Suppression of T cell costimulator ICOS by Δ^9-tetrahydrocannabinol

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Abstract: Inducible costimulator (ICOS), a prototypic T cell costimulator, is induced on activated T cells. ICOS regulates T cell activation and Th cell differentiation and is principally involved in humoral immune responses. Previous work showed that T cell accessory function is modulated by the plant-derived cannabinoid, delta-9-tetrahydrocannabinol (Δ^9-THC). In light of an emerging role by ICOS in T cell-mediated immunity, the objective of this study was to investigate the effect of Δ^9-THC on ICOS in activated mouse T cells. Induction of ICOS mRNA levels by phorbol ester (PMA) plus ionomycin (Io) activation in mouse splenocytes was attenuated by Δ^9-THC in a concentration-related manner. Similar results were obtained in the mouse T cell line, EL4.IL-2. Anti-CD3/CD28 induced ICOS expression on $CD4^+$ splenic T cells, which was suppressed by Δ^9-THC in a time- and concentration-related manner. The PMA/Io-induced *icos* promoter luciferase reporter activity was also down-regulated by Δ^9-THC, suggesting that the suppression of ICOS expression by Δ^9-THC occurs at the transcriptional level. Moreover, transcriptional activation of the NFAT was also down-regulated by Δ^9-THC as shown by a NFAT luciferase reporter assay, which is consistent with a putative role of NFAT in regulating ICOS expression. Collectively, Δ^9-THC suppresses ICOS expression in activated T cells, and this suppression may be related, in part, to its modulation of NFAT signaling. The emerging role of ICOS in a wide range of immune-related diseases also suggests that it may represent a potential therapeutic target, which could be modulated by cannabinoid compounds. *J. Leukoc. Biol.* 85: 322–329; 2009.

Key Words: *cannabinoid · splenocyte · NFAT · immunomodulation*

INTRODUCTION

It is well established that cannabinoid compounds modulate the immune system with T cells identified as sensitive cellular targets [1]. Among more than 60 plant-derived cannabinoid compounds, delta-9-tetrahydrocannabinol (Δ^9-THC) is the prototypic cannabinoid compound and the primary psychoactive component in marijuana. Previous investigations from this laboratory have demonstrated that Δ^9-THC modulates T cell-associated immune responses including those in which the T cell functions as an accessory cell. For example, humoral immune responses to T cell-dependent antigens such as sheep erythrocytes (sRBC) are markedly suppressed by Δ^9-THC [2]. The specific reason precluding productive T cell accessory function in the presence of Δ^9-THC has been elusive.

Within the last decade, the critical role of the T cell costimulatory system has been emerging [3, 4]. It is now well established that signals through a family of T cell costimulators are required for optimal T cell activation. Inducible costimulator (ICOS) is a T cell costimulatory molecule that binds to the B7 family member, B7-related protein-1 (or ICOS ligand), which is widely expressed on APCs as well as nonlymphoid cells [5]. ICOS expression is minimal on resting T cells but up-regulated upon T cell activation [6]. ICOS plays a crucial role in Th cell differentiation by regulating the production of IL-4, IL-5, IL-10, and IFN-γ and may particularly promote Th2 cell development [7–12]. Blockade of ICOS was found to impair Ig class-switching and germinal center formation, suggesting an indispensable role of ICOS in T cell-dependent B cell responses [11, 13, 14]. The major role of ICOS in T cell costimulation is illustrated by the association found between ICOS deficiency and common variable immunodeficiency, which is demonstrated by impaired T-dependent humoral immune responses [15, 16].

The modulation of T cell activation as well as its accessory and effector function by cannabinoids has been confirmed in vitro and in vivo. Immunomodulatory cannabinoids impair T cell activation, as evidenced by suppression of IL-2 production [17, 18]. The mechanism for IL-2 suppression by Δ^9-THC and cannabinol was found to be associated, at least in part, with altered transcriptional activity of the NFAT and AP-1, as well as the disruption of intracellular calcium regulation in resting T cells [19–22]. In *Legionella pneumophila* challenge animal models, Δ^9-THC was found to suppress the Th1-polarizing function by targeting dendritic cells [23, 24]. Morever, Δ^9-THC suppressed cytolytic function of mouse cytotoxic T cells in vitro

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and in vivo [25]. In a mouse model for host resistance against influenza virus, Δ^9-THC reduced the magnitude of inflammation but increased the viral load, partially through a decrease in recruitment of macrophage and T cells to the lung [26]. In allergic airway responses, Δ^9-THC and cannabinol were found to attenuate the induction of IL-2, IL-4, IL-5, and IL-13 expression as well as serum IgE levels in A/J mice challenged with OVA [27].

Although cannabinoid compounds are well known to produce a broad range of effects on T cell function, to date, the effect of cannabinoid compounds on the T cell costimulatory molecules has not been investigated. Thus, the objective of the present study was to characterize the effects of prototypic cannabinoid compound Δ^9-THC on ICOS. In particular, we demonstrate for the first time that Δ^9-THC suppressed ICOS mRNA levels and cell-surface expression in activated mouse splenocytes and T cells, which appears to occur through a decrease in ICOS transcription, as demonstrated by suppressed *icos* promoter activity. We also provide direct evidence for Δ^9-THC-mediated suppression of NFAT transcriptional activity, which may be involved in the suppression of ICOS.

MATERIALS AND METHODS

Δ^9-THC

The National Institute on Drug Abuse (Bethesda, MD, USA) provided Δ^9-THC.

Animals and cell cultures

Virus-free, female C57BL/6 mice (6 weeks of age) were purchased from Charles River (Portage, MI, USA). Mice were randomized, transferred to plastic cages containing sawdust bedding (five mice per cage), and quarantined for 1 week. Mice were given food (Purina certified laboratory chow) and water ad libitum and were not used for experimentation until their body weight was 17–20 g. Animal holding rooms were kept at 21–24°C and 40–60% humidity with a 12-h light/dark cycle. Mice were used in accordance with guidelines set forth by the Michigan State University Institutional Animal Care and Use Committee (East Lansing, MI, USA). Spleens were isolated and made into single-cell suspensions aseptically. The splenocytes were depleted of erythrocytes by incubation with ammonium phosphate lysis buffer (10 μM EDTA, 10 mM $KHCO_3$, and 150 mM NH_4Cl). The EL4.IL-2 T cell line was obtained from American Type Culture Collection (Manassas, VA, USA). EL4.IL-2 cells were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% bovine calf serum (BCS; HyClone, Logan, UT, USA), 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 100 mM nonessential amino acids (Invitrogen), and 1 mM sodium pyruvate (Invitrogen). In all cases, cells were cultured at 37°C in 5% CO_2 unless otherwise noted.

Lymphocyte activation

Splenocytes were cultured in triplicate (1×10^6 c/ml) in RPMI supplemented with 2% BCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. Splenocytes were activated with 40 nM PMA and 0.5 μM ionomycin (Io; Sigma Chemical Co., St. Louis, MO, USA) or 1 μg/ml immobilized anti-mouse CD3 and 4 μg/ml soluble anti-mouse CD28 antibodies (BD PharMingen, San Diego, CA, USA). For treatment with sRBC (Colorado Serum, Denver, CO, USA), splenocytes were cultured in triplicate (1×10^7 c/ml) in RPMI supplemented with 10% BCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. sRBC were added to the culture at 6.5×10^6 sRBC per 5×10^6 splenocytes. EL4.IL-2 cells were cultured in triplicate (5×10^6 c/ml) in culture medium as described for splenocytes and activated with PMA and Io (40 nM and 0.5 μM). Splenocytes and EL4.IL-2 cells were pretreated with Δ^9-THC and/or vehicle (VH; 0.1% ethanol) 30 min prior to activation.

In vitro T cell-dependent antibody response

After sRBC treatment, splenocytes were cultured for 24 h or 3 or 5 days in a Bellco stainless tissue-culture chamber pressurized to 5.5 pounds per square inch with a gas mixture containing 10% O_2, 7% CO_2, and 83% N_2. The culture chamber was incubated at 37°C and rocked continuously for the duration of the culture period.

Real-time PCR

Total RNA from splenocytes was isolated using Tri Reagent (Sigma Chemical Co.) or from EL4.IL-2 cells using the SV Total RNA isolation system, according to the manufacturers' protocols (Promega, Madison, WI, USA). Known amounts of total RNA were reverse-transcribed into cDNA using the High-Capacity cDNA RT kit (Applied Biosystems, Foster City, CA, USA). TaqMan primers for ICOS (Mm00497600_m1) were purchased from Applied Biosystems. The relative steady-state levels of ICOS were determined by TaqMan real-time multiplex PCR using the ABI PRISM^R 7900HT sequence detection system (Applied Biosystems). Relative steady-state ICOS mRNA levels were calculated as described previously and normalized to the endogenous reference, 18S rRNA [28].

Cell viability determinations

Splenocyte and EL4.IL-2 T cell viability was determined routinely in parallel with measurements of effector responses. Specifically, for in vitro T cell-dependent antibody response experiments, cell viability was determined at the end of culture by the pronase activity assay as described previously [2]. In all of the other experiments, cell viability was determined by trypan blue exclusion.

Flow cytometry (FCM) analysis

Splenocytes were resuspended in FCM staining buffer: 1× HBSS (Invitrogen) containing 1% BSA (Calbiochem, La Jolla, CA, USA) and 0.1% sodium azide (Sigma Chemical Co.), pH 7.6. Splenocytes were incubated with 2.4G2 antibody specific for FcγRII/III (BD PharMingen) for 15 min to prevent nonspecific binding. Cells were then incubated with PE-conjugated rat anti-mouse ICOS and FITC-conjugated rat anti-mouse CD4 mAb, as well as their isotype control antibodies (BD PharMingen) for 30 min. In all cases, antibodies were added at 0.5 μg/10^6 cells, which were washed with FCM staining buffer and incubated in BD Cytofix^{TM} buffer (BD PharMingen) for 15 min. Cells were kept on ice in all steps. Cells were resuspended in FCM staining buffer and assessed using the BD FACSCalibur^{TM} system (BD Biosciences, San Jose, CA, USA). Data were acquired and analyzed using CellQuest Pro^{TM} (BD Biosciences).

Plasmids

ICOS luciferase reporter gene plasmids were generous gifts from Dr. Kong-Peng Lam's laboratory (Agency for Science, Technology, and Research, Singapore). The construction of these plasmids was described previously [29]. Briefly, the mouse *icos* gene promoter, spanning from −1478 to −1 relative to the first nucleotide of the mouse cDNA, was amplified from C57BL/6 mouse genomic cDNA and cloned into the promoterless luciferase plasmid vector pGL3-Basic (pGL3B, Promega) to create the ICOS-1478. A 5′ deletion mutant of this promoter fragment was generated after restriction enzyme digestion and religated into pGL3B to create ICOS-288. The pGL3B plasmid served as a vector control for ICOS-288 and ICOS-1478 plasmids. Plasmid NFAT-luciferase (pNFAT-luc) and pTA-luc reporter gene plasmids were purchased from Clontech (Mountain View, CA, USA). The pTA-luc plasmid served as a vector control and is identical to pNFAT-luc except for the absence of the NFAT response elements.

Transient transfection assays

EL4.IL-2 cells were incubated with transfection reagents [1 μg plasmid and 3 μl Lipofectamine 2000 (Invitrogen) per 1×10^6 cells] for 4 h in RPMI-1640 medium supplemented with 2% BCS. The transfected EL4.IL-2 cells were pretreated with Δ^9-THC and then activated with PMA/Io as described above. Twelve hours after PMA/Io activation, luciferase activity was assayed using the Promega luciferase assay system according to the manufacturer's protocol (Promega). Fluorescence was measured using the BioTek Synergy^{TM} HT au-

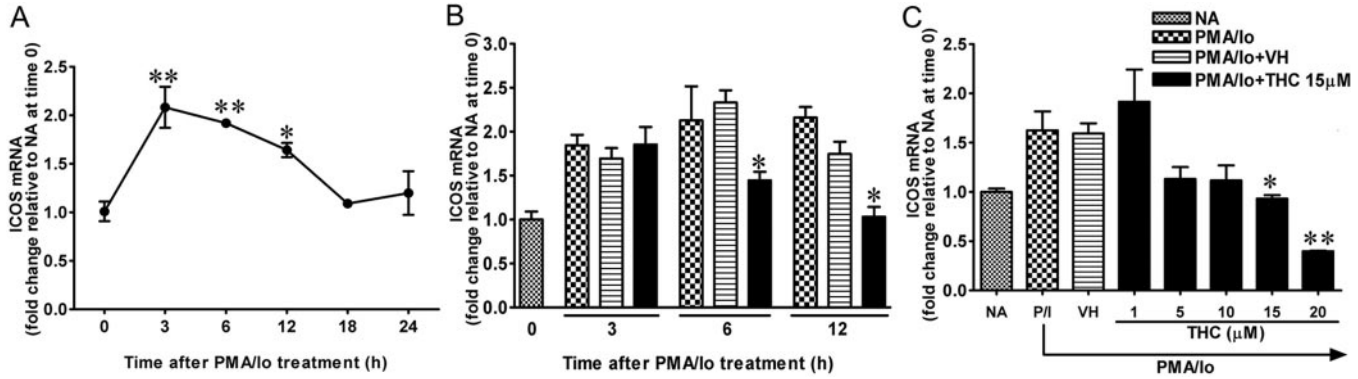

Fig. 1. Effect of Δ^9-THC on ICOS mRNA levels in PMA/Io-activated primary splenocytes. (A) Primary splenocytes (5×10^6 cells/ml) were treated with PMA (40 nM) and Io (0.5 μM) for 3, 6, 12, 18, or 24 h. (B) Pretreated with Δ^9-THC (15 μM) and/or VH (0.1% ethanol) for 30 min and then treated with PMA (40 nM) and Io (0.5 μM) for 3, 6, or 12 h. (C) Pretreated with Δ^9-THC at indicated concentrations and/or VH (0.1% ethanol) for 30 min and then treated with PMA (40 nM) and Io (P/I; 0.5 μM) for 12 h. Total RNA was isolated, and steady-state ICOS mRNA levels were determined by real-time PCR analysis. Cellular viability was ≥85% for all treatment groups as assessed by trypan blue dye exclusion. Data are presented as fold change relative to the nontreatment (NA) group at Time 0. The results are the mean ± SE, as determined for each group. (A) *, $P < 0.05$; **, $P < 0.01$, compared with the nontreatment group at Time 0. (B) *, $P < 0.05$, compared with the VH + PMA/Io group at the same time-point. (C) *, $P < 0.05$; **, $P < 0.01$, compared with the VH + PMA/Io group. These data are representative of two separate experiments with three replicates per treatment group.

toreader, and data were analyzed using KC4 software (BioTek Instruments, Highland Park, VT, USA).

ELISpot assay

ELISpot assay was performed using MultiScreen^{TM}-HA filter plates (Millpore, Billerica, MA, USA), which were precoated with goat anti-mouse IgM antibody (Sigma Chemical Co.) at 10 μg/ml and preblocked with PBS containing 5% BSA. Splenocytes were washed and diluted in RPMI supplemented with 10% BCS and then loaded into the wells. Typically, 2×10^3–3×10^3 cells in 50 μl cell culture medium were incubated on precoated plates overnight at 37°C. Biotin-conjugated goat anti-mouse IgM antibody (Sigma Chemical Co.) was added to plates at 1 μg/ml and incubated for 2 h at room temperature. To develop spots, streptavidin-HRP conjugate (Sigma Chemical Co.) was used, followed by the aminoethylcarbazole staining kit reaction (Sigma Chemical Co.). Data were acquired and analyzed using the CTL ImmunoSpot^{TM} system (Cellular Technology Ltd., Shaker Heights, OH, USA).

Statistical analysis

The mean ± SE was determined for each treatment group in the individual experiments. Homogeneous data were evaluated by one-way ANOVA, and Dunnett's post-hoc test was used to compare treatment groups with the VH control when significant differences were observed. Two-way ANOVA was used for data evaluation in temporal studies, and Bonferroni's post-hoc test was used to compare treatment groups with the VH control.

RESULTS

Suppression by Δ^9-THC of ICOS mRNA levels in primary splenocytes

To address whether Δ^9-THC modulates ICOS mRNA levels in activated splenocytes, we first characterized the kinetics of ICOS induction as measured by ICOS steady-state mRNA levels at different times within the first 24 h postactivation by PMA/Io treatment. As presented in **Figure 1A**, ICOS mRNA levels were transiently up-regulated in splenocytes, and the peak levels of expression occurred between 3 h and 12 h after PMA/Io treatment. Pretreatment of splenocytes with Δ^9-THC (15 μM) 30 min before PMA/Io attenuated the up-regulation of ICOS mRNA levels at 6 h and 12 h (Fig. 1B). The suppression by Δ^9-THC was not observed at 3 h after PMA/Io treatment, suggesting that the effect of Δ^9-THC on ICOS mRNA levels in splenocytes is time-dependent. In addition, at 12 h after PMA/Io treatment, Δ^9-THC suppressed ICOS mRNA levels in a concentration-related manner (Fig. 1c). Δ^9-THC at 15 and 20 μM caused a significant decrease in ICOS mRNA levels when compared with the VH-treated group.

Suppression by Δ^9-THC of ICOS mRNA levels in EL4.IL-2 T cells

Although it is well established that T cells are the major cell type that express ICOS [6], we investigated whether the effect

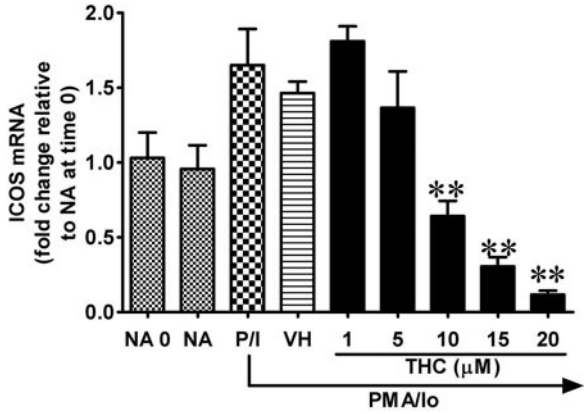

Fig. 2. Effect of Δ^9-THC on ICOS mRNA levels in PMA/Io-activated EL4.IL-2 T cells. EL4.IL-2 cells (5×10^5 cells/ml) were pretreated with Δ^9-THC at indicated concentrations and/or VH (0.1% ethanol) for 30 min and then treated with PMA (40 nM) and Io (0.5 μM) for 12 h. Total RNA was isolated, and steady-state ICOS mRNA levels were determined by real-time PCR analysis. Cellular viability was ≥90% for all treatment groups as assessed by trypan blue exclusion. Data are presented as fold change relative to the nontreatment group at Time 0. The results are the mean ± SE as determined for each group. **, $P < 0.01$, compared with the VH + PMA/Io group. These data are representative of three separate experiments with three replicates per treatment group.

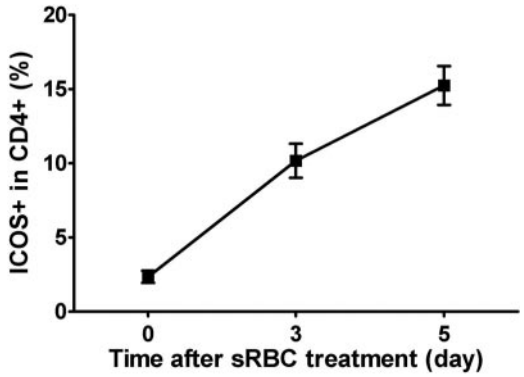

Fig. 3. ICOS cell-surface expression in Th cells activated with sRBC. Splenocytes (1×10^7 cells/ml) were treated with sRBC (6.5×10^6 cells) for 3 days or 5 days. Cells were stained with fluorescence-conjugated anti-CD4 or anti-ICOS antibodies and assessed by FCM for cell-surface expression of CD4 and ICOS. $CD4^+$ cells (Th cells) were analyzed, and ICOS cell-surface expression in this subpopulation of cells was measured. Data are presented as the percentage of $ICOS^+$ cells within the total Th cell pool. The results are the mean ± SE as determined for each group. These data are representative of two separate experiments with four replicates per treatment group.

of Δ^9-THC on ICOS could be demonstrated in a homogenous T cell preparation. Toward this end, EL4.IL-2 cells were pretreated with Δ^9-THC at different concentrations, and ICOS steady-state mRNA levels were measured after stimulation with PMA/Io. As illustrated in **Figure 2**, Δ^9-THC treatment suppressed ICOS mRNA levels in PMA/Io-activated EL4.IL-2 cells in a concentration-related manner.

Suppression by Δ^9-THC of the primary antibody response but not ICOS cell-surface expression induced by a T cell-dependent antigen

ICOS is crucial to T cell-dependent humoral immune responses [11], and consistent with this premise, the T cell-dependent antigen sRBC was able to induce ICOS expression on $CD4^+$ cells (Th cells). Flow cytometric analysis showed that on Days 3 and 5 after sRBC stimulation, there was a two- to three-fold increase in the number of $ICOS^+$ Th cells when compared with the freshly isolated, naive cells on Day 0 (**Fig. 3**). We further demonstrated by ELISpot that on Day 5, Δ^9-THC suppressed IgM antibody-secreting cell (ASC) formation induced by sRBC stimulation (**Fig. 5B**), confirming a previous observation from this laboratory that sRBC-induced, T cell-dependent antibody responses are suppressed by Δ^9-THC, as assessed by the plaque-forming cell assay [2]. However, when the surface expression of ICOS was assessed on splenocytes in the same experimental setting, the effect of Δ^9-THC on ICOS surface expression (Fig. 4A) on Days 3 and 5 was modest compared with the magnitude of suppression of IgM ASC formation by Δ^9-THC (Fig. 4B). The modulation of ICOS surface expression by Δ^9-THC, 24 h after sRBC stimulation, was similar to Days 3 and 5 (data not shown).

Attenuation by Δ^9-THC of ICOS cell-surface expression induced by anti-CD3/CD28

Above, we have shown that Δ^9-THC suppressed ICOS mRNA expression induced by PMA/Io, a T cell stimulus that bypasses TCR signaling, yet Δ^9-THC only modestly modulated ICOS cell-surface expression induced by sRBC, arguably a more physiologically relevant activation stimulus. The modest modulation of ICOS by Δ^9-THC is most likely a result of a small number of splenic T cells that were capable of recognizing antigenic epitopes on sRBC. To activate T cells in a physiologically relevant manner and activate a large proportion of the T cell pool, we investigated the effect of Δ^9-THC on ICOS cell-surface expression induced by anti-CD3/CD28, a polyclonal T cell activation stimulus. Freshly isolated, naive splenocytes were treated with anti-CD3/CD28 and assayed by FCM for ICOS expression. Anti-CD3/CD28-induced expression of ICOS on Th cells was observed as early as 6 h and

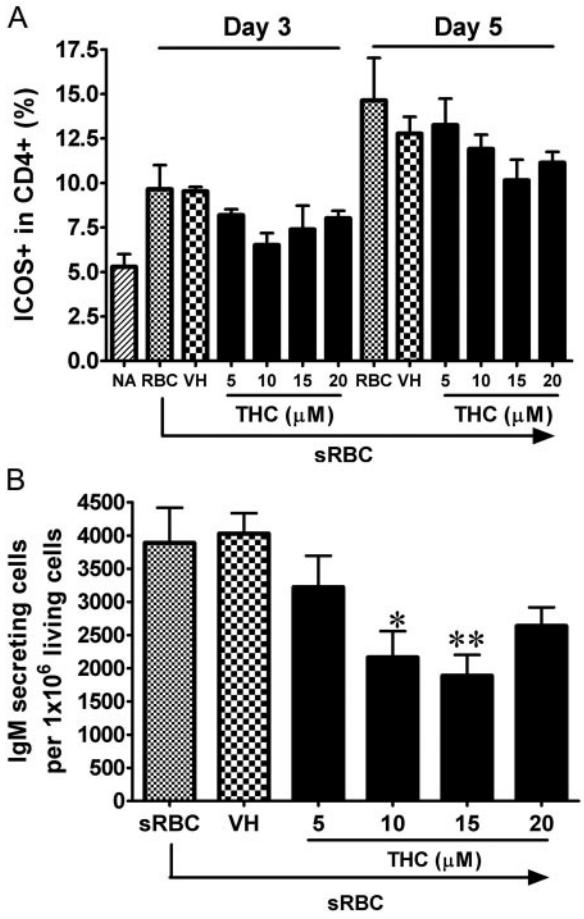

Fig. 4. Effect of Δ^9-THC on primary splenocytes activated with sRBC. Splenocytes (1×10^7 cells/ml) were pretreated with Δ^9-THC (at indicated concentrations) and/or VH (0.1% ethanol) for 30 min and then treated with sRBC (RBC; 6.5×10^6 cells) for 3 days or 5 days. (A) Cells were stained with fluorescence-conjugated anti-CD4 or anti-ICOS antibodies and assessed by FCM for cell-surface expression of CD4 and ICOS. $CD4^+$ cells (Th cells) were analyzed, and ICOS cell-surface expression in this subpopulation of cells was measured. Data are presented as the percentage of $ICOS^+$ cells within the total Th cell pool. The results are the mean ± SE as determined for each group. These data are representative of two separate experiments with three replicates per treatment group. (B) On Day 5, IgM-secreting cells in total cells were enumerated by ELISpot analysis. The cell viability was determined by pronase viability assay. Data are presented as the number of IgM-secreting cells per 1×10^6 living cells. The results are the mean ± SE as determined for each group. *, $P < 0.05$; **, $P < 0.01$, compared with the VH + sRBC group. These data are representative of three separate experiments with three replicates per treatment group.

peaked between 24 h and 48 h, consistent with previous observations (Fig. 5) [12, 29]. Pretreatment with a single concentration of Δ^9-THC (15 μM) appeared to show a trend toward a decrease in the percentage of $ICOS^+$ Th cells in the splenocyte preparation activated with anti-CD3/CD28 at 24 h and 48 h (**Fig. 6**). Using multiple concentrations of Δ^9-THC, we examined more rigorously the effect of Δ^9-THC on ICOS-expressing $CD4^+$ T cells at 24 h and 48 h. Δ^9-THC treatment decreased the percentage of the $CD4^+$ Th-expressing ICOS as well as the average ICOS cell-surface expression, as indicated by a decrease in mean fluorescent intensity (**Table 1**). Collectively, Δ^9-THC attenuated ICOS cell-surface expression in a concentration-related manner, and the attenuation was predominantly specific to the $CD4^+$ Th cell population.

Suppression by Δ^9-THC of *icos* promoter activity in EL4.IL-2 T cells

To further examine the underlying mechanism responsible for Δ^9-THC-mediated attenuation of ICOS expression, EL4.IL-2 cells were transiently transfected with an *icos* promoter luciferase reporter. ICOS-1478 is the putative, full-length *icos* promoter, and ICOS-288 is the putative minimal essential *icos* promoter. Both reporters have been shown previously to maintain similar levels of response in activated T cell [29]. In Figure 6, we show that PMA/Io induced *icos* promoter luciferase reporter activity of ICOS-288 and ICOS-1478 and had no significant effect on the pGL3B vector control. This induction of ICOS-288 promoter activity was attenuated by Δ^9-THC at concentrations between 5 and 20 μM and ICOS-1478 between 10 and 20 μM.

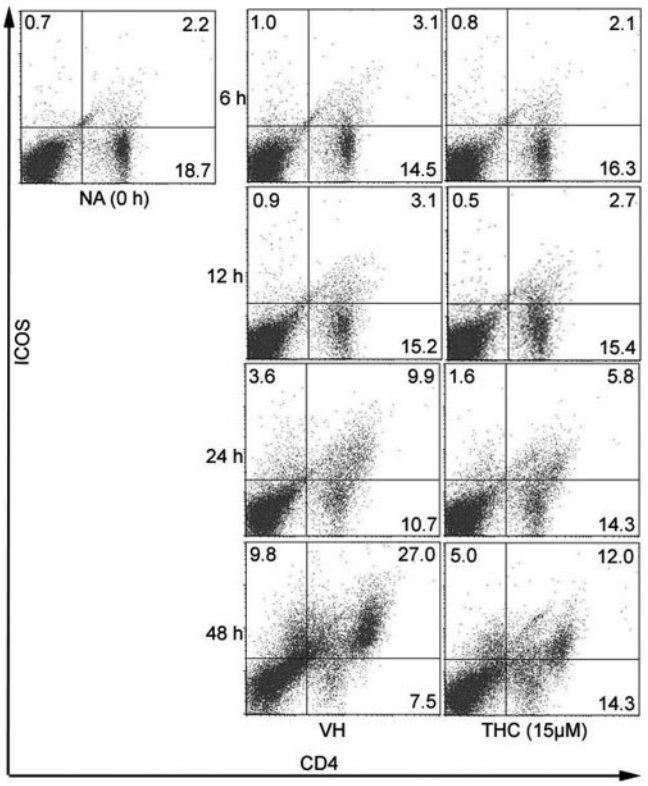

Fig. 5. Effect of Δ^9-THC on ICOS cell-surface expression in primary splenocytes activated with anti-CD3/CD28. Splenocytes (1×10^6 cells/ml) were pretreated with Δ^9-THC (15 μM) and/or VH (0.1% ethanol) for 30 min and then treated with immobilized anti-CD3 (1 μg/ml) and soluble anti-CD28 (4 μg/ml) for 6, 12, 24, and 48 h. Cells were stained with fluorescence-conjugated anti-CD4 or anti-ICOS antibodies and assessed by FCM for cell-surface expression of CD4 and ICOS. These data are presented as dot plots, with CD4 and ICOS as the *x*- and *y*-axes, respectively. The number in the upper-right corner of each dot plot indicates the percentage of $ICOS^+CD4^+$ cells ($ICOS^+$ Th cells) in total cells. These data are representative of two separate experiments, and each dot plot is representative of three replicates per treatment group.

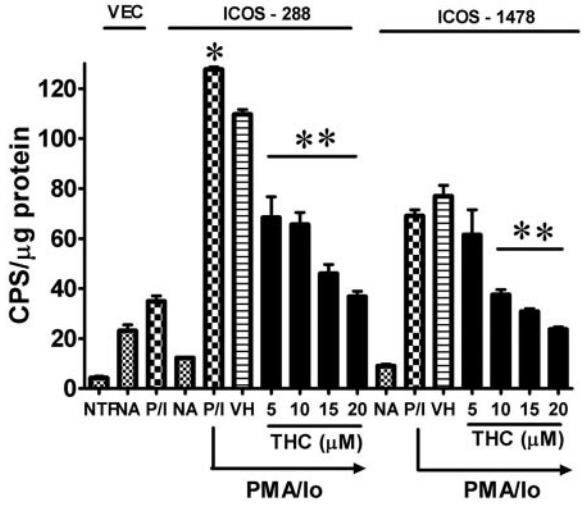

Fig. 6. Effect of Δ^9-THC on *icos* promoter activity in PMA/Io-activated EL4.IL-2 T cells, which were transiently transfected with pGL3B vector control (VEC), ICOS-288, or ICOS-1478 or treated with transfection reagents in the absence of plasmid (NTF). After 5 h of incubation, cells (1×10^6 cells/ml) were pretreated with Δ^9-THC at indicated concentrations and/or VH (0.1% ethanol) for 30 min and then treated with PMA (40 nM) and Io (0.5 μM) for 12 h. Luciferase activity was quantified in count per second (CPS) and normalized to total protein (μg). The results are the mean ± SE as determined for each group. **, $P < 0.01$, compared with the VH + PMA/Io group. These data are representative of two separate experiments with three replicates per treatment group.

Impairment by Δ^9-THC of NFAT transcriptional activity in EL4.IL-2 T cells

Based on the above findings showing that Δ^9-THC alters *icos* transcription, we sought to address which transcription factor might be involved. Recent findings have suggested that the transcription factor, NFAT, plays a crucial role in the regulation of ICOS [29]. Therefore, we investigated the effect of Δ^9-THC on NFAT transcriptional activity. PMA/Io treatment significantly induced NFAT reporter activity in EL4.IL-2 T cells (**Fig. 7**). In contrast, Δ^9-THC treatment, at all concentrations between 5 and 20 μM, attenuated NFAT reporter activity in a concentration-related manner.

DISCUSSION

The objective of the present investigation was to further characterize the effects of Δ^9-THC on T cell function by characterizing the costimulator, ICOS, which contributes to the T cell accessory function by interacting with the ICOS ligand on

TABLE 1. The Concentration-Related Effect of Δ^9-THC on ICOS Cell-Surface Expression in Primary Splenocytes Activated with Anti-CD3/CD28

Time (h)	Treatment	$ICOS^+CD4^+$ % in total preparation	$ICOS^+$% in CD4+ preparation	MFI of ICOS on $CD4^+$ preparation
0	NA	1.887 ± 0.2691	11.65 ± 1.129	3.48 ± 0.6930
24	Anti-CD3/CD28	8.24 ± 0.3587	50.63 ± 1.695	22.10 ± 1.415
	VH	8.22 ± 0.5232	51.23 ± 2.411	22.39 ± 1.911
	Δ^9-THC 5 μM	7.62 ± 0.5399	46.40 ± 1.582	18.65 ± 1.011
	Δ^9-THC 10 μM	6.58 ± 0.4877	41.05 ± 2.175	15.57 ± 1.103
	Δ^9-THC 15 μM	4.56 ± 0.1217[a]	32.72 ± 0.3007[a]	10.70 ± 0.1968
	Δ^9-THC 20 μM	1.69 ± 0.1572[b]	17.02 ± 0.5391[b]	4.313 ± 1.420[b]
48	Anti-CD3/CD28	13.49 ± 1.830	67.62 ± 1.534	44.54 ± 3.078
	VH	16.15 ± 0.1266	70.11 ± 1.147	47.29 ± 1.898
	Δ^9-THC 5 μM	14.53 ± 1.896	59.13 ± 9.771	33.29 ± 9.397[a]
	Δ^9-THC 10 μM	7.36 ± 0.5535[b]	51.27 ± 1.335[a]	22.21 ± 1.372[b]
	Δ^9-THC 15 μM	4.12 ± 0.3700[b]	41.63 ± 7.653[b]	16.16 ± 5.782[b]
	Δ^9-THC 20 μM	0.70 ± 0.06658[b]	16.56 ± 0.7881[b]	3.853 ± 0.2345[b]

Splenocytes (1×10^6 cells/ml) were pretreated with Δ^9-THC at indicated concentrations and/or VH (0.1% ethanol) for 30 min, and then treated with immobilized CD3 (1 μg/ml) and soluble CD28 (4 μg/ml) for 24 or 48 h. Cells were stained with fluorescence-conjugated anti-CD4 or anti-ICOS antibodies and assessed by flow cytometry for cell surface expression of CD4 and ICOS. The values represent the mean ± SE of three replicates per treatment group. [a]$P < 0.05$; [b]$P < 0.01$ compared with the VH-treated group at the same point, calculated using two-way ANOVA. Percentages were transformed into log scale prior to statistical analysis. MFI, Median fluorescence intensity.

APCs. Studies from this laboratory have demonstrated previously that cannabinoid compounds impair T cell activation, culminating in the suppression of IL-2 production in response to a variety of T cell stimuli including anti-CD3/CD28 as well as phorbol ester plus Io [17, 19, 30]. In the present study, we sought to address whether ICOS is another downstream target affected by Δ^9-THC during T cell activation. In light of the synchronous and measurable level of ICOS induction reported in murine as well as human T cells by PMA/Io treatment, we capitalize on this mechanism of T cell activation in the present investigation [7, 29].

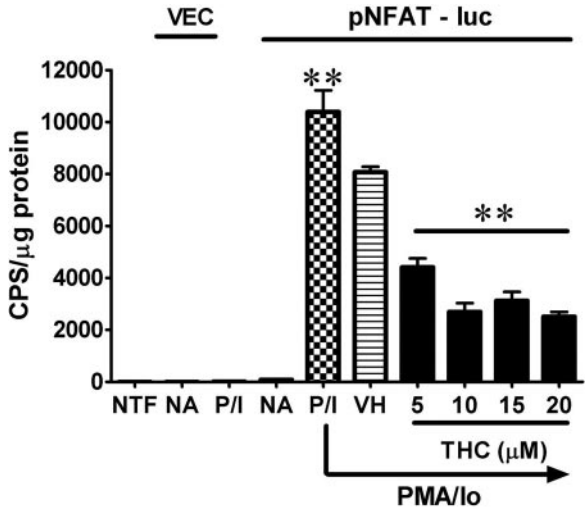

Fig. 7. Effect of Δ^9-THC on NFAT transcriptional activity in PMA/Io-activated EL4.IL-2 T cells, which were transiently transfected with pTA-luc vector control, NFAT-luc, or treated with transfection reagents in the absence of plasmid. After 4 h of incubation, cells (1×10^6 cells/ml) were pretreated with Δ^9-THC at indicated concentrations and/or VH (0.1% ethanol) for 30 min and then treated with PMA (40 nM) and Io (0.5 μM) for 20 h. Luciferase activity was quantified in CPS and normalized to total protein (μg). The results are the mean ± SE as determined for each group. **, $P < 0.01$, compared with the VH + PMA/Io group. These data are representative of two separate experiments with three replicates per treatment group.

Using murine splenocytes and the murine T cell line, EL4.IL-2, we found that PMA/Io transiently up-regulated ICOS mRNA levels. Splenocytes are a mixed population containing ~40% T cells, and EL4.IL-2 T cells were reported previously to have a relatively high basal level of ICOS mRNA [29], explaining the somewhat modest induction levels of ICOS in both cell preparations. Δ^9-THC suppressed ICOS mRNA levels robustly in PMA/Io-activated cells, and the significant, suppressive effect was achieved at lower Δ^9-THC concentrations in EL4.IL-2 T cells when compared with splenocytes. Interestingly, in splenocytes and EL4.IL-2 T cells, Δ^9-THC, at 10–20 μM, decreased the steady-state ICOS mRNA approximately to, or even below, the basal level [(nontreatment, naive) at time 0], suggesting that Δ^9-THC may also have a suppressive effect on the basal background transcription of ICOS.

Using anti-CD3/CD28, which provides a more physiologically relevant activation stimulus than phorbol ester plus Io, we were able to increase the percentage of $ICOS^+CD4^+$ cells in the splenocyte preparation, as well as the percentage of $ICOS^+$ cells within the Th cell population. The peak time of induction for ICOS cell-surface expression was between 40 h and 48 h, and the effect of Δ^9-THC only became significant after 24 h. Although presently unclear, one possible explanation for the delayed effect of Δ^9-THC on ICOS may be that the cannabinoid-mediated effects on immune modulation are dependent on the activation status of T cells. Consistent with this premise are previous findings from our laboratory demonstrating that the same concentration of Δ^9-THC can suppress as well as enhance IL-2 production. Specifically, optimal or supra-optimal activation of T cells with a variety of different stimuli, including anti-CD3/anti-CD28 as well as phorbol ester plus Io, suppressed IL-2 production at concentrations of Δ^9-THC that produced enhancement of IL-2 when T cells were suboptimally activated with the same stimulus [18, 31]. The different mag-

nitude of activation may also account for other effects of Δ^9-THC on T cells that have been observed. Although our findings confirmed that the suppressive effect of Δ^9-THC on ICOS was specific for the Th cell subpopulation, our flow cytometric analysis showed that Δ^9-THC treatment may also diminish the $ICOS^+CD4^-$ subpopulation. Potential targets for the Δ^9-THC-mediated decrease in ICOS may include $CD8^+$ T cells. Anti-CD3/CD28 induced ICOS cell-surface expression in $CD8^+$ T cells [32], and cytotoxic T cell responses were found to be enhanced greatly by ICOS costimulation [33]. It has also been reported that Δ^9-THC attenuated cytotoxic T cell responses [25, 34]. Therefore, it remains to be investigated whether ICOS is, at least in part, involved in the underlying mechanism responsible for Δ^9-THC-mediated suppression of $CD8^+$ T cell responses.

The effect of Δ^9-THC on ICOS cell-surface expression was also examined in splenic Th cells activated with sRBC in vitro. Although sRBC treatment modestly induced ICOS expression in Th cells, the suppression by Δ^9-THC was not statistically significant. In contrast, but consistent with previous results from this laboratory as assessed using the plaque assay [2], Δ^9-THC suppressed the IgM antibody response as assessed using ELISpot. The modest effect of Δ^9-THC on Th cells is likely a result of the fact that only a small fraction of T cells in the spleen is capable of recognizing antigenic epitopes on sRBC, therefore resulting in only a small number of T cells becoming activated. Alternatively, Δ^9-THC may regulate ICOS in a different manner in the context of sRBC stimulation as compared with PMA/Io or anti-CD3/CD28 stimulation, which are considered as more robust activators of T cells, especially as a result of the fact that they are polyclonal activators. Data from previous studies, mainly using ICOS knockout mice, also suggested that ICOS seems to be more critical in regulating Ig isotype switching rather than primary antibody responses [8, 11, 14, 35]. Interestingly, it was also shown by a recent study that the cannabinoid receptor 2 agonist induced Ig class-switching from IgM to IgE in mouse B cells in vitro [36]. These results suggest that ICOS does not play an essential role in the suppression of the T cell-dependent primary antibody response mediated by Δ^9-THC, or ICOS is regulated in a manner that is different in response to PMA/Io or anti-CD3/CD28 stimulation.

To date, only a few studies have been conducted to gain an understanding about how ICOS is regulated at the level of transcription. One recent study proposed a critical role of NFAT cytoplasmic, calcineurin-dependent 2 (NFATc2) in regulating ICOS, showing the binding of NFATc2 to the *icos* minimal promoter [29]. Using *icos* promoter luciferase reporters, we demonstrated that Δ^9-THC also robustly suppressed *icos* promoter luciferase activity induced by PMA/Io in EL4.IL-2 T cells. The effect of Δ^9-THC on *icos* transcription could, at least partially, be explained by suppression of NFAT transcriptional activity, as shown for the first time in the present study. Cannabinol, another cannabinoid compound, was also found to inhibit DNA binding activity of NFAT, leading us to hypothesize that altered regulation of NFAT may be one common mechanism underlying immune modulation by cannabinoid compounds [19, 20]. Nevertheless, the regulation of *icos* transcription seems to extend beyond the minimal essential promoter. Recently, it was reported that NFATc2 cooperated with two other transcription factors, T-bet and GATA-3, to regulate *icos* transcription in differentiated Th cells by binding to the 3′-untranslated region (3′-UTR) of its mRNA [37]. The 3′-UTR may play a critical role in regulating ICOS expression, as it was involved in promoting degradation of ICOS mRNA through a post-transcriptional mechanism [38]. Therefore, although our results suggest a role for NFAT in Δ^9-THC-mediated ICOS suppression, it is unlikely that this accounts for the entire mechanism.

ICOS-mediated T cell costimulation is suggested to play an important role in a number of diseases, including allergy, certain autoimmune diseases, and pulmonary inflammation. The blockade of ICOS attenuated antigen-specific IgE levels, as well as production of cytokines, including IL-4 and IL-13 [9]. Interestingly, in an OVA-induced allergic airway response model, administration of Δ^9-THC or cannabinol attenuated the elevation of serum IgE and IL-4, IL-5, and IL-13 mRNA levels [27]. Moreover, ICOS may also be involved in the regulation of autoimmunity, as demonstrated in different autoimmune disease models [38, 39]. Δ^9-THC was also able to reduce the autoimmune response in an established model of multiple low-dose, streptozotocin-induced autoimmune diabetes [40]. Pulmonary inflammation in response to influenza virus infection was reduced by inhibition of ICOS with a mAb [41]. Similarly, administration of Δ^9-THC also attenuated the magnitude of inflammation in the lung after influenza virus challenge [26].

In conclusion, Δ^9-THC treatment attenuated ICOS promoter activity to decrease ICOS mRNA levels and cell-surface expression in activated T cells, in part, through deregulation of transcription factor NFAT. Future studies will be required to delineate the detailed molecular mechanisms underlying the modulation of ICOS by Δ^9-THC, as well as how it contributes to the immune-modulating effects of cannabinoid compounds in the aforementioned disease processes. Likewise, further delineation will be needed to understand the role of cannabinoid receptors in the cannabinoid-mediated modulation of ICOS.

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