as by endogenous cannabinoids. Arachidonoylethanolamide (anandamide or AEA) has been identified as the major endogenous ligand for cannabinoid receptors (Devane et al., 1992). Recent studies have demonstrated the involvement of endocannabinoid system in chronic liver diseases (Julien et al., 2005; Siegmund and Schwabe, 2008; Teixeira-Clerc et al., 2006). It has been shown that endocannabinoid signaling through CB1 and CB2 receptors may produce pro- and anti-fibrogenic responses respectively in the liver during fibrosis (Julien et al., 2005; Teixeira-Clerc et al., 2006).

The aim of the present study was to determine the immunomodulatory effect of THC in the murine model of ConA-induced hepatitis. We demonstrate that a single injection of THC significantly ameliorates ConA-induced T cell-mediated liver injury by up regulating $Foxp3^+$ regulatory T cells, and down regulating inflammatory cytokines. Using select cannabinoid receptor agonists and antagonists we demonstrate that THC mediates immune modulation in this model by signaling through both CB1 and CB2 receptors. Importantly, we also demonstrate that anandamide, an endocannabinoid can also effectively attenuate the disease.

Materials and Methods

Reagents. Concanavalin A, JWH-133 and methylarachidonyl fluorophosphate (MAFP) were purchased from Sigma-Aldrich (St. Louis, MO). Arachidonyl-2'-chloroethylamide (ACEA), AM251, AM630, CP55,940 and WIN55212 were from Tocris Bioscience (Ellisville, MO). THC, AEA and SR144528 were provided by NIDA, NIH (Bethesda, MA). 4-Benzyloxyphenyl-n-butylcarbamate (URB532) was from Calbiochem. The monoclonal antibodies (mAbs), FITC-conjugated anti-CD3, anti-CD25, PE-conjugated anti-CD4, anti-CD19, anti-Mac3, anti-CD11a, anti-CD11b, anti-Gr-1, anti-F4/80 and PE-Cy7 conjugated anti-NK1.1 were purchased from BD Bioscience (San Diego, CA). Anti-mouse CD25 mAb was a product of Cedarlane Laboratories (Hornby, ON, Canada).

Mice. Female C57BL/6 mice (8-12 weeks old) were purchased from NCI, NIH, Frederick, MA. Fatty acid amide hydrolase (FAAH) knockout mice have been described before (Cravatt et al., 2001). Animals were housed in specific pathogen-free conditions and all experiments were approved by Institutional Animal Care and Use Committee.

Induction of ConA-Hepatitis and Drug Administration. Concanavalin A was dissolved in pyrogen-free PBS at a concentration of 2.5 mg/ml and injected intravenously at a dose of 12.5 mg/kg body weight (in 0.1 ml PBS) to induce hepatitis as described (Chen et al., 2001). THC was dissolved in ethanol and diluted in PBS and administered i.p. 5 or 30 min after ConA injection. Anandamide dissolved in ethanol, JWH-133 dissolved in DMSO were diluted in PBS and injected i.p. 5 min after injecting ConA. Cannabinoid receptor antagonists AM251, AM630 or SR144528 were dissolved in DMSO containing drop of Tween 80 (SigmaAldrich), further

diluted in PBS and were injected i.p. 1 h before injecting ConA and THC. Control groups received vehicle ethanol, DMSO or DMSO+Tween-80 vehicle similarly diluted in PBS.

Analysis of Transaminase Activities. Blood from individual mice were obtained at various time points after ConA injection. Liver enzymes aspartate transaminase (AST) or alanine transaminase (ALT) activities in the serum were determined by spectrophotometric method using commercially available assay kits (Pointe Sci. Inc.), as described previously (Chen et al., 2001).

Liver Histology and TUNEL Staining. Mice were euthanized 24 h after ConA injection. Livers were fixed in 10% buffered paraformaldehyde (pH 7.4), and embedded in paraffin. Sections (5 μm) on slides were deparaffinized in xylene, rehydrated in decreasing concentrations of ethanol and stained with hematoxylin-eosin (H&E). Slides were examined by light microscopy for infiltrating leukocytes and tissue injury. TUNEL staining on the sections was done by dead end colorimetric method using In Situ Cell Death Detection Kit (Promega).

Cytokine and Chemokine Measurement. Sera from individual mice were collected 6, 12 and 24 h after ConA injection and stored below −20°C. IL-2, TNF-α, IFN-γ, IL-1α, IL-1β, IL-3, IL-4, IL5, IL-6, IL-10, IL-12p40, IL-12p70, IL-17, GM-CSF, G-CSF, KC, MIP-1α, RANTES and eotaxin concentrations were determined by Biolplex chemiluminescence assay (Biorad).

Cell Preparation and Flow Cytometric Analysis. Hepatic mononuclear cells (MNCs) were isolated using Histopaque density gradient as described earlier (Chen et al., 2001). Briefly, livers were flushed with cold PBS, crushed using a tissue homogenizer and passed through sterile mesh

(70 micron). Cell suspension washed once with PBS was layered over 15 mL of histopaque 1077 (Sigma-Aldrich) and centrifuged at 2000 rpm for 15 min at room temperature. Cells at the interface were transferred to a fresh tube and washed twice with PBS. Contaminating RBCs were lysed using RBC-lysis solution (Sigma). For FACS analysis, cells were blocked using mouse Fc-block (anti-CD16/CD32) and stained for various cell surface markers using fluorescently labeled mAbs (10 μg/mL, in PBS containing 2% FBS). After washing, stained cells were analyzed in a flow cytometer (FC500, Beckman Coulter). Only live cells were counted by setting gates on forward and side scatters to exclude debri and dead cells. Isotype antibody-treated cells served as staining controls. Data obtained were analyzed in Cytomics CXP software (Beckman Coulter).

Preparation of ConA supernatant. Murine splenocytes ($5x10^6$/well) were seeded onto a 24-well plate and cultured with complete RPMI-1640 medium containing 4 μg/mL ConA. After 72 h the culture supernatant was harvested, filtered, aliquoted, and stored at -20°C until use.

Detection of apoptosis by TUNEL assay. Cells were fixed at room temperature with 4% paraformaldehyde for 30 min, and stained for apoptosis using the TUNEL assay (Roche) as described earlier (Kamath et al., 1997). Briefly, the cells were permeabilized on ice for 2 min with 100 μl of 0.1% Triton X-100 in 0.1% sodium citrate, washed and incubated for 1 h at 37°C with 25 μl of TUNEL reaction mixture. The cells were analyzed in a flow cytometer.

Cell Depletion. $CD25^+$ T cells were depleted using anti-CD25 (IL-2Rα) mAb (clone: PC61, rat IgG1; Cedarlane Labs) as described elsewhere (Haeryfar et al., 2005). To deplete $CD25^+$ cells one dose (500 μg) of the ascites was injected intraperitoneally, 12h before ConA administration.

This procedure resulted in >96% decrease in $CD25^+$ cells as determined by flow cytometry using FITC-conjugated anti-CD25 mAb (BD BioSci).

RT-PCR for Foxp3. This was performed by standard protocol. Briefly, total RNA was prepared using the total RNeasy kit (Qiagen) and cDNA was prepared using random hexamer primers (Invitrogen). Foxp3 message was determined by PCR using gene specific primers (forward, 5'GGG GAA GCC ATG GCA ATA GTT3' and reverse, 5'TGA AGT AGG CGA ACA TGC GAG TAA3'). Amplified products were visualized by electrophoresis using 1.5% agarose gels containing ethidium bromide.

Statistical Analysis. Data are expressed as mean ± S.E.M. Student's t test was used to compare two data sets and $p<0.05$ was considered statistically significant.

Results

THC Ameliorates ConA-induced Liver Injury. THC has been shown to possess significant immunomodulatory and anti-inflammatory properties. To see if THC treatment prevents ConA-induced liver injury, various doses of THC were administered intraperitoneally into ConA-injected mice. Mice injected with ConA+vehicle developed acute hepatitis as indicated by elevated serum AST and ALT levels at 16h when compared to vehicle alone (Fig 1A). THC administration resulted in a THC-dose dependent decrease in ConA-induced AST and ALT levels. Although, 20mg/kg THC had a significant effect, the dose of 50mg/kg was most effective. We used this dose of THC and determined AST and ALT levels at various time points after ConA challenge (Fig 1B). As can be seen liver enzyme levels peaked at about 12 hours and started to subside by 24 h in ConA injected mice. Mice treated with 50mg/kg body weight THC, immediately after ConA challenge, showed significant decrease in AST levels at 6, 12 and 24 hours and ALT levels at 12 and 24 hours post-treatment. Even when administered 30 min after ConA injection, THC significantly reduced the serum AST levels (data not shown). It is important to note that mice injected with THC alone did not develop hepatitis and showed AST and ALT levels in the serum similar to vehicle injected group.

Liver histological examination was also used to determine the protective effect of THC in ConA-induced hepatitis. Light microscopy showed normal tissue histology for vehicle injected mice, whereas, dramatic leukocyte infiltration, massive tissue damage in ConA-injected mice. ConA-injected mice administered with THC showed dramatic decrease in hepatic tissue injury (Fig 1C). THC alone did not cause any liver damage. TUNEL staining showed massive hepatocyte apoptosis in mice challenged with ConA when compared with control group, which was markedly reduced upon THC treatment (Fig 1D).

THC Protects Against ConA-hepatitis by Suppressing Cytokines and Chemokines. ConA-induced hepatitis is associated with the production of various cytokines. In order to determine if THC was acting by modulating cytokine and chemokine levels, we measured various cytokine and chemokines at different time points (6, 12 and 24 hours). As shown in Fig 2, ConA challenge lead to elevation of most of the cytokines and chemokines tested when compared with vehicle injected control group including IL-2, TNF-α, IFN-γ, IL-1α, IL-1β, IL-5, IL-6, IL-10, IL-12, IL-17, GM-CSF, G-CSF, KC, MIP-1α, and RANTES. Administration of THC into ConA-injected mice resulted in significant suppression of most of the inflammatory cytokines and chemokines, except IL-2, IL-4, and IL-5.

Analysis of T cell Subpopulations and T Cell Apoptosis in Liver. Earlier studies from our laboratory have shown that THC can trigger apoptosis in T cells (McKallip et al., 2005). Also, T cells and NKT cells play critical roles in ConA-hepatitis. Thus, we investigated if THC-induced suppression of hepatic injury results from apoptosis of T or NKT cells in liver. To this end, we isolated intrahepatic mononuclear cells 12 hours post-ConA challenge and stained them for CD3 and NK1.1 markers. ConA administration resulted in significant increase in percentage and absolute number of T cells in liver compared to vehicle (Fig 3A, B). ConA+Vehicle injected mice showed a decrease in the percentage of hepatic NKT cells when compared to vehicle alone group (Fig 3A). These data are consistent with earlier reports that ConA administration in vivo leads to early activation and elimination of NKT cells in liver (Takeda et al., 2000). Although, there was not much difference in percentages (Fig 3A), interestingly, we noted that ConA+THC treated groups showed significant increase in the absolute numbers of hepatic T and NK cell numbers when compared to ConA+Vehicle treated groups (Fig 3B). We have shown earlier that

THC triggers apoptosis in T cells. We therefore, investigated the levels of apoptosis in hepatic T cells harvested 12 hour post-ConA challenge *in vivo*, and found that significantly higher percentage of apoptotic hepatic T cells were detected in ConA+THC group (58%) when compared to ConA+Vehicle injected mice (34.5%) (Fig 3C). The fact that ConA+THC treated mice showed increased levels of apoptosis in T cells while also demonstrating an increase in their numbers in the liver suggested that additional mechanisms such as increased migration of cells into the liver may play a role in ConA+THC groups or that certain subsets of T cells may be more susceptible to THC-induced apoptosis than others.

Similar to immune cells, hepatocytes are also known to express cannabinoid receptors (Biswas et al., 2003; Osei-Hyiaman et al., 2005). To investigate if the effect of THC on hepatocytes played a role in ConA-induced hepatitis, we cultured a normal mouse hepatocyte cells (BNL.CL.2) with various concentrations of THC for 24 hours and determined the percentage of viable cells by trypan blue exclusion. We also measured cells for apoptosis by TUNEL staining and flow cytometry. Mouse splenocytes served as controls. Splenocytes were susceptible to THC-induced apoptosis as indicated by dose-dependent decrease in viable cell number and increase in the percentage of apoptotic cells (Fig 4B, C). However, BNL.CL.2 cells were unresponsive to similar concentrations of THC (Fig 4A, C) indicating that unlike lymphocytes, murine hepatocytes may be resistant to THC-induced apoptosis. These studies also ruled out the possible direct toxic effects of THC on hepatocytes. Next, we tested if THC could protect hepatocytes against apoptosis induced by culture supernatant from ConA-activated splenocytes (CS) *in vitro*. CS induced significant apoptosis in hepatocytes when compared to medium control (Fig 4D). However, percentage of cells undergoing apoptosis in response to CS was decreased in the presence of THC in a dose dependent manner (Fig 4D). These data

suggested that THC may afford protection to the hepatocytes from apoptosis induced by activated immune cell-derived factors.

THC Treatment Increases $CD4^+CD25^+$ Cells in Liver in ConA-Hepatitis. Although the number of T and NKT cells was significantly increased in the liver upon THC administration in ConA-injected mice, they seemed to be functionally suppressed based on the cytokine data. One possibility was that THC treatment in ConA-injected mice triggered increased levels of $CD4^+CD25^+$ T regs in the liver that were responsible for decreased hepatitis. To this end, we investigated the levels of these cells in liver. ConA+vehicle injection resulted in significant increase in the percentage of $CD4^+CD25^+$ cell population in the liver over vehicle injected controls. Also, ConA+THC treated mice did not show any significant increase in the percentage of $CD4^+CD25^+$ cells. However, when we enumerated the total number of $CD4^+CD25^+$ cells, we noted that there was a significant increase in ConA+THC treated groups when compared to ConA+vehicle treated mice (Fig 5A, B).

THC Treatment Increases $Foxp3^+$ regulatory T Cells in Liver in ConA-Hepatitis. In mice injected with ConA, the $CD4^+CD25^+$ population includes both effector and regulatory T cell populations. Forkhead helix transcription factor (foxp3) is the intracellular marker unique to regulatory T cells (Fontenot et al., 2003). Therefore, in order to further confirm the role of regulatory T cells in THC-induced immune suppression in ConA-hepatitis we looked at $CD4^+Foxp3^+$ cells in liver. ConA+THC injected mice showed a slight decrease in the percentage of $CD4^+Foxp3^+$ regulatory T cells when compared to ConA+Vehicle injected mice (Fig 5C). However, they showed a significant increase in the absolute number of $CD4^+Foxp3^+$ regulatory

T cells (Fig 5D) due to an increase in the absolute number of T cells seen following ConA+THC treatment (Fig 3B). It should be noted that ConA injected mice showed a significant increase regulatory T cells over vehicle controls. This is in accordance with recent reports that T regulatory cells play a crucial role in natural tolerance in ConA-induced hepatitis (Erhardt et al., 2007). Nonetheless, we observed that THC treatment further significantly enhanced the number of T regulatory cells in this model.

To further corroborate the role of Tregs, we also analyzed Foxp3 mRNA expression by RTPCR in splenocytes and liver cells obtained 4 hours post-ConA challenge in vivo (Fig 5E). ConA+THC treated mice showed significantly higher levels of Foxp3 mRNA in the liver and spleen, when compared to ConA+Vehicle treated groups. Mice treated with THC alone showed slight decrease in Foxp3 mRNA in the liver and an increase in spleen.

$CD25^+$ Cells are Required for THC-mediated Suppression of ConA-Hepatitis. To further understand the crucial nature of regulatory T cells in ConA-induced hepatitis and THC-mediated suppression, we used anti-mouse CD25 mAbs. We noted that these Abs depleted $CD25^+$ cells very effectively (Fig 6B). Mice pretreated with anti-CD25 and injected with ConA showed significantly higher serum AST levels when compared to mice pretreated with control IgG (Fig 6A). These data suggested that in ConA-induced hepatitis, regulatory T cells do play a critical role in trying to reduce the severity of hepatitis. In these studies, mice receiving anti-CD25 alone showed normal AST levels. In the ConA+THC group, pre-injection with anti-CD25 significantly reversed the modulatory effect of THC as opposed to control IgG pretreatment indicating that $CD25^+$ regulatory cells are essential for THC mediated suppression of hepatitis.

Modulatory Effect of THC in ConA-Hepatitis is mediated through Cannabinoid Receptors. THC is known to act through cannabinoid receptors, CB1 and CB2. To delineate the role of cannabinoid receptors involved in the modulation of ConA-induced hepatitis by THC, we used CB1 and CB2 select agonists. Interestingly, CB1 (ACEA) and CB2 (JWH-133) select agonists independently or in combination did not decrease AST levels in ConA-induced hepatic injury as shown by serum AST levels (Fig 7A, B). In fact, ConA+ACEA, at higher concentrations (20mg/kg), caused an increase in AST levels when compared to ConA+vehicle groups. It should be noted that these cannabinoid receptor agonists by themselves were not hepatotoxic.

We next tested CB1/CB2 mixed agonists (CP55,940 and WIN55212) which bind to both CB1 and CB2 receptors, similar to THC. We found that these compounds were very effective in suppressing ConA-induced hepatitis in a dose-dependent manner (Fig 7C). Next, we addressed if THC acts through CB1 or CB2 receptors. To this end, we pretreated mice with CB1 (AM251) or CB2 antagonists (SR144528) before injecting ConA+THC and determined if this would reverse the THC-mediated suppression of hepatitis (Fig 7D). It was noted that blocking either one of the receptors could reverse the THC-mediated decrease in AST levels in ConA-treated mice. Also, control groups receiving antagonists alone showed normal AST levels indicating that these compounds were not toxic to the liver at the indicated doses. These data demonstrate that immunomodulatory function of THC in ConA-hepatitis is mediated by signaling through both CB1 and CB2 receptors, and blocking either of these receptors was sufficient to reverse the effects of THC.

Endocannabinoid Anandamide attenuates ConA-induced hepatitis. Anandamide an endocannabinoid acts as a partial agonist towards CB1 and CB2 receptors (Devane et al., 1992).

In order to see if anandamide can attenuate hepatitis, we administered exogenous anandamide into mice injected with ConA. ConA+Anandamide treatment resulted in decreased AST and ALT levels (Fig 8A) and less liver damage (Fig 8B) when compared to ConA+Vehicle injected mice, suggesting that anandamide can also effectively block ConA-induced hepatitis similar to THC. We also analyzed cytokine and chemokine levels 12 hours after anandamide treatment in ConA-injected mice and observed that there was a significant decrease in inflammatory cytokines TNF-α, IL-1β, IL-6, IL-9, IL-12, IL-17 and chemokines KC, MCP-1 and eotaxin (Fig 8C). Interestingly, anandamide treatment in ConA-injected mice, similar to THC treatment, failed to decrease IL-2 levels when compared to ConA+Vehicle-injected mice. We also tested if the suppressive effect of anandamide is mediated by cannabinoid receptors using CB1 and CB2 specific antagonists. Blocking of CB1 or CB2 receptors by pre-treating mice with antagonists AM251 or AM630 respectively resulted in abrogation of anandamide mediated suppression of AST levels induced by ConA (Fig 8D) indicating that anandamide is acting in a CB1 and CB2 dependent manner.

Absence or inhibition of FAAH makes mice resistant to ConA-induced hepatitis. Endogenous cannabinoids such as anandamide are rapidly hydrolyzed by fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996). Thus, inhibition of FAAH is known to raise anandamide levels (Chang et al., 2006). Also, FAAH-KO mice are known to have increased levels of endocannabinoids including anandamide (Cravatt et al., 2001). We therefore investigated if FAAH KO mice are more resistant to ConA-induced hepatitis. To test this, we injected wild type and FAAH-deficient mice with ConA and measured AST levels in the serum after 12 hours and also analyzed liver tissue injury by histology after 24 hours. As shown in Fig

9A, FAAH-deficient mice showed significantly reduced AST levels when compared to wild type mice challenged with ConA. Moreover, FAAH-deficient mice showed less severe liver tissue damage and less leukocyte infiltration upon ConA challenge (Fig 9B). We also tested the effect of pharmacological inhibition of FAAH using inhibitors, methyl arachidonyl fluorophosphates (MAFP) or 4-Benzyloxyphenyl-n-butylcarbamate (URB532). URB532 has been shown to be a very potent and selective inhibitor of FAAH (Kathuria et al., 2003). Administration of MAFP or URB532 before ConA challenge in wild type mice resulted in significant decrease in AST levels (Fig 9C). These data suggest that deficiency or pharmacological inhibition of FAAH imparts mice with some level of resistance to the development of ConA-induced hepatitis.

Discussion

In vivo administration of THC alleviated ConA-induced hepatitis as indicated by decreased transaminase levels and markedly attenuated inflammatory lesions in liver. THC at 20 and 50mg/kg bd.wt. could significantly suppress AST/ALT levels induced by ConA. A chronic study by the National Toxicology Program (Chan et al., 1996) concluded that a daily dose of up to 150mg/kg THC for 13 weeks in mice did not produce significant adverse effects. Based on body surface area normalization (Center for Drug Evaluation and Research, 2002), 20 and 50mg/kg THC doses convert to 60 and 150mg/m^2 respectively. THC may cause central side effects at these doses in humans. However, this is within the range of synthetic THC recommended for clinical use. For example, in case of dronabinol (Marinol) used as an antiemetic during chemotherapy, 15mg/m^2 is recommended for up to 6 doses/day which equates to 90mg/m^2/day. Unlike the prolonged use of dronabinol during chemotherapy, we noted that in our study, a single dose of THC was effective thereby suggesting that psychotropic effects may not pose a problem if a similar regimen is used in humans to treat autoimmune hepatitis.

TNF-α, IFN-γ and IL-2 play crucial roles in ConA-induced hepatitis (Gantner et al., 1995; Tagawa et al., 1997). We noted that THC could suppress TNF-α and IFN-γ in ConA injected mice to almost basal levels. IFN-γ-inducing cytokine IL-12 was also significantly decreased after THC treatment. A recent study showed that IL-6 makes CD25+CD4+ effector T cells resistant to the suppression by Tregs (Pasare and Medzhitov, 2003). Decreased IL-6 levels observed in ConA+THC injected mice may be contributing to suppression of effector T cell function by Tregs. We also observed that chemokines GM-CSF, G-CSF, KC, MIP-1α and RANTES that were elevated after ConA challenge, were significantly suppressed upon THC treatment. This is

especially important since chemotaxis and activation of macrophages and eosinophils contribute to ConA-induced hepatitis (Bonder et al., 2004; Louis et al., 2002; Schumann et al., 2000).

Previous studies showed that activated T and NKT cells are increased in liver after ConA challenge. However, these cells rapidly undergo apoptosis, which may be crucial for mice to recover from ConA-hepatitis (Russell, 1995). We have earlier noted that THC induces apoptosis thymic and splenic T cells (McKallip et al., 2002). In the current study, the frequency of hepatic T lymphocytes undergoing apoptosis was increased in ConA-injected mice upon THC treatment. Nonetheless, absolute numbers of hepatic T cells in THC-treated hepatitis-induced mice was significantly higher. This may result from induction of Tregs as shown, as well as possible increased migration of T cells into the liver. It is interesting to note that IL-2 was not decreased upon THC treatment and may be contributing to proliferation of T cells. However, T cells were functionally suppressed by THC as indicated by decreased serum cytokine levels and liver injury. This could be due to direct suppressive effect of THC on activated T cells as well as increase in $CD4^{+}Foxp3^{+}$ Tregs. IL-2 is an essential growth and survival factor for Tregs and is required for their function (Furtado et al., 2002). Sufficient levels of IL-2 observed in ConA-injected mice treated with THC or anandamide could contribute to increased Treg function resulting in significant suppression of inflammatory cytokines. The crucial role of Tregs was further confirmed by the fact that THC was not able to prevent hepatitis in $CD25^{+}$ Treg-depleted mice. It is important to note that $CD25^{+}$ cell depleted mice showed increased AST levels after ConA-injection compared to naïve mice. One possible explanation for this would be, depletion of Tregs resulted in uncontrolled hepatotoxicity upon polyclonal activation of T cells and other inflammatory cells by ConA. This supports the recent observation that Tregs mediate tolerance in ConA-induced hepatitis model (Erhardt et al., 2007). Previous studies have shown that THC

suppresses cytokine secretion by its direct action on lymphocytes (Blanchard et al., 1986; Klein et al., 2000). In the current model, THC may be acting directly on T cells and also by enhancing Treg function in the presence of ConA to effect cytokine suppression and disease attenuation.

There is growing interest in recent years to target cannabinoid receptors to treating liver diseases (Mallat and Lotersztajn, 2008; Pacher and Gao, 2008). In the current study, CB1 or CB2 activation alone had no anti-inflammatory effect on hepatitis. However, cannabinoids which bind to both CB1 and CB2 receptors (THC, CP55,940, WIN55212 and anandamide), effectively attenuated hepatitis. The fact that CB1/CB2 mixed agonists could suppress the disease but not the co-administered CB1 and CB2 agonists indicates that both the cannabinoid receptors need to be activated simultaneously in order to produce the observed effect and that the different pharmacokinetics of the two co-administered agonists may not allow this to happen. Signaling through both the receptors is important since blocking either CB1 or CB2 could reverse the effect of THC. Activation through CB1 per se worsened the effect of ConA which is in agreement with previous studies showing CB1 may contribute towards liver inflammatory disorders (Teixeira-Clerc et al., 2006). However, activation of both CB1 and CB2 not only prevented the worsening effect of CB1 stimulation alone, but also resulted in a strong counteraction of inflammation. The need for simultaneous activation of CB1 and CB2 in order to see the beneficial effect is consistent with some earlier observations (Van Sickle et al., 2005). However, these results are contradictory to previous understanding that anti-inflammatory properties of cannabinoids are mainly mediated by CB2. The finding that hepatocytes (Biswas et al., 2003; Osei-Hyiaman et al., 2005) and dendritic cells (Do et al., 2004; Matias et al., 2002) express CB1 and CB2 receptors may explain the contribution of both receptors towards protection observed in this study.

The endocannabinoid system plays a protective role in various inflammatory diseases, and is considered an attractive therapeutic target (Pacher et al., 2006). Recently, activation of endocannabinoid system has been shown to attenuate cutaneous hypersensitivity (Karsak et al., 2007). Anandamide could attenuate hepatitis similar to THC in the current study. Anandamide levels are elevated in the absence of FAAH activity (Cravatt et al., 1996). Increased endogenous anandamide levels may be causing decreased hepatic injury upon ConA-challenge in FAAH-KO mice as well as wild-type mice administered with FAAH inhibitors. Anandamide and 2-AG induce cell death in hepatic stellate cells (HSCs) (Siegmund et al., 2007; Siegmund et al., 2006), and increase in HSC apoptosis is associated with the resolution of liver fibrosis (Iredale et al., 1998; Oakley et al., 2005). However, $FAAH^{-/-}$ mice displayed increased hepatocellular injury upon bile duct ligation (Siegmund et al., 2006). In contrast, we observed that in ConA-induced immune-mediated hepatitis $FAAH^{-/-}$ mice were at least partially resistant when compared to wild types. Studies on the role of endocannabinoids in liver diseases have so far mainly focused on hepatic fibrosis, a result of chronic liver disease. $CB1^{-/-}$ mice showed decreased fibrogenesis (Teixeira-Clerc et al., 2006), whereas $CB2^{-/-}$ mice displayed increased fibrogenesis following CCL4 treatment, a chemical-induced liver injury (Julien et al., 2005). This was attributed to opposing effects of CB1 and CB2 in cell death and proliferation of myofibroblasts (Julien et al., 2005). However, other cell types and mechanisms may also play crucial roles because CB1 and CB2 are highly expressed in other hepatic cell types, and CB1 is expressed on the vascular endothelium and in hepatocytes in the injured liver (Siegmund and Schwabe, 2008). The induction of endocannabinoids and their receptors constitute complementary mechanisms that render the liver responsive towards endocannabinoids in hepatic fibrosis (Siegmund and Schwabe, 2008). Also, endocannabinoids exert numerous and sometimes opposite effects on

target cells. Strong upregulation of CB1 occurs only in some types of liver injury whereas CB2 is highly induced in most liver diseases (Siegmund and Schwabe, 2008). The regulation of endocannabinoids and their receptors on immune and hepatic cell types during ConA-induced immune-mediated acute liver injury is not known. In contrast to the studies on liver fibrosis, we find that THC and anandamide act through both CB1 and CB2 receptors to produce the observed effect in ConA-hepatitis. The levels of endocannabinoids and related receptors on various cell types and hence their effect, as well as other mechanisms of protection that may be involved in different types of liver injury may be distinct and yet to be clearly elucidated. This may explain varying response of anandamide and $FAAH^{-/-}$ mice in different liver disease models. Modulation of immune response by cannabinoids seems to play a crucial role in immune-mediated liver injury.

Taken together our data suggest that exogenous cannabinoids such as THC upon binding to CB1 and CB2 receptors on immune cells, induce apoptosis in effector T cells, up regulate Treg function and suppress inflammatory cytokines there by preventing ConA-induced activated T cell-mediated liver injury. The observation that the anandamide treatment ameliorates ConA-induced hepatitis, together with the fact that FAAH deficiency or inhibition leads to increased resistance to the disease, strongly suggests that the endocannabinoid system serves to attenuate the inflammatory response in ConA-induced acute hepatitis. These findings raise the promising potential of developing novel pharmacological treatments for T-cell mediated liver diseases.

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Footnotes

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Legends to Figures

Figure 1. THC ameliorates ConA-induced hepatitis in mice. **A.** C57BL/6 mice (6 per group) were administered with vehicle, ConA+vehicle or ConA and 5, 10, 20 or 50 mg/kg bd wt THC. Serum was collected 16h post-ConA injection. AST and ALT were determined by spectrophotometric method. **B.** Mice were injected with vehicle, ConA+vehicle or ConA+50mg/kg bd wt THC or 50mg/kg THC alone. Sera were collected after various time points. AST and ALT levels were determined as before **C.** Forty eight hours after ConA treatment, livers were subjected to histological examination. Representative pictures from H&E stained sections are shown (10X objective). a) Vehicle, b) THC(50mg/kg), c) ConA+vehicle, d) ConA+THC(50mg/kg). Damaged areas surrounded by infiltrating leukocytes are indicated by arrows. **D.** Sections were stained for apoptosis by TUNEL (brown) and counterstained with hematoxylene (blue). *, $p<0.05$ and **, $p<0.01$ compared to ConA+veh.

Figure 2. Analysis of cytokines and chemokines in ConA-induced hepatitis. Mice (4 per group) were injected with vehicle, THC, ConA+vehicle or ConA+THC(50mg/kg). Serum cytokines at 6, 12 and 24 hr post-ConA injection were analyzed by chemiluminescence method using Bioplex system. Significant decrease in the levels of cytokines in ConA+THC group when compared with ConA+Veh group at 6 and 12h are indicated (*, $p<0.05$).

Figure 3. Enumeration of lymphocyte subpopulations in the liver of ConA injected mice. Mice (4 per group) were injected with vehicle, THC, ConA+vehicle, or ConA+ THC(50mg/kg). After 12 hours, hepatic MNCs were harvested and cells were stained for various markers and analyzed by FACS. **A.** Representative double-stain flow profiles showing percentages of each

cell population. **B.** Absolute cell numbers of various lymphocyte subpopulations were calculated by taking into consideration total number of liver MNCs isolated per mouse and percentage of cells as shown in panel A (†, $p<0.05$ compared to Vehicle; *, $p<0.05$ ompared to ConA).

C. Detection of apoptosis in liver MNCs using TUNEL assay. MNCs isolated 12 hour post-ConA injection were analyzed by TUNEL for apoptotic cells by flow cytometry. Histograms represent TUNEL staining on $CD3^+$ cells. The last panel shows an overlay of 3 histograms.

Figure 4. Murine hepatocyte cells are resistant to THC-induced apoptosis. Normal murine hepatocyte cells (BNL.CL.2) (**A**) and splenocytes (**B**) were cultured with various doses of THC in complete medium containing 5% FBS for 24 hours and percentage of viable cells was determined by trypan blue exclusion (*, $p<0.05$). **C.** Cells were stained by TUNEL for apoptosis and analyzed by flow cytometry. Numbers on histograms indicate percentage of apoptotic cells. **D.** THC protects hepatocyte cells against apoptosis. BNL.CL.2 cells were preincubated with various doses of THC for 1h. ConA supernatant (CS, 20% v/v) was added and cells were further cultured for 24h. Apoptosis was analyzed by TUNEL staining and flow cytometry. Percentage TUNEL positive cells are indicated on each histogram.

Figure 5. Analysis of regulatory T cells in liver. Hepatitis was induced in mice using ConA as described in Fig 1. Twelve hrs after ConA injection, liver MNCs were analyzed for $CD4^+CD25^+$ and $CD4^+Foxp3^+$ T cells by double staining. Representative flow profiles with percentage of cell population for each treatment group are shown (**A, C**) along with absolute cell numbers (**B, D**). *, $p<0.05$ compared to ConA+veh. **E.** Four hours after ConA injection, spleens and livers were

collected from each group. cDNA was synthesized from total mRNA and PCR was carried out using gene specific primers for Foxp3 and 18S genes.

Figure 6. Role of $CD4^+CD25^+$ T cells in the pathogenesis of ConA-induced hepatitis and suppression by THC. BL/6 mice (6 per group) were injected with isotype control antibody or anti-CD25 mAbs. After 12 hours, animals received ConA+vehicle or ConA+THC(50mg/kg). Plasma AST levels were determined 12 hours post-ConA injection **(A)**. Control mice received isotype IgG alone. *, $p<0.05$ between groups as indicated. **B.** Representative flow profiles showing depletion of $CD25^+$ cells in ConA+THC group.

Figure 7. Effect of CB1 or CB2 or mixed agonists on ConA-induced AST levels. A. Mice (4 per group) were injected with vehicle or ConA. ConA-injected mice were treated with vehicle, CB1 (ACEA) or CB2 (JWH-133) agonists (**A**) or both ACEA and JWH-133 (**B**) or CB1/CB2 mixed agonists CP55,940 and WIN55212 (**C**) with doses as indicated. Control groups received agonists alone as indicated. **D.** Mice received various doses of select CB1 (AM251) or CB2 (SR144528) antagonist 1 hour before injecting ConA and THC(50mg/kg). Serum AST levels in all cases were determined 12 hrs after ConA-injection. (*, $p<0.05$ and **, $p<0.01$ compared to ConA+veh; †, $p<0.05$, ††, $p<0.01$ compared to ConA+THC).

Figure 8. Effect of endocannabinoid anandamide (AEA) on ConA-induced hepatitis. Mice (6 per group) were injected with vehicle, AEA alone, ConA+vehicle or ConA+AEA. **A.** Serum AST and ALT levels determined 12 hours post-ConA challenge. **B.** Liver histology, H&E stained sections 48 hours after ConA challenge: a) Vehicle b) AEA (20 mg/kg), c) ConA +

vehicle, d) ConA + AEA(20 mg/kg). **C.** Serum cytokines and chemokines were determined 12 hours after ConA-challenge by Bioplex method. *, $p<0.05$ and **, $p<0.01$ compared to ConA+veh. **D.** Mice received 5mg/kg bd wt of CB1 (AM251) or CB2 (AM630) antagonists 1 hour before injecting ConA and AEA (10 mg/kg). Serum AST levels were determined 12 hrs after ConA-injection. *, $p<0.05$ as denoted.

Figure 9. Effect of deficiency or inhibition of FAAH on ConA-mediated hepatitis.

Wild type (BL/6) and FAAH-KO mice (4 per group) were injected with vehicle or ConA. **A.** Serum AST levels were analyzed spectrophotometrically 12 hrs post-ConA challenge. *, $p<0.05$ compared to WT mice injected with ConA. **B.** twenty four hours after ConA injection, livers were harvested and H&E stained sections were analyzed for histological changes: a) WT-vehicle, b) FAAHKO-vehicle, c) WT-ConA, d) FAAHKO-ConA. **C.** Wild type mice were injected with ConA and treated with vehicle or FAAH inhibitors, methyl arachidonyl fluorophosphate (MAFP, 10 mg/kg bd wt) and URB532. AST levels were determined 12 hrs post-ConA injection. Controls injected with vehicle or inhibitors alone are also shown. *, $p<0.05$ when compared to ConA+veh.

Figure 1

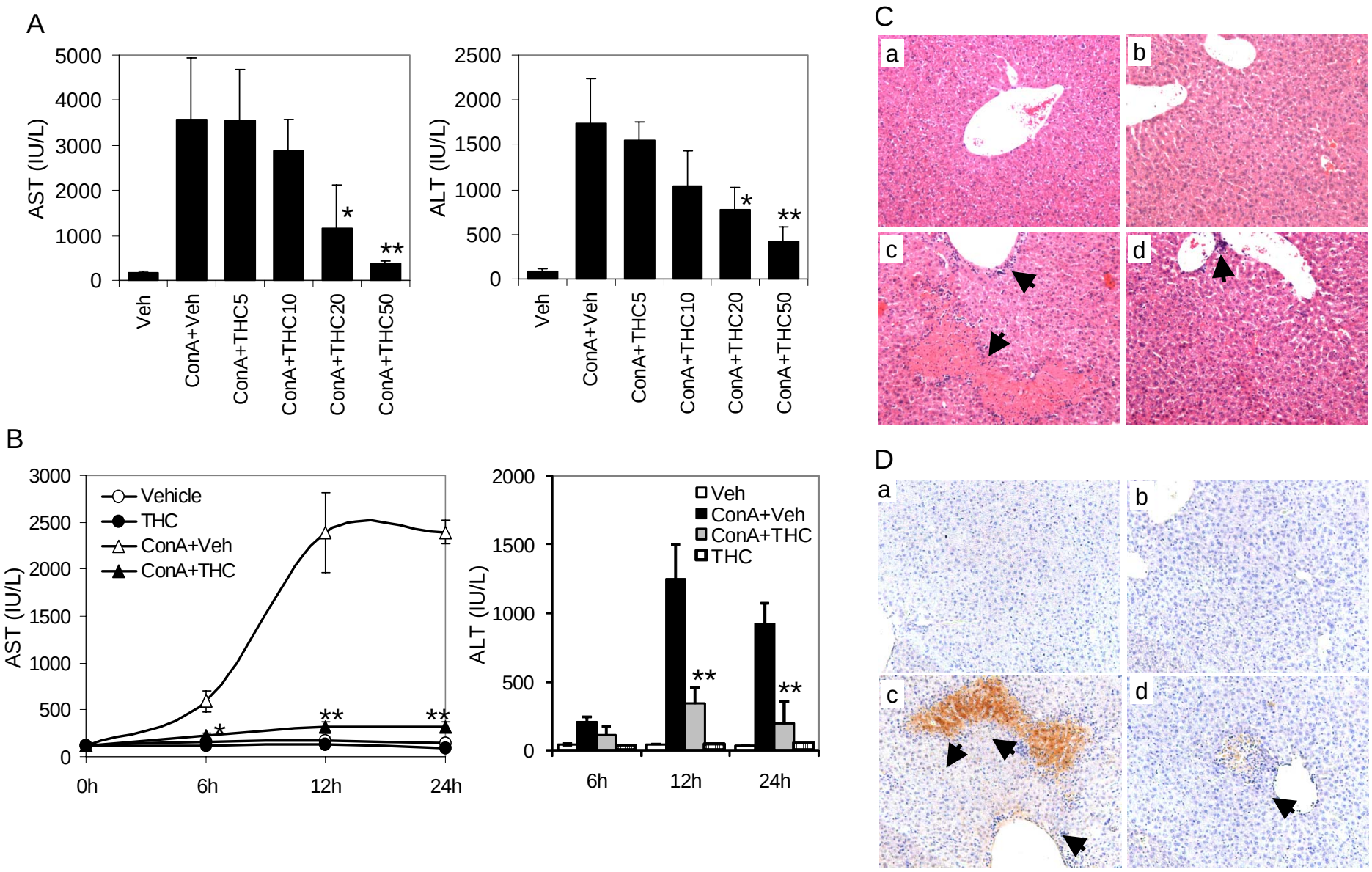
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Figure 2

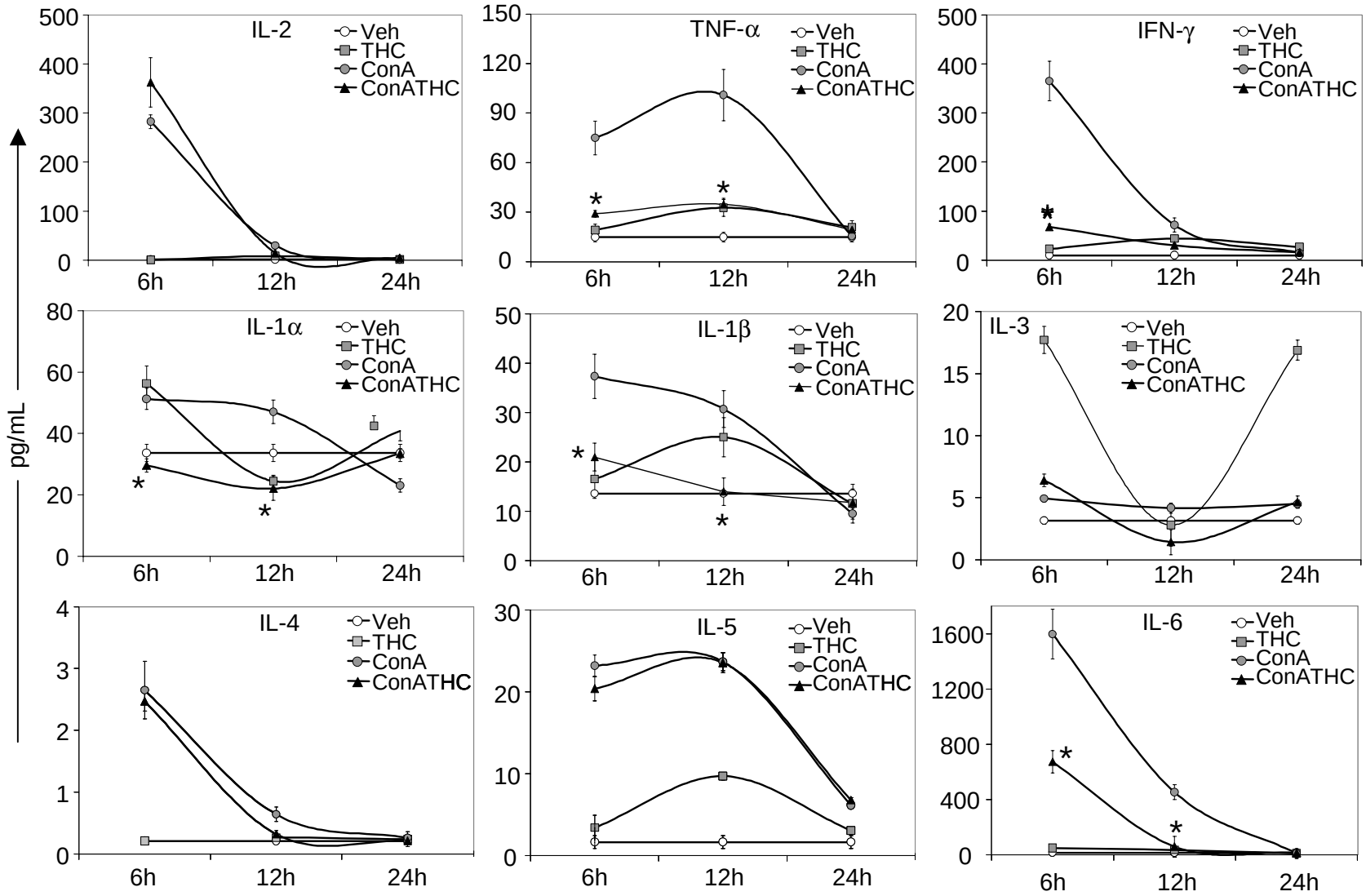
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Figure 2

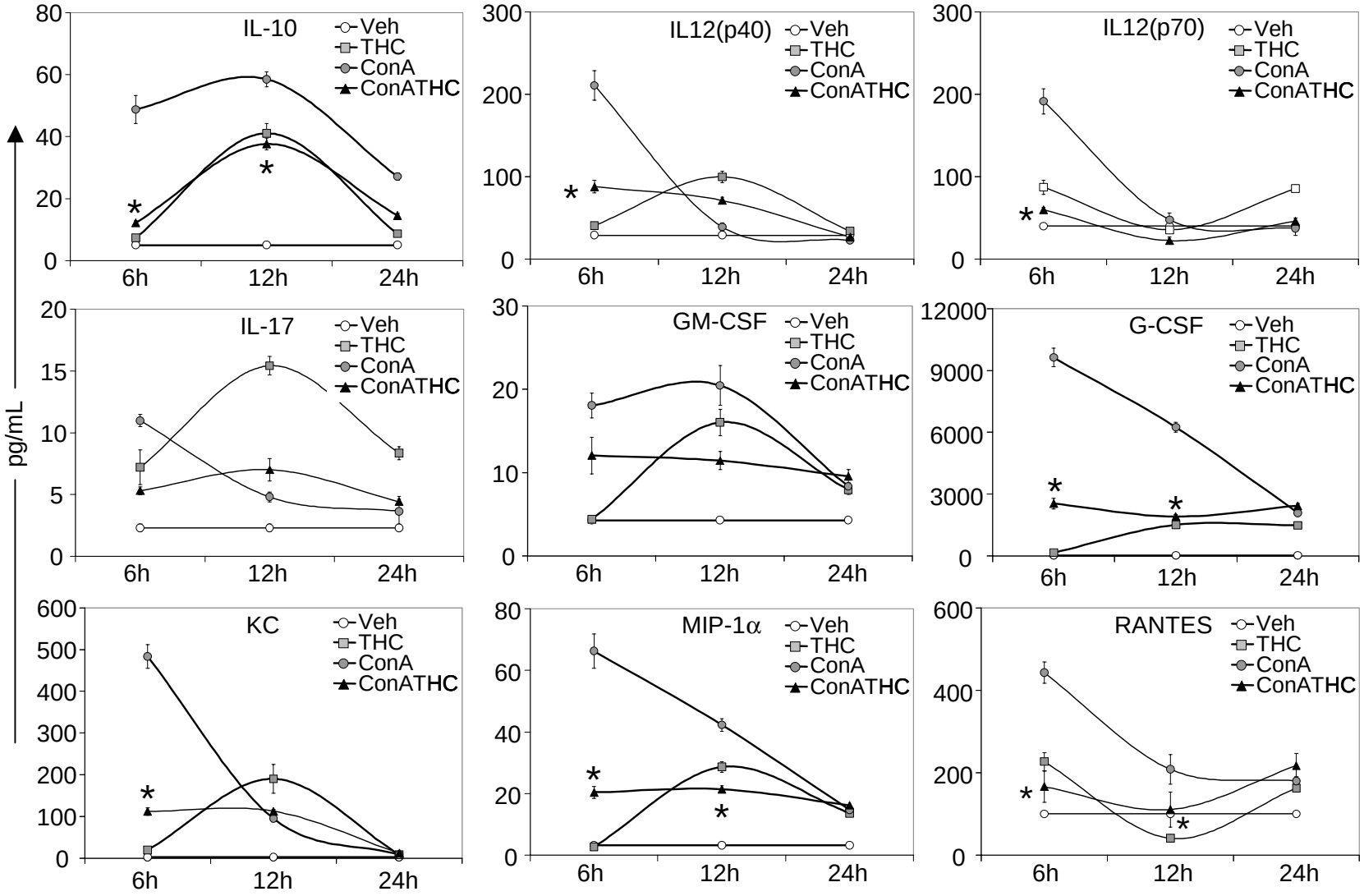
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Figure 3

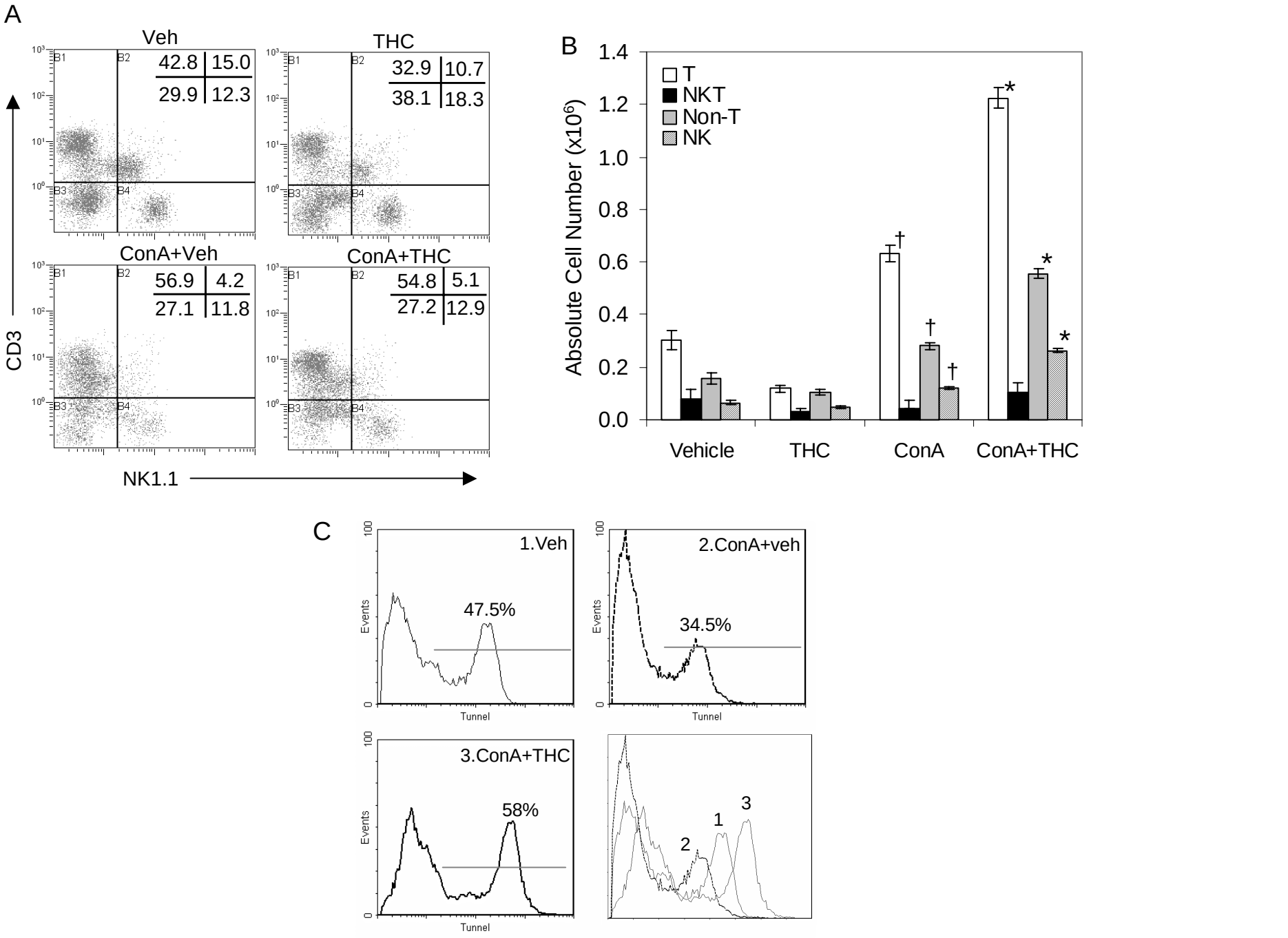
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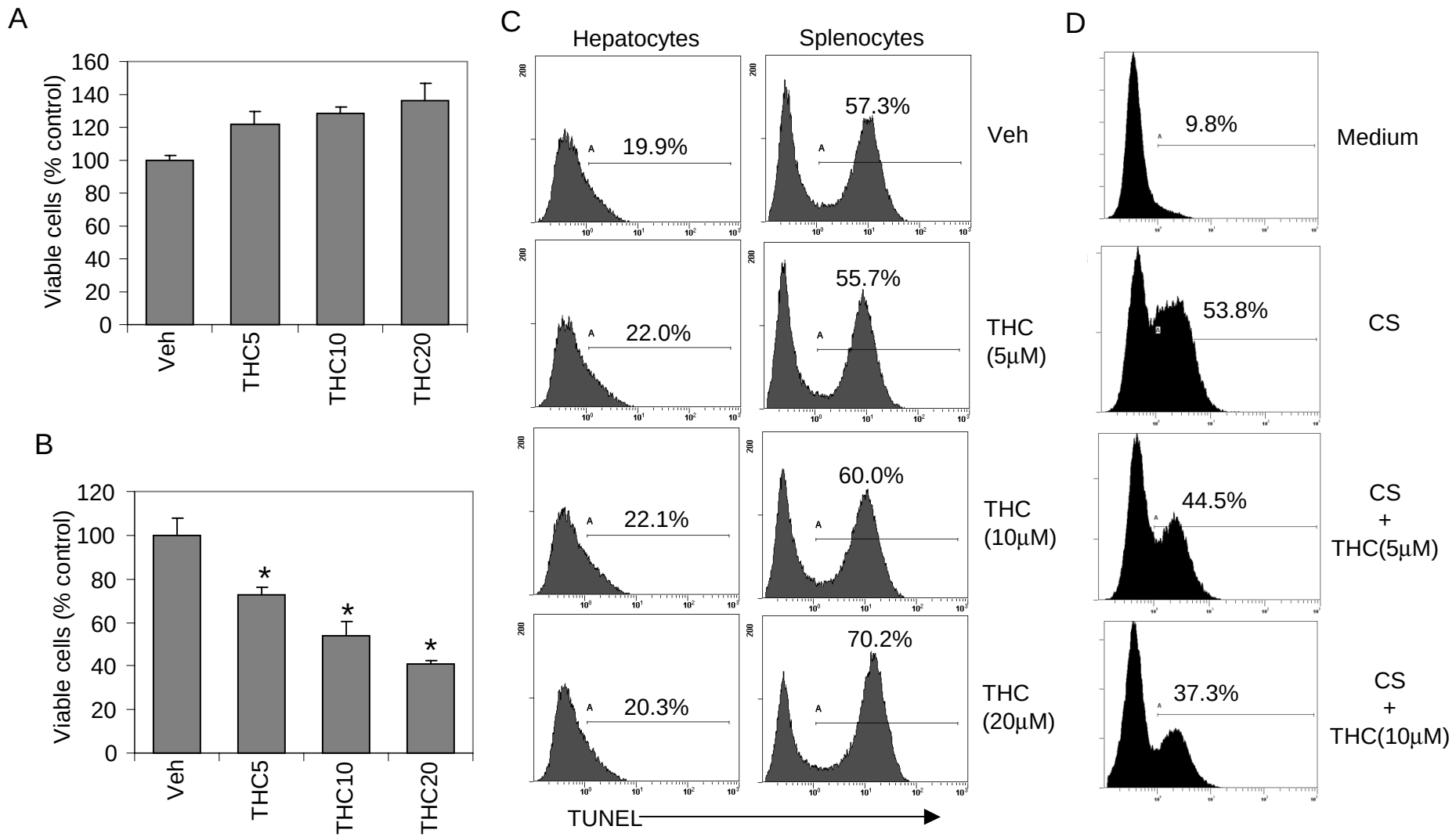
Figure 4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70.2%
THC (20μM)
TUNEL
D
9.8%
Medium
53.8%
CS
44.5%
CS + THC(5μM)
37.3%
CS + THC(10μM)

Figure 5

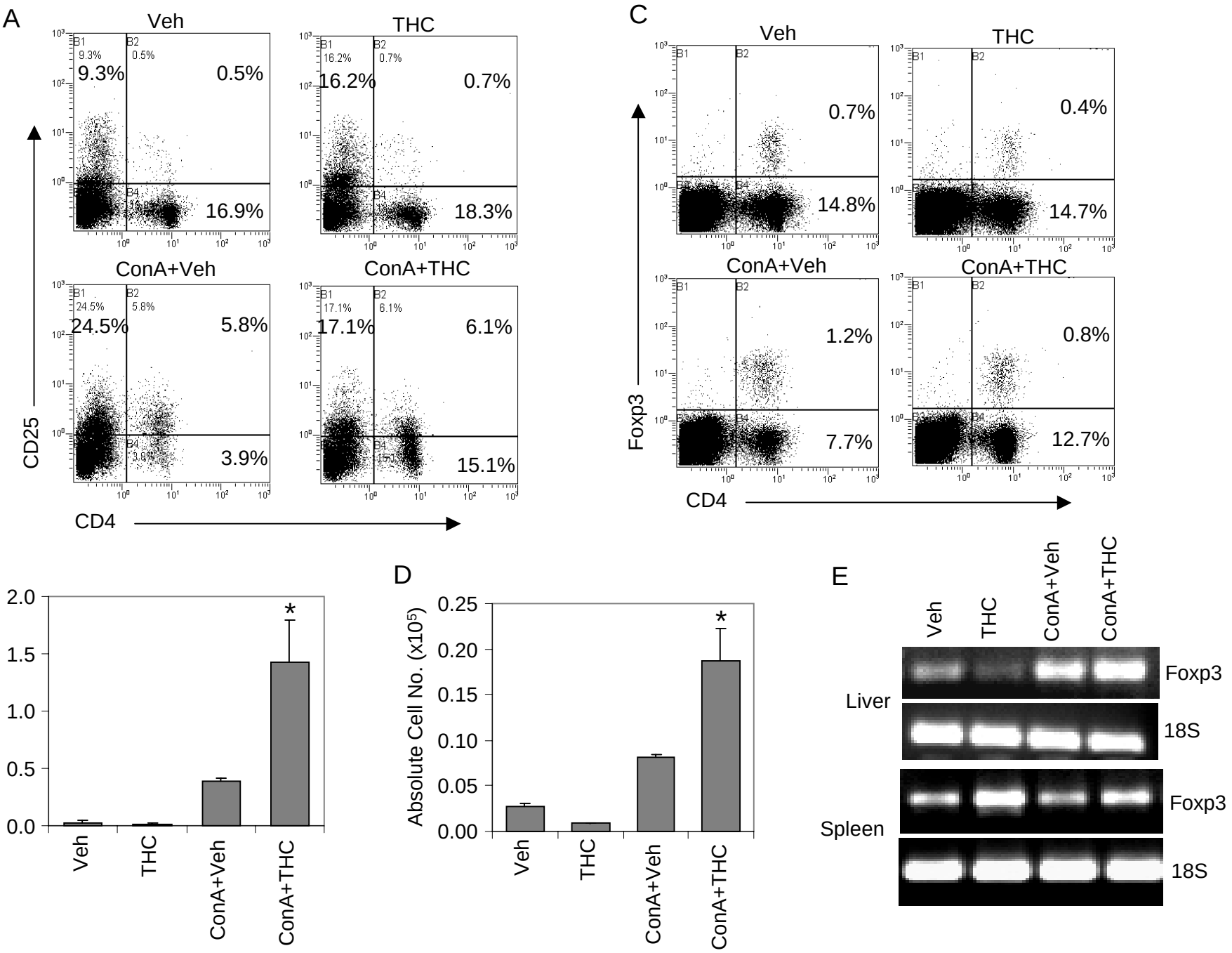
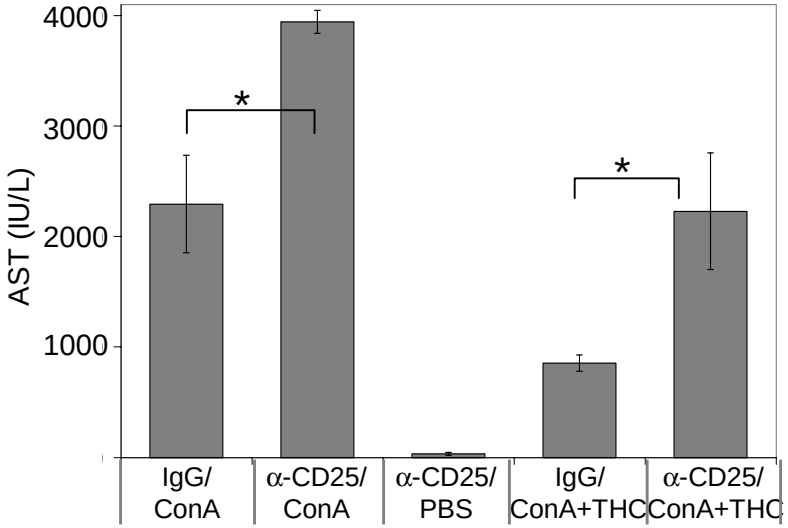

A
Veh
THC
ConA+Veh
ConA+THC
9.3%
0.5%
16.9%
16.2%
0.7%
18.3%
24.5%
5.8%
3.9%
17.1%
6.1%
15.1%
CD25
CD4
C
Veh
THC
ConA+Veh
ConA+THC
0.7%
14.8%
0.4%
14.7%
1.2%
7.7%
0.8%
12.7%
Foxp3
CD4
B
Absolute Cell No. (x10⁵)
2.0
1.5
1.0
0.5
0.0
*
Veh
THC
ConA+Veh
ConA+THC
D
Absolute Cell No. (x10⁵)
0.25
0.20
0.15
0.10
0.05
0.00
*
Veh
THC
ConA+Veh
ConA+THC
E
Veh
THC
ConA+Veh
ConA+THC
Liver
Foxp3
18S
Spleen
Foxp3
18S

Figure 6

A


4000
3000
2000
1000
AST (IU/L)
*
*
IgG/ ConA
α-CD25/ ConA
α-CD25/ PBS
IgG/ ConA+THC
α-CD25/ ConA+THC

B

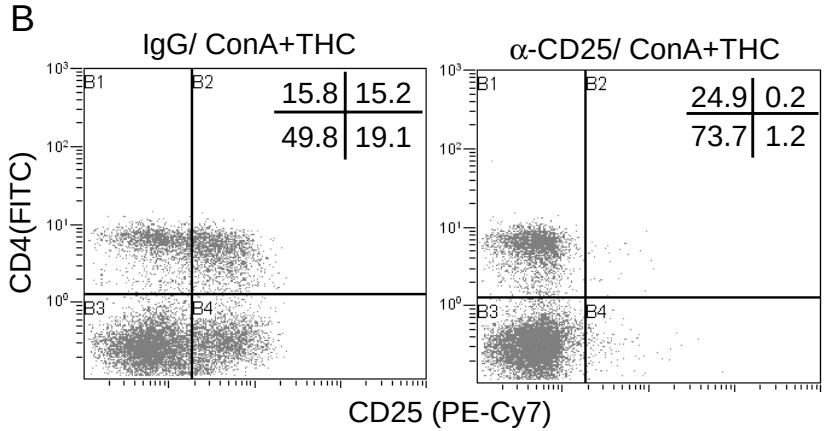

IgG/ ConA+THC
α-CD25/ ConA+THC
CD4(FITC)
CD25 (PE-Cy7)
B1
B2
B3
B4
15.8
15.2
49.8
19.1
24.9
0.2
73.7
1.2

Figure 7

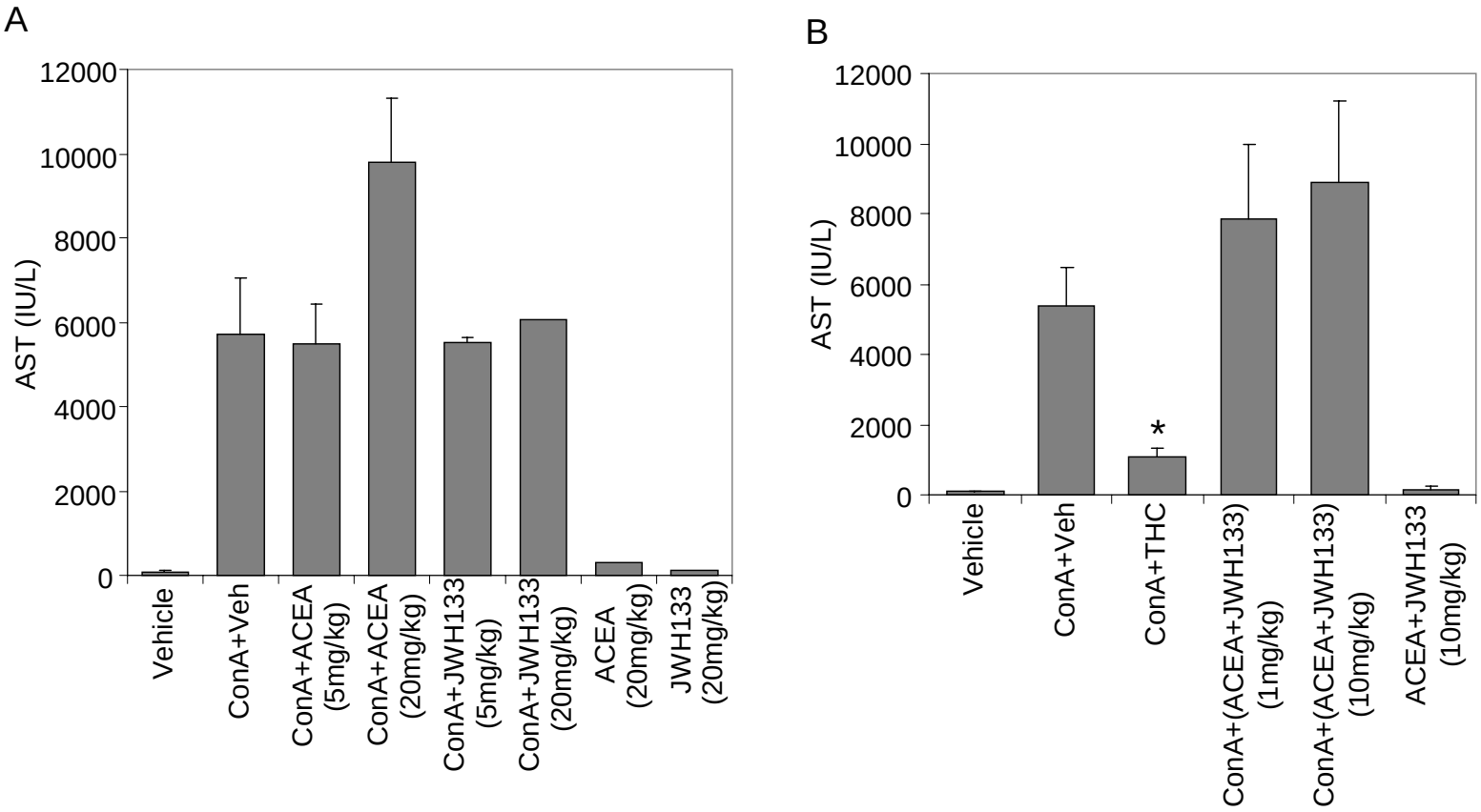
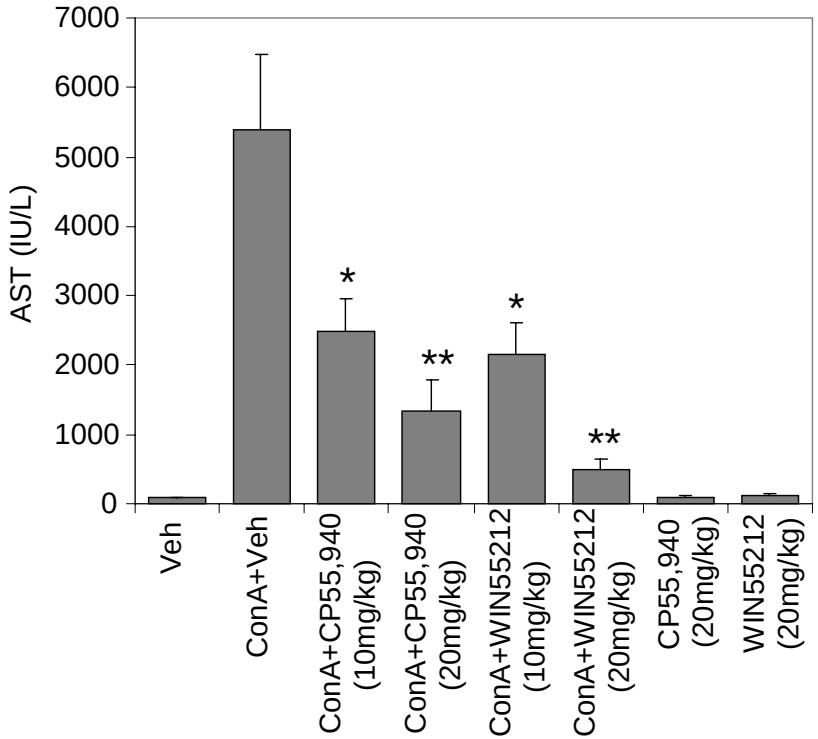

A
AST (IU/L)
12000
10000
8000
6000
4000
2000
0
Vehicle
ConA+Veh
ConA+ACEA (5mg/kg)
ConA+ACEA (20mg/kg)
ConA+JWH133 (5mg/kg)
ConA+JWH133 (20mg/kg)
ACEA (20mg/kg)
JWH133 (20mg/kg)
B
AST (IU/L)
12000
10000
8000
6000
4000
2000
0
*
Vehicle
ConA+Veh
ConA+THC
ConA+(ACEA+JWH133) (1mg/kg)
ConA+(ACEA+JWH133) (10mg/kg)
ACEA+JWH133 (10mg/kg)

Figure 7

C


AST (IU/L)
7000
6000
5000
4000
3000
2000
1000
0
*
**
*
**
Veh
ConA+Veh
ConA+CP55,940 (10mg/kg)
ConA+CP55,940 (20mg/kg)
ConA+WIN55212 (10mg/kg)
ConA+WIN55212 (20mg/kg)
CP55,940 (20mg/kg)
WIN55212 (20mg/kg)

D

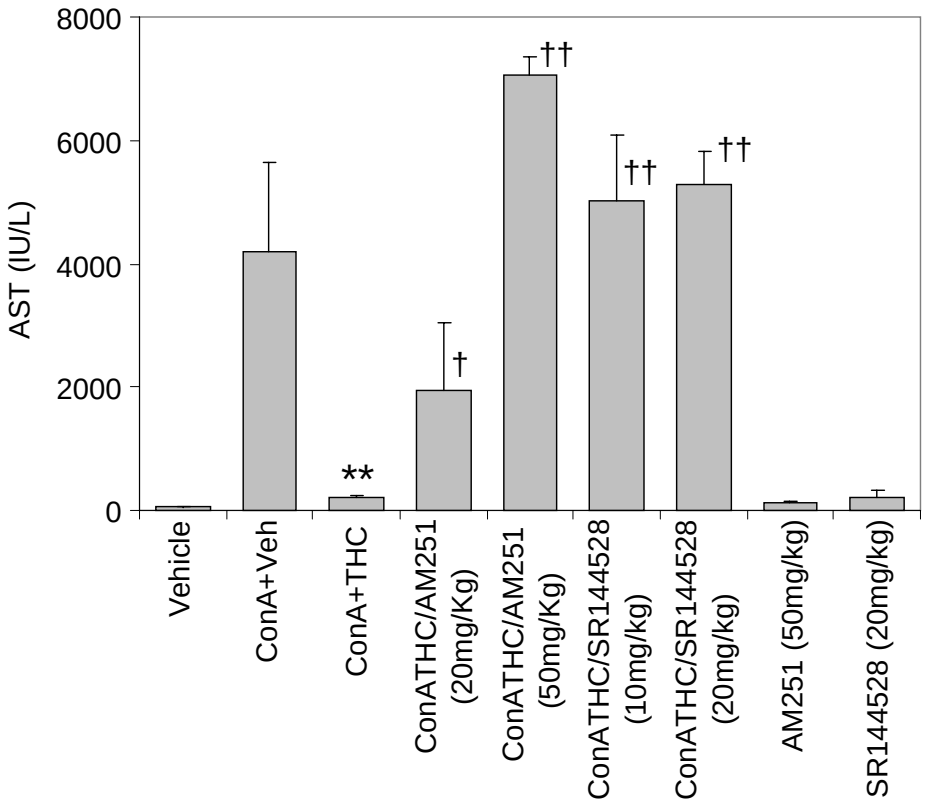
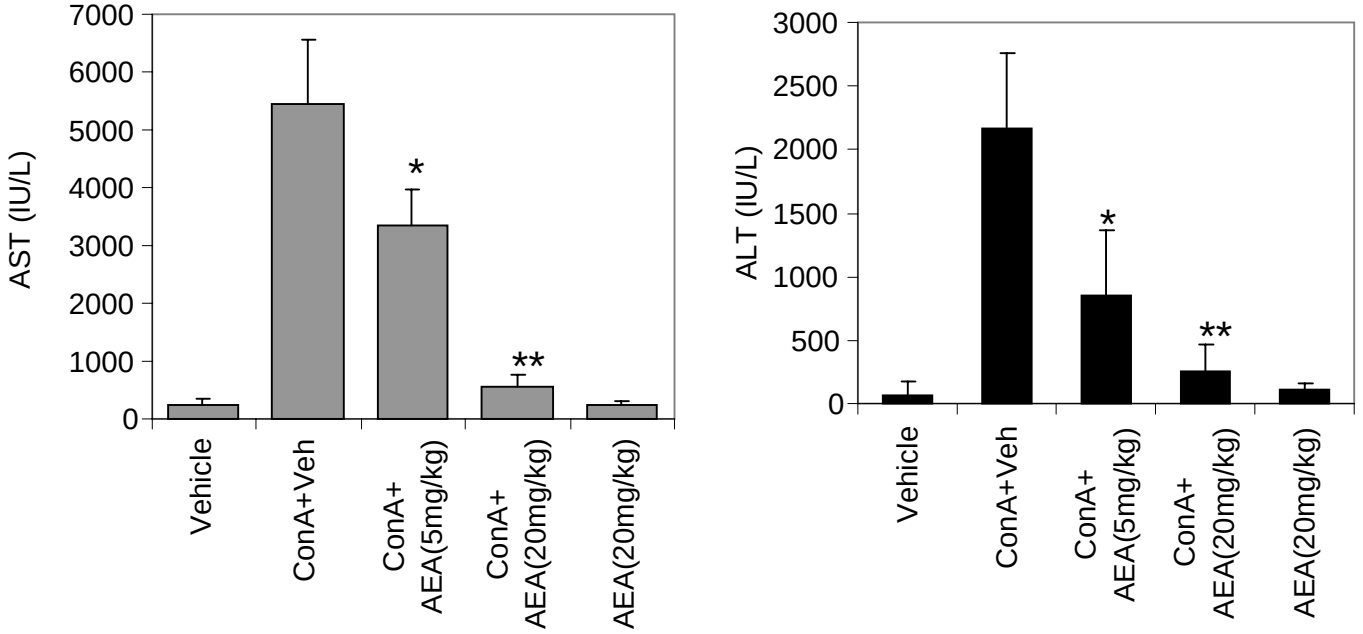

AST (IU/L)
8000
6000
4000
2000
0
**
†
††
††
††
Vehicle
ConA+Veh
ConA+THC
ConATHC/AM251 (20mg/Kg)
ConATHC/AM251 (50mg/Kg)
ConATHC/SR144528 (10mg/kg)
ConATHC/SR144528 (20mg/kg)
AM251 (50mg/kg)
SR144528 (20mg/kg)

Figure 8 A


AST (IU/L)
7000
6000
5000
4000
3000
2000
1000
0
*
**
Vehicle
ConA+Veh
ConA+ AEA(5mg/kg)
ConA+ AEA(20mg/kg)
AEA(20mg/kg)
ALT (IU/L)
3000
2500
2000
1500
1000
500
0
*
**
Vehicle
ConA+Veh
ConA+ AEA(5mg/kg)
ConA+ AEA(20mg/kg)
AEA(20mg/kg)

B

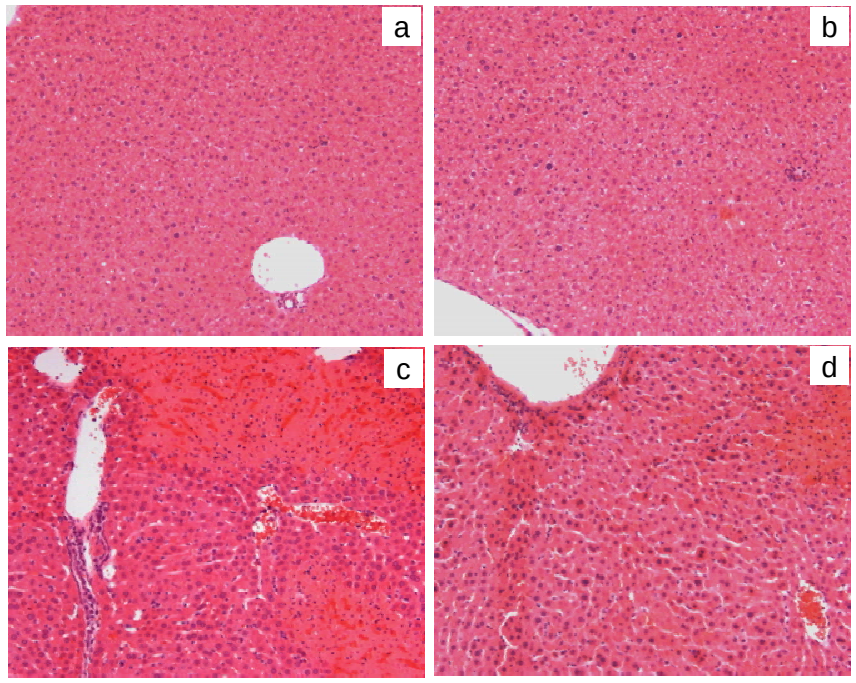

a
b
c
d

Figure 8c

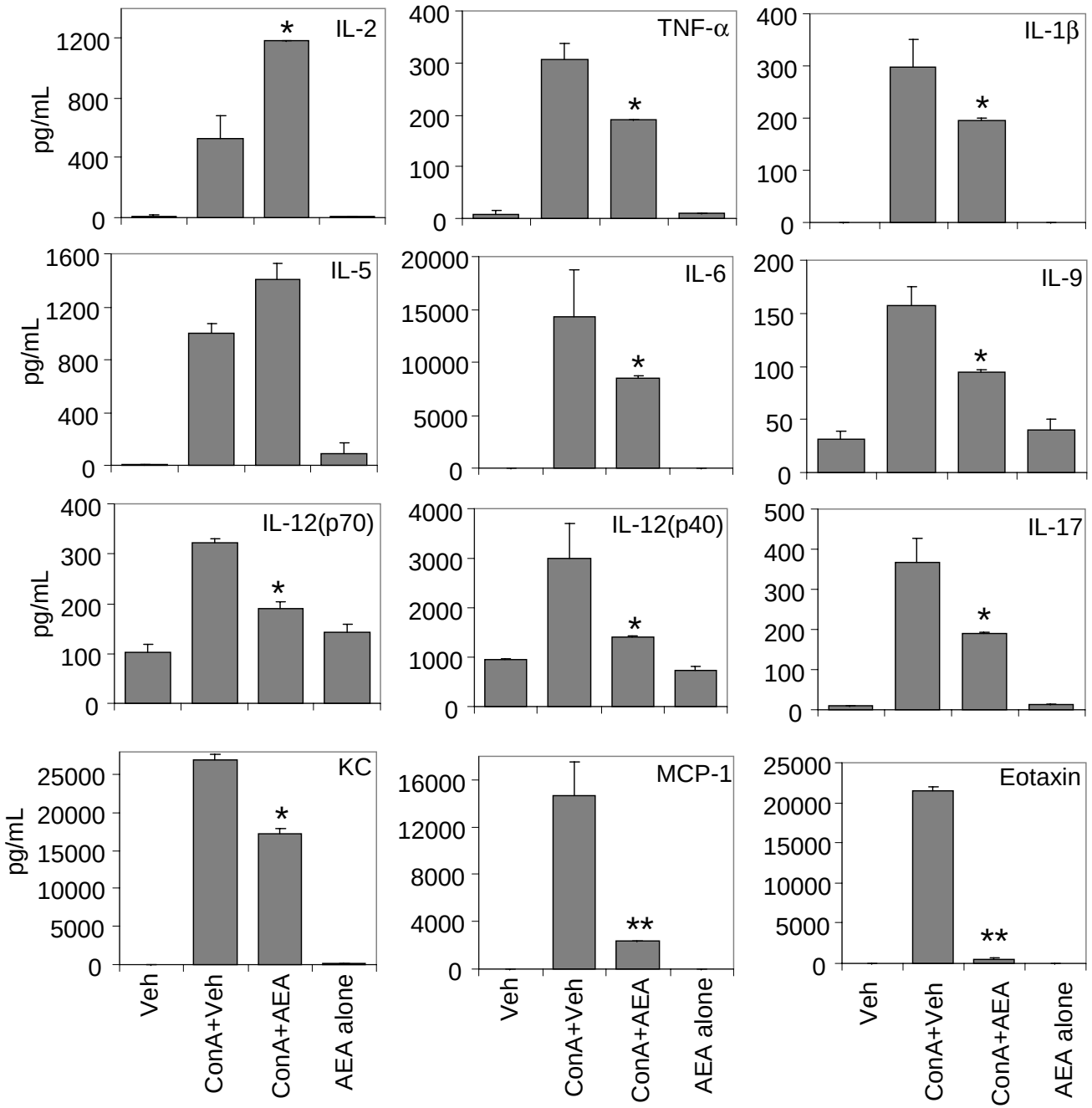

IL-2
TNF-α
IL-1β
IL-5
IL-6
IL-9
IL-12(p70)
IL-12(p40)
IL-17
KC
MCP-1
Eotaxin
pg/mL
Veh
ConA+Veh
ConA+AEA
AEA alone

Figure 8D

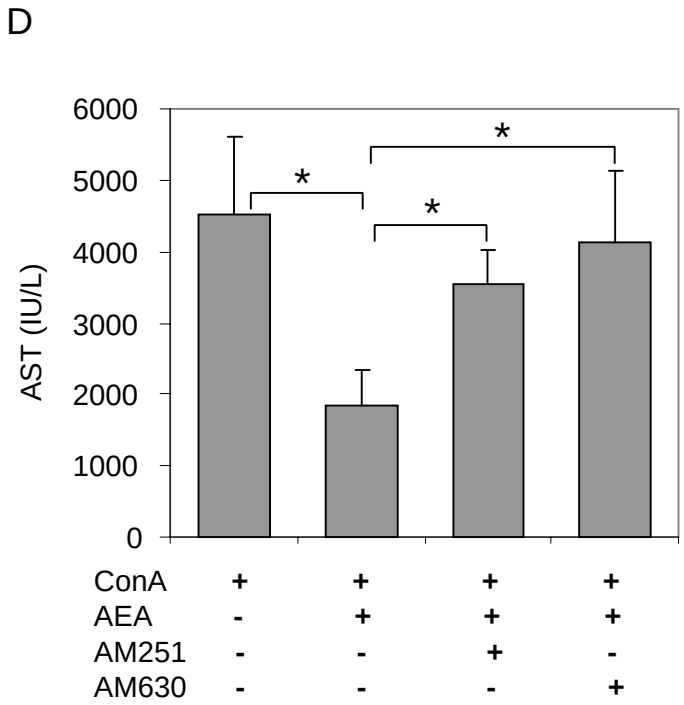
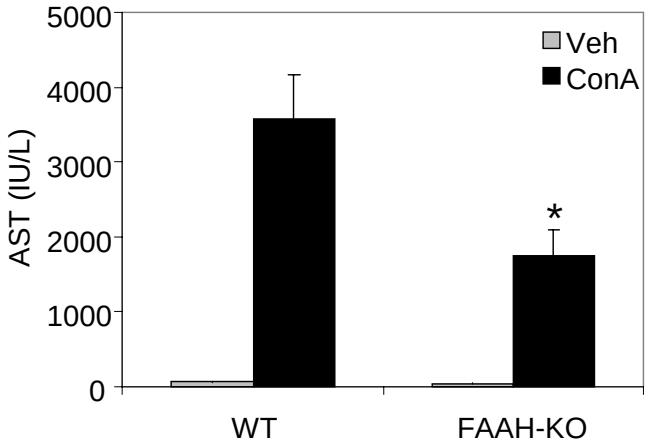
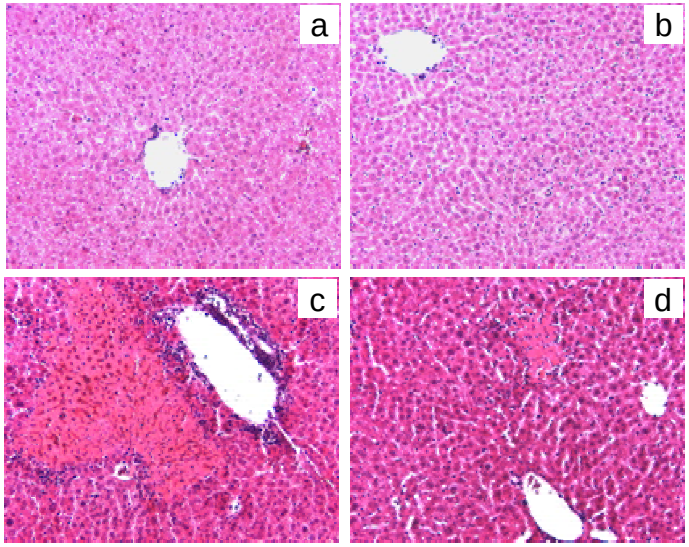


Figure 9

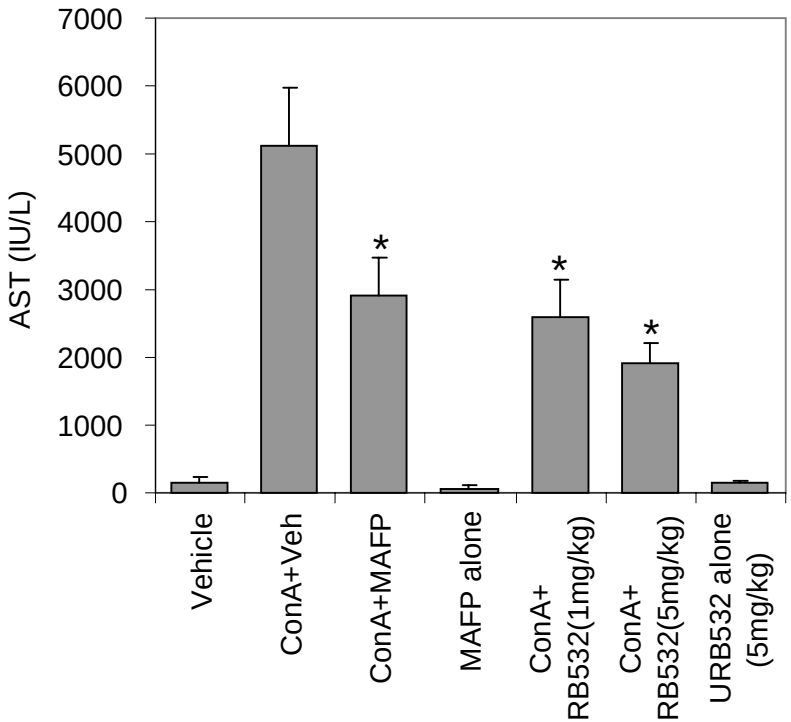
A


5000
4000
3000
2000
1000
0
AST (IU/L)
Veh
ConA
*
WT
FAAH-KO

B


a
b
c
d

C


7000
6000
5000
4000
3000
2000
1000
0
AST (IU/L)
*
*
*
Vehicle
ConA+Veh
ConA+MAFP
MAFP alone
ConA+ URB532(1mg/kg)
ConA+ URB532(5mg/kg)
URB532 alone (5mg/kg)