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Impaired NFAT and NF κ B Activation are Involved in Suppression of CD40 Ligand Expression by Δ^9 -Tetrahydrocannabinol in Human CD4⁺ T cells

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Abstract

We have previously reported that Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the main psychoactive cannabinoid in marijuana, suppresses CD40 ligand (CD40L) expression by activated mouse CD4⁺ T cells. CD40L is involved in pathogenesis of many autoimmune and inflammatory diseases. In the present study, we investigated the molecular mechanism of Δ^9 -THC-mediated suppression of CD40L expression using peripheral blood human T cells. Pretreatment with Δ^9 -THC attenuated CD40L expression in human CD4⁺ T cells activated by anti-CD3/CD28 at both the protein and mRNA level, as determined by flow cytometry and quantitative real-time PCR, respectively. Electrophoretic mobility shift assays revealed that Δ^9 -THC suppressed the DNA-binding activity of both NFAT and NF κ B to their respective response elements within the CD40L promoter. An assessment of the effect of Δ^9 -THC on proximal T cell-receptor (TCR) signaling induced by anti-CD3/CD28 showed significant impairment in the rise of intracellular calcium, but no significant effect on the phosphorylation of ZAP70, PLC γ 1/2, Akt, and GSK3 β . Collectively, these findings identify perturbation of the calcium-NFAT and NF κ B signaling cascade as a key mechanistic event by which Δ^9 -THC suppresses human T cell function.

Keywords

Δ^9 -tetrahydrocannabinol (Δ^9 -THC); T cell-receptor signaling; Intracellular calcium; NFAT; NF κ B; CD40L

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Conflicts of interest

The authors declare that they have no conflicts of interest.

Introduction

CD40L, also termed CD154, is a type II transmembrane protein and member of the tumor necrosis factor (TNF) gene superfamily. Although CD40L can be expressed by many cell types such as eosinophils, basophils, macrophages, and natural killer cells, the highest level of CD40L expression is present on activated CD4⁺ T cells [reviewed in (Schonbeck et al., 2000)]. CD40L normally binds to its cognate receptor, CD40, which is constitutively expressed on a variety of cells, such as B cells, activated macrophages, dendritic cells, vascular endothelial cells, astrocytes and microglial cells [reviewed in (van Kooten and Banchereau, 2000; Schonbeck and Libby, 2001; Chen *et al.*, 2006)]. Ligation of CD40L on CD40 expressing cells enhances the function of the interacting effector cells, for instance, promoting antigen presentation, cytokine production, as well as antibody production. Thus, both CD40L and CD40 serve as important molecular targets for therapeutic intervention of diseases [reviewed in (Daoussis *et al.*, 2004; Chatzigeorgiou *et al.*, 2009)].

Due to the significant role of CD40-CD40L interaction in the adaptive immune response, the expression of CD40L on CD4⁺ T cells is tightly regulated and controlled by similar mechanisms to those that control the production of T cell-derived cytokines, which mainly occurs at the transcriptional level [reviewed in (Cron, 2003)]. T cell activation requires two independent signals, the first is antigen-specific and mediated through TCR, and the second from costimulatory receptors, particularly CD28 [reviewed in (Frauwirth and Thompson, 2002)]. Specific peptide recognition, within the context of the MHC complex, by the TCR leads to phosphorylation of TCR ζ chains, which results in phosphorylation and recruitment of Zeta-chain-associated protein kinase 70 (ZAP70) to TCR complex. Activation of ZAP70 then contributes to the activation of phospholipase C- γ (PLC γ) enzymes, resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 increases intracellular calcium (Ca²⁺) concentration, which is mainly responsible for activation of NFAT, whereas DAG activates protein kinase C- θ (PKC- θ), which is mainly responsible for activation of NF κ B [reviewed in (Bromley et al., 2001)]. Importantly, Ca²⁺ elevation is also crucial for TCR-induced NF κ B activation [reviewed in (Feske et al., 2003)]. Both transcription factors are critically involved in transactivation of *CD40L* (Schubert *et al.*, 1995; Smiley *et al.*, 2000; Srahna *et al.*, 2001; Mendez-Samperio *et al.*, 2003). Ligation of CD28 leads to activation of both phosphoinositide 3-kinase (PI3K)-dependent and -independent pathways. The activation of the PI3K pathway results in the phosphorylation of protein kinase B (Akt), an important effector protein in CD28 signaling (Boomer and Green, 2010). Glycogen synthase kinase-3 β (GSK3 β), a negative regulator of NFAT, is one downstream target of Akt [(Appleman et al., 2002), reviewed in (Grimes and Jope, 2001)]. Further, the binding of adapter proteins to the cytoplasmic tail of CD28 activates PLC γ and its downstream signaling pathways (Boomer and Green, 2010).

Cannabinoids are a family of compounds derived from *Cannabis sativa*, more commonly known as marijuana, which have been used recreationally and medicinally for centuries [reviewed in (Guzman, 2003)]. There are at least 60 structurally-related plant-derived cannabinoids present in marijuana, of which Δ^9 -THC is the primary psychoactive constituent (Turner *et al.*, 1980). Medically, synthetic Δ^9 -THC (Marinol®) has been

approved by the Food and Drug Administration (FDA) since the mid-1980's for chemotherapy-induced nausea and vomiting in cancer patients and cachexia in AIDS patients (Klein, 2005). Crude marijuana is not FDA-approved, although 18 states and the District of Columbia have legalized its medical use (NIDA, 2011). Moreover, a whole-plant cannabis extract composed of a 1:1 ratio of Δ^9 -THC and cannabidiol (Sativex®), was recently approved for treatment of neuropathic pain and spasticity in multiple sclerosis in the United Kingdom, Spain, Germany, Denmark, the Czech Republic, Sweden, New Zealand and Canada, and is currently in phase III trials for cancer pain treatment in the US (Karschner *et al.*, 2011).

In addition to treatment of nausea, weight loss and pain, many studies have shown that Δ^9 -THC also modulates immune function [reviewed in (Croxford and Yamamura, 2005)]. Specifically, our laboratory has shown that Δ^9 -THC impairs production of cytokines such as interleukin 2, expression of costimulatory molecules such as inducible costimulatory molecules (ICOS) and CD40L by activated mouse splenic T cells (Springs *et al.*, 2008; Lu *et al.*, 2009b; Ngaotepprutaram *et al.*, 2012). With the increased use of marijuana recreationally and medically, the potential impact on immune competence is a concern, especially when used by patients whose immune system has already been compromised. Therefore, an increased understanding of the mechanisms by which Δ^9 -THC suppresses T cell function can significantly contribute to making science-based decisions concerning the risk to benefit ratio for patients considering this therapeutic course. In light of the crucial role CD40L plays in inflammatory responses, the objective of the present study was to investigate whether Δ^9 -THC suppresses CD40L expression by activated primary human CD4⁺ T cells and, if so, to begin elucidating the critical molecular events that are involved.

Materials and Methods

Reagents

Δ^9 -THC was provided by the National Institute on Drug Abuse (Bethesda, MD) and dissolved in 100% ethanol (EtOH). Unless otherwise noted, all other chemicals were obtained from Sigma-Aldrich (St Louis, MO).

Antibodies

The following antibodies obtained from BD Pharmingen (San Diego, CA) were utilized to induce T cell activation; purified NA/LE anti-human CD3 (anti-CD3, clone UCHT1), purified NA/LE anti-human CD28 (anti-CD28, clone 28.2), purified polyclonal goat anti-mouse IgG (IgG crosslinker), purified NA/LE anti-mouse CD3 (clone 145-2C11), and purified NA/LE anti-mouse CD28 (clone 37.51). Antibodies used for surface staining of CD40L expression on CD4⁺ T cells were purchased from Biolegend (San Diego, CA); PE anti-human CD154 (CD40L; clone 2431) and PerCP-Cy5.5-anti-human CD4 (clone OKT4). The following antibodies were used for staining of intracellular phosphorylated kinases: V450 mouse anti-human CD4 (clone RPA-T4), V450 rat anti-mouse CD4 (clone RM4-5), Alexa Fluor 647 mouse anti-ZAP70 (pY319)/Syk (pY352) antibody, PE mouse Anti-Akt (pS473) antibody, PE mouse anti-PLC- γ 2 (pY759) (clone K86-689.37), which is crossreactive to PLC γ 1 in T cells (Landrigan *et al.*, 2011), and purified anti-mouse CD16/

CD32 were obtained from BD; whereas FITC-conjugated anti-Phospho-GSK-3 β (S9) was obtained from R&D (Minneapolis, MN). Mouse IgG was obtained from Invitrogen (Frederick, MD). APC anti-human CD4 (clone RPA-T4; BD Pharmingen) was used for intracellular Ca²⁺ measurements in CD4⁺ T cells.

Isolation and culturing of human peripheral blood mononuclear cells (PBMCs)

Human leukocyte packs were obtained commercially from anonymous donors (Gulf Coast Regional Blood Center, Houston, TX). Primary human PBMCs were isolated from buffy coats by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ) as previously described (Lu et al., 2009a). PBMCs were cultured at a final concentration of 1×10^6 cells/mL in RPMI complete media [RPMI medium (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated bovine calf serum (HyClone, Logan, UT), and the antibiotics Penicillin (100 units/mL)/Streptomycin (100 μ g/mL) (Gibco Invitrogen)] at 37°C in 5% CO₂.

Isolation of human peripheral blood naïve CD4⁺ T cells

Negative selection was used to isolate human naïve CD4⁺ T cells from PBMCs using MACS Naïve Human CD4⁺ T Cells Isolation Kits per the manufacturer's protocol (Miltenyl Biotec, Auburn, CA) with some modifications. RPMI medium was used instead of EDTA-containing MACS buffer to minimize perturbation of intracellular Ca²⁺ during the isolation of naïve CD4⁺ T cells. Briefly, primary human PBMC were isolated and resuspended in RPMI medium before incubating with the antibody cocktail. The cell suspension was then applied onto the magnetic column. Unlabeled naïve CD4⁺ T cells were eluted from the column, collected and maintained in RPMI complete media at 37°C in 5% CO₂.

Animals

Female C57BL/6 mice (6 weeks of age) were purchased from Charles River Laboratories (Portage, MI). Mice were maintained as previously described (Kaplan et al. 2003). All experimental mouse protocols were reviewed and approved by the Institutional Animal Care and Use Committee at MSU. Spleens were aseptically isolated and made into single-cell suspensions in RPMI complete media.

TCR stimulation

Cells were subjected to either short-term (5 min) or prolonged (16 to 48 h) stimulation. For short-term stimulation, TCR stimulation was performed by anti-CD3/CD28 crosslinking. PBMCs, 1×10^6 cells, were first incubated with anti-CD3 and anti-CD28, 10 μ g/mL each, for 15 min on ice. Subsequently, cells were washed once with cold 1X Hank's Balanced Salt Solution (HBSS, Invitrogen) and centrifuged at $350 \times g$ for 5 min at 4°C. After discarding the supernatant, cells were resuspended in complete media followed by incubation with 10 μ g/mL IgG crosslinker for another 15 min on ice. TCR-mediated signaling cascades were activated by transferring the tubes to a 37°C water bath and incubating for respective time periods as indicated in the results. For prolonged stimulation, PBMCs, 2×10^5 cells, were stimulated with immobilized anti-CD3 (5 μ g/mL) plus soluble anti-CD28 (5 μ g/mL) in precoated 96-well plates (100 μ L/well) overnight at 4°C. Before addition of the cells, plates

were washed twice with 1X RPMI followed by addition of 5 µg/mL anti-CD28 (20 µL/well). Cells were activated in the presence or absence of ⁹-THC. Stock solutions of ⁹-THC were used at a final EtOH concentration of 0.1% and were added 30 min before TCR stimulation. 0.1 % EtOH was used as a vehicle control (VH). ⁹-THC or VH were present throughout the course of incubation process by adding them back to the culture after performing a washing step in the case of short-term stimulation.

Flow cytometric analysis

At the indicated time points, 1×10^6 cells were harvested and, when necessary, dead cells were detected by staining with Live/Dead Fixable Dead Cell Stain Kit (Near-infrared dye, Invitrogen) per the manufacturer's protocol prior to all the staining steps. Briefly, cells were washed once with 1X HBSS following by incubation with Near-infrared dye for 20 min at 4°C. The amount of antibodies used varied in staining of each specific antigen based on preliminary antibody titration and were typically pre-diluted in FACS buffer (1X HBSS containing 1% BSA and 0.1% sodium azide, pH 7.4–7.6) at appropriate amounts prior to addition to the cells. Staining for intracellular phosphorylated kinases was conducted on the same day and immediately followed by data analysis. In all cases, stained cells were analyzed on FACSCanto II cell analyzer (BD Biosciences) and data were analyzed using Kaluza software (Beckman Coulter, Miami, FL).

Cell surface expression of CD40L on CD4⁺ T cells was assessed by simultaneously staining with PE-anti CD154 and PerCP-Cy5.5-anti-CD4 for 30 min at 4°C in the dark. Unbound antibodies were removed by washing once with FACS buffer. Cells were fixed with BD CytoFix™ Buffer (BD Biosciences) for 10 min at 4°C in the dark, followed by washing once with FACS buffer. Stained cells were then resuspended in FACS buffer for analysis.

For staining of intracellular phosphorylated kinases, PBMCs or splenocytes were equilibrated at 37°C in 5% CO₂ for 3 h to normalize baseline kinase activation prior to pretreatment with ⁹-THC or VH. TCR stimulation was performed at 37 °C in a water bath. Cells were fixed in 1.5% formaldehyde by direct dilution in cell culture from 32% stock (electron microscopy grade, Electron Microscopy Sciences, Hartfield, PA) for 10 min at 37 °C followed by centrifugation at 600×g for 6 min at 4°C. Cells were then permeabilized by drop-wise addition of ice-cold 100% methanol while vortex mixing at medium speed. Cells were stored in methanol at –80°C until staining with indicated antibodies. Cells were washed 3 times with FACS buffer. Prior to staining of intracellular phosphorylated kinases, surface Fc receptors and unbound IgG crosslinker in human samples were blocked by incubating with 20% human AB serum (Invitrogen) and 15 µg/mL mouse IgG; whereas surface Fcγ receptors in mouse samples was blocked by incubating with anti-mouse CD16/CD32 for 15 min at room temperature. The level of phosphorylated kinases in CD4⁺ T cells was assessed by simultaneously staining with V450 anti-CD4 and antibodies specific for phosphorylated epitopes on Zap70, Akt, and GSK3β or with V450 anti-CD4 and antibodies specific for phosphorylated epitopes on PLCγ1/2 for 60 min at room temperature in the dark. Unbound antibodies were removed by washing twice with FACS buffer. Stained cells were then resuspended in FACS buffer for analysis.

Real time polymerase chain reaction (Real-time PCR)

Total RNA was isolated from activated human PBMC using RNeasy Kit (Qiagen, Valencia, CA). RNA was quantified using a Nanodrop 1000 (Thermo Scientific, Wilmington, DE). Total RNA was reverse-transcribed into cDNA using random primers with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). TaqMan® Gene Expression Assay primers for human CD40L (Hs99999102_m1) were purchased from Applied Biosystems. The relative steady-state levels of CD40L mRNA were determined using ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems). The fold-change value of relative steady-state CD40L mRNA levels were calculated using the $-C_t$ method as described previously (Livak and Schmittgen, 2001) and normalized to the endogenous reference, 18s rRNA.

Nuclear protein isolation

Human PBMCs (approximately 2×10^7 cells/mL) were stimulated with anti-CD3/CD28 in the presence or absence of 9 -THC for 16 h. PBMCs were collected and washed once with PBS. Nuclear proteins was isolated as previously described with some modifications (Faubert Kaplan and Kaminski, 2003). Briefly, cells were lysed with HB buffer (10 mM HEPES and 1.5 mM $MgCl_2$), containing protease inhibitors (1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 μ g/mL aprotinin, and 0.2 μ g/mL leupeptin) for 15 min on ice. Nuclei were pelleted by centrifugation at $1000 \times g$ for 5 min. The supernatant containing cytoplasmic protein was discarded. The nuclear pellet was washed twice with MDHN buffer (25 mM HEPES, 3 mM $MgCl_2$, and 100 mM NaCl) containing proteinase inhibitors. Nuclei were lysed using a hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.3 mM EDTA, and 0.1% Igepal) plus protease inhibitors by rocking for 30 min on ice. The nuclear fraction in supernatant was separated by centrifugation at $17500 \times g$ for 15 min. The salt concentration was reduced by adding equal amount of a hypotonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA and 10% glycerol). Protein concentrations were determined using the bicinchoninic acid assay.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts (1 μ g) were incubated with 0.25 μ g poly(dI-dC) in binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 0.2 μ g/mL aprotinin, and 0.2 μ g/mL leupeptin) for 10 min on ice. The corresponding ^{32}P -labeled probe (30,000 count per minute) was added, followed by incubation at room temperature for 30 min. Samples were resolved on a 5% polyacrylamide gel in 1X TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) as previously described (Condie et al., 1996). Unlabeled probes were added in 100-fold molar excess to detect the specificity of DNA-protein complexes. After electrophoresis, gels were dried and analyzed by standard autoradiograph. The sequences of the oligonucleotide probes used are: NFAT 5'-AAGCACATTTTCCAGGA-3' (Lindgren et al., 2001) and NF κ B probe, 5'-TGAGGTAGGGATTTCACAGCTG-3' (Srahna et al., 2001). The corresponding binding site for each transcription factor is underlined.

Intracellular Ca²⁺ measurements

Purified naïve CD4⁺ T cells (1×10⁶ cells/mL, 15 mL) were allowed to equilibrate at 37°C in 5% CO₂ for 3 h. Naïve CD4⁺ T cells were then labeled with the Ca²⁺ indicator dyes (Invitrogen), Fura-3 (2 µM) and Fluo-red (5 µM), for 60 min at room temperature prior to pretreatment with ⁹-THC or VH (2×10⁶ cells per treatment groups). T cells were activated by incubation with anti-CD3 and anti-CD28 for 15 min on ice (10 µg/mL each). Excess antibodies against CD3 and CD28 were removed by washing once with complete RPMI. Cells were then resuspended in complete media containing Ca²⁺ dyes and ⁹-THC or VH. The intracellular Ca²⁺ measurement were then acquired by using the time parameter on the FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and analyzed for Fura-Red (FL1) and Fluro-3 (FL3) fluorescence. Following 60 sec of data acquisition, cells were activated by adding IgG crosslinker (10 µg) to crosslink the anti-CD3/CD28. Cells were analyzed for a total of 300 seconds. Post-collection analysis was performed using FlowJo software (TreeStar, Ashland, OR). The ratio of FL1:FL3 was derived and plotted over time. Kinetic plots are expressed as median of the FL1:FL3 ratio. Data are presented in arbitrary units as a function of fluorescence (relative intracellular Ca²⁺) versus time.

Statistical analyses

GraphPad Prism 4.00 (Graphpad Software, San Diego, CA) was used for all statistical analysis. Data acquired as percentages from flow cytometry were transformed into log scale before performing statistical analysis. In the case of mRNA data, the transformed fold-change values were used in statistical analysis. For comparisons among treatment groups, one-way ANOVA was used. Dunnett's post-hoc test was used to test for significance between treatment groups and control. Outliers were eliminated using the Grubb's test. A value of $p < 0.05$ was considered significant.

Results

1. ⁹-THC attenuated anti-CD3/CD28-induced CD40L expression by activated human T cells at the transcriptional level

Based on our previous finding that ⁹-THC attenuated the upregulation of CD40L expression in activated mouse splenic T cells (Ngaotepprutaram *et al.*, 2012), the effect of ⁹-THC on anti-CD3/CD28-induced CD40L expression by activated primary human T cells was investigated. Human PBMCs were activated with anti-CD3/CD28 in the presence or absence of ⁹-THC, and peak expression of CD40L occurred between 36–48 h (Fig. 1) following activation. As shown in Figure 1, 2a and 2b, ⁹-THC significantly decreased the percentage of double positive CD4⁺CD40L⁺ cells in a concentration-dependent manner at 48 h post activation. In addition, the mean fluorescence intensity (MFI), which corresponds to the total expression of surface CD40L on each activated CD4⁺ T cell was markedly attenuated in the presence of ⁹-THC (Fig. 2c). The concomitant decrease in both percentage of double positive cells and MFI of CD40L clearly indicates that ⁹-THC impaired the upregulation of surface CD40L expression on activated human CD4⁺ T cells. It is well established that CD40L expression is almost exclusively regulated at the level of transcription [reviewed in (Cron, 2003)]. Therefore, the steady-state level of CD40L mRNA was assessed by quantitative real time PCR in the presence or absence of ⁹-THC at 24 h

post stimulation. As shown in Figure 3, 9 -THC significantly attenuated anti-CD3/CD28-induced CD40L mRNA expression in activated human T cells. The suppression of CD40L mRNA expression by 9 -THC also occurred in a concentration-dependent manner, which corresponds to its effect at the protein level.

2. 9 -THC impaired anti-CD3/CD28-induced DNA-binding activity of NFAT and NF κ B in activated human T cells

The transactivation of the *CD40L* is tightly regulated by several transcription factors (NFAT, CD28RE, NF κ B, TFE3/TFEB, EGR, AKNA, and AP1) that bind in the promoter region (Steiper et al., 2008). Although NFAT is the key transcription factor found in the minimal CD40L promoter (Schubert et al., 1995), several reports demonstrated significant involvement of NF κ B in the upregulation of CD40L expression in both activated mouse and human T cells (Smiley *et al.*, 2000; Srahna *et al.*, 2001; Mendez-Samperio *et al.*, 2003). Previous studies from our laboratory demonstrated that cannabinoids suppressed T cell cytokine production in mice partly through the impairment of NFAT and NF κ B activation (Herring and Kaminski, 1999; Lu *et al.*, 2009b). In light of the above, the effect of 9 -THC on anti-CD3/CD28-induced DNA binding activities of NFAT and NF κ B to CD40L promoter was investigated by EMSA. Both NFAT and NF κ B oligonucleotide probes containing the respective binding sites were derived from the human *CD40L* promoter (Lindgren *et al.*, 2001; Srahna *et al.*, 2001). Human PBMCs were activated with anti-CD3/CD28 in the presence or absence of 9 -THC for 16 h, which was the period of greatest DNA-binding activity (data not shown). The specificity of NFAT-DNA complexes (as indicated by arrows) was determined by addition of 100-fold molar excess of unlabelled probe (Fig. 4a, lane 9). Upon T cell activation, there was an increase in DNA binding activity of specific NFAT-DNA complexes (lane 3) compared to the nuclear protein from nonactivated PBMCs (NA) (lane 2). Pretreatment with 9 -THC (1, 5, 10, and 15 μ M - lane 5–8 followed by stimulation with anti-CD3/CD28 decreased the intensity of specific NFAT-DNA complexes, thereby indicating a decrease in NFAT-DNA binding activity. For NF κ B, only one complex, indicated by an arrow, was competed completely in the presence of 100-fold molar excess of unlabelled probe (Fig. 4b, lane 9). Upon activation, there was an increase in NF κ B-DNA binding activity (lane 3) when compared to the nuclear protein from NA cells. Pretreatment with 9 -THC (1, 5, 10, and 15 μ M - lane 5–8) followed by anti-CD3/CD28 activation decreased the intensity of specific NF κ B-DNA complexes, thereby indicating a decrease in NF κ B-DNA binding activity. These results suggest that 9 -THC impaired both NFAT- and NF κ B-DNA binding activity in activated human T cells.

3. 9 -THC did not alter GSK3 β activity in activated human CD4 $^+$ T cells

GSK3 β , a serine/threonine protein kinase, has been shown to act as a negative regulator to limit the activation of many transcription factors, particularly NFAT. The phosphorylation of NFAT by GSK3 β inhibits NFAT activation by retaining NFAT in the cytoplasm as well as promoting the export from the nucleus [reviewed in (Grimes and Jope, 2001)]. GSK3 β is constitutively active in resting cells and becomes inactive upon stimulation through TCR by phosphorylation at serine residue 9 (pGSK3 β) [reviewed in (Grimes and Jope, 2001)]. To further elucidate the mechanism by which 9 -THC suppresses NFAT-DNA binding activity, we investigated the effect of 9 -THC pretreatment on the activity of GSK3 β in activated T

cells. Human PBMCs were activated using anti-CD3/CD28 crosslinking. The level of pGSK3 β , which corresponds to its inactive state, was assessed by flow cytometry at 5 min post activation. As shown in Figure 5, there was a significant increase in the amount of pGSK3 β in activated human CD4⁺ T cells as demonstrated by an increase of MFI upon activation. Pretreatment with Δ^9 -THC did not alter the level of pGSK3 β suggesting that Δ^9 -THC did not perturb anti-CD3/CD28-mediated GSK3 β inactivation in human CD4⁺ T cells.

4. Δ^9 -THC attenuated anti-CD3/CD28-induced elevation of intracellular Ca²⁺, but did not impair PLC γ activation

The inhibitory effect of Δ^9 -THC on the DNA binding activity of both NFAT and NF κ B strongly implicated altered intracellular Ca²⁺ regulation. To investigate the effects of Δ^9 -THC on anti-CD3/CD28-induced Ca²⁺ elevation in human CD4⁺ T cells, naïve CD4⁺ T cells were incubated with the Ca²⁺ indicator dyes Fluo-3 and Fura-red for 30 min, following by treatment with Δ^9 -THC for 30 min. Cells were then incubated with anti-CD3/CD28 on ice. Upon TCR stimulation with an IgG crosslinker, there was a pronounced rise in intracellular Ca²⁺ (Fig. 6). Pretreatment with Δ^9 -THC resulted in a concentration-dependent attenuation of elevated Ca²⁺ following anti-CD3/CD28 crosslinking in activated human CD4⁺ T cells with nearly a complete loss of the response at 15 μ M Δ^9 -THC (Fig. 6). The increase in the intracellular Ca²⁺ following TCR stimulation is mainly regulated by the activation of PLC γ [reviewed in (Feske et al., 2003)]. Furthermore, PLC γ also controls NF κ B activation through the PKC θ pathway [reviewed in (Schmitz et al., 2003)]. We next tested whether Δ^9 -THC affects the activation of PLC γ . The phosphorylation status of PLC γ 1/2 (pPLC γ 1/2), which corresponds to its active state, was assayed by flow cytometry at 5 min post stimulation with anti-CD3/CD28 crosslinking. Upon activation, there was a robust increase in the amount of pPLC γ 1/2 as demonstrated by the increase of MFI in activated human CD4⁺ T cells. However, pretreatment with Δ^9 -THC did not significantly affect the anti-CD3/CD28-mediated increase of pPLC γ 1/2 in activated human CD4⁺ T cells (Fig. 7). These results suggest that suppression of anti-CD3/CD28-induced elevation of Ca²⁺ influx by Δ^9 -THC in human CD4⁺ T cells is not mediated through the activation of PLC γ 1/2.

5. Δ^9 -THC does not modulate anti-CD3/CD28-mediated phosphorylation of ZAP70 or Akt in activated human CD4⁺ T cells

Our previous study using mouse splenocytes suggested that Δ^9 -THC affected the proximal signaling of TCR based on the fact that Δ^9 -THC-mediated suppression of CD40L expression occurred following T cell activation by anti-CD3/CD28 antibodies, but not phorbol ester/ionomycin (Ngaotepprutaram *et al.*, 2012). Concordantly, a study reported by Borner and coworkers demonstrated that the T-cell inhibitory effect of Δ^9 -THC is, partly, due to the impairment of proximal TCR signaling cascades (Borner et al., 2009). To test this hypothesis, we examined the effect of Δ^9 -THC on the proximal TCR signaling cascades. The level of phosphorylated ZAP70 (pZAP70), which is mediated through CD3 activation, and the level of phosphorylated Akt (pAkt), which is mediated through CD28 activation, was measured by flow cytometry at 5 min post activation with anti-CD3/CD28 crosslinking. Upon activation, there was a significant increase in the amount of both pZAP70 (Fig. 8a) and pAkt (Fig. 8b) in activated human CD4⁺ T cells. However, pretreatment with Δ^9 -THC

did not significantly affect anti-CD3/CD28-induced phosphorylation of either ZAP70 or Akt in activated human CD4⁺ T cells (Fig. 8a and 8b, respectively). Interestingly, pretreatment with ⁹-THC increased the variability in the phosphorylation of ZAP70 among the human subjects (Fig. 8a). Thus a comparable mouse model was used to investigate the effect of ⁹-THC on the activation of ZAP70. In these murine studies, ⁹-THC did not affect the phosphorylation level of Zap70 in activated CD4⁺ T cells (data not shown). Collectively, these results suggest that ⁹-THC does not impair proximal TCR-associated signaling in activated human CD4⁺ T cells.

Discussion

In this present study, we clarified the mechanism underlying ⁹-THC-mediated suppression of CD40L expression by activated primary human T cells. Consistent with our previous study demonstrating the suppression of CD40L upregulation by ⁹-THC in mouse splenic T cells (Ngaotepprutaram *et al*, 2012), ⁹-THC attenuated anti-CD3/CD28-induced CD40L expression by human T cells. It is noteworthy that the decrease of surface CD40L expression by ⁹-THC could be the result of increasing the generation of soluble CD40L by proteolysis in the microsome (Pietravalle *et al.*, 1996); however, this possibility is unlikely in light of the concomitant decrease in the *CD40L* mRNA levels by ⁹-THC treatment. It is also notable that the kinetics of CD40L upregulation in human observed here is different from previous findings in mouse T cells and several other reports, in which the peak induction was early, approximately 6–8 h post activation (Roy *et al*, 1993; Fuleihan *et al*, 1994; Nusslein *et al*, 1996; Ford *et al*, 1999; Ngaotepprutaram *et al*, 2012). Although the rapid induction of CD40L by activated CD4⁺ T cells is well-established, more recent studies support a biphasic kinetic profile of CD40L induction in both mouse and human activated CD4⁺ T cells (Lee *et al.*, 2002; McDyer *et al*, 2002; Snyder *et al*, 2007; Kaminski *et al*, 2009). Here we report that the peak time of induction of surface CD40L expression on activated human CD4⁺ T cells was between 36 and 48 h post activation with anti-CD3/CD28. This is in accordance with reports showing that the second peak occurred at 48 h. In contrast, we did not observe biphasic kinetics, which could possibly be due to differences in the *in vitro* culture model. Although our study and the studies conducted by McDyer *et al.* and Snyder *et al.* all used PBMCs as the source of T cells, the clones and concentrations of antibodies directed against CD3 and CD28 were different from those used here (McDyer *et al.*, 2002; Snyder *et al*, 2007).

Next, we investigated the mechanisms by which ⁹-THC attenuated anti-CD3/CD28-induced CD40L expression. It should be noted that the concentrations of ⁹-THC used in these studies have not been shown to produce toxicity in mouse or human studies *in vitro* (Rao *et al*, 2004; Springs *et al*, 2008; Chen *et al*, 2012b). As compared to concentrations found in serum of marijuana users, the concentrations of ⁹-THC used in the present studies are higher. For instance, peak ⁹-THC plasma concentration after smoking marijuana is typically 100–200 ng/mL (approximately 0.3–0.6 μ M) within first 10 minutes, although plasma ⁹-THC concentrations can vary with the potency of marijuana and the manner in which the drug is smoked (Huestis *et al*, 1992). Following oral administration, peak ⁹-THC plasma concentration is approximately 3–4 ng/mL at 1–5 hours due to extensive first pass metabolism [reviewed in (Grotenhermen, 2003)]. Due to its lipophilicity, ⁹-THC

immediately distributes from blood to tissues resulting in a large volume of distribution (Wall *et al*, 1983; Kelly and Jones, 1992). Two additional points must be emphasized. First, marijuana contains more than 60 structurally-related cannabinoids, a number of which are well-established to be immune modulatory, including cannabinol and cannabidiol (Jan *et al*, 2002; Chen *et al*, 2012a), and which also likely impact the pharmacokinetics and/or effects of 9 -THC *in vivo* (Bornheim *et al*, 1995). Second, the lipophilic properties of 9 -THC promote nonspecific binding with serum lipids and proteins (Springs *et al*, 2008), and even plastic surfaces (Christophersen, 1986). Together, these observations suggest that the 9 -THC concentrations used in this current *in vitro* study are physiologically relevant.

Mechanistically, we demonstrate that 9 -THC impaired the DNA-binding activity of both NFAT and NF κ B, two critical transcription factors involved in regulating CD40L expression (Schubert *et al*, 1995; Smiley *et al*, 2000; Srahna *et al*, 2001; Mendez-Samperio *et al*, 2003). Consistent with several reports demonstrating that there are two specific complexes formed at the proximal NFAT site, we also observed two specific NFAT-DNA complexes, which are likely composed of NFATc (NFATc1) and NFATp (NFATc2) (Tsytyskova *et al*, 1996; Lindgren *et al*, 2001). 9 -THC-mediated suppression of NFAT-DNA complexes observed here is consistent with a previous study in which we demonstrated that 9 -THC impaired NFAT-driven luciferase (Lu *et al*, 2009b). In addition, cannabinol, another immunomodulatory plant-derived cannabinoid compound, 2-arachidonyl-glycerol (2-AG), an endogenous cannabinoid, and 15-deoxy- 12,14 -PGJ $_2$ -glycerol ester, a putative metabolite of 2-AG, were all found to inhibit the DNA-binding activity of NFAT in activated T cells (Ouyang and Kaminski, 1999; Faubert and Kaminski, 2000; Yea *et al*, 2000; Raman *et al*, 2012). 2-AG also inhibited the nuclear translocation of both NFATc1 and NFATc2 (Kaplan *et al*, 2005). In order to characterize the molecular target responsible for 9 -THC-mediated suppression of NFAT, the possible involvement of GSK3 β , a kinase regulating NFAT activation, was investigated. These results suggested that GSK3 β is not involved in cannabinoid-mediated suppression of NFAT activity. For NF κ B, we observed three complexes, of which only one complex exhibited specific NF κ B-DNA binding activity, and is consistent with a report by Shrena *et al*, who also identified p65 containing NF κ B-DNA binding complexes in the absence of p50 (Srahna *et al*, 2001). Again, these results are consistent with another study from our laboratory demonstrating that cannabinol primarily suppressed the DNA binding of p65 and c-Rel in mouse T cells (Herring and Kaminski, 1999). Collectively, these findings suggest that suppression of NFAT and NF κ B DNA binding activity are common mechanisms underlying the impairment of T cell activity by cannabinoids.

We demonstrated for the first time that 9 -THC significantly impaired anti-CD3/CD28-induced Ca $^{2+}$ elevation in activated primary human CD4 $^{+}$ T cells. Ca $^{2+}$ elevation is crucial for both TCR-induced NFAT and NF κ B activation [reviewed in (Bromley *et al*, 2001) and (Feske *et al*, 2003)]. Therefore, 9 -THC-mediated suppression of NFAT and NF κ B DNA binding activity suggests the involvement of impaired intracellular Ca $^{2+}$ elevation. This observation is somewhat in contrast to our prior finding that immunomodulatory cannabinoids, 9 -THC, cannabinol, and HU-210, which robustly increased the influx of extracellular Ca $^{2+}$ in resting T cells (Rao *et al*, 2004; Rao and Kaminski, 2006a). It is

noteworthy that although this study investigated the effect of 9 -THC on Ca^{2+} signaling in response to anti-CD3/CD28 activation; pretreatment with 9 -THC and/or preincubation with anti-CD3 and anti-CD28 in the absence of the IgG crosslinker did not increase intracellular Ca^{2+} concentration. The previous studies were performed using either purified mouse splenic T cells and/or the human peripheral blood acute lymphoid leukemia (HPB-ALL) T cell line, and therefore, these differences might account for the differential effect of 9 -THC on Ca^{2+} elevation. On the other hand, our results are consistent with a report demonstrating that pretreatment with 9 -THC suppressed the increase of Ca^{2+} induced by concanavalin A in mouse thymocytes (Yebra et al., 1992). Our data are also consistent with a study demonstrating that calcium alone is sufficient to induce *Cd40l* mRNA expression in mouse megakaryocytes (Crist et al., 2008). The exact mechanism by which 9 -THC exerts its suppressive effect on anti-CD3/CD28-induced Ca^{2+} elevation in primary human CD4^+ T cells has not been fully elucidated. In the present study, we demonstrated that 9 -THC did not affect anti-CD3/CD28-induced phosphorylation of $\text{PLC}\gamma 1/2$, the active forms of $\text{PLC}\gamma$ that generate IP_3 to release Ca^{2+} from intracellular stores. To date, the activation of $\text{PLC}\gamma$ is the major pathway responsible for the IP_3 production; therefore it is unlikely that 9 -THC affects IP_3 production without changes in the activation of $\text{PLC}\gamma$. In contrast, if 9 -THC altered the capacity of IP_3 receptors to bind IP_3 , a similar profile of activity could be observed. Another possibility is that 9 -THC may affect distal steps in receptor-mediated Ca^{2+} mobilization. In fact, not only Ca^{2+} channels, but voltage- and Ca^{2+} -dependent potassium channels, play critical roles in promoting the sustained increase of intracellular Ca^{2+} [reviewed in (Lewis and Cahalan, 1995)]. Therefore, 9 -THC might directly or indirectly target one of the aforementioned ion channels in T cells. Indeed, previous studies from our laboratory showed that the 9 -THC-induced increase in intracellular Ca^{2+} is mediated, at least partially, through transient receptor potential cation channel, subfamily C, member 1 (TRPC1) (Rao and Kaminski, 2006b). Additional studies are required to decipher the detailed molecular mechanisms of Ca^{2+} regulation by 9 -THC in T cells.

In conjunction with the absence of an inhibitory effect of 9 -THC on phosphorylation of $\text{PLC}\gamma 1/2$, 9 -THC did not attenuate anti-CD3/CD28-induced phosphorylation of pZAP70 and Akt, key regulators in early events of TCR and CD28 signaling, respectively. These results suggest that 9 -THC does not interfere with the proximal events of TCR signaling in primary human CD4^+ T cells. This is contrasted with a study utilizing the human Jurkat T cell line in which 9 -THC suppressed TCR-induced phosphorylation of ZAP70 (Borner et al., 2009). The divergent results might be due to the differences in the cell model and/or experimental conditions. The Jurkat E6.1 T cell line likely has aberrant signaling pathways that facilitate its immortalized state. For instance, Jurkat E6.1 T cells exhibit lower phosphorylation of ZAP70, but have higher phosphorylation of $\text{PLC}\gamma$ upon TCR stimulation (Bartelt et al., 2009). In addition, we demonstrated in the present studies that a 30 min pretreatment with 9 -THC significantly suppressed TCR-induced CD40L expression; whereas Borner and coworkers did not observe any effect of 9 -THC in any measurement unless the cells were pretreated with 9 -THC for 2 h. Differences in clones and concentration of anti-CD3 and anti-CD28, as well as the differences in concentration of 9 -THC, might also account for the differential effect of 9 -THC on proximal TCR signaling (Borner et al., 2009).

In summary, these studies are the first to provide mechanistic insights by which Δ^9 -THC attenuates CD40L induction in activated human primary T cells. Our findings demonstrate that Δ^9 -THC suppressed TCR-induced NFAT and NF κ B-DNA binding activity, likely in part through impairment of Ca^{2+} elevation. To our knowledge, we provide the first conclusive evidence that Δ^9 -THC does not interrupt proximal TCR signaling events (e.g. tyrosine phosphorylation of ZAP70, Akt, and PLC γ 1/2) in primary human CD4 $^{+}$ T cells. These results suggest that PLC γ activation, which is the major pathway regulating the increase of intracellular Ca^{2+} , is not involved in suppression of anti-CD3/CD28-induced Ca^{2+} elevation by Δ^9 -THC. Furthermore, we have ruled out the possible involvement of GSK3 β in altered NFAT regulation by Δ^9 -THC. An understanding of the mechanisms by which Δ^9 -THC attenuates CD40L induction in activated human T cells might allow its expansion for therapeutic use and provide additional information when weighing risk to benefit for use in immunocompromised patients.

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Highlights

- Δ^9 -tetrahydrocannabinol (Δ^9 -THC), a plant-derived cannabinoid, impairs CD40L expression by activated primary human CD4⁺ T cells.
- Δ^9 -THC suppresses the DNA-binding activity of NFAT and NF κ B to their response elements in CD40L promoter.
- Δ^9 -THC attenuates increase of intracellular calcium in activated primary human CD4⁺ T cells.
- Δ^9 -THC does not interrupt proximal T cell signaling cascades in activated primary human CD4⁺ T cells.

Fig. 1. Δ^9 -THC attenuated TCR-induced surface CD40L expression on human T cells at all times following activation

PBMC (1×10^6 cells) were treated with Δ^9 -THC (15 μ M) for 30 min and then activated with anti-CD3/CD28 for various times. Cell surface CD40L expression on activated CD4⁺ T cells was determined by flow cytometry. Numbers in parentheses represent the percentage of CD4⁺CD40L⁺ cell population $\pm$ SEM from triplicates. Results represent at least two different donors with three replicates per treatment group.

a

b

C

Fig. 2. Δ^9 -THC attenuated TCR-induced surface CD40L expression on activated human T cells in a concentration-dependent manner

PBMC (1×10^6 cells) were treated with Δ^9 -THC (1, 5, 10, and 15 μ M) for 30 min and then activated with anti-CD3/CD28. (a) Cell surface CD40L expression on activated CD4⁺ T cells was determined by flow cytometry 48 h after activation. Numbers in parentheses represent the percentage of CD4⁺CD40L⁺ cell population $\pm$ SEM from triplicates. (b) Bar graph is a representation of percentage of CD4⁺CD40L⁺ cells. (c) Bar graph represents MFI of CD40L gated on CD4⁺ T cells. The * or ** indicates significant effect compared to

vehicle control (VH = 0.1% EtOH), at $p = 0.05$ or $p = 0.01$, respectively. Results represent five different donors with three replicates per treatment group.

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Fig. 3. Δ^9 -THC suppressed TCR-induced CD40L mRNA expression in activated human T cells PBMC (1×10^6 cells) were treated with Δ^9 -THC (1, 5, 10, and 15 μ M) for 30 min and then activated with anti-CD3/CD28. Steady-state expression of CD40L mRNA was determined by real-time PCR 24 h after activation. The fold difference of CD40L mRNA expression relative to nonactivated resting cells (naïve, NA) was normalized using the endogenous reference, 18s rRNA. The * or ** indicates significant effect compared to vehicle control (VH = 0.1 % EtOH) at $p = 0.05$ or $p = 0.01$, respectively. Results represent three different donors with four replicates per treatment group.

a

b

Fig. 4. ⁹-THC impaired TCR-induced DNA binding activity of NFAT and NFκB in activated human T cells

PBMC (2×10^7 cells) were treated with VH (lane 4) or ⁹-THC (1, 5, 10, and 15 μM; lane 5, 6, 7, 8) for 30 min and then activated with anti-CD3/CD28, for 16 h. The basal DNA binding activity was measured in nonactivated cells (lane 2), whereas the formation of NFAT- or NFκB-DNA complexes was measured in TCR-activated cells (lane 3). The specificity of either NFAT- or NFκB-DNA complexes was determined by adding 100-fold molar excess of the unlabeled probes using the same protein as loaded in lane 3 (lane 9). Lane 1 is radiolabeled probe alone. The nuclear proteins (1 μg) were incubated with

oligonucleotide probes derived from human CD40L promoter containing either NFAT or NF κ B binding site. The DNA-binding complexes were resolved by gel electrophoresis. **(a)** EMSA analysis of NFAT binding to the CD40L promoter. **(b)** EMSA analysis of NF κ B binding to the CD40L promoter. Arrows indicate the specific DNA-protein complexes. The data are representative of three independent experiments from different donors.

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Fig. 5. The effect of Δ^9 -THC on the activity GSK3 β in activated human CD4 $^+$ T cells PBMC (1×10^6 cells/100 μ L) were equilibrated at 37°C in 5% CO $_2$ for 3 h, treated with Δ^9 -THC (1, 5, 10, and 15 μ M) and/or VH for 30 min, and then incubated with anti-CD3, anti-CD28, and IgG crosslinker on ice. T cell activation was initiated by transferring to 37°C water bath for 5 min. Cells were fixed with 1.5% paraformaldehyde and permeabilized with ice-cold 100% methanol, followed by staining with anti-CD4 and anti-pGSK3 β . The intracellular expression of pGSK3 β in CD4 $^+$ T cells was assessed by flow cytometry. Results depicted are MFI of pGSK3 β gated on CD4 $^+$ T cells. Data were normalized to the VH-treated group and presented as percent of control. The mean $\pm$ SEM between human subjects from VH group was 9.9 ± 0.5 . Each symbol represents data from one individual donor.

Fig. 6. Δ^9 -THC suppressed TCR-induced elevation of intracellular Ca^{2+} in activated human CD4^+ T cells

Naïve CD4^+ T cells (1×10^6 cells/mL) were incubated with the indicator dyes Fura-3 and Fluo-red for 60 min. Cells were treated with Δ^9 -THC (1, 5, 10, and 15 μM) and/or VH for 30 min before incubating with anti-CD3 and anti-CD28. Intracellular Ca^{2+} mobilization was observed by flow cytometry. After 60 sec of data acquisition, TCR activation was initiated by adding IgG crosslinker followed by measuring the intracellular Ca^{2+} mobilization for the total of 300 sec. Kinetic plots are expressed as median of the FL1:FL3 ratio. Results represent two separate experiments. Data are presented in arbitrary units as a function of fluorescence (relative intracellular Ca^{2+}) versus time.

Fig. 7. The effect of Δ^9 -THC on the activation of PLC γ in activated human CD4 $^+$ T cells PBMC (1×10^6 cells/100 μ L) were equilibrated at 37°C in 5% CO $_2$ for 3 h, treated with Δ^9 -THC (1, 5, 10, and 15 μ M) and/or VH for 30 min, and then incubated with anti-CD3, anti-CD28, and IgG crosslinker on ice. T cells activation was initiated by transferring to 37°C water bath for 5 min. Cells were fixed with 1.5% paraformaldehyde and permeabilized with ice-cold 100% methanol, followed by staining with anti-CD4 and anti-pPLC γ 2. The intracellular expression of pPLC γ 1/2 in CD4 $^+$ T cells was assessed by flow cytometry. Results depicted are MFI of pPLC γ 1/2 gated on CD4 $^+$ T cells. Data were normalized to the VH-treated group and presented as percent of control. The mean $\pm$ SEM between human subjects from VH group was 193 ± 14.8 . Each symbol represents data from one individual donor.

a

Fig. 8. The effect of Δ^9 -THC on the proximal T cell receptor signaling molecules in activated human CD4⁺ T cells

PBMC (1×10^6 cells/100 μ L) were equilibrated at 37°C in 5% CO₂ for 3 h, treated with Δ^9 -THC (1, 5, 10, and 15 μ M) and/or VH for 30 min, and then incubated with anti-CD3, anti-CD28, and IgG crosslinker on ice. T cells activation was initiated by transferring to 37°C water bath for 5 min. Cells were fixed with 1.5% paraformaldehyde and permeabilized with ice-cold 100% methanol, followed by staining with anti-CD4, anti-pZAP70, and anti-pAKT. The intracellular expression of pZAP70 or pAkt in CD4⁺ T cells was assessed by flow cytometry. (a) Scatter plot represents the level of pZAP70 in CD4⁺ T cells. Data were normalized to the VH-treated group and presented as percent of control. The mean $\pm$ SEM between human subjects from VH group was 3.2 ± 0.5 . The coefficient of variance between

human subjects from each treatment group were: NA (13.7%), anti-CD3/CD28 (16.9%), VH (0%), 1 μ M (12.3%), 5 μ M (34.9%), 10 μ M (26.3%), and 15 μ M (36.7%). **(b)** Scatter plot represents the level of pAkt. Results depicted are MFI of pZAP70 or pAkt gated on CD4⁺ T cells. Data were normalized to the VH-treated group and presented as percent of control. The mean $\pm$ SEM between human subjects from VH group was 2.0 ± 0.2 . Each symbol represents data from one individual donor.

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