

SUPPRESSION OF THE T-CELL DEPENDENT HUMORAL IMMUNE
RESPONSE BY Δ^9 -TETRAHYDROCANNABINOL INVOLVES IMPAIRMENT
OF CD40-CD40 LIGAND INTERACTION

By

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ABSTRACT

SUPPRESSION OF THE T-CELL DEPENDENT HUMORAL IMMUNE RESPONSE BY Δ^9 -TETRAHYDROCANNABINOL INVOLVES IMPAIRMENT OF CD40-CD40 LIGAND INTERACTION

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Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the main psychoactive congener in marijuana, modulates a variety of immunological responses, of which humoral immune responses against T cell antigens are particularly sensitive to suppression by Δ^9 -THC. Among different types of contact-mediated B cell activation, the CD40-CD40L interaction between B cells and activated CD4⁺ T cells plays an important role in all stages involved in plasma cell differentiation. Thus, the hypothesis tested in this dissertation research is that Δ^9 -THC attenuates the human T cell-dependent IgM antibody response by suppression of CD40L upregulation in activated CD4⁺ T cells and impairment of the CD40-mediated B cell activation. These studies showed that Δ^9 -THC significantly impaired the upregulation of surface CD40L on mouse splenic CD4⁺ T cells activated by anti-CD3/CD28, but not by phorbol ester plus calcium ionophore (PMA/Io). Further, suppression of anti-CD3/CD28-induced CD40L expression in mouse splenic CD4⁺ T cells by Δ^9 -THC likely occurred at the transcriptional level, independently of cannabinoid

receptor (CB) 1, CB2 or the glucocorticoid receptor. Mechanistically, Δ^9 -THC suppressed anti-CD3/CD28-induced DNA binding activity of NFAT and NF κ B, two transcription factors critical is involved in the upregulation of CD40L in activated human CD4⁺ T cells. The inhibitory effect of Δ^9 -THC on the activation of NFAT and NF κ B in primary human CD4⁺ T cells also involved impairment of Ca²⁺ elevation without perturbation of proximal T cell receptor signaling events. Additional findings using an *in vitro* T cell-dependent antibody response model to induce B cell responses showed that Δ^9 -THC significantly decreased the number of IgM secreting cells, which correlated with the impairment of plasma cell differentiation as evidenced by suppression of immunoglobulin joining chain (IgJ) mRNA expression, B cell activation, and proliferation of activated B cells. Moreover, pretreatment with Δ^9 -THC was accompanied by a robust decrease of STAT3 phosphorylation, whereas the phosphorylation of p65 NF κ B subunit was not affected in activated B cells. Collectively, this dissertation research demonstrated that Δ^9 -THC exhibits stimulation- and/or cell type-specific selectivity of NF κ B inhibition, and identified several aspects of the multi-faceted mechanism by which Δ^9 -THC suppresses T cell-dependent humoral immunity in humans. The significance of this work is that it provides novel insights into the mechanism of cannabinoid-mediated suppression of human humoral immunity.

DEDICATION

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LIST OF ABBREVIATIONS

Δ^9 -THC	delta ⁹ -tetrahydrocannabinol
2-AG	2-arachidonyl glycerol
AF	Alexa fluor
Akt	protein kinase B
ANOVA	analysis of variance
AP-1	activator protein 1
APC	allophycocyanin
BCL-6	B-cell lymphoma
Blimp1	B lymphocytes maturation protein 1
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP	cyclic adenosine monophosphate
CB1	cannabinoid receptor1
CB2	cannabinoid receptor 2
CBD	cannabidiol
CBN	cannabinol
CD	cluster of differentiation
CD40L	CD40 ligand
CD40L-L	mouse fibroblast line expressing human CD40L

cDNA	complementary DNA
CFSE	carboxyfluorescein succinimidyl ester
CRAC	calcium release activated calcium channels
CsA	cyclosporin A
DMSO	dimethyl sulfoxide
ELISPOT	enzyme-linked immunospot
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
GSK3 β	glycogen synthase kinase 3 β
HIV	human immunodeficiency virus
IFN γ	interferon gamma
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IgJ	immunoglobulin joining chain
IL	interleukin
IP ₃	1,4,5-inositol triphosphate
JAK	Janus family kinase
JNK	c-Jun N terminal kinase
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinases
MFI	mean fluorescences intensity

mRNA	messenger ribonucleic acid
NA	naïve or unstimulated cells
NFAT	nuclear factor of activated T cells
NF κ B	nuclear factor kappa B
PAX5	paired box protein 5
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PE	phycoerythrin
PI3K	phosphoinositide 3-kinase
PLC γ	phospholipase C γ
PPAR	peroxisome proliferator activated receptors
PRDM1	PR domain zinc finger protein 1
sRBC	sheep erythrocyte
STAT	signal transducer and activator of transcription
TCR	T cell receptor
Th	helper T cell
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor associated factor
TRCP1	transient receptor potential cation channel, subfamily A, member 1
TRPV	transient receptor potential vanilloid
XBP-1	X-box binding protein 1

ZAP70

zeta-chain-associated protein kinase 70

LITERATURE REVIEW

I. T-cell dependent humoral immune response and plasma cell differentiation

A. T-cell dependent humoral immune response

Humoral immunity refers to immune responses that are mediated by secreted antibodies. The main function of humoral immunity is to defend against extracellular microbes, parasites, viruses, and pathogenic foreign macromolecules. In addition, the aberrant production of antibodies is associated with many types of autoimmune diseases and hypersensitivity (reviewed in [1]). Antibodies are produced by plasma cells, which are differentiated B cells. The generation of antibody producing cells requires distinct sequential phases: B cell activation, proliferation, and differentiation. The activation phase is initiated by the binding of antigen to Ig surface receptors of B cells. However, the binding of most protein antigens, which contain only one copy of epitope per molecule, is not sufficient to initiate the response. Such molecules require accessory cells, in particular helper T cell ($CD4^{+}$ T cells), to deliver activating signals. The presented antigen by B cells is recognized by specific T cell receptor (TCR) on the surface of $CD4^{+}$ T cells and thereby activates T cells. Activated $CD4^{+}$ T cells upregulate expression of many co-stimulatory molecules and secrete several cytokines important in development of plasma cells. Co-stimulatory molecules provide the contact-dependent signals requiring for B-cell activation. Cytokines

serve two principle functions in antibody response: 1) by further amplifying signaling cascades involved in B cell proliferation and differentiation and 2) by determining the type of antibodies produced by isotype switching. The activated B cells then undergo clonal expansion, isotype switching, affinity maturation in germinal center (GC) prior differentiate to plasma cells or memory cells. The antibodies that are secreted initially are predominantly of the IgM isotype (reviewed in [2]). IgM is normally secreted in the pentameric form, of which each subunit is linked by immunoglobulin joining chian (IgJ) (Figure 1).

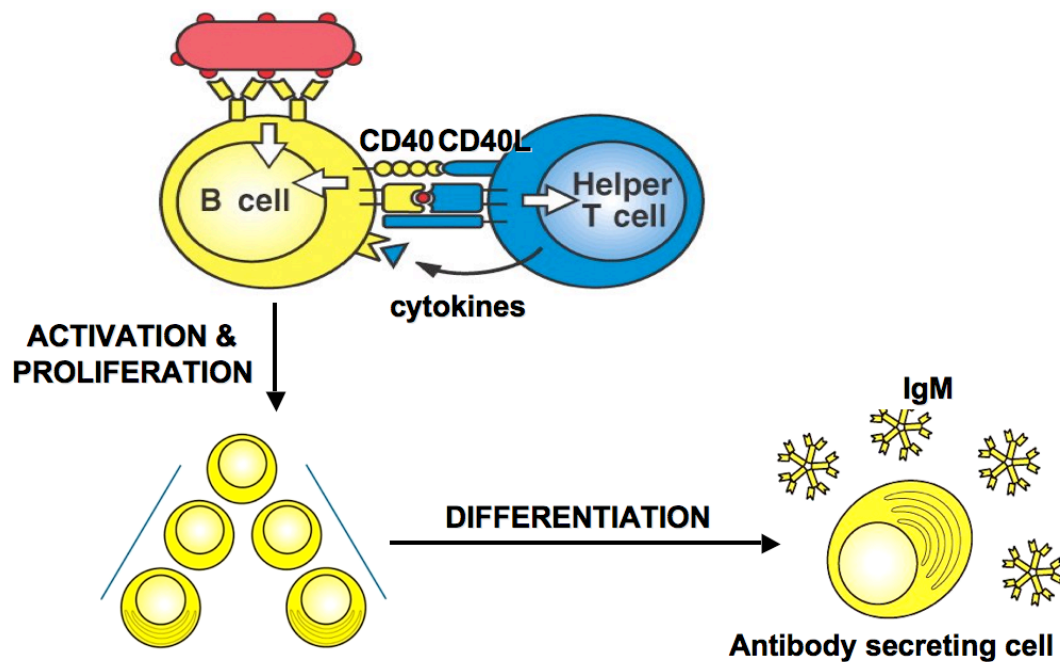


Figure 1. Schematic diagram of the T cell-dependent humoral immune response.

The interaction between B cells presenting processed antigen to specific primed helper T cells allows the primed helper T cell to delivery the activation signals that are involved in their differentiation into plasma cells. “For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.”

B. The genetic network controlling plasma cell differentiation

The differentiation of mature B cells into plasma cells involves a complex regulatory network of transcription factors that are only partially understood. Included in this process are the down-regulation of PAX5 and Bcl-6, critical transcription factors in maintaining B cell identity and the up-regulation of IRF4, Blimp1, and XBP1, crucial transcription factors in promoting plasma cell fate (reviewed in [3]). PAX5 is essential for B cell commitment and development as its expression was found only in committed B cells beginning at the pro-B cells stage (reviewed in [4]). For instance, PAX5 controls B cell characteristics through the induction of the genes encoding for B cell receptor and co-receptor such as IgM heavy chain, CD19, CD21 [5,6], and, at the same time, repression of the genes involved in plasmacytic differentiation such as *PRDM1* encoding for Blimp1 and *IGJ* encoding for IgJ [7]. Bcl-6 is highly expressed in GC B cells [8]. The function of Bcl-6 is to prevent premature differentiation of plasma cells by facilitating proliferation and allowing somatic hypermutation [9]. Further, both PAX5 and Bcl-6 prevent plasma cell differentiation by repressing the expression of Blimp1, often referred as the master regulator of B cell plasmacytic differentiation (reviewed in [10]). Blimp1 can drive plasma cell differentiation by directly repressing the expression of genes important for B cell receptor signaling, germinal cell function, and proliferation, whereas allowing the expression of XBP1 [11]. XBP1 is also necessary for the effective plasma cell formation [12]; however, the underlying mechanism remains to be resolved. As

XBP1 plays an important role in unfolded protein response [13], it seems to regulate chaperones involved in handling load of the increased Ig synthesis (reviewed in [3]). IRF4 was demonstrated to induced Blimp1 expression [14]. Taken together, the mutually exclusive transcriptomes of B cells and plasma cells are maintained by the antagonistic influences of two groups of transcription factors, those that maintain the B cell program and those that promote and facilitate plasma cell differentiation (Figure 2).

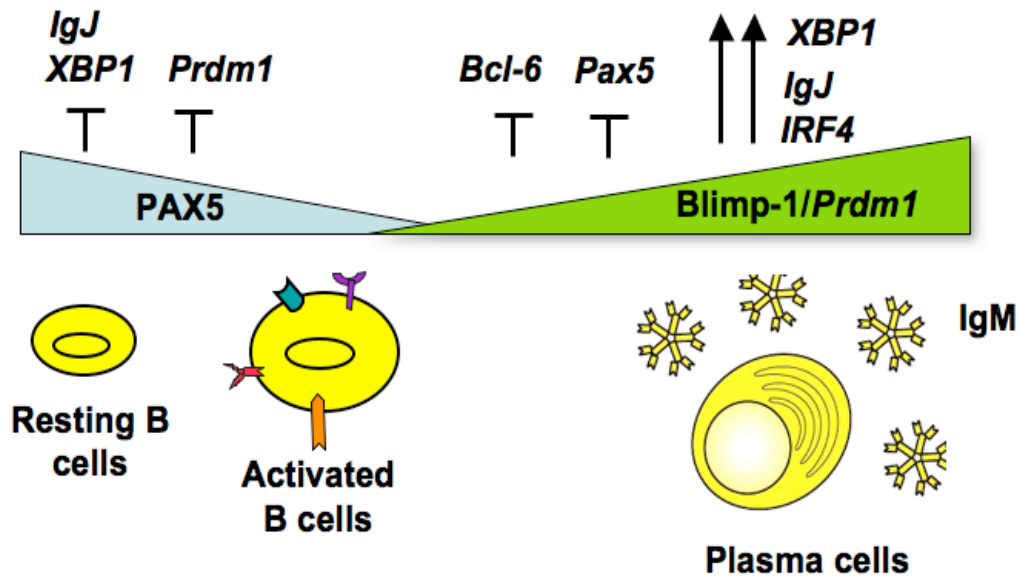


Figure 2. Schematic diagram of the cellular stages and genetic network involved in plasma cell differentiation.

The stages of cellular differentiation from a resting B cell to an antibody-secreting plasma cell are indicated, as are the relative levels of PAX5, Blimp-1 and key target genes. The pre-plasmablast stage (resting and activated B cells) is characterized by high PAX5, but low Blimp1, XBP1, and IgJ expression. Plasmablasts have intermediate Blimp1 expression, proliferate rapidly and secrete antibody. Plasma cells that secrete large quantities of antibody have high Blimp1, XBP1, and IgJ, but low PAX5 and Bcl-6. Positive influence on gene expression is indicated by (→) and repressive activity by (⊥).

II. Signaling cascades in T cells

A. T cell receptor signaling

T cell activation requires two independent signals; the first is antigen-specific and mediated through TCR, and the second is from costimulatory receptors, particularly CD28. Engagement of TCR along with co-stimulation through the co-receptor, CD28, leads to the activation of non-receptor protein tyrosine kinases (PTKs) of which Lck and Fyn are responsible for the phosphorylation of immunoreceptor-based tyrosine activation motifs (ITAMs) in the cytoplasmic domain of CD3 in the TCR complex (reviewed in [15]). Further, 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) [16]. Phosphorylated ITAMs then serve as docking sites promoting the recruitment and the activation of many adapter proteins, including Zeta-chain-associated protein kinase 70 (ZAP70), another member of PTKs. Activation of ZAP70 by phosphorylation (pZAP70) increases both tyrosine phosphorylation and thereby catalytic activity of phospholipase C- γ (PLC γ) [17]. Phosphorylated PLC γ (pPLC γ) then promotes the generation of two secondary messengers, inositol triphosphate (IP₃) and diacylglycerol (DAG) by hydrolysis of phosphatidylinositol 4,5-bisphosphate in the plasma membrane. IP₃ increases intracellular calcium concentration ($[Ca^{2+}]_i$), which is mainly responsible for activation of NFAT, whereas DAG activates protein kinase C-

theta (PKC θ), which is mainly responsible for activation of NF κ B (reviewed in [18]).

B. Ca²⁺ signaling in T cells

Ca²⁺ is essential for optimal T cell activation and is regulated by two mechanisms (reviewed in [19]). The initial increase in [Ca²⁺]_i occurs in response to stimulation of TCR is mediated by release of Ca²⁺ from intracellular stores such as the endoplasmic reticulum (ER) and mitochondria. The ER is the primary source of this initial increase in [Ca²⁺]_i in T cells (reviewed in [20]). Although several intracellular messengers (IP₃, cyclic ADP ribose, and nicotinic acid adenine dinucleotide phosphate) have been implicated in Ca²⁺ release from ER in T cells, IP₃ which binds to the IP₃ receptor, is the main mechanism and has been extensively characterized (reviewed in [20]). This transient elevation of [Ca²⁺]_i is necessary, but not sufficient for optimal T cell activation. Depletion of ER Ca²⁺ stores evokes a sustained increase in [Ca²⁺]_i through the activation of Ca²⁺ release activated Ca²⁺ (CRAC) channels located on the plasma membrane (reviewed in [21]). CRAC channels are composed of ORAI proteins, which are the pore subunits and are activated by the ER Ca²⁺ sensors, stromal interaction molecules (STIM) 1 and 2, (reviewed in [22]). Upon ER store depletion, STIM

forms multimers in the ER membrane and translocates to sites of ER-plasma membrane apposition where they bind to and activate ORAI channels resulting in the sustained increase in $[Ca^{2+}]_i$ [23-26].

Although IP_3 is the main mechanism that controls the sustained increase in $[Ca^{2+}]_i$, other mechanisms mediated by potassium (K^+) channels, plasma membrane Ca^{2+} ATPase (PMCA), and mitochondria Ca^{2+} homeostasis, have been extensively studied. For example, K^+ channels control entry of Ca^{2+} through CRAC channels by regulating the membrane potential in which depolarization decreases Ca^{2+} entry whereas hyperpolarization increases Ca^{2+} entry [27]. Voltage-dependent ($Kv1.3$) and Ca^{2+} activated K^+ channels ($IKCa1$) are major K^+ channels controlling the membrane potential in effector $CD4^+$ T cells and upregulated during T-cell activation [28]. PMCA, the major Ca^{2+} clearance mechanism in T cells, regulates the amplitude of Ca^{2+} through its delayed upregulation, which allows larger Ca^{2+} rise [29]. Mitochondria are involved in the control of CRAC channel activity by reducing Ca^{2+} -dependent inactivation of CRAC channels. Ca^{2+} entry through CRAC channels is immediately imported to the lumen of mitochondria located in the vicinity of

CRAC entry, thereby increasing CRAC activity and the amplitude of Ca^{2+} signal (reviewed in [30]).

The sustained increase of Ca^{2+} through the CRAC channel is essential for the activation of transcription factors necessary for T cells to expand clonally and to acquire effector functions, for instance, through the production of cytokines and the upregulation of costimulatory molecules (reviewed in [19]). At least three transcription factors: NFAT, $\text{NF}\kappa\text{B}$, and activator protein 1 (AP1), are regulated by Ca^{2+} signaling. Among them NFAT is a pivotal target of Ca^{2+} signaling, whereas the role of Ca^{2+} may be regarded as being more indirect for $\text{NF}\kappa\text{B}$ and AP1 activation (reviewed in [31]).

C. NFAT

The NFAT family consists of five members; NFAT1 (also known as NFATc2 or NFATp), NFAT2 (also known as NFATc1 or NFATc), NFAT3 (also known as NFATc4), NFAT4 (also known as NFATc3 or NFATx) and NFAT5 (also known as tonicity enhancer binding protein) (reviewed in [32]). Four NFAT proteins are expressed in T cells: NFAT1, which is constitutively expressed in resting T cells and is the predominant NFAT protein [33], NFAT2, which is inducible upon activation [33], NFAT4, which is very weakly expressed in unstimulated T cells and its expression is not inducible [33], and NFAT5, which is highly expressed in the thymus, is undetectable in mature T cells. However, the expression of NFAT5 is inducible upon activation [34].

With the exception of NFAT5, NFAT1-4 are regulated by Ca^{2+} /calcineurin-dependent signaling [35]. In resting T cells, NFAT is phosphorylated by several NFAT kinases and localized in the cytosol. The sustained increase in $[\text{Ca}^{2+}]_i$ activates calmodulin, which then activates a large number of calmodulin-dependent proteins including, but not limited to, calcineurin, a serine/threonine phosphatase (reviewed in [36]). Activated calcineurin dephosphorylates NFAT resulting in nuclear translocation, stimulation of NFAT-DNA binding activity and increased transcription of NFAT-regulated genes [37,38]. Inhibition of the phosphatase activity of calcineurin by FK506 or cyclosporin A (CsA) results in relocalization of NFAT to the cytosol and loss of its DNA-binding activity (reviewed in [39]). Once the calcium-calcineurin signaling is terminated, rephosphorylation of NFAT by NFAT kinases is required for its nuclear export [40-42].

D. NF κ B

The NF κ B family consists of five genes coding for NF κ B1 (p105/p50), NF κ B2 (p100/p52), RelA (p65), RelB and c-Rel. RelA, Rel B, and cRel are synthesized as mature products that do not require proteolytic processing, whereas NF κ B1 and NF κ B2 are synthesized as large precursors that require proteolytic processing to produce the mature proteins, p50 and p52, respectively. These proteins share a common structural Rel homology domain that is important for dimerization, interaction with I κ B inhibitory proteins, and DNA binding. However only p65, RelB and c-Rel contain a transactivation domain,

therefore p50 and p52 homodimers can function as transcriptional repressors (reviewed in [43]). P50 and p65 are expressed widely in various cell types, whereas the expression of Rel B and c-Rel are restricted to lymphoid organs and haematopoietic cells (reviewed in [44]).

With a diversity of stimuli leading to the activation of NF κ B, the specific biological responses are associated with different combinations of homo- and heterodimers that distinctly regulate target gene transcription. The main activated form of NF κ B is a heterodimer of the p65 subunit associated with either a p50 or p52 subunit. In the resting state, NF κ B dimers are inactive in the cytoplasm because they are bound to inhibitory I κ B proteins or I κ B-like proteins, such as p100 and p105. Two different pathways, canonical and non-canonical, regulate NF κ B activation. The canonical pathway depends on ubiquitination-dependent degradation of I κ B proteins. The phosphorylation of I κ B by I κ B kinases (IKK) upon activation promotes the rapid ubiquitination and degradation of I κ B resulting in the release of active NF κ B dimers. The noncanonical pathway depends on proteolytic cleavage of the precursor p100 that mainly dimerizes with RelB. The liberated NF κ B dimers are transported to the nucleus and thereby activating gene expression [45]. However, to achieve the maximal NF κ B transcription response, the NF κ B dimers, particularly the p65 subunit, must undergo additional post-translational modification involving site-specific phosphorylation [46].

TCR ligation induces NF κ B activation mainly through the canonical pathway. The activation of IKK upon T cell activation depends on the formation of the “CBM” complex, which is composed of caspase recruitment domain, CARD, membrane-associated guanylate kinase, MAGUK, protein 1 (CARMA1), B cell lymphoma 10 (BCL10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1) (reviewed in [47]). The recruitment of CARMA1 to the constitutively interacting BCL10 and MALT1 complex is largely dependent on its phosphorylation (reviewed in [48]). PKC θ is the major kinase responsible for the phosphorylation of CARMA1 after TCR/CD28 costimulation [49]. Importantly, $[Ca^{2+}]_i$ elevation is also crucial for TCR-induced NF κ B activation (reviewed in [50]). Calcineurin and calcium/calmodulin-dependent protein kinase II (CaMKII) were involved in the regulation NF κ B [51]. Calcineurin was demonstrated to facilitate the assembly of the CBM complex by dephosphorylation of BCL10 [52]; whereas CaMKII was identified as a BCL10 and CARMA1 kinase [53,54].

E. GSK3

GSK3 is a protein serine/threonine kinase, which is ubiquitously expressed in almost all cell types. There are two isoforms of mammalian GSK3; GSK3 α and GSK3 β [55]. Unlike most kinases, GSK3 is constitutively active and regulated by inhibitory phosphorylation at Ser21 in GSK3 α and Ser9 in GSK3 β (reviewed in [56]). In T cells, CD28 co-stimulation was found to facilitate the inactivation of GSK3 through the activation of Akt (protein kinase B), one of the

inhibitory GSK3 kinases (reviewed in [56]). GSK3 β serves as an NFAT kinase and plays an important role in regulating NFAT activity [40]. Inhibition of GSK3 β increased IL-2 production in both CD4 $^{+}$ and CD8 $^{+}$ T cells [57,58]. Although GSK3 β was also shown to regulate NF κ B activation; its role is much less established in T cells following TCR stimulation (reviewed in [59]).

III. CD40 and CD40 ligand (CD40L)

A. CD40

The CD40 receptor is a type I transmembrane glycoprotein and is a member of the tumor necrosis factor (TNF) receptor superfamily. The human *CD40* gene, located on chromosome 20, encodes for a 258 amino acid polypeptide with molecular weight of 50 kDa. CD40 is constitutively expressed on a variety of cells, both immune and non-immune cells. For example, CD40 are found on surface of B cells, activated macrophages, dendritic cells, vascular endothelial cells, astrocytes and microglial cells (reviewed in [60-62]).

B. CD40L

CD40L, also termed CD154, is a type II transmembrane protein and is a member of the TNF superfamily. The human *CD40L* gene is located on the X-chromosome and encodes for a 261 amino acid polypeptide. CD40L exists in at least two forms: the transmembrane form, which has a molecular weight of 39 kDa, and the soluble form, which has a molecular weight of 18. Similar to its receptor, CD40, CD40L can be expressed by many cell types including

eosinophils, basophils, macrophages, and natural killer cells, of which CD4⁺ T cells have the highest level of CD40L expression. In contrast to the constitutively expressed CD40, the expression of CD40L under physiological conditions is inducible and transient (reviewed in [63]).

C. Cellular response of CD40-CD40L interaction

CD40-CD40L interaction plays a crucial role in various aspects of the immune response. Ligation of CD40L on CD40⁺ cells enhances the function of the interacting effector cells. In antigen-presenting cells, the upregulation of co-stimulatory molecules [e.g. CD69, CD80, CD86, and Major Histocompatibility Complex (MHC)-II] as well as adhesion molecules [e.g. intercellular adhesion molecules-1 (ICAM1) and vascular cell adhesion molecules-1 (VCAM-1)] enhances antigen presentation. CD40 and/or CD40L-induced production and secretion of cytokines such as interleukin (IL)-1, IL-2, IL-6, IL-10, TNF- α , and interferon- γ (IFN- γ), as well as chemokines, such as monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein 1 α (MIP-1 α) and regulated on activation, normal T expressed and secreted (RANTES), involved in inflammatory response. Further, CD40-induced expression of matrix metalloproteinases (MMPs) by endothelial cells was associated with atherosclerosis (reviewed in [64,65]) and tumor metastasis in cervical cancer [66]. The implication of CD40 and/or CD40L in both physiological and pathophysiological processes emphasizes the possible targets for therapeutic intervention (reviewed in [67,68]).

IV. Regulation of CD40L expression in activated T cells

In T cells, CD40L expression is rapidly but transiently induced after T cell activation. Upon *in vitro* stimulation either by treatment with phorbol ester plus calcium ionophore (PMA/Io) or antibodies directed against CD3 and CD28 (anti-CD3/CD28), the level of surface CD40L on activated T cells is maximal between 6 and 8 h after activation followed by a decline over the next 12-18 h (reviewed in [69,70]). However, recent evidence suggests biphasic expression of CD40L on activated CD4⁺ T cells, in which the second peak occurred at 48 h post stimulation [71-74]. Interestingly, elevated expression of surface CD40L is down-regulated by endocytosis upon binding to its receptor, CD40 [75]. The expression of CD40L is tightly regulated and occurs at the transcriptional, post-transcriptional, and/or post-translational level, of which the regulation at the transcriptional level is the main mechanism (reviewed in [69]). At the transcriptional level, NFAT is the key transcription factor found in the minimal CD40L promoter [76]. However, optimal transcription of the *CD40L* gene requires cooperatively binding of other transcription factors such as CD28RE, NFκB, TFE3/TFEB, EGR, AKNA, and AP1 [77]. Among them, NFκB has shown to be critically involved in the up-regulation of CD40L expression in both activated mouse and human T cells [78-80]. The signaling pathways are likely to be relevant for transcribing the *CD40L* gene upon T cell activation are schematically represented in Figure 3.

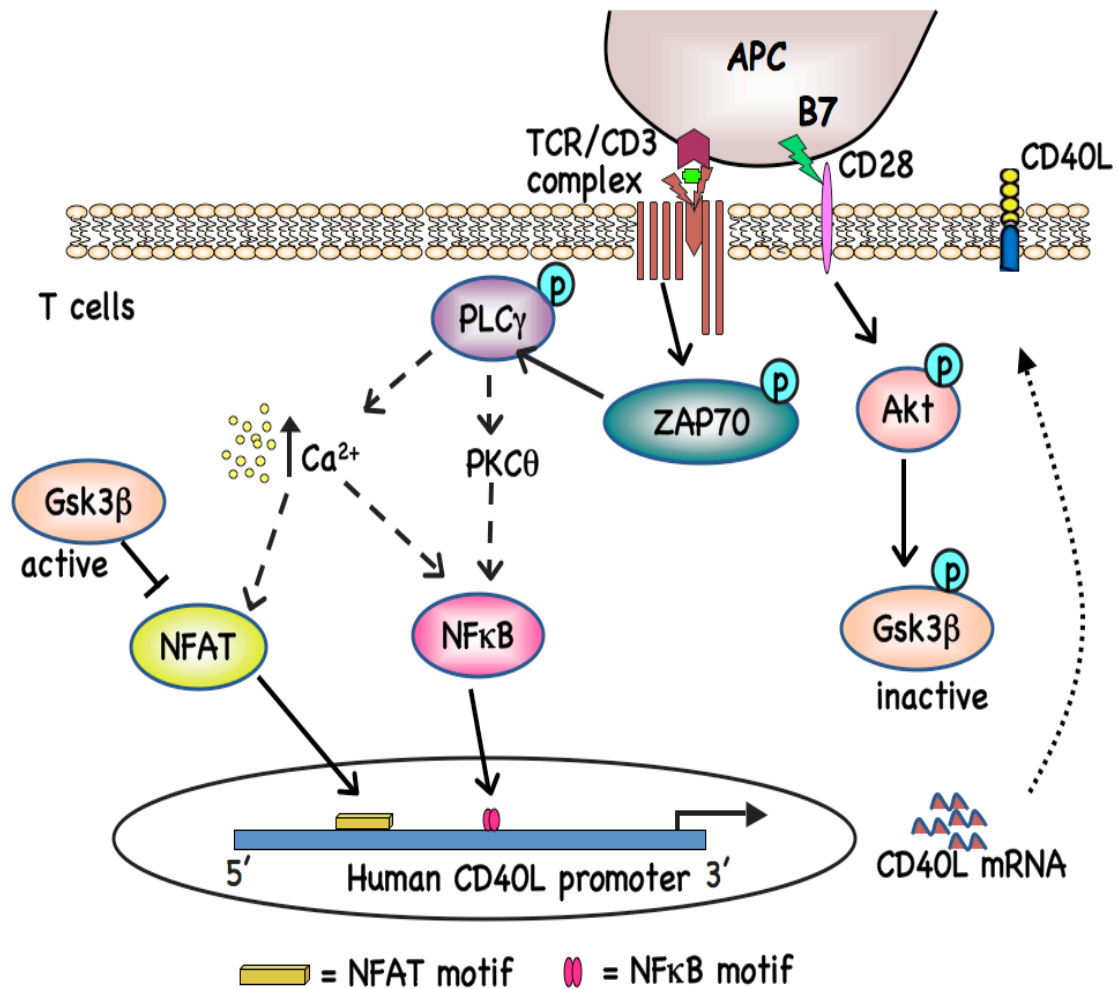


Figure 3. Schematic diagram represents the proposed signaling cascades and mechanisms involved in transcriptional regulation of CD40L promoter in T cells.

The engagement of TCR in combination with its co-receptor, CD28, leads to the activation of ZAP70 by phosphorylation. The activated ZAP70 then phosphorylates and activates PLCγ. The activation of PLCγ leads to an increase in $[Ca^{2+}]_i$ and activation PKCθ, which ultimately results in activation of two transcription factors, NFAT and NFκB. The activated NFAT and NFκB then translocate into the nucleus and binds to the CD40L promoter. *CD40L* transcripts are translated and exported to the surface. The activation of NFAT is also regulated by GSK3β, which is constitutively active in resting T cell, but becomes inactive upon phosphorylation with Akt.

V. Role of CD40 in plasma cell differentiation

A. CD40 and T cell-dependent humoral immune response

The importance of cell-contact in T cell-induced B cell proliferation and differentiation was initially identified since plasma membrane fractions from activated T helper cells, but not cytokines, were able to reconstitute the requirement of T cell help in B cell function [81-86]. Among the different surface molecules, the engagement between CD40 and its ligand, CD40L, has been well established as a key signal for driving B cells to become plasma cells by promoting the activation, proliferation and differentiation of B cells [87,88]. Subsequently, the physiologic relevance of the CD40-CD40L interaction for normal humoral immunity was demonstrated in humans, in which mutations in the gene encoding CD40L associates with X-linked hyper-IgM syndrome. Patients suffering with X-linked hyper-IgM syndrome showed deficiencies in antibody class switching and germinal center formation [89-92]. Further, administration of anti-CD40L antibodies to mice abrogated antibody responses against T cell-dependent, but not T cell-independent, antigens [93]. Similar results were also demonstrated in CD40L knockout mice [94].

B. TRAF-dependent CD40 signaling

Upon binding to CD40L, CD40 molecules, which were once distributed evenly throughout the plasma membrane, rapidly trimerize in microdomains. The cytoplasmic tail of CD40, which lacks intrinsic enzymatic activity, delivers signals to the cells by recruitment of TNFR-associated factors (TRAFs) (reviewed in

[95]). TRAFs serve as adapter proteins, which in turn deliver signals to B cells through the activation of different signaling pathways: NF κ B, PI3K, and MAPKs (Figure 4). These signaling pathways then regulate B-cell fate in humoral immunity through the upregulation of cell adhesion molecules, co-stimulatory molecules, immunoglobulin, cytokines and lymphokines that are involved in activation, proliferation, differentiation, antibody isotype switching, as well as generation of memory B cells (reviewed in [96,97]). Among different TRAF-dependent CD40 signal cascades, NF κ B is the major transcription factor involved in CD40-mediated B cell proliferation [98] and IgM production [99], whereas p38 MAPK is required for CD40-induced B cell proliferation [100]. There are currently seven types of TRAFs, TRAF1 through TRAF7. All types of TRAFs, except TRAF4 and TRAF7, are directly or indirectly recruited to the cytoplasmic domain of CD40 during CD40L engagement (reviewed in [95]). The consensus binding sites for TRAF1, TRAF2, and TRAF3, are overlapping and located at the distal domain, whereas the binding site of TRAF6 is located at the proximal domain of CD40 [101]. Interestingly, TRAF2 was shown to bind to the non-canonical binding site at the carboxy-terminus of CD40, which responsible for CD40-induced B-cell activation, proliferation and differentiation through the activation of NF κ B [98,102].

C. TRAF-independent CD40 signaling

Janus family kinases (JAKs), in particular JAK3, is also constitutively bound to the proximal region of CD40 cytoplasmic tail [103]. To date, JAKs are

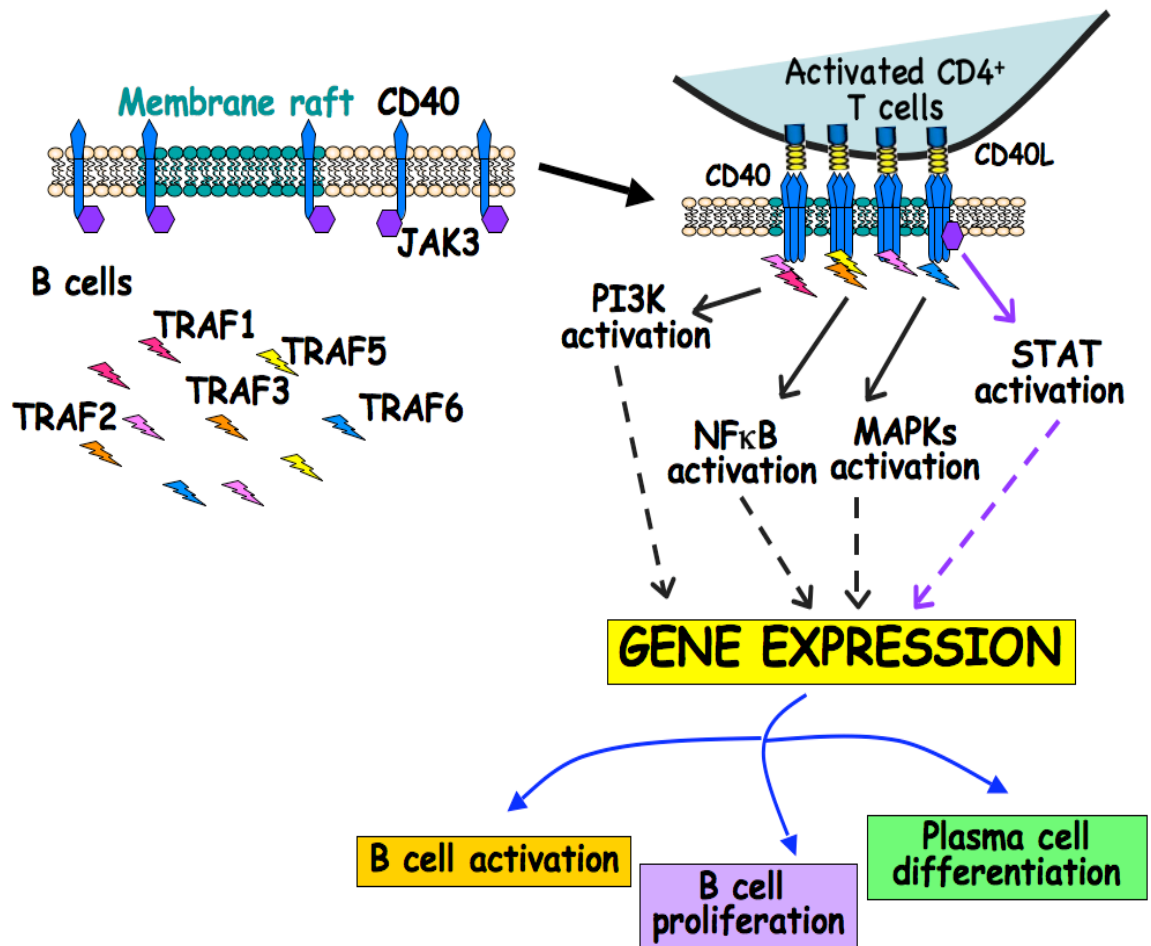


Figure 4. Schematic diagram represents the proposed downstream signaling pathways initiated by the engagement of CD40 in B cells.

The ligation of CD40 by CD40L promotes the trimerization of CD40 in the membrane raft. The trimerization of CD40 signals to B cells through two mechanisms, TRAF-dependent and -independent pathways. In TRAF-dependent pathway, the trimerized CD40 recruits the binding of TRAFs to their cytoplasmic tails. TRAFs then activate several signaling cascades such as PI3K, NFκB, and MAPKs. In the TRAF-independent pathway, the trimerization of CD40 leads to the activation of JAKs, which are constitutively bound to their cytoplasmic tails. The activated JAKs then phosphorylate STATs. The activation of these signaling pathways regulates the expression of genes involved in B cell activation, proliferation, and differentiation.

composed of four nonreceptor tyrosine kinases (JAK1, JAK2, JAK3, and TYK2) [104,105]. Activated JAKs then phosphorylate signal transducer and activator of transcription (STAT) proteins, which then dimerize and translocate to the nucleus and regulates gene expression. There are at least seven STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5b and STAT6) (reviewed in [106]). The ligation of CD40 induces activation of associated JAK3 leading to the phosphorylation and activation of STAT3 [103] (Figure 4). However, the role of JAK3 in CD40-mediated B cell responses is controversial. For instance, B cells transfected with mutant CD40 that lacks the bind site for JAK3 failed to induce surface expression of CD23, ICAM-1, and LT- α upon activation suggesting a critical role of JAK3 in CD40-mediated gene expression [103]. By contrast, in studies using purified B cells from JAK3-deficient patients, JAK3 is not essential for CD40-mediated B-cell proliferation, isotype switching, or upregulation of aforementioned surface molecules [107]. Further, CD40 ligation results in phosphorylation and activation of STAT6, but not STAT1, in mouse B cells [108].

VI. Role of cytokines, interleukin (IL)-2, -6, and -10, in plasma cell differentiation

Although the CD40 signaling is absolutely required for the development of T-cell dependent humoral immune response, sustained engagement of CD40 prevents the terminal differentiation of activated mature B cells into plasma cells [109,110]. Several studies demonstrated that plasma cell differentiation in

humans requires the presence of several cytokines including, but not limited to, IL-2, -6, and -10 [109,111-115]. Most cytokine receptors lack intrinsic kinase activity, and hence often employ JAKs that are associated with cytokine receptors to activate STAT proteins, which are the main downstream signaling of cytokine receptors (reviewed in [116]).

A. IL-2

IL-2 has been shown to play essential role from the initial stage of B cell activation through the final stage of B cell differentiation into plasma cells. The addition of IL-2 at the initiation of culture was able to reverse the suppression of pokeweed mitogen-induced T cell-dependent differentiation of B cells by cyclosporin A [117]. Further, exogenous IL-2 enhanced the proliferation and differentiation of B cells into antibody secreting cells after antigenic stimulation by *Staphylococcus aureus* Cowan strain I (SAC) [118,119], influenza virus [120], and hepatitis B virus in humans [121].

IL-2 receptor (IL-2R) is comprised of three subunits: IL-2R α , IL-2R β , and IL-2R γ (the common cytokine receptor γ chain). There are three classes of IL-2R, the low, intermediate, and high affinity receptors, which are defined by combinations of aforementioned subunits. The low affinity receptors contain only the IL-2R α ; intermediate affinity receptors contain IL-2R β and IL-2R γ ; high affinity receptors contain all three chains (reviewed in [122]). All three subunits of IL-2R were upregulated in human B cells and several B cell lines upon activation with different stimuli [118,123-125]. The binding of IL-2 to high affinity IL-2R

expressed on antigen activated B cells induced expression of IgJ mRNA mediated by downregulation of PAX5 mRNA, a negative regulatory motif in the *IgJ* promoter [7,126,127]. Recently, IL-2 treatment induced phosphorylation of STAT5 and ERK MAPK in primed human B cells with CD40L, CpG, and F(ab')₂ anti-Ig (IgA+IgG+IgM) [125]. However, IL-2-induced ERK signaling, but not STAT5, was responsible for plasma cell differentiation, at least in part, through the downregulation of BACH2, a transcriptional repressor of *PRDM1*, the gene that encodes Blimp1 [125].

B. IL-6

IL-6 has been identified as a B cell-differentiation-inducing factor, which is important in the terminal differentiation of B cells to antibody secreting cells [128,129]. IL-6-deficient mice were defective in the T-cell dependent IgG antibody response against vesicular stomatitis virus [130]. Consistent with the aforementioned observation, IL-6 overexpressing transgenic mice developed plasmacytosis and a massive increase in polyclonal IgG1 [131]. Further, IL-6 was shown to regulate the growth and development of plasma cell neoplasia [132].

The IL-6 receptor is comprised of two polypeptide chains, a ligand specific receptor and a common signal transducer, gp130 [133]. IL-6 treatment induced the phosphorylation of STAT3 in primary human B cells [134] and ERK MAPK in human B cell line AF10 [135] likely through the activation of gp130-associated JAKs (JAK1, JAK2, and Tyk2) [136-141]. In addition, SHP-2 has been

demonstrated to be responsible for the activation of ERK MAPK induced by IL-6 in murine pro B-cell line (BAF-B03) [142], and hepatocellular carcinoma cell line (HepG2) [143,144]. Although the molecular mechanism by which IL-6 induces plasma cell differentiation are not well established, STAT3 activation is thought to play a central role in cell survival and cell-cycle progression induced by the IL-6 family of cytokines (reviewed in [145]). STAT3 activation results in the upregulation of various genes involved in cell survival and proliferation; such as Pim-1, Bcl-2, Bcl-XL Mcl-1, cyclin D, and c-Myc [146-148]. Further, STAT3 is involved in the upregulation of Blimp1 [149].

C. IL-10

IL-10 is a potent cofactor for proliferation of human B cells activated either by anti-CD40 plus anti-IgM or CD40 cross-linking [112,150]. The effect of IL-10 on B cell survival was also demonstrated as IL-10 treatment prevented the spontaneous death of human splenic B cells in vitro [151]. IL-10 also acts as a plasma cell differentiation factor for B cells stimulated by either anti-CD40 [152], activated T cells [153] or follicular dendritic cell-like cell line [154]. Further, blocking IL-10 suppressed plasma cell differentiation and isotype switching induced by anti-CD40 plus anti-IgM by decrease IgG, IgA, and IgM production [111].

The IL-10 receptor is composed of at least two subunits, IL-10R1 or IL-10R α and IL-10R2 or IL-10R β , that are members of the interferon receptor (IFNR) family (reviewed in [155]). The IL-10/IL-10R interaction activated STAT1,

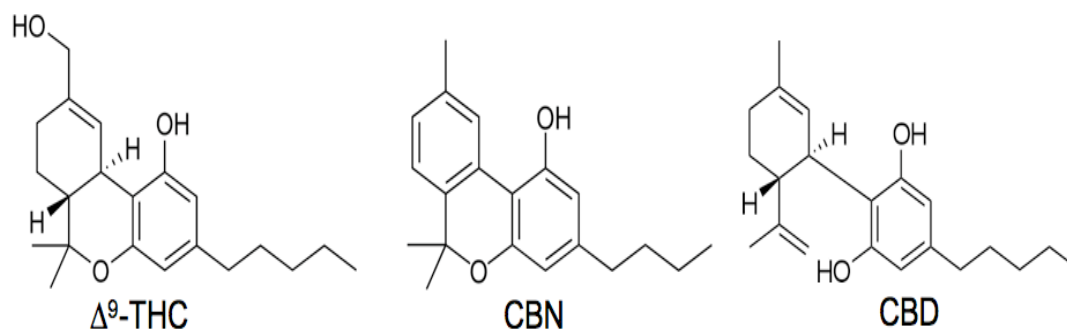
STAT3, and STAT5, most likely through the activation of JAK1 and TYK2, but not JAK2 and JAK3 in murine pro-B cell line [156,157]. In humans, IL-10 treatment resulted in the activation of several transcription factors, of which STAT1 and STAT3 in B cell chronic lymphoid lymphoma [158] and c-fos in human B cells [159]. IL-10 treatment also induces the expression of anti-apoptotic protein, bcl-2, in resting human B cells [151] or SAC-activated human B cells [160]. Further, naïve B cells derived from patients with an inactivating mutation in STAT3, but not STAT1, showed the profound defects in plasma cells differentiation induced by CD40L plus IL-10 or IL-21 through impairment of Blimp1 and XBP1 upregulation [161].

VII. Δ^9 -tetrahydrocannabinol (Δ^9 -THC) and cannabinoid receptors

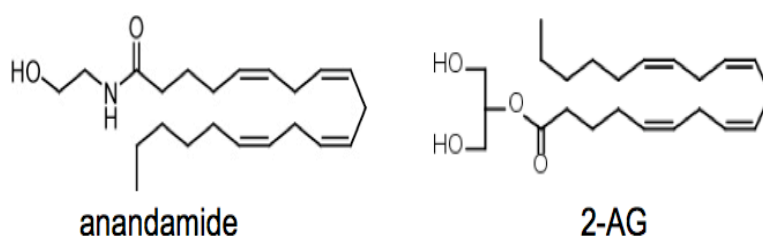
A. Cannabinoids and Δ^9 -THC

The term cannabinoids originally described compounds extracted from the plant, *Cannabis sativa*. In 1964, Gaoni and Mechoulam first elucidated the chemical structure of Δ^9 -THC, the main psychoactive congener present in the cannabis plant [162]. The term cannabinoids refer to a group of compounds that are structurally related to Δ^9 -THC or that bind to cannabinoid receptors. There are three general types of cannabinoids: plant-derived, synthetic and endocannabinoids (Figure 5).

Plant-derived cannabinoids



Endocannabinoids



Synthetic cannabinoids

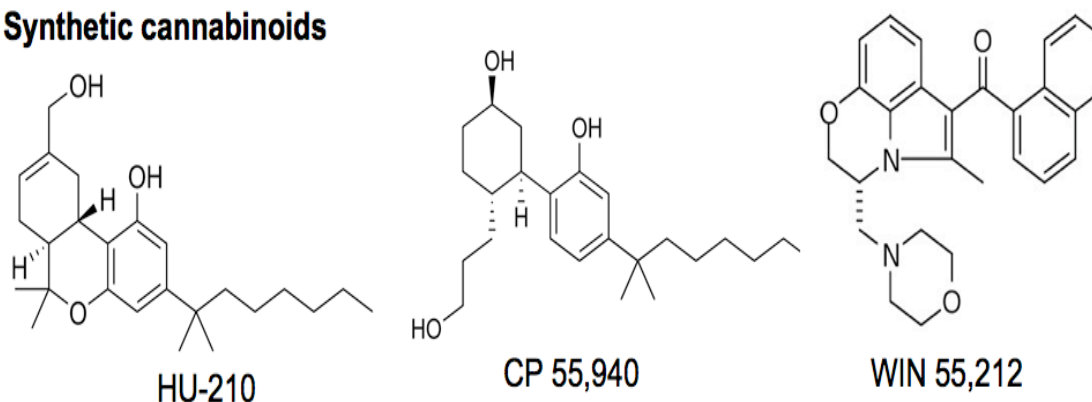


Figure 5. Chemical structures of cannabinoid compounds.

Most plant-derived cannabinoids, such as Δ^9 -THC, CBN, and CBD, contain dibenzopyran ring as a base structure. Endocannabinoids, anandamide and 2-AG, are derivatives of long-chain polyunsaturated fatty acids. Synthetic cannabinoids are classified into two groups. The first group shares structural similarity with naturally cannabinoids such as HU-210 and CP 55,940. Another group contains aminoalkylindole structure such as WIN 55,212.

There are at least 60 plant-derived cannabinoids, of which Δ^9 -THC, cannabinol (CBN), and cannabidiol (CBD) are the most abundant and most extensively characterized [163]. These naturally occurring plant-derived cannabinoids vary extensively in their biological activity, which is attributable in part, to their respective binding affinity for cannabinoid receptors. Endocannabinoids, the endogenous ligands of cannabinoid receptors, are fatty acid amides produced by humans and animals. Thus far, N-arachidonylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG) are the most extensively characterized endocannabinoids (reviewed in [164]). Synthetic cannabinoids are compounds that have structural similarity to natural cannabinoids or substances with structural features, which allow binding to one of the known cannabinoid receptors such as HU-210 and CP 55,940, which share structural similarity to Δ^9 -THC, and WIN 55,212, which has markedly different structure, but is more potent than naturally-occurring compounds [165].

For centuries, the preparations from *Cannabis sativa* such as marijuana, hashish, and hashish oil, have been used both medicinally and recreationally (reviewed in [166]). However, marijuana, hashish, and hashish oil are schedule I control substances in the U.S. since 1914 [167]. To date, marijuana is the most widely used illicit drug worldwide of which the USA is one of the countries with the highest prevalence of marijuana use (14.1%) among population aged 15 to 64 [168]. Furthermore, the Food and Drug Administration (FDA) has approved a synthetic Δ^9 -THC (Dronabinol or Marinol[®]) for treatment of chemotherapy-

induced nausea and vomiting in cancer patients as well as for treatment of weight loss in AIDS patients [169]. While marijuana is not an FDA-approved medicine, 18 states and the District of Columbia have currently legalized its medical use [170]. Sativex[®], a whole-plant cannabis extract, 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. In the US, Sativex is currently in phase III trials for cancer pain treatment [171].

B. Cannabinoid receptors

The first cannabinoid receptor, now referred to as CB1, was identified and cloned from rat cerebral cortex cDNA library in 1990 [172]. Soon after, the second cannabinoid receptor, termed CB2, was cloned from the human leukemia cell line, HL-60, in 1993 [173]. Although much evidence supports the existence of non-CB1/CB2 cannabinoid receptors, only CB1 and CB2 are definitively classified as cannabinoid receptors currently (reviewed in [174,175]).

Both CB1 and CB2 are members of the G protein-coupled receptor (GPCR) superfamily. Human CB1 and CB2 share an overall identity of 44% and ranging from 35% to 82% in the transmembrane regions [176]. CB1 is more conserved between species. For example, human CB1 shares 90% and 96% identity with mouse and rat, respectively; whereas human CB2 shares 82% and 56% identity to mouse and rat, respectively [177-179]. CB1 is found throughout the body, but is most abundantly expressed in the central nervous system,

especially in hippocampus, basal ganglia, and cerebellum, which correlates well with the psychotropic effects of Δ^9 -THC and have the highest expression levels [180,181]. CB2 is mainly found in peripheral tissues and is highly expressed in spleen and cells of the immune system. Human B cells have the highest level of *CNR2* mRNA, whereas CD4⁺ T cells have relative low level of *CNR2* mRNA [180]. Importantly, under certain condition and disease states, both CB1 and CB2 can be up- and down-regulated in particular cell types (reviewed in [182]). Examples include induction of *CNR1* gene transcripts (mRNA) in activated human T cells [183], down-regulation of CB1 in human colorectal cancer [184], and up-regulation of CB2 in human inflammatory bowel disease [185]. In addition, there is induction of both *CNR1* and *CNR2* mRNA in peripheral blood mononuclear cells (PBMCs) derived from chronic marijuana smokers [186].

In human populations, several reports demonstrated the divergence of the genes encoding for CB1 and CB2. To date, at least five distinct variant exonic structures were identified in human CB1 gene [187]. Both the CB1a and CB1b show altered ligand binding activity compared with CB1 [188]. The length of microsatellite (AAT)_n and single nucleotide polymorphisms at intron 2 in the *CNR1* gene was associated to susceptibility to drug abuse [26,189]. Polymorphisms in the human *CNR2* gene have been identified as well, of which 188-189 GG/GG dinucleotide polymorphism was associated to osteoporosis in several ethnic groups [190,191] and autoimmune diseases [192].

C. Signal transduction activated by CB1 and/or CB2

As members of GPCR superfamily, CB1 and CB2 were initially reported to couple to inhibitory heterotrimeric G proteins ($G_{\alpha_{i/o}}$) since those cannabinoid-mediated responses were blocked with pertussis toxin (PTX) treatment [172,193-197]. Stimulation of CB1 and CB2 by various cannabinoids inhibited adenylyl cyclase (AC) activity resulting in decreased levels of cAMP in several cell and tissue preparations [172,193,194,196-201]. However, CB1, but not CB2, was shown to also interact with stimulatory heterotrimeric G proteins (G_{α_s}) resulting in activation of AC [202-207]. The differential effects of CB1-mediated AC activity could also be attributed to the specific isoform(s) of AC, of which AC types I, V, VI and VIII were inhibited, whereas types II, IV, and VII were activated following treatment with HU-210 and WIN 55,212-2 [208].

The coupling of CB1 and CB2 to mitogen-activated protein kinase (MAPK) pathway has been demonstrated in several cell types. Treatment with CP-55940, Δ^9 -THC and WIN 55212-2 activated p42/p44, the extracellular signal-regulated kinase subgroup of the mitogen-activated protein kinases (ERK MAPK), in CHO cells expressing the CB1 receptor and this effect was blocked by a CB1 antagonist or PTX [209]. Similarly, CB2 is involved in cannabinoid-mediated activation of ERK MAPK in CHO cells expressing the CB2 receptor [210] and HL-60 cells [211]. Further, CB1 and CB2 activation in human prostate epithelial PC-3 cells facilitated the phosphorylation of ERK MAPK [212]. Interestingly, WIN

WIN 55,212-2 and CBN suppressed ERK MAPK activation in stimulated mouse splenocytes [213]. The differential effect of cannabinoids on the regulation of the MAPK signaling cascade may be explained by the requirement of cellular activation. Although the mechanisms underlying the regulation of MAPK signaling by cannabinoids have not been fully elucidated, MAPK activation by cannabinoids mediated, at least in part, through activation of PI3K/Akt pathway, and the subsequent activation of Raf, a MAPKKK [212,214,215]. In addition, a decrease in PKA activity as a result of cannabinoid-mediated decreases in cAMP level have shown to enhance MAPK activation in breast cancer cells (MCF-7) [216], neuroblastoma cells (N1E-115) [217], and rat hippocampal slices [218].

Voltage-gated ion channels, primarily calcium (Ca^{2+}) and/or potassium (K^+) channels, were also shown to be a target of CB1 stimulation. For instance, WIN 55,212-2 and/or CP 55,940 inhibited N-type Ca^{2+} channels in neuroblastoma cells (NG108-15 cells), CB1-transfected rat ganglion neurons, and hippocampus [196,219-222]. Anandamide inhibited P/Q-type Ca^{2+} channels in pituitary tumor cells (AtT-20) [223] and rat cerebellar brain slices [224]. WIN 55,212-2 inhibited L-type Ca^{2+} channels in cat cerebral arterial smooth cells [225] and retinal slices from larval tiger salamander [226]. Additionally, WIN 55,212-2 activated A-type, but inhibited D-type and M-type K^+ channels in hippocampal neurons [227,228]. G protein-coupled inwardly rectifying K^+ (GIRK) channels

were also activated by cannabinoids in a CB1-dependent manner [223,229,230]. It is noteworthy that CB2 receptors are believed not to directly modulate ion channel function, as WIN 55,212-2 did not alter the GIRK1/4 current in both AtT-20 cells and *Xenopus* oocytes transfected with CB2 and GIRK1/4 [229,231]. CB2 also did not modulate the activity of Q-type Ca^{2+} channels in AtT-20 cells expressing CB2 [231].

Ca^{2+} signaling has been demonstrated to be modulated by the activation of CB1 and/or CB2 in several models. CB1 coupled to $\text{G}_{i/o}$ was shown to be involved in cannabinoid-mediated increases in $[\text{Ca}^{2+}]_i$ in neuroblastoma cells (NG108-15) [232], primary culture of striatal astrocytes [233], human endothelial cells [234], canine kidney cells [235], and hamster smooth muscle cell line (DDT₁ MF-2 cells) [236]. Further, anandamide and 2-AG, acting via PTX-sensitive CB2, induced the increase of $[\text{Ca}^{2+}]_i$ in calf pulmonary endothelial cells [237] and HL-60 cells [238], respectively. Anandamide-mediated increase in $[\text{Ca}^{2+}]_i$ was mediated, at least in part, by the G protein-dependent activation of PLC β and thereby IP3-mediated release of Ca^{2+} from internal stores [237]. However, CB1 and CB2 were not involved in Δ^9 -THC- or CBN-induced increases in $[\text{Ca}^{2+}]_i$ in resting mouse splenic T cells and/or the human peripheral blood acute lymphoid leukemia (HPB-ALL) T cell line [239,240].

D. Pharmacokinetics of Δ^9 -THC

Inhalation through smoking a cannabis cigarette, mouth spray of Sativex, or oral administration of Marinol in capsules or baked foods or liquid are the major routes of administration and delivery of Δ^9 -THC (reviewed in [241]). Absorption through inhalation is very rapid with peak Δ^9 -THC plasma concentration of 100-200 ng/mL within first 10 minutes after smoking. However, plasma Δ^9 -THC concentrations vary depending on the potency of marijuana and the manner in which the drug is smoked [242]. With oral administration, absorption is slower with much lower (approximately 3-4 ng/mL) and peak Δ^9 -THC plasma concentration is delayed to between 1-5 hours due to extensive first pass metabolism (reviewed in [241]). Due to its lipophilicity, Δ^9 -THC immediately distributes from blood to tissues resulting in a large volume of distribution ranging from 1 to 10 L/kg [243,244]. In addition, Δ^9 -THC rapidly crosses the placenta [245] and also distributed to breast milk [246]. The Δ^9 -THC concentration in human milk was 8.4 times higher than in plasma [246].

Δ^9 -THC is primarily metabolized by hydroxylation and oxidation via cytochrome P450 in the liver [247-249]. 11-hydroxy-THC, a monohydroxylated compound, is the major metabolite found in humans and has equipotent psychoactivity. The 11-hydroxy-THC is further oxidized to the 11-nor-9-carboxy-THC (THC-COOH), which is not psychoactive [250,251]. The acid metabolites of

Δ^9 -THC are mainly excreted in feces (approximately 65%) [244,252], and in the urine as conjugated glucuronic acids and free THC-hydroxylated metabolites [253-255].

E. Pharmacodynamics of Δ^9 -THC

The physiological effects of Δ^9 -THC are mediated through its agonistic and antagonistic actions at the specific sites (reviewed in [241]). Δ^9 -THC binds both known cannabinoid receptors with similar K_i values in the nanomolar range. However, Δ^9 -THC has relatively low cannabinoid receptor efficacy with higher efficacy at CB1 than CB2, and behaves as a partial agonist to both receptors (reviewed in [256]). In addition, the binding of Δ^9 -THC to cannabinoid receptors may interfere the physiological responses mediated by endocannabinoids, the endogenous ligands of cannabinoid receptors in humans and animals (reviewed in [257]). Some effects of Δ^9 -THC have been demonstrated that are not mediated by CB1 and/or CB2, supporting the presence of cannabinoid receptor subtypes that have not yet been identified [257]. This section focuses on the effect of Δ^9 -THC on central nervous system (CNS), cardiovascular system, reproductive system, and gastrointestinal system. The effects of Δ^9 -THC on the immune system are discussed in more details in the following section.

In the CNS, Δ^9 -THC was shown to modulate the release or reuptake of neurotransmitters. For instance, Δ^9 -THC increased the CB1-mediated release of acetylcholine in rat hippocampus, of acetylcholine, glutamate and dopamine in rat

prefrontal cortex, and of dopamine in mouse and rat nucleus accumbens [258-262], but decreased gamma-aminobutyric acid uptake in the globus pallidus [263]. The release of dopamine by Δ^9 -THC most likely explains the euphoria produced by Δ^9 -THC in humans. In animal models, a characteristic “tetrad” including antinociception, hypothermia, hypomotility, and catalepsy, represents the CNS effect after *in vivo* administration of Δ^9 -THC [264]. Additionally, Δ^9 -THC caused impairment in psychomotor functions including learning and memory (reviewed in [265,266]) and driving a car or piloting an aircraft [267-269].

In the cardiovascular system, single dose administration of Δ^9 -THC induced tachycardia [270], increased cardiac output [271], and reduced platelet aggregation [272]. However, prolonged Δ^9 -THC ingestion significantly decreased heart rate and blood pressure [270].

In the reproductive system, Δ^9 -THC was associated with impairment of menstrual cycle and ovulation through a decrease in follicle-stimulating hormone and luteinizing hormone (reviewed in [273]). Further, testosterone synthesis from Leydig cells and sperm count was reduced by Δ^9 -THC (reviewed in [273]). In pregnant rhesus monkeys, administration of Δ^9 -THC (2.5 mg/kg/day) increased abortions, as well as produced a rapid decrease in chorionic gonadotropin and progesterone concentrations, and stillbirths [274].

In the gastrointestinal system, Δ^9 -THC was shown to prevent emesis [275,276]. Δ^9 -THC also inhibited gastrointestinal motility in both rodents [277] and humans [278]. Additionally, Δ^9 -THC at doses used for preventing chemotherapy-induced nausea and vomiting significantly delays gastric emptying [279].

F. Toxicity of Δ^9 -THC

Acute oral administration of Δ^9 -THC caused lethality only in rat at a median lethal dose of 800-900 mg/kg. In rats toxicity was characterized by severe hypothermia, and other central effects such as ataxia, muscle tremors, prostration, and rapid weight loss. Although no deaths were associated with oral administration of maximum oral Δ^9 -THC doses in dogs and monkeys, both species developed toxic signs including drowsiness, ataxia, and abnormal eating pattern [280]. Acute fatalities following cannabis use in human has been inconclusive; however, Δ^9 -THC was associated possibly with sudden death caused by myocardial infarction [281,282]. Adverse effects of marijuana use are controversial, but are mainly associated with decreased motor coordination, impaired cognitive function, and alteration in cardiovascular system including tachycardia, postural hypotension, and supine hypertension [283-286]. Chronic administration of Δ^9 -THC results in development of reversible tolerance to the

cardiovascular, psychological, skin hypothermic, and behavioral effects of Δ^9 -THC in human [252,287].

VIII. Immunomodulatory properties of Δ^9 -THC

Administration of Δ^9 -THC *in vivo* results in perturbation of host immune resistant against viral infection [288], bacterial infection [289,290], or tumor challenges [291]. These studies show that Δ^9 -THC modulates multiple immune cell populations. However, this section focuses principally on the effect of Δ^9 -THC on T cell and B cell function, which are both critical in mounting T cell-dependent humoral immune responses.

A. T cells

Many studies have shown that Δ^9 -THC has anti-inflammatory activity, partly through suppression of T cell function (reviewed in [292]). Specifically, this laboratory has identified a number of T cell-mediated responses modulated by Δ^9 -THC including proliferation, production of cytokines, expression of co-stimulator molecules, and T cell responses to specific antigens [116,288,293,294]. Importantly, the effects of Δ^9 -THC on T-cell functions depend on experimental conditions, such as concentrations of Δ^9 -THC, cell types, and specific or magnitude of immune stimuli.

Numerous laboratories have reported that lymphoproliferative responses of mouse splenocytes to T cell specific mitogens (concanavalin A, ConA; or phytohemagglutinin, PHA) were inhibited by cannabinoids, including Δ^9 -THC [295-298]. In addition, Δ^9 -THC was shown to suppress the proliferation of mouse splenic CD4⁺ T cells stimulated by allogeneic class II histocompatibility antigens [298]. Interestingly, Δ^9 -THC positively or negatively modulated proliferation of mouse splenic CD8⁺ T cells. For example, Δ^9 -THC (< 15.9 μ M) can either enhance or suppress proliferation of mouse splenic CD8⁺ T cells stimulated by anti-CD3 antibodies or mitogens (ConA or PHA), respectively [297]. The same group also reported that CD8⁺ T cells derived from lymph nodes were more resistant to the modulation by Δ^9 -THC, as demonstrated by the fact that Δ^9 -THC did not affect the proliferation of lymph node-derived T cells upon stimulation with anti-CD3 antibody or PHA [296]. Another study reported that Δ^9 -THC enhanced mitogen-induced proliferation of mouse splenocytes or human lymphocytes at low concentration (< 3.2 μ M), and was suppressive at high concentration (6.4 – 25.6 μ M) [295]. In addition, the magnitude of stimulation plays an important role in Δ^9 -THC-mediated modulation of mouse splenic T cell proliferation, in which Δ^9 -THC decreased or increased the percentage of proliferating CD8⁺ T cells under condition where CD8⁺ T proliferation was high or

low, respectively [299]. CB1 and CB2 did not play a role in Δ^9 -THC-mediated suppression of PHA-induced lymphoproliferative response in mouse [294].

Activation of helper $CD4^+$ T cells induces expression of many co-stimulatory molecules as well as cytokines [300]. Cytokines derived from helper $CD4^+$ T cells are classified into 2 groups, Th1 (e.g. IL-2, IFN- γ and TNF- α) or Th2 (e.g. IL-4, IL-5 and IL-10). Th1 cytokines promote and regulate cell-mediated immunity to protect the host against intracellular pathogens; whereas Th2 cytokines promote and regulate humoral immunity which confers protection against extracellular pathogens such as bacteria [300]. Disruption of the Th1/Th2 cytokine balance by Δ^9 -THC was demonstrated in both animal and human models. *In vivo*, a single dose or chronic treatment with Δ^9 -THC in mice resulted in lower serum concentration of IL-2 and IFN- γ [290,301]. Treatment with Δ^9 -THC decreased steady-state levels of mRNA encoding for Th1 cytokines, while increasing mRNA levels for Th2 cytokines in human T cells stimulated with allogeneic dendritic cells [302]. Interestingly, cytokine production by T cells has been shown to be positively or negatively regulated by Δ^9 -THC depending on the magnitude of stimulation. For instance, Δ^9 -THC suppressed both IL-2 and IFN- γ production by mouse splenocytes induced by optimal stimulation with PMA/Io or anti-CD3/CD28 [299]. In contrast, Δ^9 -THC enhanced IL-2 production by mouse

splenocytes induced by suboptimal stimulation with anti-CD3/CD28 [299]. CB1 and CB2 did not play a role in Δ^9 -THC-mediated suppression of PMA/Io-induced IL-2 and IFN- γ production [294]. In addition, Δ^9 -THC suppressed the expression of inducible co-stimulatory molecules (ICOS) in activated T cells regardless of the mode of cellular activation [303].

The functions of cytotoxic T lymphocytes, which are effector CD8⁺ T cells that play an important role in antiviral and antitumor immune responses, also exhibited marked sensitivity to modulation by Δ^9 -THC. For example, administration of Δ^9 -THC *in vivo* suppressed the cytolytic activity of mouse splenic cytotoxic T lymphocytes to herpes simplex virus type 1-infected cells [304,305]. Δ^9 -THC also decreased the number of CTL effector cells, but not IFN- γ production from CTL induced by *in vitro* allogenic model using tumor cells independently of CB1 and CB2 [306]. Interestingly, CTL responses directed specifically against HIVgp120 antigens were differentially modulated by Δ^9 -THC depending on the magnitude of stimulation [299].

Several contributing mechanisms responsible for immune suppression induced by cannabinoids have been demonstrated. For one, immediate (< 15 min) suppression of intracellular cAMP by Δ^9 -THC have been suggested as a mechanism responsible for cannabinoid-mediated immunosuppression [307]. In contrast, Δ^9 -THC-mediated increase of cAMP at later time points, 2 h after T cell

stimulation, decreased the dephosphorylation of Lck in response to T cell receptor activation, which is necessary for the subsequent initiation of T cell receptor signaling [308]. CB1 and CB2 were shown to be involved in Δ^9 -THC-mediated impairment of proximal TCR signaling, as evidenced by attenuation of the suppressive effect of Δ^9 -THC, in the presence of CB1 and CB2 antagonists [308]. Further, cannabinoids, including Δ^9 -THC, were shown to increase $[Ca^{2+}]_i$ in resting T cells and mouse splenocytes [240,299,309] or further augment Ca^{2+} elevation in activated mouse splenocytes [299]. The elevation of $[Ca^{2+}]_i$ by cannabinoids occurred primarily via influx of extracellular calcium, which was mediated by TRPC1 [309,310]. The specific downstream signaling pathways affected by Δ^9 -THC-mediated Ca^{2+} elevation remain to be elucidated, but likely involve MAPK. Indeed, CBN, another cannabinoid sharing structural similarity to Δ^9 -THC, was shown to suppress the phosphorylation of ERK MAPK [213]. Interestingly, Δ^9 -THC increased $[Ca^{2+}]_i$ independently of CB1 and CB2 [239], T cell activation [240] or when activated the magnitude of stimulation [299]. Additionally, impairment of transcription factors, NFAT, NF κ B, and AP1, by cannabinoids was shown to be a potential mechanism responsible for suppression of both cytokine production and expression of co-stimulatory molecules by activated T cells. For instance, the differential effects of CBD on cytokine production were correlated with effects on NFAT nuclear translocation

[299]. Attenuation of ICOS expression in activated mouse T cells were mediated, at least in part, through impairment of NFAT signaling, as evidenced by decreased NFAT reporter gene activity after treatment with Δ^9 -THC [303]. Impairment of IL-2 production by CBN was attributed, at least in part, to suppression in the DNA binding activity of NF κ B, p65 and c-Rel, in mouse T cells [311].

B. B cells

In general, the majority of attention concerning immune modulation by cannabinoids has been on T cells and cells of the myeloid lineage with investigations of B cells being relatively limited. However, cannabinoids have been shown to modulate B cell function including proliferation and humoral immune response. Similar to T cells, the effect of Δ^9 -THC on B-cell function depends on the specific experimental conditions.

Δ^9 -THC at micromolar concentrations suppressed mouse splenic B cell proliferation induced by polyclonal B cell activator, lipopolysaccharide (LPS) [312]. Interestingly, LPS-induced B cell proliferation is more sensitive to suppression by Δ^9 -THC than ConA- or PHA-induced T cell proliferation in mouse splenocytes [312]. In addition, Δ^9 -THC at nanomolar concentrations enhanced human B-cell proliferation induced by cross-linking of surface Igs or ligation of the CD40. This enhancement was not mediated by CB1, as a CB1 antagonist did not reverse the observed effect [313]. Moreover, CP 55,940 enhanced the

proliferation of both naïve and GC B cells induced by the ligation of CD40. This enhancement was blocked by the CB2 receptor antagonist, but not by the CB1 receptor antagonist supporting an involvement of CB2 receptors during B cell differentiation [314].

The effect of cannabinoids on humoral immune responses, an effector function of B cells, has been extensively characterized, of which the primary humoral immune responses against T-cell dependent antigens, sheep red blood cells (sRBC), is one of the most sensitive immune responses to suppression by cannabinoids [298,315]. These initial studies suggested that T cells are more sensitive target to modulation by cannabinoids than B cells, and are also primarily responsible for Δ^9 -THC-mediated suppression of IgM response against sRBC. Importantly, Δ^9 -THC was found not to suppress the number of IgM antibody forming cells induced either by T-cell independent antigen, DNP-Ficoll, or the polyclonal B cell activator, LPS [298]. However, subsequent study demonstrated that B cells can be modulated by Δ^9 -THC in response to ligation of CD40 in the presence of cytokines, IL-2, -6, and -10 (CD40L plus cytokine) [294]. Additionally, studies in humans emphasized that humoral immune responses, either primary or secondary, are sensitive to modulation by cannabinoids, although there is controversy concerning which immunoglobulin isotypes. The first study showed that chronic marijuana smokers had normal serum IgG, IgA, and IgM levels, although IgE levels were increased [316]. In contrast, a subsequent study showed that chronic marijuana use was associated with a

decrease in serum IgG, an increase in serum IgD, but no change in serum IgA and IgM [317]. One additional study demonstrated that oral ingestion of marijuana decreased serum IgG and IgM levels [318].

The involvement of CB1/CB2 in modulation of primary humoral immunity by Δ^9 -THC depends on the types of stimuli. Δ^9 -THC-mediated suppression of IgM responses induced by either sRBC *in vivo* or by CD40L plus cytokine *in vitro* was dependent of CB1/CB2 [294]. Interestingly, LPS-induced IgM responses were refractory to suppression by Δ^9 -THC, regardless of genotype [294]. It is noteworthy that the expression of CB2 was modulated during B cell differentiation from naïve to memory B cells. The lowest expression of CB2 at both the protein and mRNA level was observed in GC proliferating B cells [314]. Moreover, CB2-deficient mice (CB2^{-/-}) have defect in T cell-independent humoral immune response; however, the authors did not include T cell-dependent antigens [319]. Collectively, these findings strongly supported an involvement of CB2 during B cell differentiation.

The molecular mechanisms responsible for Δ^9 -THC-mediated modulation of B-cell function remains unclear in spite of a number of signaling pathways in B cells being identified to be altered in the presence of cannabinoids. For example, Δ^9 -THC suppressed adenylate cyclase activity in B cells, which presumably is due to negative coupling of CB1 and CB2 to this membrane associated enzyme. Moreover, treatment with the membrane-permeable cAMP analogs (dibutyryl-

cAMP and 8-bromo-cAMP) reversed Δ^9 -THC-mediated suppression of the anti-sRBC IgM response. These finding suggested that suppression of T-cell dependent IgM response by Δ^9 -THC was mediated, at least in part, through impairment of adenylate cyclase [311].

IX. Rationale

In addition to the fact that marijuana is the most commonly used illegal drug in the United States, the Food and Drug Administration has approved a synthetic Δ^9 -THC (Marinol[®]) for treatment of chemotherapy-induced nausea and vomiting in cancer patients as well as for treatment of weight loss in AIDS patients [169]. This raises concerns about: 1) the human health impact as demonstrated by *in vivo* and *in vitro* studies suggesting cannabinoids can influence regulation of the immune system; and 2) undesirable immunosuppressive side effect(s) of these drugs in patients whose immune system has already been compromised.

As described earlier, Δ^9 -THC has been demonstrated to modulate a variety of immune responses, of which the primary IgM response against the T cell-dependent antigen, sheep erythrocytes (sRBC), is one the most sensitive immune response by Δ^9 -THC [294,298]. Importantly, Δ^9 -THC was found not to suppress the number of IgM antibody forming cells induced either by T cell-independent antigen, DNP-Ficoll, or the polyclonal B cell activator, LPS [298].

These initial studies suggested that T cells are a more sensitive targets to modulation by cannabinoids than B cells, and are also primarily responsible for Δ^9 -THC-mediated suppression of IgM response against sRBC. However, a subsequent study demonstrated that B cells can be modulated by Δ^9 -THC in response to ligation of CD40 in the presence of cytokines, IL-2, -6, and -10 in the absence of T cells [294]. This study suggested that B cells are also affected by Δ^9 -THC, but only under the condition that requiring CD40-CD40L interaction.

To date, there is still only a remarkably small set of studies that have assessed the effect of Δ^9 -THC on effector function of both primary human T cells and B cells. Furthermore, the molecular basis of Δ^9 -THC-mediated suppression of T cell-dependent antibody responses remains largely unknown. Therefore, this dissertation research was particularly focused on elucidating, at the molecular level, how Δ^9 -THC suppresses T cell-dependent humoral immune response in humans with emphasis on CD40-CD40L interaction. Taken together, the overarching hypothesis of this dissertation was Δ^9 -THC attenuates the human T cell-dependent IgM antibody response by suppression of CD40L upregulation in activated CD4⁺ T cells and impairment of the CD40-mediated B cell activation. To test this hypothesis, three studies were performed; of which the first two studies focused on T cells, whereas the third study focused on B cells. The first subhypothesis was Δ^9 -THC suppresses CD40L expression by activated primary

mouse CD4⁺ T cells in CB1/CB2-dependent mechanism. The second subhypothesis was Δ^9 -THC suppresses CD40L expression in activated primary human CD4⁺ T cells by impairment of NFAT and NF κ B activity. The third subhypothesis was attenuation of the primary IgM response by Δ^9 -THC in human is mediated by impairment of CD40-induced activation of B cells. An understanding of the mechanisms by which Δ^9 -THC affects the function of human T cells and B cells might allow its expansion for therapeutic use and also provide additional information when making risk to benefit decisions for use in immunocompromised patients.

MATERIALS AND METHODS

I. Reagents

Δ^9 -THC dissolved in 100% ethanol was provided by the National Institute on Drug Abuse (Bethesda, MD) and was used in the studies focused on T cells. Preliminary data demonstrated that human B cells are very sensitive to ethanol (Figure 6). Therefore, Δ^9 -THC in this study was dissolved in 100% dimethyl sulfoxide (DMSO) for human B cell studies. Unless otherwise noted, all other chemicals were obtained from Sigma-Aldrich (St Louis, MO).

II. Plasmids

GRE-luciferase reporter gene plasmid (pGRE-luc) and the vector control plasmid (pTAL-luc) were purchased from Clontech (Mountain View, CA).

III. Cell culture

HEK293T were purchased from Open Biosystems and maintained in DMEM media (Gibco Invitrogen, Carlsbad, CA) supplemented with 5% Charcoal/Dextran Treated Fetal Bovine Serum (CD-FBS, Hyclone, Logan, UT), 1X HT supplement (Gibco Invitrogen), and the antibiotics Penicillin (100 units/mL)/Streptomycin (100 μ g/mL) (Gibco Invitrogen). Jurkat T cells (clone E6-1) were obtained from American Type Culture Collection (Manassas, VA) and

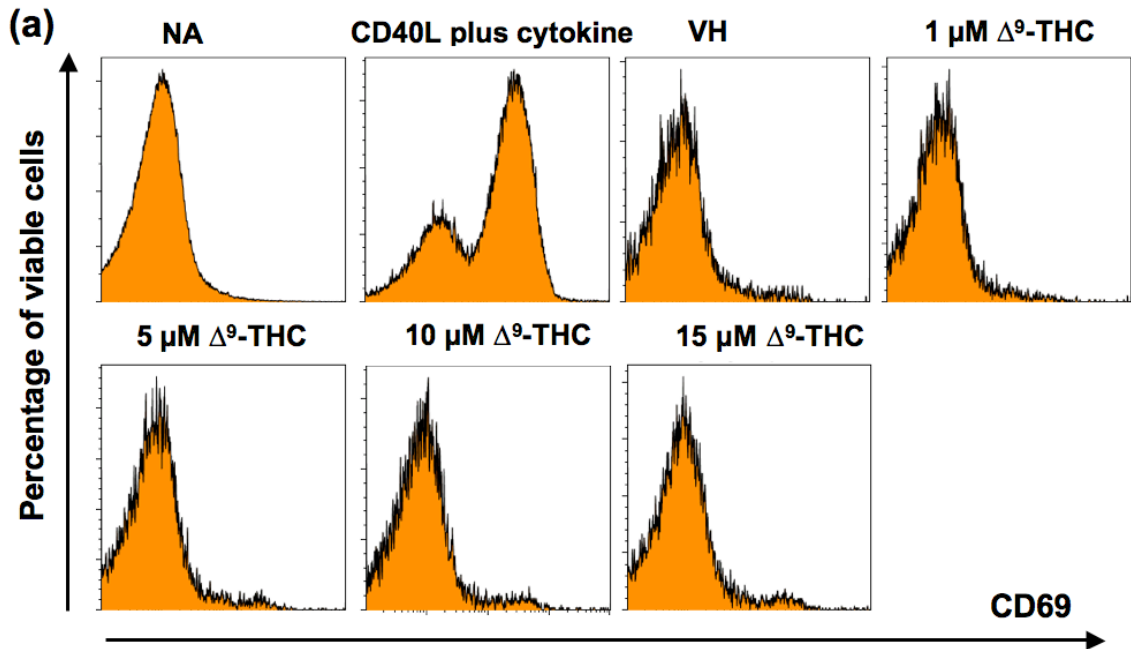
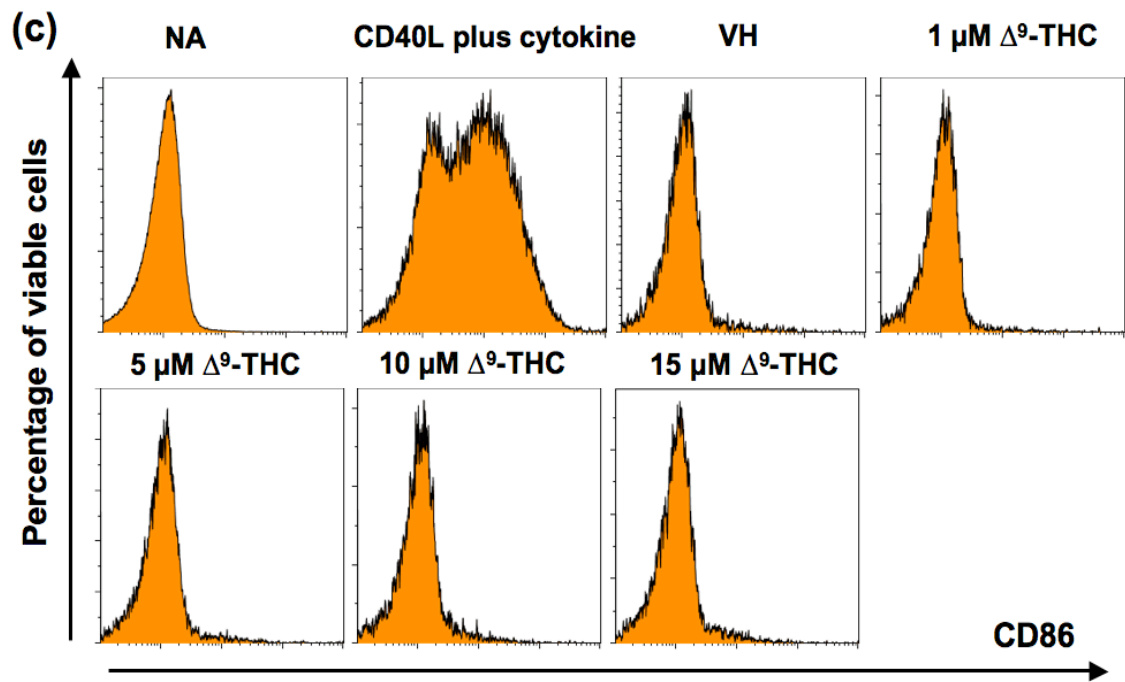
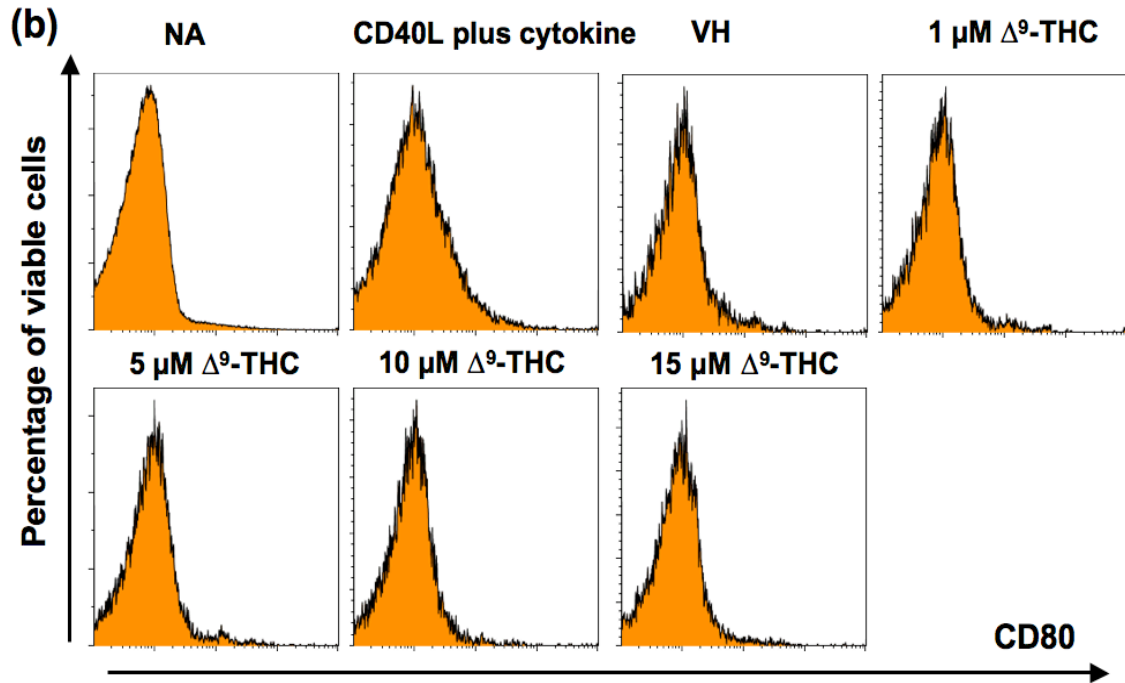


Figure 6. The demonstration that human B cells are sensitive to ethanol.

Studies characterize the effect of $\Delta^9\text{-THC}$ demonstrated that 0.01% ethanol (VH in T cell studies) suppressed B cell activation assessed by measuring the surface expression of CD80, CD86, and CD69 (a-c). These results suggested that primary human B cells are sensitive to EtOH treatment. HPB naïve B cells (1×10^6 cells) were treated with $\Delta^9\text{-THC}$ at indicated concentrations or VH (0.1% Ethanol) for 30 min and then cultured with irradiated CD40L -L cells (1.5×10^3 cells/well) in the presence of recombinant human IL-2 (10 U/mL), IL-6 (100 U/mL), and IL-10 (20 ng/mL). Surface expression of B-cell activation markers induced by CD40L plus cytokine were assessed by flow cytometric analysis on day 3 for expression of CD80, CD86, and CD69. Dead cells were identified by staining with Live/Dead near-infrared Staining Kit, and are excluded. (a) Histogram plot represents the mean fluorescent intensity of CD69. (b) Histogram plot represents the mean fluorescent intensity of CD80. (c) Histogram plot represents the mean fluorescent intensity of CD86. Results represent three different donors with three replicates per treatment group.

Figure 6 (cont'd)



maintained in RPMI media (Gibco Invitrogen, Carlsbad, CA) supplemented with 5% CD-FBS, 1X non-essential amino acids (Gibco Invitrogen), 1X sodium pyruvate (Gibco Invitrogen), and the antibiotics Penicillin (100 units/mL)/Streptomycin (100 μ g/mL). In all cases, cells were cultured at 37°C in 5% CO₂.

The stably transfected mouse fibroblast line expressing human CD40L (CD40L-L cells) was a generous gift from Dr. David Sherr (Boston University School of Public Health) and was prepared for the *in vitro* IgM activation model as previously described [320]. Briefly, CD40L-L cells were cultured in DMEM complete media [Dulbecco's modified Eagle Medium (Gibco Invitrogen, Carlsbad, CA) supplemented with 10% bovine calf serum (Hyclone), 50 μ M 2-mercaptoethanol, 1X HT supplement, and the antibiotics Penicillin (100 units/mL)/Streptomycin (100 μ g/mL)] at 37°C in 5% CO₂ 3-4 days prior to irradiation. CD40L-L cells were trypsinized with 0.25% Trypsin-EDTA (Gibco Invitrogen) for 2-3 min at 37°C. After washing once with complete media, CD40L-L cells were resuspended in 500 μ L complete media and X-ray-irradiated at 3500 Gy (XRAD-320, Precision X-Ray, North Branford, CT). The irradiated CD40L-L cells were washed once with complete media, seeded into 96-well plate at 1.5×10^3 cells/well, and incubated at 37°C in 5% CO₂ 1 day prior to experimentation.

IV. Animals

Female C57BL/6 mice (6 weeks of age) were purchased from Charles River Laboratories (Portage, MI). CB1/CB2 null mice (CB1^{-/-}/CB2^{-/-}) on C57BL/6 background were a generous gift from Dr. Andreas Zimmer (University of Bonn, Germany) and were bred at Michigan State University (MSU). Mice were maintained under specific pathogen-free conditions as previously described [309]. All experimental mouse protocols were reviewed and approved by the Institutional Animal Care and Use Committee at MSU.

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

Human leukocyte packs were obtained commercially from anonymous healthy 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 [320]. PBMCs were cultured at a final concentration of 1×10^6 cells/mL in RPMI complete media [RPMI medium supplemented with 2% bovine calf serum and the antibiotics Penicillin (100 units/mL)/Streptomycin (100 μ g/mL)] at 37°C in 5% CO₂.

VI. Isolation of human peripheral blood (HPB) 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.

VII. Isolation of HPB naïve B cells

Negative selection of human naïve B cells were isolated from human PBMCs using MACS Naïve Human B Cells Isolation Kits following the manufacturer's protocol (Miltenyl Biotec, Auburn, CA) and have been described previously [320]. HPB naïve B cells were maintained in RPMI complete media [RPMI medium supplemented with 10% heat inactivated bovine calf serum (Hyclone), and the antibiotics Penicillin (100 units/mL)/Streptomycin (100 µg/mL)] at 37°C in 5% CO₂.

VIII. Mouse T cell activation

Spleens were aseptically isolated and made into single-cell suspensions in RPMI media (Gibco Invitrogen, Carlsbad, CA) supplemented with either 2% bovine calf serum or 5% CD-FBS, 50 μ M 2-mercaptoethanol, and the antibiotics Penicillin (100 units/mL)/Streptomycin (100 μ g/mL). Splenocytes, 5×10^6 cells/mL, were cultured in 48-well plates at 37°C in 5% CO₂. Cells were stimulated with 40 nM PMA plus 0.5 μ M ionomycin (PMA/Io) or with 1 μ g/mL immobilized anti-mouse CD3 (clone 145-2C11; BD Pharmingen, San Diego, CA) plus 1 μ g/mL soluble anti-mouse CD28 (clone 37.51; BD Pharmingen) antibodies (anti-CD3/CD28) in the presence or absence of Δ^9 -THC. The immobilized CD3 was obtained by precoated 48-well plates (100 μ L/well) with 1 μ g/mL anti-mouse CD3 overnight at 4°C. Before addition of the cells, plates were washed twice with 1X RPMI followed by addition of 10 μ g/mL anti-CD28 (100 μ L/well). Stock solutions of Δ^9 -THC, 0.1 μ M cyclosporin A (CsA) and 0.5 μ M dexamethasone (DEX) were used at a final EtOH concentration of 0.1% and were added 30 min before T cell stimulation. 0.1% EtOH was used as a vehicle control (VH).

IX. Human TCR activation

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

(clone UCHT1; BD Pharmingen, San Diego, CA) and anti-CD28 (clone 28.2; BD Pharmingen, San Diego, CA), 10 $\mu\text{g/mL}$ each, for 15 min on ice. Subsequently, cells were washed once with cold 1X Hank's Balanced Salt Solution (HBSS, Invitrogen) and centrifuged at 350xg for 5 min at 4°C. After discarding the supernatant, cells were resuspended in complete media followed by incubation with 10 $\mu\text{g/mL}$ IgG crosslinker (purified polyclonal goat anti-mouse IgG, BD Pharmingen, San Diego, CA) 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 plus soluble anti-CD28. 96-well plates were precoated with 100 μL /well of anti-CD3 (5 $\mu\text{g/mL}$) overnight at 4°C. Before addition of the cells, plates were washed twice with 1X RPMI followed by addition of 5 $\mu\text{g/mL}$ anti-CD28 (20 μL /well). Cells were activated in the presence or absence of Δ^9 -THC. Stock solutions of Δ^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). Δ^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.

X. Flow cytometry analysis

At the indicated time points, 0.5 to 1×10^6 cells were harvested and washed once with FACS buffer [1X HBSS containing 1% bovine serum albumin (Calbiochem, San Diego, CA) and 0.1% sodium azide, pH 7.4-7.6]. When necessary, dead cells were detected by staining with Live/Dead Fixable Dead Cell Stain Kit (near-infrared dye, Invitrogen). Briefly, cells were washed once with 1X Hank's Balanced Salt Solution (HBSS, Gibco Invitrogen) following by incubation with near-infrared dye for 20 min at 4°C per manufacturer's protocol. Prior staining with respective antibodies (Appendix A), Fc γ III/II receptors on mouse cells were blocked using purified anti-mouse CD16/CD32; whereas Fc receptors on human cells were blocked by incubating with 20% human AB serum (Invitrogen) for 15 min at 4°C. The amount of antibodies used varied in staining for each specific antigen based on preliminary antibody titration and were typically pre-diluted in FACS buffer at appropriate amounts prior to addition to the cells. Staining for phosphorylated kinases was conducted on the same day and immediately followed by flow cytometry analysis. In all cases, stained cells were analyzed using a FACSCanto II cell analyzer (BD Biosciences). Data were analyzed using Kaluza (Bechman Coulter, Miami, FL) or FlowJo software (TreeStar, Ashland, OR).

Cell surface expression of CD40L on mouse CD4⁺ T cells was assessed by simultaneously staining with APC-anti-CD40L and FITC-anti-CD4; whereas cell surface expression of CD40L on human CD4⁺ T cells was assessed by

simultaneously staining with PE-anti-CD40L 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 in CD4⁺ T cells, PBMCs or splenocytes were equilibrated at 37°C in 5% CO₂ for 3 h to normalize baseline kinase activation prior to pretreatment with Δ⁹-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 600xg 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. The unbound IgG crosslinker in human samples were blocked by incubating with 15 μg/mL mouse IgG (Invitrogen). 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.

For staining of intracellular phosphorylated kinases in B cells, HPB naïve B cells were equilibrated at 37°C in 5% CO₂ for 3 h to normalize baseline kinase activity. Cells were then pretreated with VH or Δ^9 -THC for 30 min, followed by activation with recombinant CD40L (Enzo Life Sciences, Inc, Farmingdale, NY) plus IL-2, -6, and -10 for 10 min at 37°C in a water bath. Cells were fixed in 1.5% formaldehyde by directly diluting in cell culture from 32% stock (electron microscopy grade, Electron Microscopy Sciences, Hartfield, PA) for 10 min at 37°C followed by centrifugation at 600xg for 6 min at 4°C. Cells were then permeabilized by drop-wise adding ice-cold 100% methanol while vortex mixing at medium speed and were stored in methanol at -80°C until ready to stain with indicated antibodies. Cells were washed 3 times with FACS buffer. The level of phosphorylated STAT3 (pSTAT3), and phosphorylated p65 (pp65) was assessed by simultaneously staining with Alexa Fluor 647 Mouse Anti-STAT3 and Alexa Fluor 647 Mouse Anti-NF κ B p65 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.

XI. Real Time Polymerase Chain Reaction

Total RNA was isolated from activated mouse splenocytes using TRI Reagent, and isolated from activated human PBMC or activated human B cells

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 target genes were purchased from Applied Biosystems (Appendix B). The relative steady-state levels of target mRNA were determined using ABI PRISM[®] 7900HT Sequence Detection System (Applied Biosystems). The fold change value of relative steady-state mRNA levels of target gene were calculated using the $\Delta\Delta$ -Ct method as described previously [321] and normalized to the endogenous reference, 18s rRNA.

XII. Transient transfection assay

HEK293T, 1×10^6 cells, were cultured in 6-well plates at 37° C in 5% CO₂ overnight before transfection. Cells were incubated with transfection reagents [1 μ g of plasmid and 10 μ L of Lipofectamine 2000 (Invitrogen)] for 3 h. The transfected HEK293T cells were treated with either Δ^9 -THC or DEX alone and the combination of Δ^9 -THC and DEX. 24 h after treatment, luciferase activity was assayed using the Promega luciferase assay system according to the manufacturer's protocol (Promega). Fluorescence was measured using the BioTek Synergy[™] HT autoreader. Data were analyzed using KC4 software (BioTek Instruments, Highland Park, VT) and presented as count per second

(CPS) normalized to total protein. Protein determinations were performed using a Bicinchoninic Acid Assay.

XIII. Nuclear protein isolation

Human PBMCs (approximately 2×10^7 cells/mL) were stimulated with anti-CD3/CD28 in the presence or absence of Δ^9 -THC for 16 h. PBMCs were collected and washed once with PBS. Nuclear proteins was isolated as previously described with some modifications [213]. 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 $\mu\text{g/mL}$ aprotinin, and 0.2 $\mu\text{g/mL}$ leupeptin) for 15 min on ice. Nuclei were pelleted by centrifugation at 1000xg 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 17500xg 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.

XIV. 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 [322]. 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' [323] and NF κ B probe, 5'-TGAGGTAGGGATTTCCACAGCTG-3' [80]. The corresponding binding site for each transcription factor is underlined.

XV. Calcium determination

Purified naïve CD4⁺ T cells (1×10^6 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 Ca^{2+} indicator dyes (Invitrogen), Fura-3 ($2\ \mu\text{M}$) and Fluo-red ($5\ \mu\text{M}$), for 60 min at room temperature prior to pretreatment with Δ^9 -THC or VH (2×10^6 cells per treatment group). T cells were activated by incubation with anti-CD3 and anti-CD28 for 15 min on ice ($10\ \mu\text{g/mL}$ each). Excess antibodies against CD3 and CD28 were removed by washing once with complete RPMI. Cells were then resuspended in complete media containing Ca^{2+} dyes and Δ^9 -THC or VH. The intracellular Ca^{2+} measurement was 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 ($10\ \mu\text{g}$) 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 Ca^{2+}) versus time.

XVI. *In vitro* CD40L-dependent polyclonal IgM antibody response

HPB naïve B cells (1×10^6 cells/mL) were pretreated with Δ^9 -THC or vehicle (0.02% DMSO) for 30 min prior inducing primary IgM response *in vitro* as

previously described [320]. Briefly, pretreated HPB naïve B cells (1.5×10^5 cells) were co-cultured with irradiated CD40L-L cells preseeded in 96-well plate. The culture was supplemented with 10 U/mL of recombinant human IL-2 (Roche Applied Science, Indianapolis, IN, USA), 100 U/mL of recombinant human IL-6 (Roche Applied Science, Indianapolis, IN, USA), and 20 ng/mL of recombinant IL-10 (BioVision, Inc., Milpitas, CA). After 4 days of culture, B cells were transferred to a new 96-well plate without CD40L-L cells, and were cultured for an additional 3 days. The cells were harvested for IgM ELISPOT. The cell viability was determined by the pronase activity assay as described previously [298].

XVII. IgM ELISPOT

The number of IgM secreting cells were determined by IgM ELISPOT as previously described [320]. Briefly, ELISPOT wells were coated with purified anti-human IgM antibody (BD Biosciences) overnight at 4°C. Plates were washed with phosphate buffered saline (PBS) containing 0.1% Tween-20 and water. ELISPOT wells were then blocked with PBS containing 5% bovine serum albumin (BSA; Calbiochem, San Diego, CA) for 30 min at 37°C. Harvested cells were washed, diluted to the appropriate density, and incubated in the ELISPOT wells for 16-20 h at 37°C in 5% CO₂. Cells were removed from the wells and plates were washed with PBS containing 0.1% Tween-20 and water. For detection, Biotin-conjugated anti-human IgM antibody and streptavidin-

horseradish peroxidase were added to the wells. The spots were developed with the aminoethylcarbazole staining kit. Data were collected and analyzed using the CTL ImmunoSpot system (Cellular Technology Ltd, Shaker Heights, OH).

XVIII. Proliferation assay

The B cell proliferation studies were conducted as described previously [320]. In brief, HPB naïve B cells were labeled by incubation with 5 μ M of carboxyfluorescein succinimidyl ester (CFSE) (CellTrace Cell Proliferation Kits, Invitrogen) at 5×10^6 cells/mL following manufacturer's protocols. The labeled cells were washed in complete medium, and then adjusted to the desired cell density prior to pretreatment with Δ^9 -THC or vehicle (0.02% DMSO) for 30 min. Cells were then co-cultured with irradiated CD40L-L cells plus IL-2, -6, and -10. After 4 days of culture, B cells were transferred to a new 96-well plate without CD40L-L cells, and were cultured for an additional 3 days. The cells were harvested on day 7 for flow cytometric analysis.

XIX. Statistical analysis

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. The outliers were eliminated using the Grubb's test and only one outlier was allowed per treatment group. A value of $p < 0.05$ was considered significant.

RESULTS

I. Effect of Δ^9 -THC on the upregulation of CD40L on activated mouse CD4⁺ T cells

A. Expression kinetics of CD40L on activated T cells

Expression of CD40L is readily induced on T cells upon activation both *in vivo* and *in vitro* [324-329]. Here the peak time of CD40L expression in response to two different T cell activation stimuli, PMA/Io and anti-CD3/CD28, was defined at both the protein (cell surface expression) and mRNA level in splenic T cells. As shown in figure 7a and 7b, upregulation of surface CD40L by PMA/Io-activated CD4⁺ T cells was detectable as early as 4 h ($23.0 \pm 4.8\%$), maximal at 8 h ($30.0 \pm 3.0\%$), and was declining by 18 h ($19.2 \pm 1.3\%$). In concordance with surface expression, induction of steady-state *CD40L* mRNA levels by PMA/Io was rapid (as early as 2 h post activation), but transient, as evidenced by a decrease as early as 4 h after activation (Figure 7c). Although the kinetic profiles of surface CD40L expression induced by either anti-CD3/CD28 or PMA/Io were similar, the proportion of CD4⁺CD40L⁺ cells induced by PMA/Io was greater than with anti-CD3/CD28. As shown in Figure 8a and 8b, the upregulation of cell surface CD40L on activated CD4⁺ T cells by anti-CD3/CD28 was detectable at 4 h ($10.0 \pm 0.3\%$), maximal at 8 h ($15.2 \pm 0.9\%$), and decreased by 18 h ($9.8 \pm 0.3\%$) after activation. By contrast, the induction of steady-state *CD40L* mRNA levels

by anti-CD3/CD28 had a different profile in which it peaked at 4 h post stimulation and then reached a plateau (Figure 8c).

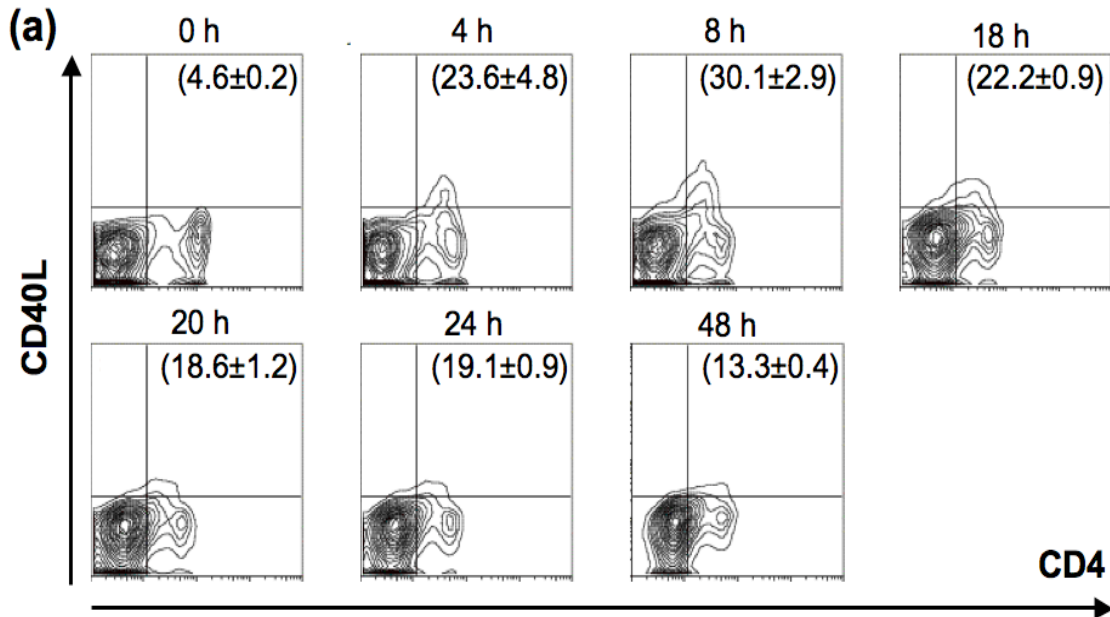
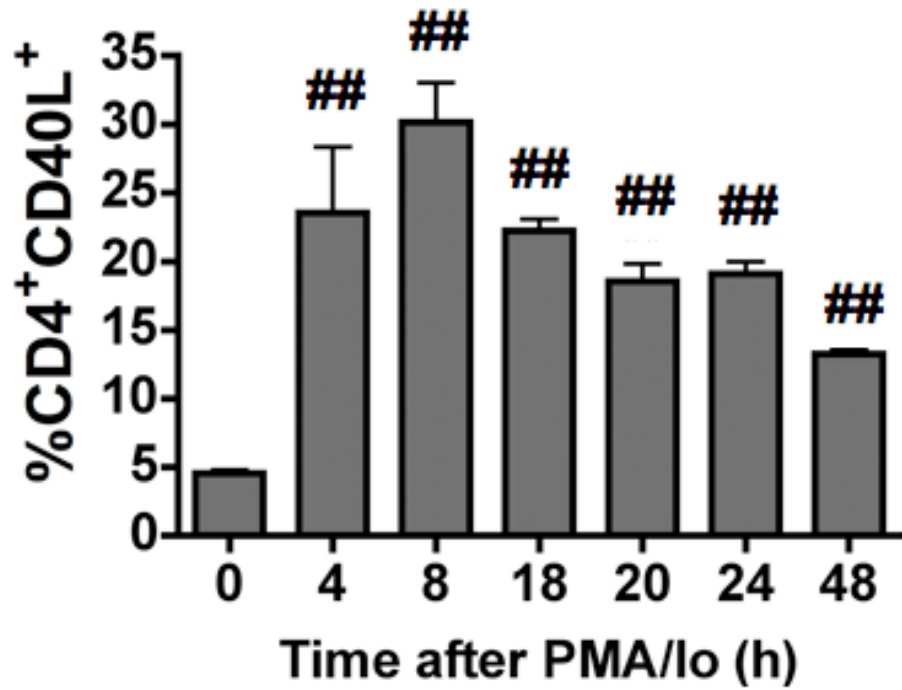


Figure 7. Kinetics of PMA/Io-induced CD40L expression in mouse splenocytes.

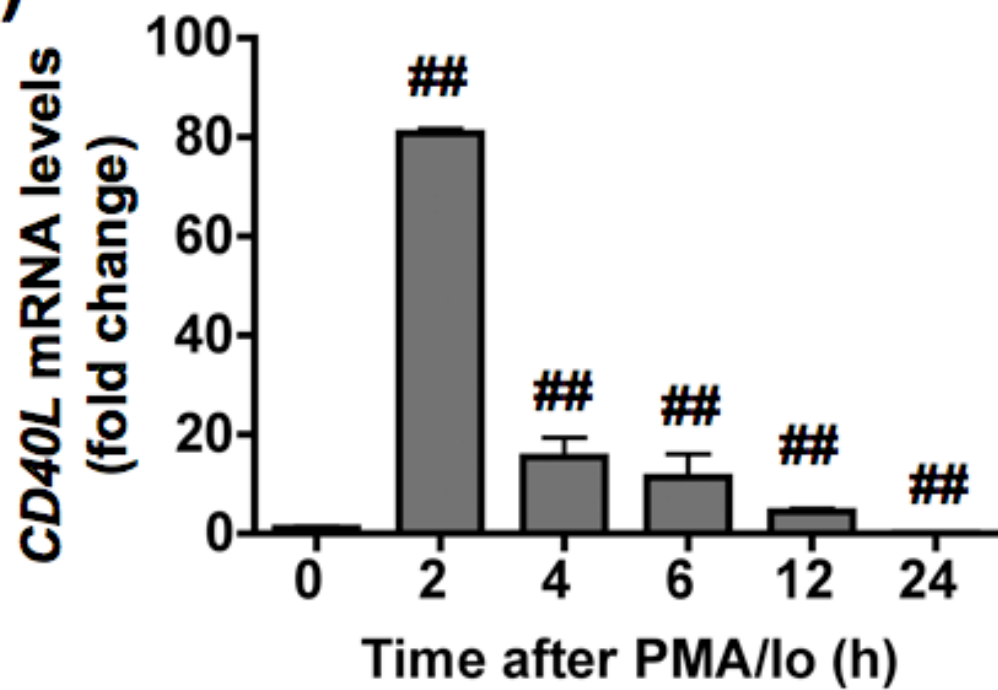
Splenocytes (5×10^6 cells) were stimulated with 40 nM PMA plus 0.5 μ M ionomycin. (a) Cell surface CD40L on activated CD4⁺ T cells was determined at indicated time points by flow cytometry. Numbers in parentheses represent the percentage of CD4⁺CD40L⁺ cell population $\pm$ SEM from triplicates. (b) Bar graph is a representation of data in (a). (c) Steady-state expression of *CD40L* mRNA was determined at indicated time points by real-time PCR. The fold difference of *CD40L* mRNA molecules relative to nonactivated resting cells (naïve, NA at day 0) was normalized using the endogenous reference, 18s rRNA. Results are the mean of triplicates per time point. The ## = $p \leq 0.01$ compared to unstimulated cells. Results represent two separate experiments.

Figure 7 (cont'd)

(b)



(c)



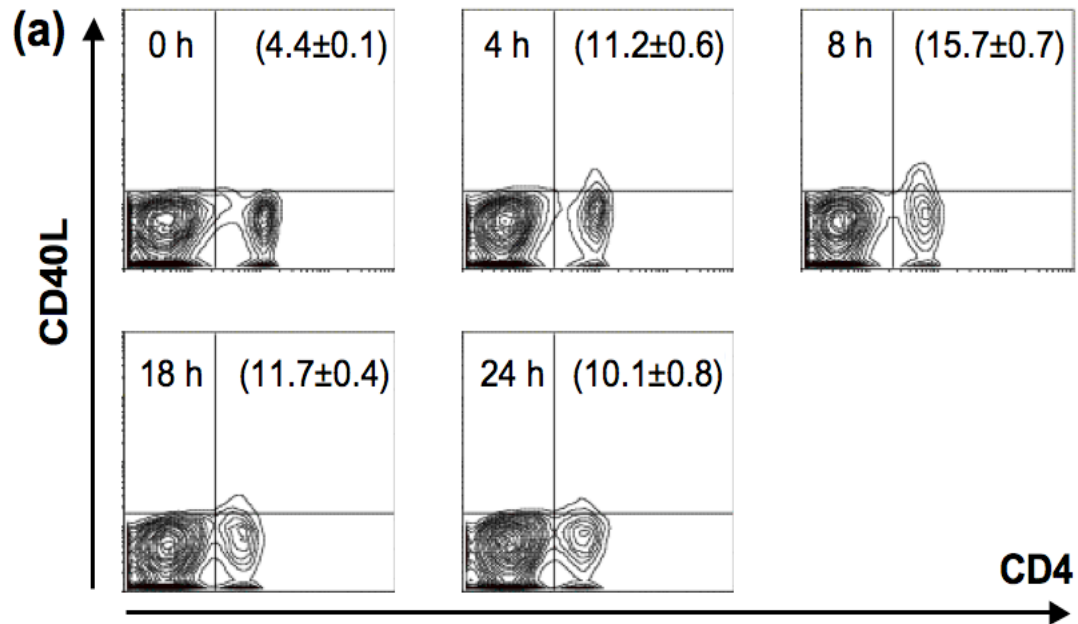
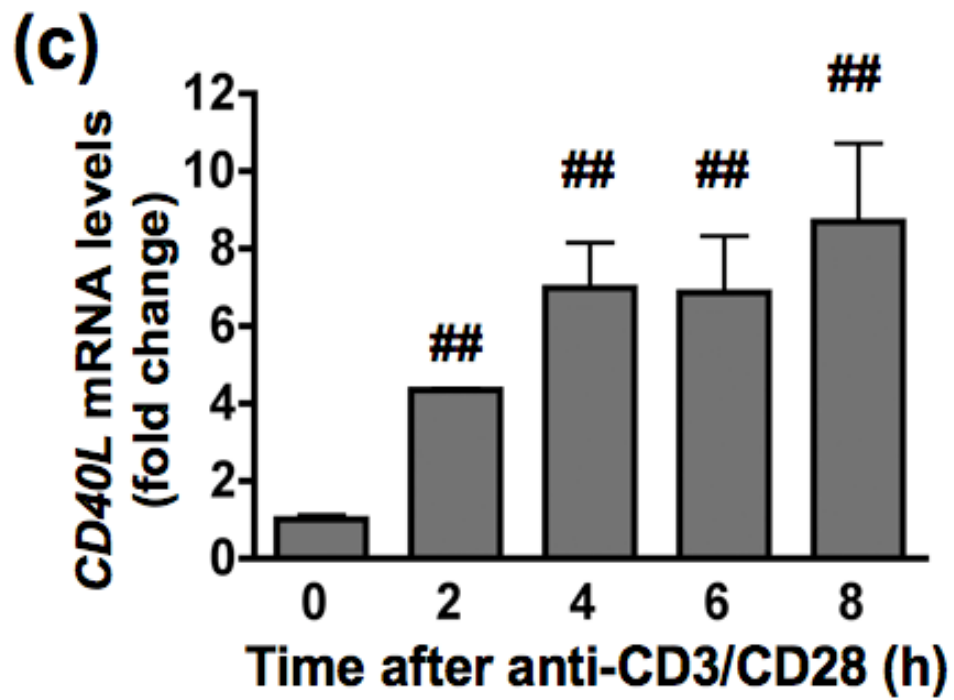
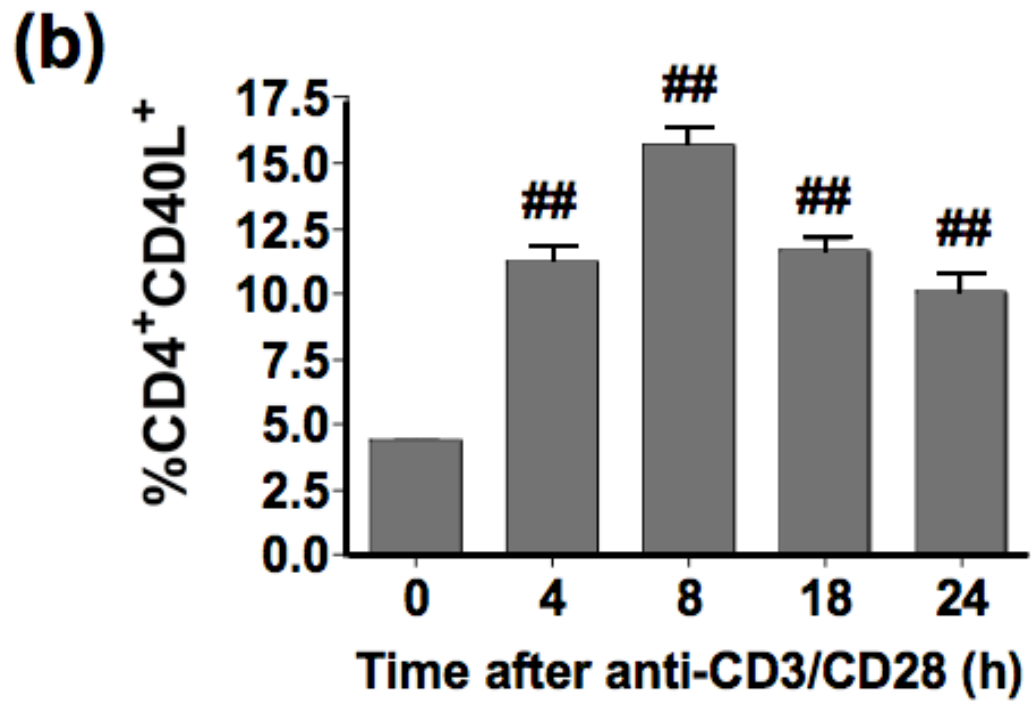


Figure 8. Kinetics of anti-CD3/CD28-induced CD40L expression in mouse splenocytes.

Splenocytes (5×10^6 cells) were stimulated with immobilized anti-CD3 plus soluble anti-CD28 antibodies, $1 \mu\text{g/ml}$ each. (a) Cell surface CD40L on activated CD4⁺ T cells was determined at indicated time points by flow cytometry. Numbers in parentheses represent the percentage of CD4⁺CD40L⁺ cell population $\pm$ SEM from triplicates. (b) Bar graph is a representation of data in (a). (c) Steady-state expression of *CD40L* mRNA was determined at indicated time points by real-time PCR. The fold difference of *CD40L* mRNA molecules relative to nonactivated resting cells (naïve, NA at day 0) was normalized using the endogenous reference, 18s rRNA. Results are the mean of triplicates per time point. The ## = $p \leq 0.01$ compared to unstimulated cells. Results represent two separate experiments.

Figure 8 (cont'd)



B. Differential effects by Δ^9 -THC on CD40L upregulation in response to different T cell activation stimuli

Based on the above kinetic studies, the effects of Δ^9 -THC on CD40L expression levels by CD4⁺ T cells activated with either anti-CD3/CD28 or PMA/Io was investigated at 8 h. CsA was included as a positive control for suppression of T cell activation. As shown in figure 9, Δ^9 -THC suppressed anti-CD3/CD28-induced CD40L surface expression by reducing the number of CD4⁺ T cells expressing CD40L (Figure 9a and 9b), but not the expression level of CD40L on each CD4⁺ T cell (no differences in MFI between treatment groups, Figure 10c). Significant suppression by Δ^9 -THC of CD4⁺CD40L⁺ double positive cells occurred in a concentration-dependent manner and was most pronounced at 10 and 15 μ M Δ^9 -THC. Although splenocytes were used as a source of T cells, we found that enrichment of T cells did not further increase the magnitude of anti-CD3/CD28-induced CD40L surface expression nor affect the sensitivity to Δ^9 -THC (Figure 10). Conversely, Δ^9 -THC treatment did not suppress PMA/Io-induced upregulation of CD40L surface expression on CD4⁺ T cells (Figure 11).

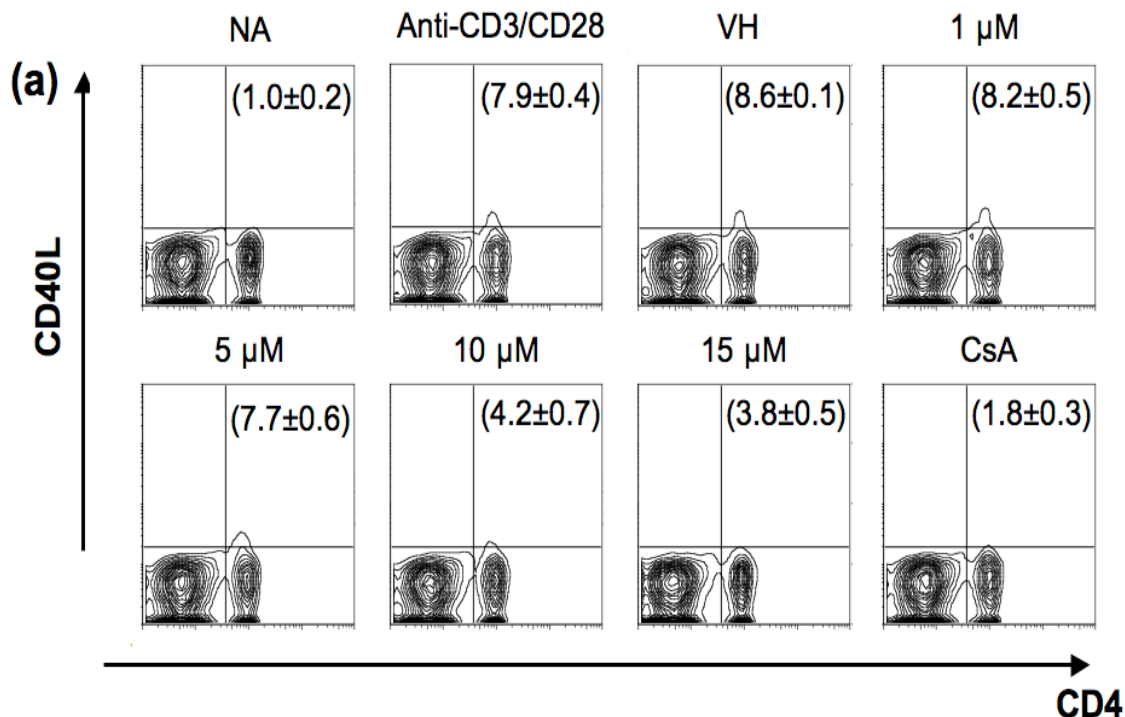
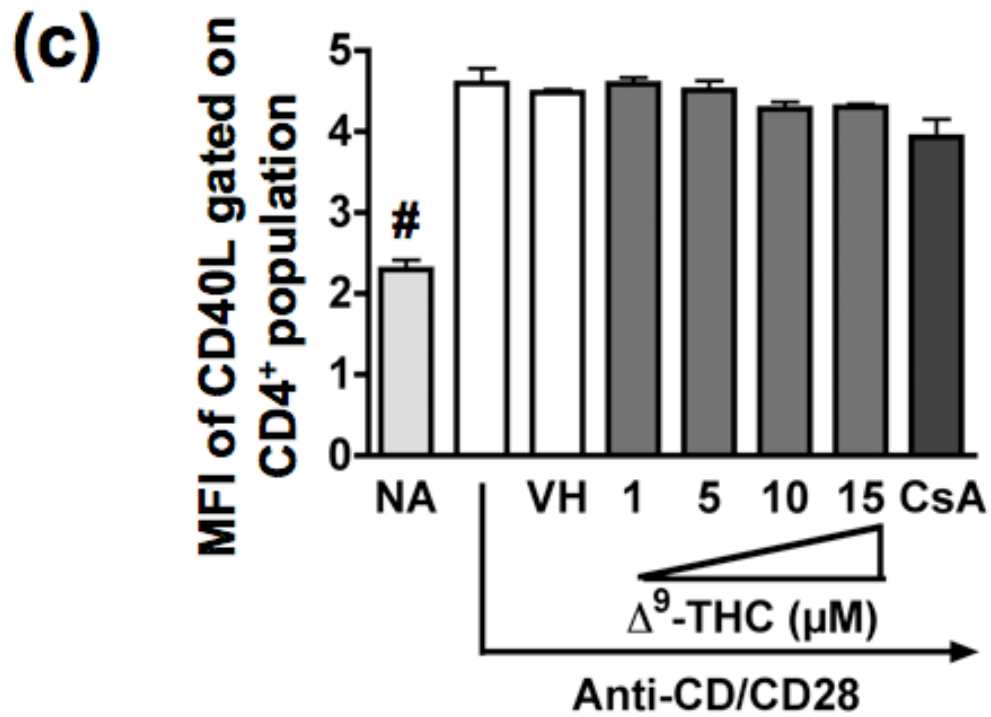
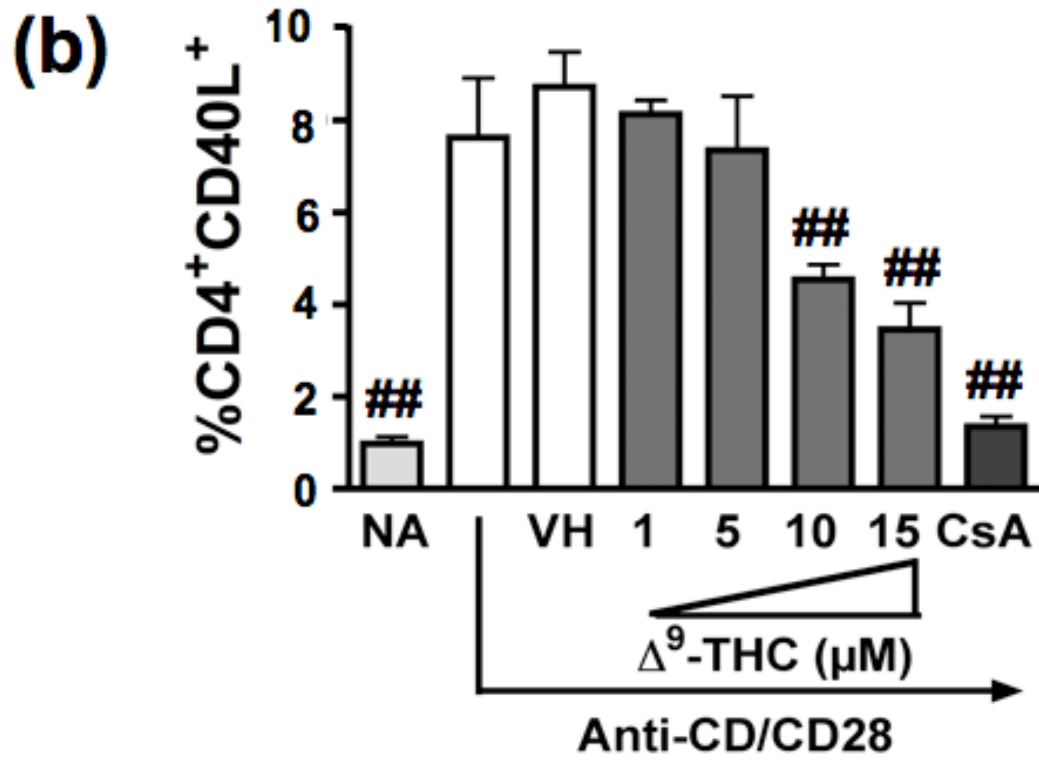


Figure 9. Δ^9 -THC suppresses anti-CD3/CD28-induced surface CD40L expression on activated mouse CD4⁺ T cells.

Splenocytes (5×10^6 cells) were treated with various concentrations of Δ^9 -THC (1, 5, 10, and 15 μ M) for 30 min and then stimulated with immobilized anti-CD3 plus soluble anti-CD28 antibodies (1 μ g/ml each). (a) Cell surface CD40L expression on activated CD4⁺ was determined by flow cytometry 8 h after stimulation. Numbers in parentheses represent the percentage of CD4⁺CD40L⁺ cell population $\pm$ SEM from triplicates. (b) Bar graph is a representation of data in (a). (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 $p \leq 0.05$ or $p \leq 0.01$, respectively. 0.1 μ M CsA was used as the positive control. Results represent two separate experiments with three replicates per treatment group.

Figure 9 (cont'd)



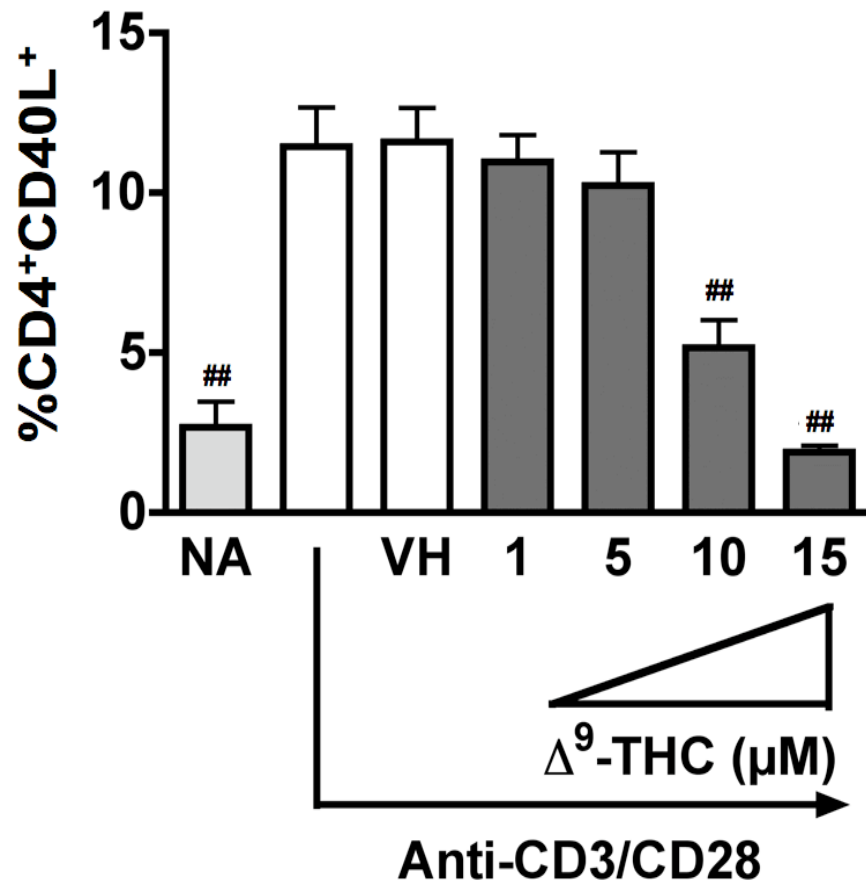


Figure 10. Δ^9 -THC suppresses anti-CD3/CD28-induced surface CD40L expression on enriched mouse CD4⁺ T cells.

Enriched CD4⁺ T cells (1×10^6 cells) were treated with various concentrations of Δ^9 -THC (1, 5, 10, and 15 μ M) for 30 min and then stimulated with immobilized anti-CD3 plus soluble anti-CD28 antibodies (1 μ g/ml each). Cell surface CD40L expression on activated CD4⁺ was determined by flow cytometry 8 h after stimulation. Each bar represents mean of the percentage of CD4⁺CD40L⁺ cell population $\pm$ SEM from triplicates. The # or ## indicates significant effect compared to vehicle control (VH = 0.1% EtOH), at $p \leq 0.05$ or $p \leq 0.01$, respectively. Results represent two separate experiments with three replicates per treatment group.

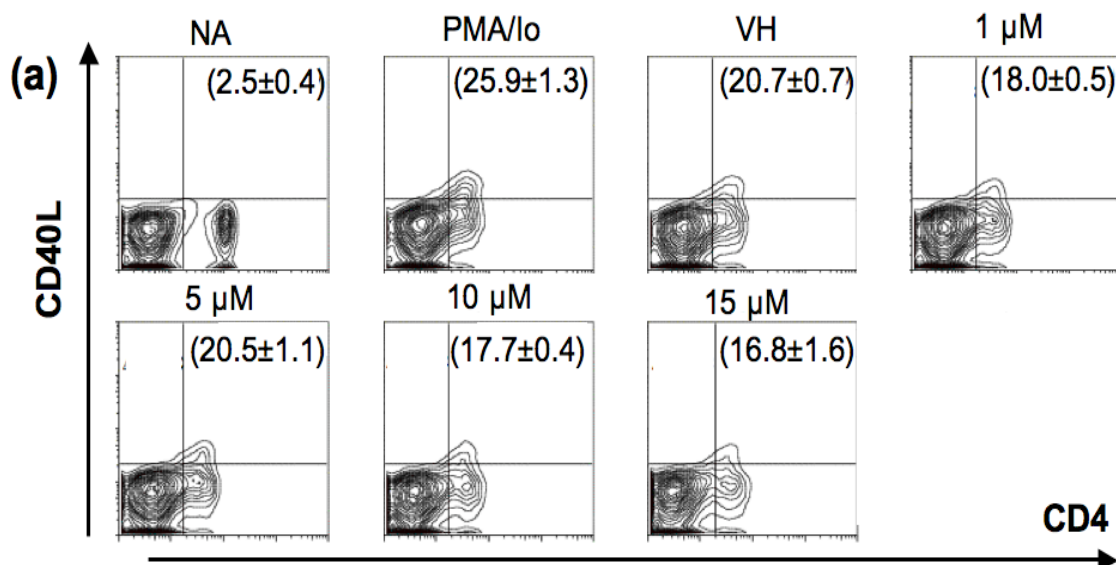
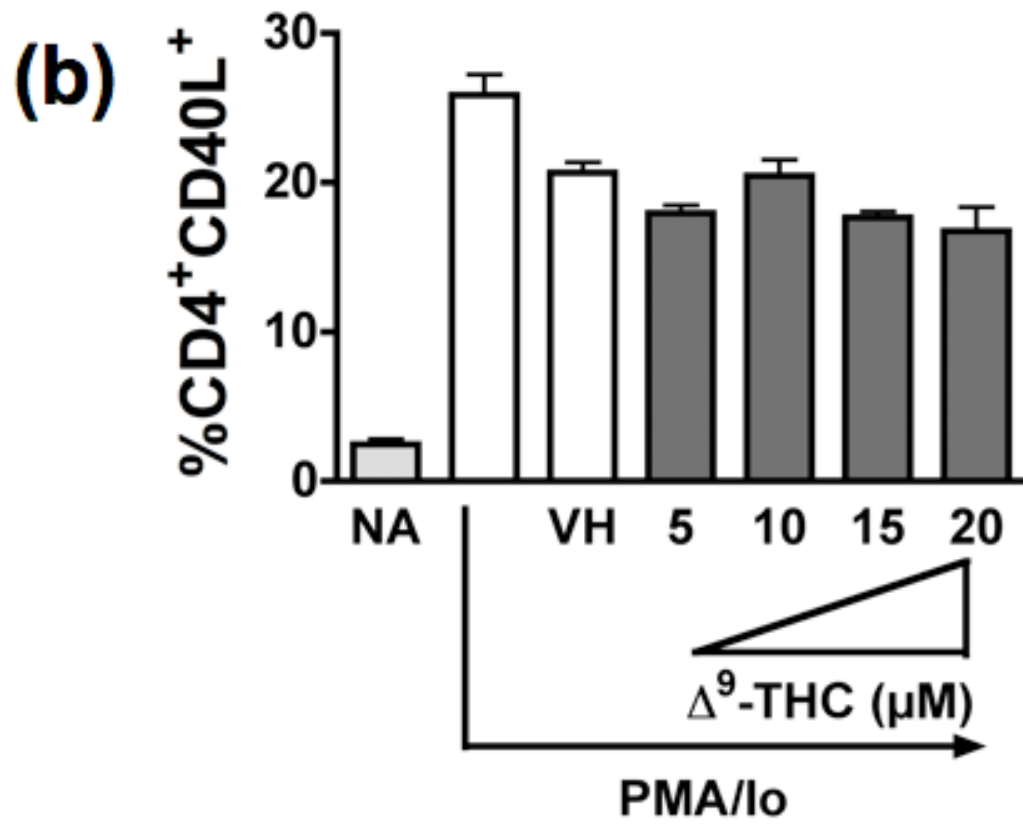


Figure 11. Δ^9 -THC does not suppress PMA/Io-induced surface CD40L expression on activated mouse CD4⁺ T cells.

Splenocytes (5×10^6 cells) were treated with various concentrations of Δ^9 -THC (5, 10, 15, and 20 μ M) for 30 min and then stimulated with 40 nM PMA plus 0.5 μ M ionomycin for 8 h. (a) Cell surface CD40L expression on activated CD4⁺ was determined by flow cytometry. Number in parentheses represents the percentage of CD4⁺CD40L⁺ cell population $\pm$ SEM. (b) Bar graph is a representation of data in (a). Results represent two separate experiments with three replicates per treatment group.

Figure 11 (cont'd)



C. Δ^9 -THC decreases steady-state mRNA levels of *CD40L* induced by anti-CD3/CD28.

The expression of surface CD40L on activated T cells is tightly controlled and occurs at the transcriptional, post-transcriptional, and/or post-translational level. However, it appears to be primarily controlled at the level of transcription and post-transcription (reviewed in [62,69,330]). Therefore the effect of Δ^9 -THC was investigated on the steady-state mRNA levels of *CD40L* induced by anti-CD3/CD28. Splenocytes were treated with increasing concentrations of Δ^9 -THC followed by anti-CD3/CD28 activation for 4 h, the peak time induction of CD40L mRNA. Δ^9 -THC suppressed the induction of *CD40L* mRNA levels by anti-CD3/CD28 in a concentration-dependent manner (Figure 12).

D. CB1 and /or CB2 are not involved in suppression by Δ^9 -THC of anti-CD3/CD28-induced CD40L expression on mouse splenic CD4⁺ T cells.

CB1 and/or CB2 -dependent and -independent mechanisms have been suggested to account for cannabinoid-mediated effects on immune function [294,309]. To investigate the involvement of CB1 and/or CB2 on Δ^9 -THC-mediated suppression of anti-CD3/CD28-induced CD40L expression, comparative studies were performed using splenocytes from C57BL/6 wild type and CB1^{-/-}CB2^{-/-} mice. As shown in figure 13, the absence of CB1 and CB2 did not affect Δ^9 -THC-mediated suppression of either anti-CD3/CD28-induced

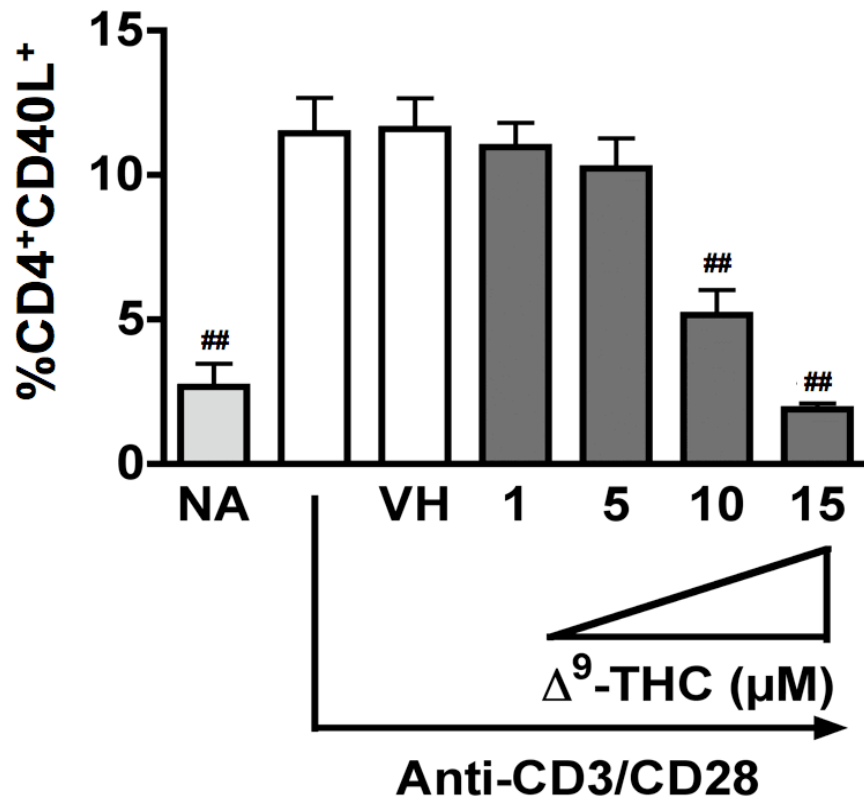


Figure 12. Δ^9 -THC suppresses anti-CD3/CD28-induced *CD40L* mRNA expression in mouse splenocytes.

Splenocytes (5×10^6 cells) were treated with various concentrations of Δ^9 -THC (1, 5, 10, and 15 μ M) for 30 min and then stimulated with immobilized anti-CD3 plus soluble anti-CD28 antibodies, 1 μ g/ml each. Steady-state expression of *CD40L* mRNA was determined by real-time PCR at 4 h after stimulation. The fold difference of *CD40L* mRNA molecules relative to unstimulated cells (naïve, NA) was normalized using the endogenous reference, 18s rRNA and results are the average of triplicates at various concentrations of Δ^9 -THC. The # or ## indicates significant effect compared to vehicle control (VH = 0.1% EtOH) at $p \leq 0.05$ or $p \leq 0.01$, respectively. 0.1 μ M CsA was used as the positive control. Results represent two separate experiments with three replicates per treatment group.

surface CD40L expression in CD4⁺ T cells (Figure 13a) or steady-state *CD40L* mRNA levels (Figure 13b). Although there was an increase of *CD40L* mRNA expression in CD4⁺ T cells derived from C57BL/6 at 5 μ M Δ^9 -THC (Figure 13b), it was not consistent across the experiments.

E. The effects of Δ^9 -THC are not mediated via GR

With the demonstration that Δ^9 -THC-mediated suppression of CD40L protein and mRNA expression was not mediated through CB1 or CB2, subsequent experiments were designed to identify an alternative receptor. One possibility is the GR since it was demonstrated that Δ^9 -THC specifically bound rat hippocampal GR *in vivo* and *in vitro* [331] and activation of GR is well known to be immune suppressive (reviewed in [332]). The initial study focused on the role of GR on CD40L expression. As shown in figure 14, the activation of GR using DEX, a potent GR agonist, suppressed CD40L expression-induced by anti-CD3/CD28. Next the interaction between Δ^9 -THC and GR using a GRE-luc reporter assay in HEK293T cells was investigated. However, treatment with Δ^9 -THC did not affect basal or DEX-induced luciferase activity (Figure 15). In addition, Δ^9 -THC did not affect the expression of *GILZ* or *I κ B* mRNA, which are GRE-dependent genes, in both HEK293T (Figure 16a and 16b) and Jurkat T cells (Figure 17a and 17b).

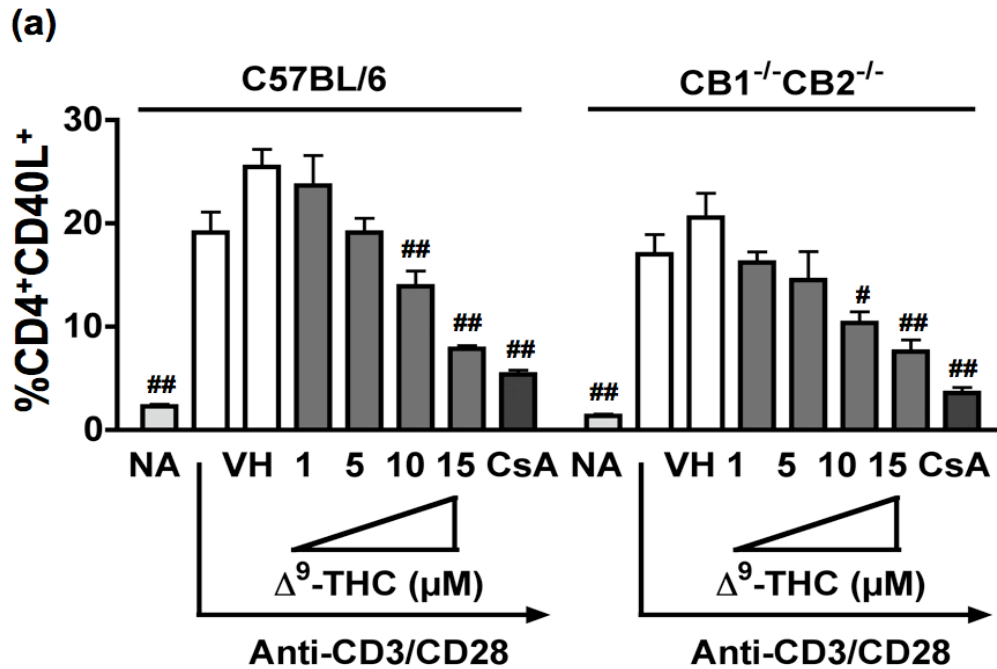
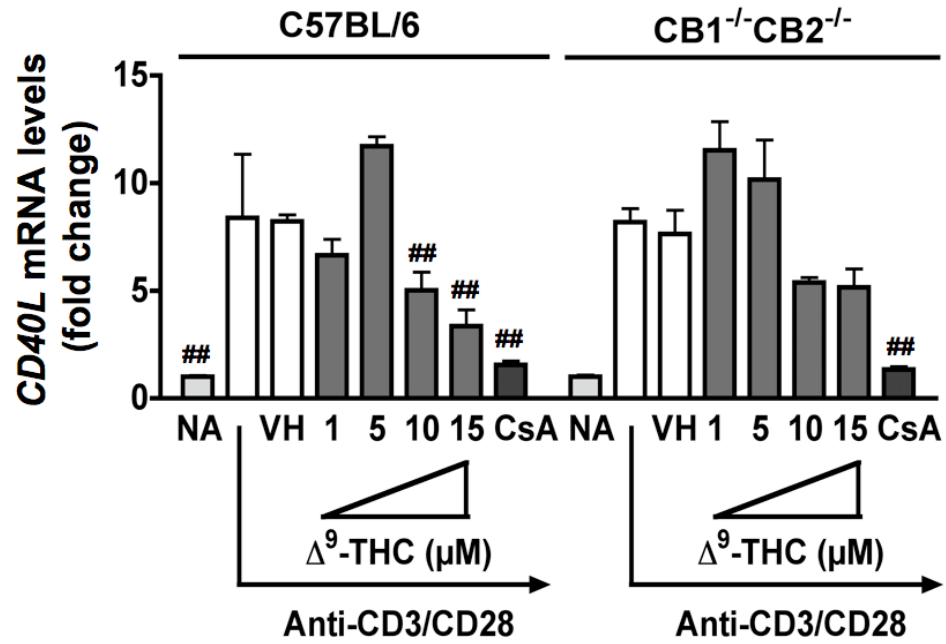


Figure 13. Comparison of the effect of Δ^9 -THC on anti-CD3/CD28-induced CD40L expression in splenic T cells derived from wildtype or CB1^{-/-}CB2^{-/-} mice.

Splenocytes (5×10^6 cells) from wildtype or CB1^{-/-}CB2^{-/-} mice were treated with various concentrations of Δ^9 -THC (1, 5, 10, and 15 μ M) for 30 min and then stimulated with immobilized anti-CD3 plus soluble anti-CD28 antibodies (1 μ g/ml each). (a) Cell surface CD40L expression on activated CD4⁺ was determined by flow cytometry at 8 h after stimulation. The bar graph represents the percentage of CD4⁺CD40L⁺ cell population $\pm$ SEM. (b) Steady-state expression of *CD40L* mRNA was determined by real-time PCR at 4 h after stimulation. The fold difference of *CD40L* mRNA molecules relative to unstimulated cells (naïve, NA) was normalized using the endogenous reference, 18s rRNA and results are the average of triplicates at various concentrations of Δ^9 -THC. The # or ## indicates significant effect compared to vehicle control (VH = 0.1% EtOH), at $p \leq 0.05$ or $p \leq 0.01$, respectively. 0.1 μ M CsA was used as the positive control. Results represent two separate experiments with three replicates per treatment group.

Figure 13 (cont'd)

(b)



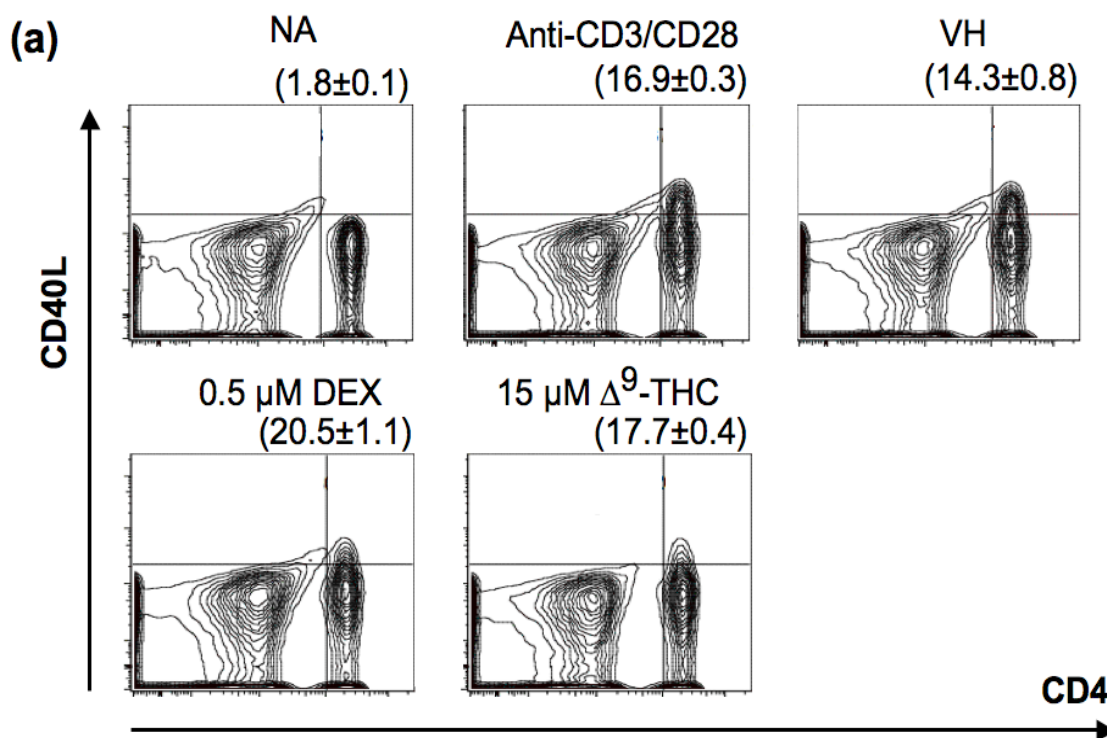
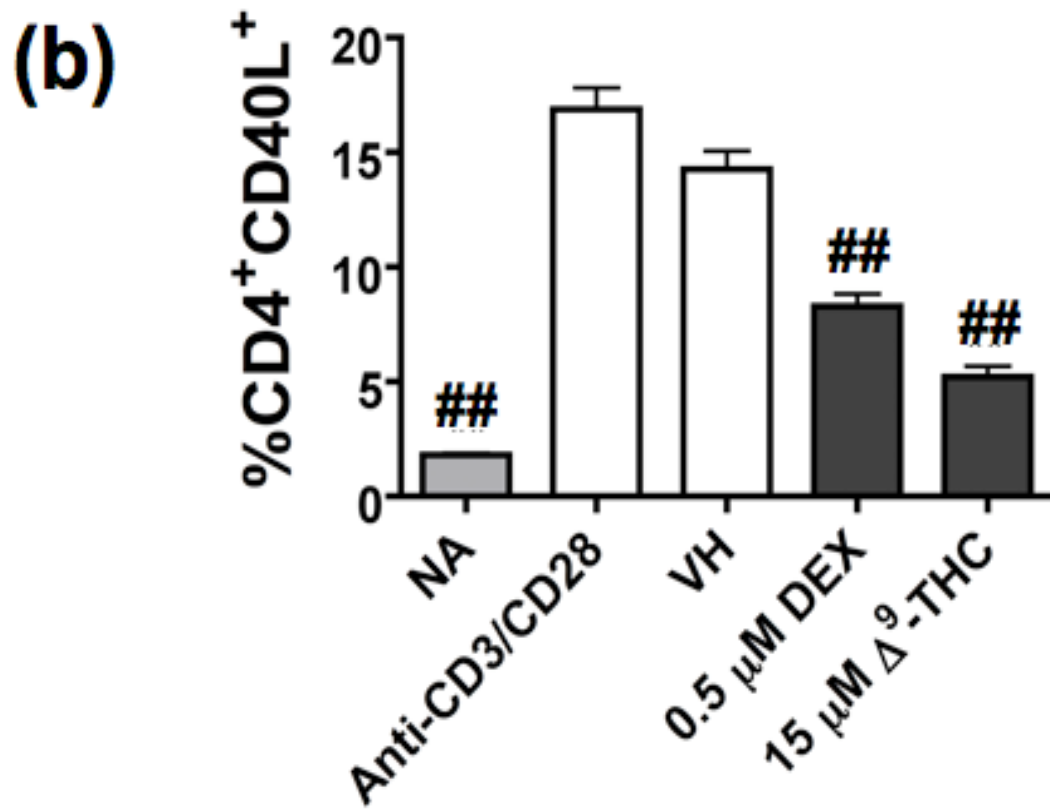


Figure 14. DEX suppresses anti-CD3/CD28-induced surface CD40L expression on activated mouse CD4⁺ T cells.

Splenocytes (5×10^6 cells) were treated with 0.5 μ M DEX or 15 μ M Δ^9 -THC for 30 min and then stimulated with immobilized anti-CD3 plus soluble anti-CD28 antibodies (1 μ g/ml each). (a) Cell surface CD40L expression on activated CD4⁺ was determined by flow cytometry at 8 h after stimulation. Number in parentheses represents the percentage of CD4⁺CD40L⁺ cell population $\pm$ SEM. (b) Bar graph is a representation of data in (a). The ## indicates significant effect compared to vehicle control (VH = 0.1% EtOH), at $p \leq 0.01$. Results represent two separate experiments with three replicates per treatment group.

Figure 14 (cont'd)



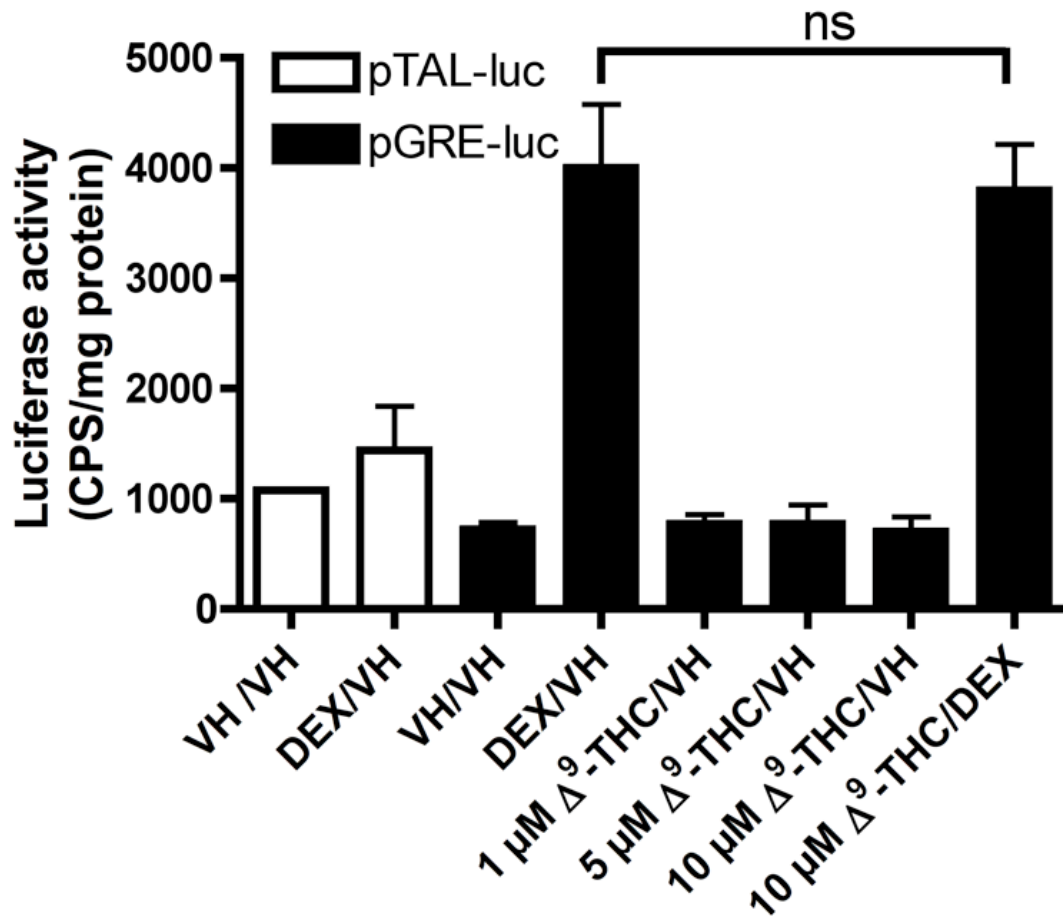


Figure 15. GRE luciferase reporter activity in HEK293T cells treated with Δ^9 -THC and/or DEX.

HEK293T cells (5×10^6 cells) were preseeded in a 6 well plate in growth medium overnight. The cells were then transiently transfected with pGRE-luc or pTAL-luc (vector control plasmid). After transfection, the cells were treated with 0.1% EtOH (VH), 0.5 μ M DEX, different concentration of Δ^9 -THC (1, 5, and 10 μ M), or the combination of 0.5 μ M DEX plus 10 μ M Δ^9 -THC. 24 h after transfection, the luciferase activity was quantified in CPS and normalized to total protein (μ g). The results are the mean+SEM. The “ns” indicates no significant difference. Results represent two separate experiments with three replicates per treatment group.

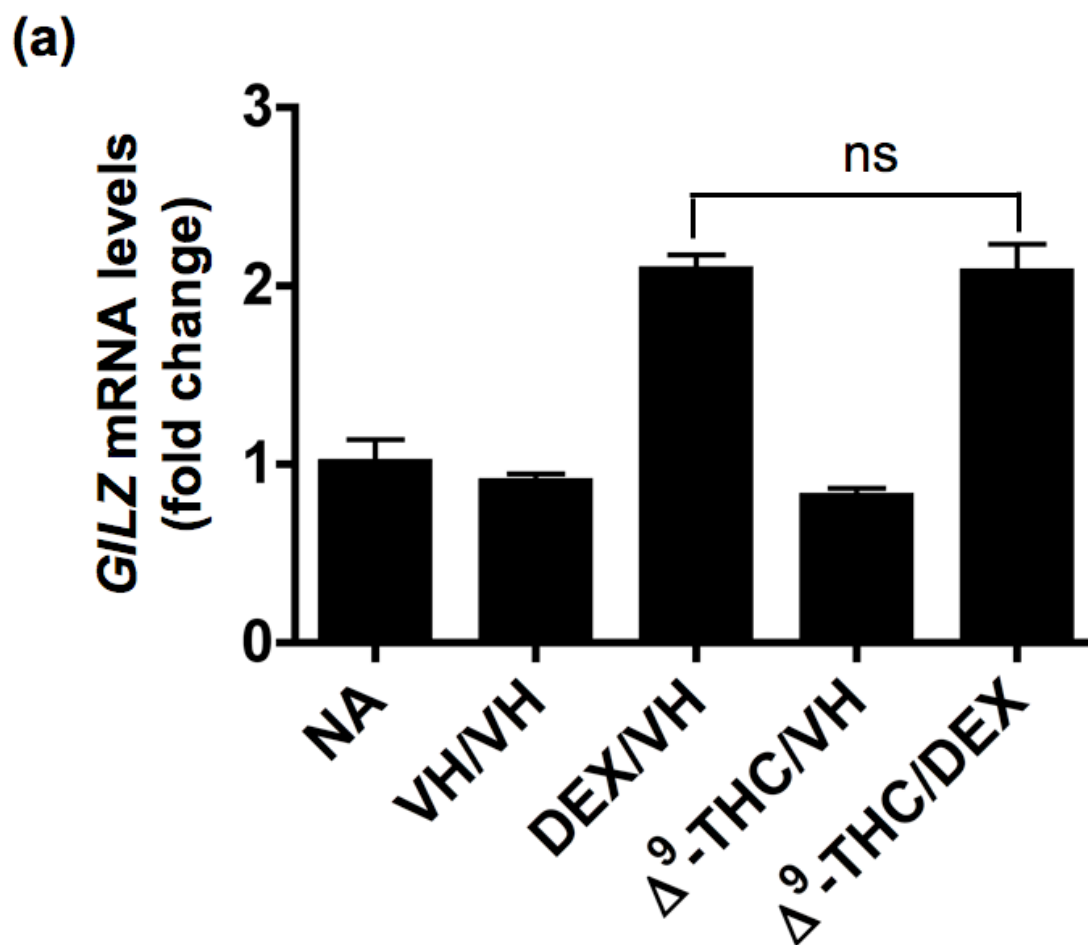
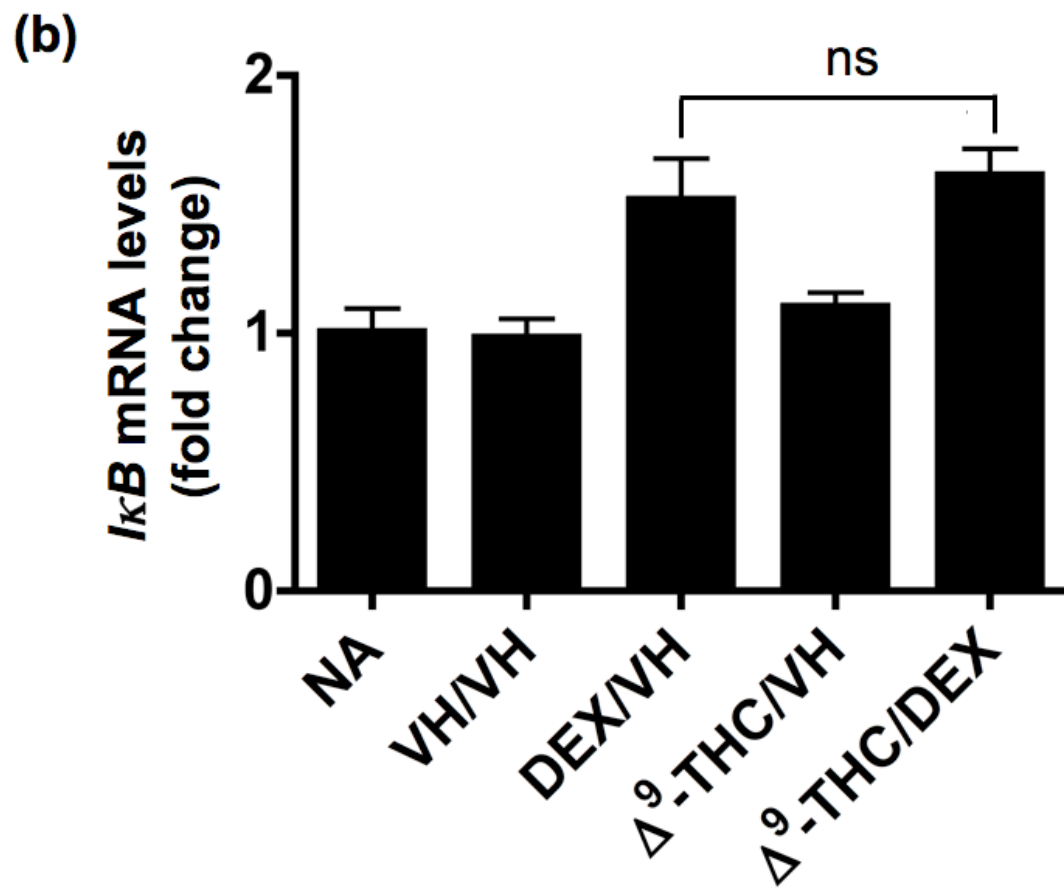


Figure 16. Effect of DEX and/or Δ^9 -THC on the mRNA expression of GR-dependent genes in HEK293T cells.

HEK293T cells (1×10^6 cells) were preseeded in a 6 well plate in growth medium overnight, followed by treatment with 0.1% EtOH (VH), 0.5 μ M DEX, 5 μ M Δ^9 -THC, or the combination of 0.5 μ M DEX plus 5 μ M Δ^9 -THC. Steady-state expression of *GILZ* or *I κ B* mRNA were determined by real-time PCR at 6 h after treatment. The fold difference of *GILZ* or *I κ B* mRNA molecules relative to unstimulated cells (naïve, NA) was normalized using the endogenous reference, 18s rRNA and results are the average of triplicates. The “ns” indicates no significant difference. Results represent two separate experiments with three replicates per treatment group.

Figure 16 (cont'd)



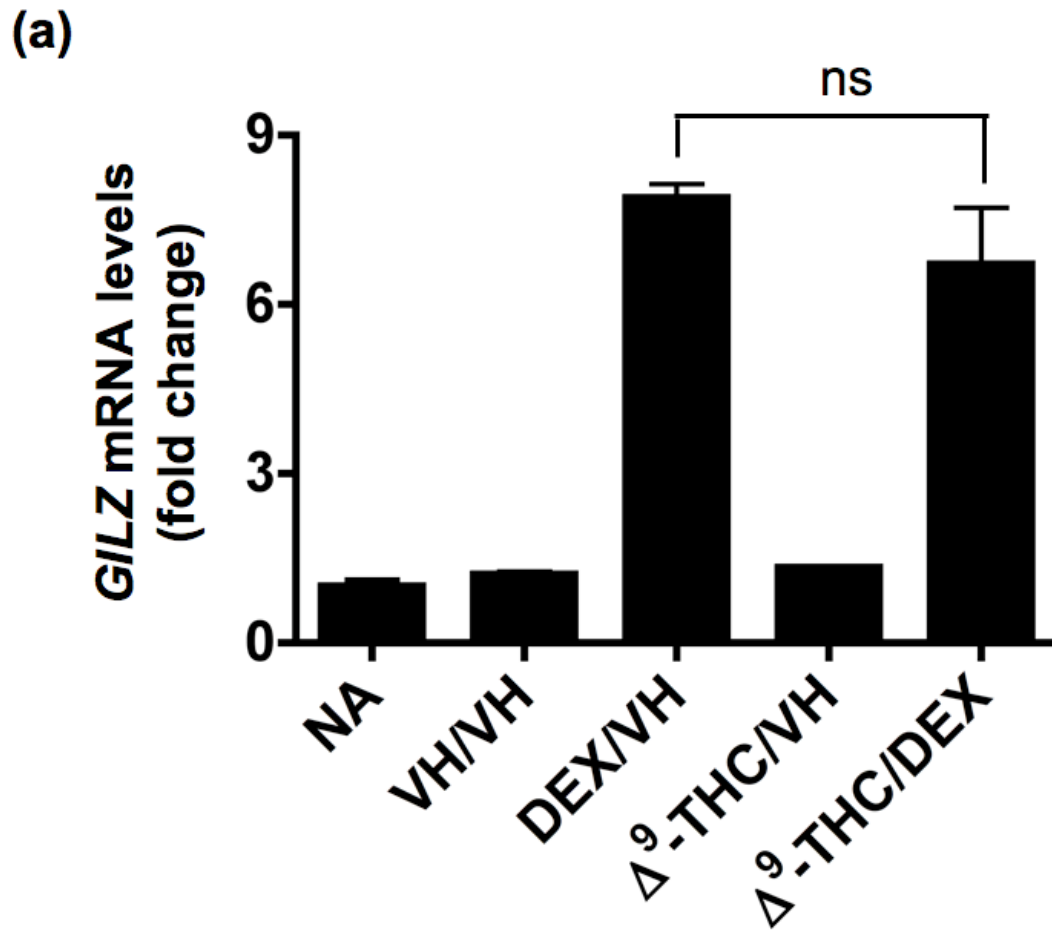
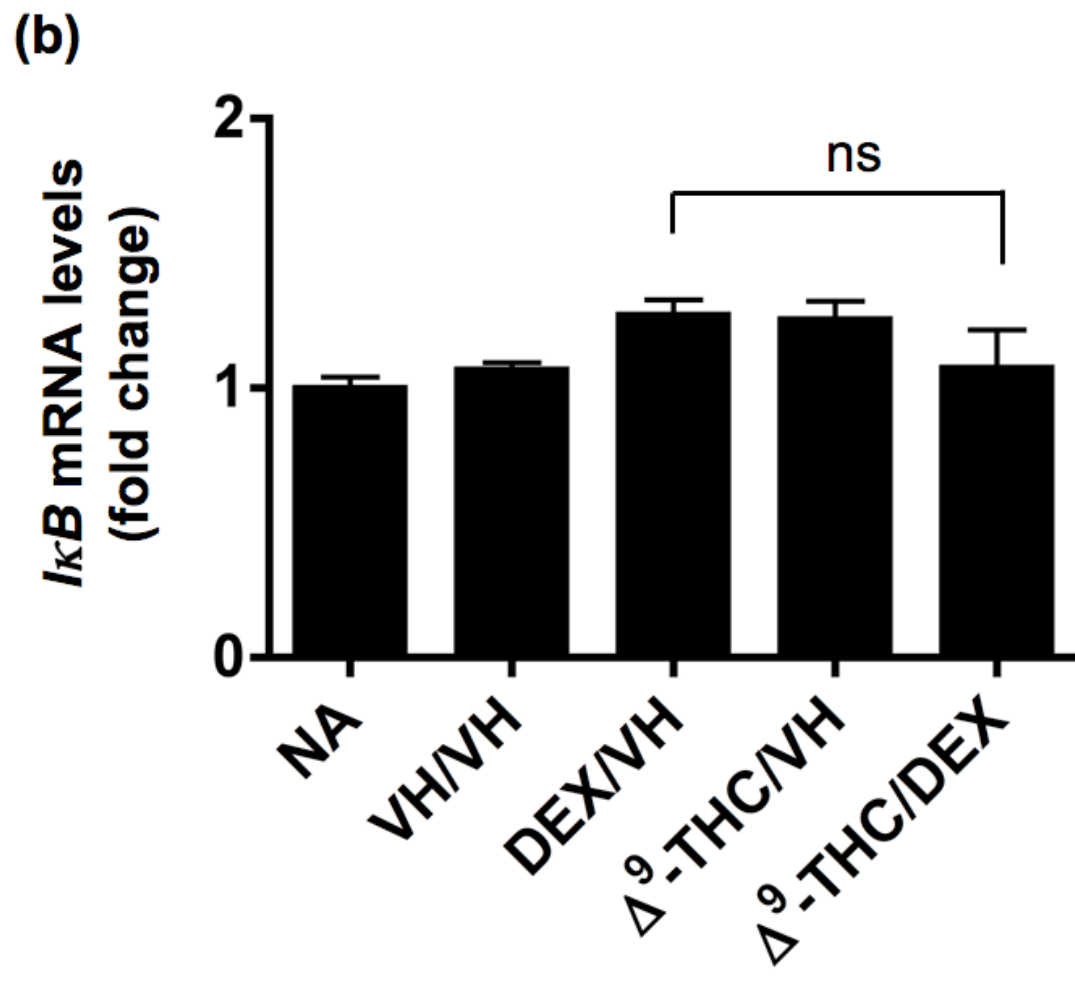


Figure 17. Effect of DEX and/or Δ^9 -THC on the mRNA expression of GR-dependent genes in Jurkat T cells.

Jurkat cells (5×10^6 cells) were preseeded in a 6 well plate in growth medium overnight, followed by treatment with 0.1% EtOH (VH), 0.5 μ M DEX, 5 μ M Δ^9 -THC, or the combination of 0.5 μ M DEX plus 5 μ M Δ^9 -THC. Steady-state expression of *GILZ* or *I κ B* mRNA were determined by real-time PCR at 6 h after treatment. The fold difference of *GILZ* or *I κ B* mRNA molecules relative to unstimulated cells (naïve, NA) was normalized using the endogenous reference, 18s rRNA and results are the average of triplicates. The “ns” indicates no significant difference. Results represent two separate experiments with three replicates per treatment group.

Figure 17 (cont'd)



II. Effect of Δ^9 -THC on the upregulation of CD40L on activated human CD4⁺ T cells

A. Δ^9 -THC attenuates anti-CD3/CD28-induced CD40L expression in activated human T cells at the transcriptional level.

Based on our previous finding that Δ^9 -THC attenuated the upregulation of CD40L expression in activated mouse splenic T cells [333], the effect of Δ^9 -THC on anti-CD3/CD28-induced CD40L expression in activated primary human T cells was investigated. Human PBMCs were stimulated with anti-CD3/CD28 in the presence or absence of Δ^9 -THC. The expression of surface CD40L was determined by flow cytometry at the indicated time points. As shown in figure 19, upregulation of surface CD40L on activated human CD4⁺ cells was detectable at 8 h, peaked at 32 h, and was declining by 72 h. Further, pretreatment with 15 μ M Δ^9 -THC attenuated the upregulation of surface CD40L expression on activated human CD4⁺ cells at all time points assessed (Figure 18). The concentration response studies were performed at 32 h post activation. Again, pretreatment with Δ^9 -THC significantly suppressed the percentage of double positive cells, CD4⁺CD40L⁺ cells in a concentration-dependent manner (Figure 19a and 19b). In addition, the MFI corresponding to total expression of surface CD40L on each activated CD4⁺ T cell was significantly attenuated in the

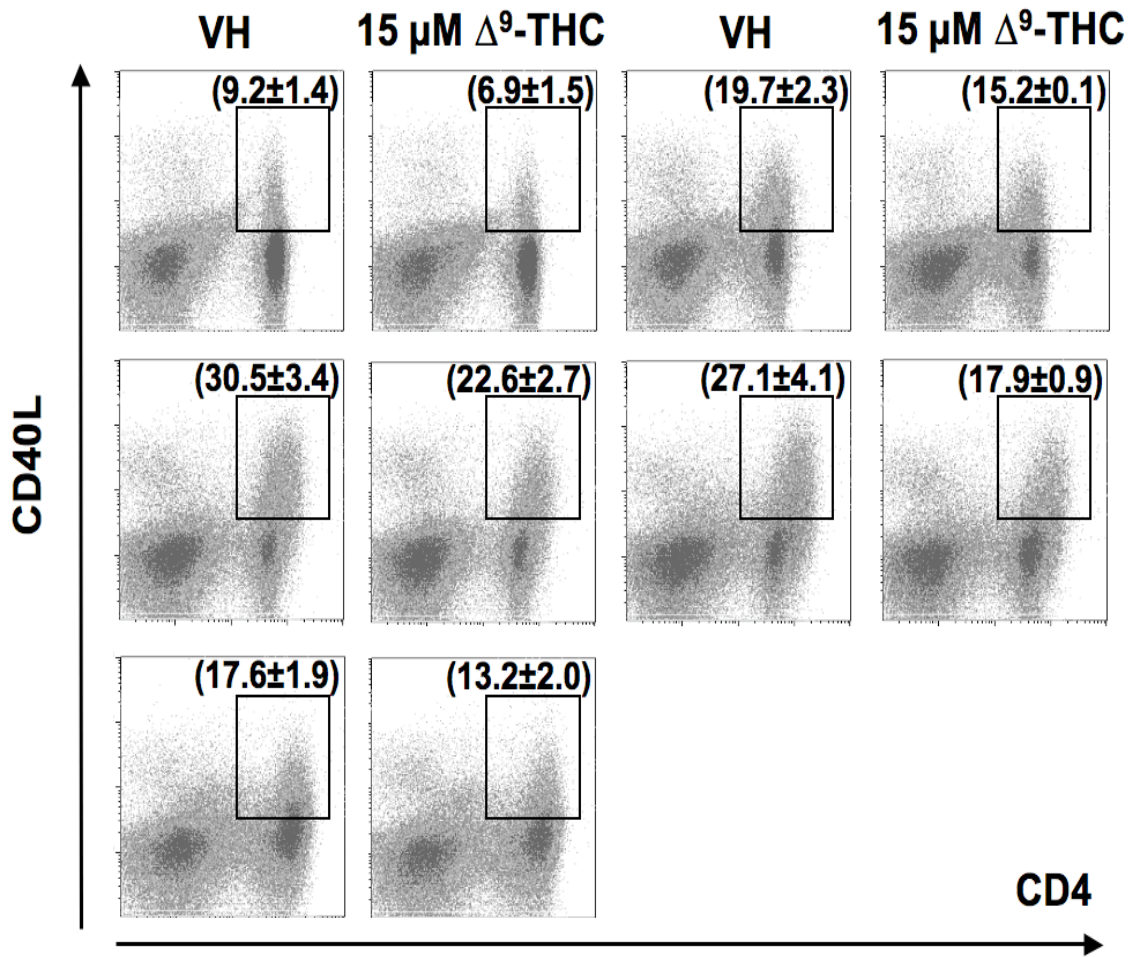


Figure 18. Effect of Δ^9 -THC on antiCD3/CD28-induced CD40L expression on activated human CD4⁺ T cells.

PBMC (1×10^6 cells) were treated with VH (0.1% EtOH) or 15 μ M Δ^9 -THC for 30 min and then activated with anti-CD3/CD28. (Cell surface CD40L expression on activated CD4⁺ was determined at indicated time points by flow cytometry. Numbers in parentheses represent the percentage of CD4⁺CD40L⁺ cell population $\pm$ SEM from triplicates. Results represent two different donors with three replicates per treatment group.

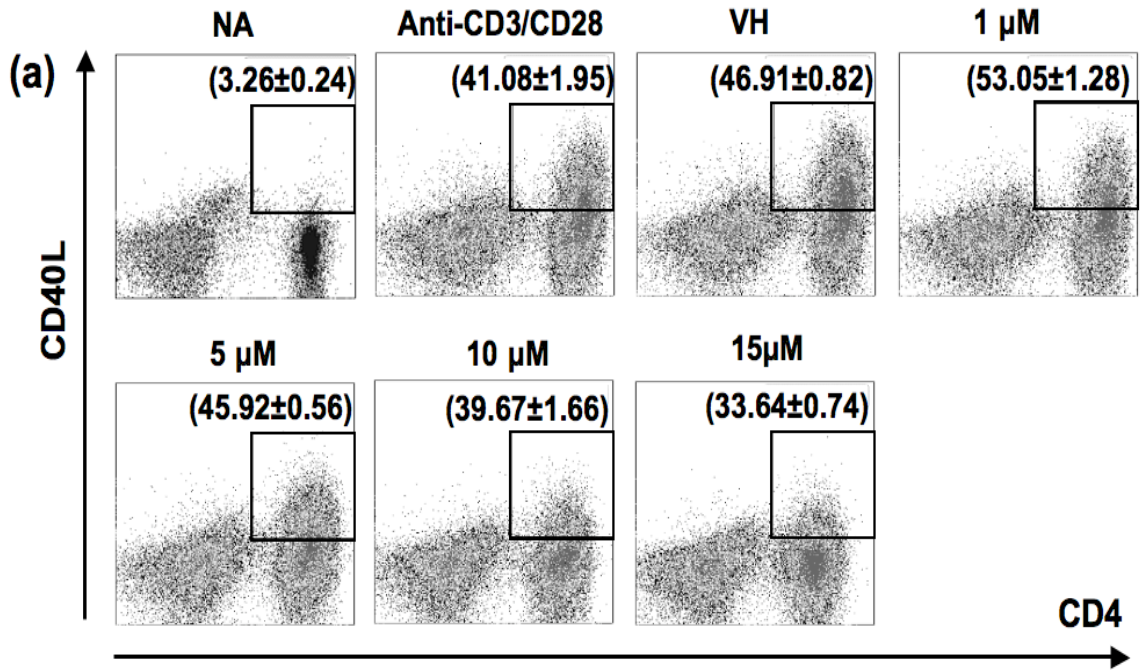
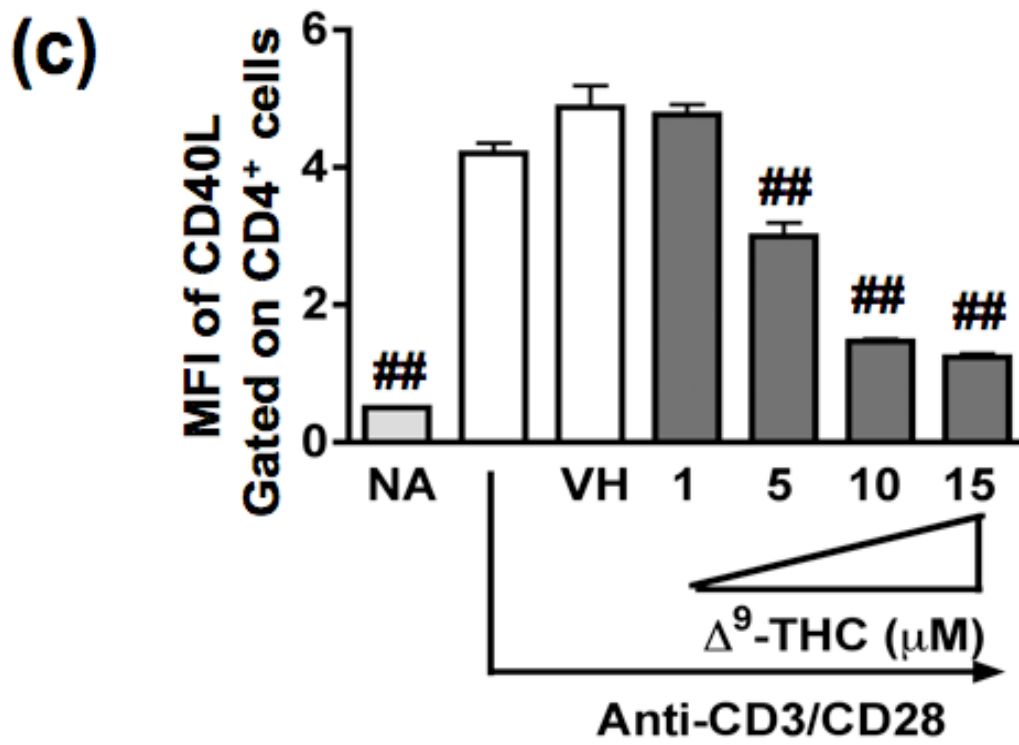
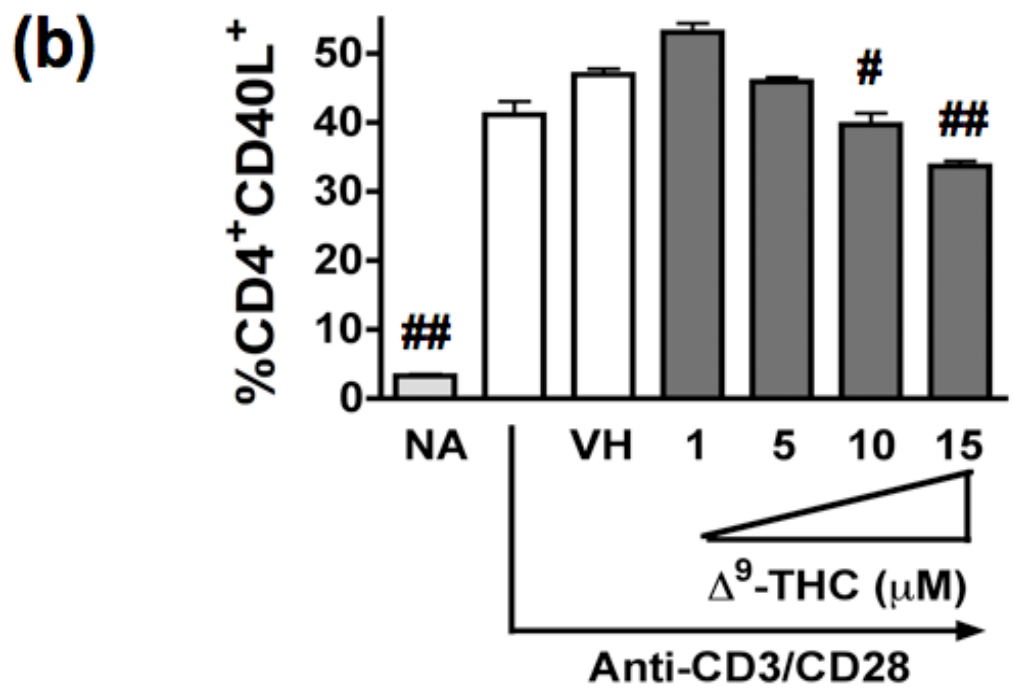


Figure 19. Δ^9 -THC attenuates TCR-induced surface CD40L expression on activated human CD4⁺ T cells.

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. (a) Cell surface CD40L expression on activated CD4⁺ 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 $p \leq 0.05$ or $p \leq 0.01$, respectively. Results represent five different donors with three replicates per treatment group.

Figure 19 (cont'd)



presence of Δ^9 -THC (Figure 19c). The concomitant decrease in both percentage of double positive cells and MFI of CD40L clearly indicates that Δ^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 [69]). Therefore, the steady-state level of CD40L mRNA was assessed by quantitative real time PCR in the presence or absence of Δ^9 -THC at 24 h post stimulation. As shown in figure 20, Δ^9 -THC significantly attenuated anti-CD3/CD28-induced CD40L mRNA expression in activated human T cells. The suppression of CD40L mRNA expression by Δ^9 -THC also occurred in a concentration-dependent manner, which corresponds to its effect on the protein level.

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

The transactivation of the *CD40L* gene is tightly regulated by several transcription factors (NFAT, CD28RE, NF κ B, TFE3/TFEB, EGR, AKNA, and AP1) that bind in the promoter region [77]. Although NFAT is the key transcription factor found in the minimal CD40L promoter [76], several reports demonstrated significant involvement of NF κ B in the upregulation of CD40L expression in both activated mouse and human T cells [78-80]. Previous studies from our laboratory demonstrated that cannabinoids suppressed T cell function, partly through the

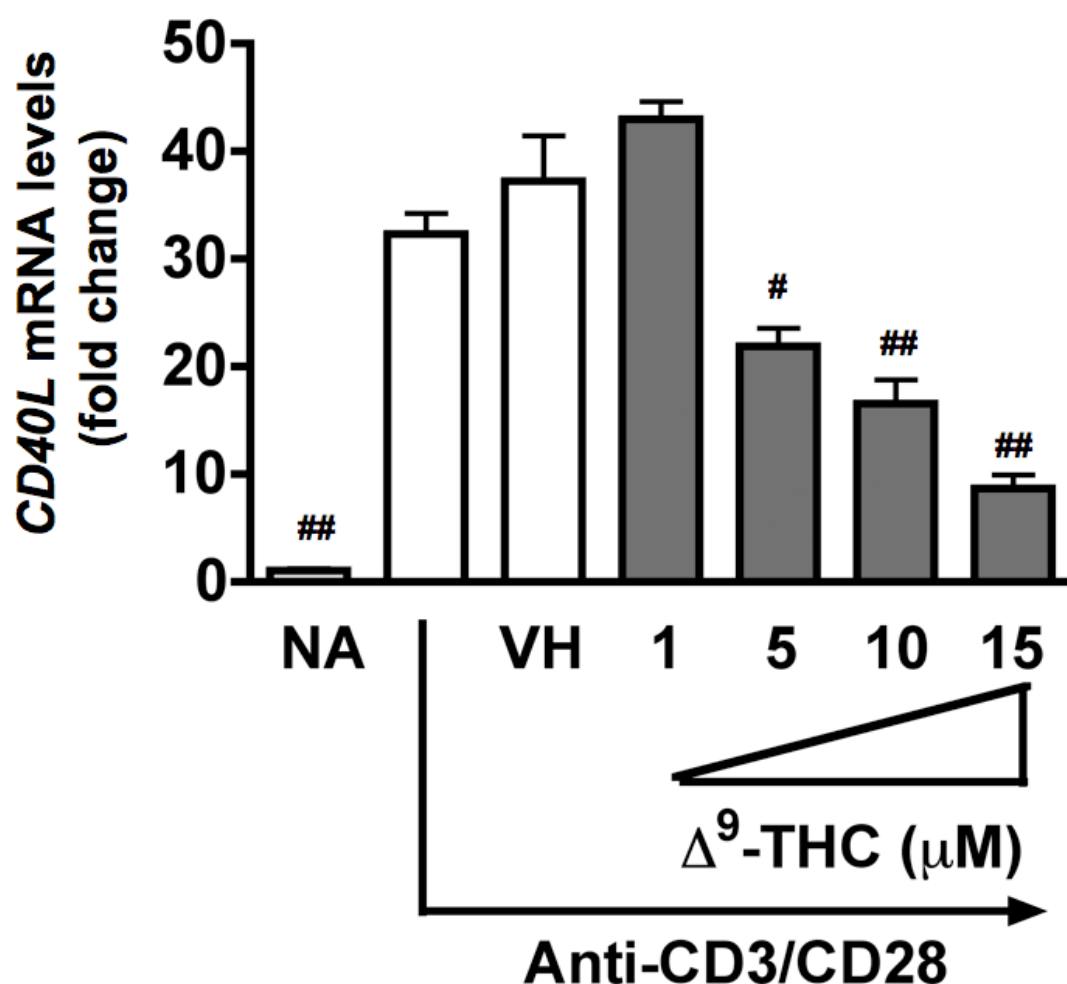


Figure 20. Δ^9 -THC suppressed TCR-induced CD40L mRNA expression in activated human CD4⁺ T cells.

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 molecules 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 $p \leq 0.05$ or $p \leq 0.01$, respectively. Results represent three different donors with four replicates per treatment group.

impairment of NFAT and NF κ B activation [303,311]. In light of the above, the effect of Δ^9 -THC on anti-CD3/CD28-induced DNA binding activity of NFAT and NF κ B to the 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 [80,323]. Human PBMCs were activated with anti-CD3/CD28 in the presence or absence of Δ^9 -THC for 16 h, which was the time of the highest magnitude of DNA-binding activity of NFAT (Figure 21). The specificity of NFAT-DNA complexes (as indicated by arrows) was determined by adding 100-fold molar excess of unlabelled probe (Figure 22a, 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 Δ^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 (Figure 22b, 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 Δ^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 Δ^9 -THC impaired both NFAT- and NF κ B-DNA binding activity in activated human T cells.



Figure 21. Peak time of anti-CD3/CD28-induced DNA-binding activity of NFAT in activated human T cells.

PBMC (2×10^7 cells) were activated with anti-CD3/CD28 at indicated time points. The nuclear proteins ($1 \mu\text{g}$) were incubated with oligonucleotide probes derived from human CD40L promoter containing NFAT. The DNA-binding complexes were resolved by gel electrophoresis. The basal DNA binding activity was measured in nonactivated cells (NA) at 0, 8, and 24 h (lane 2-4). The induction of NFAT-protein complexes in activated T cells was measured at various time points (lane 5-9). Lane 1 is radiolabeled probe alone. Arrows indicate the specific DNA-protein complexes. The data are representative of two independent experiments from different donors.

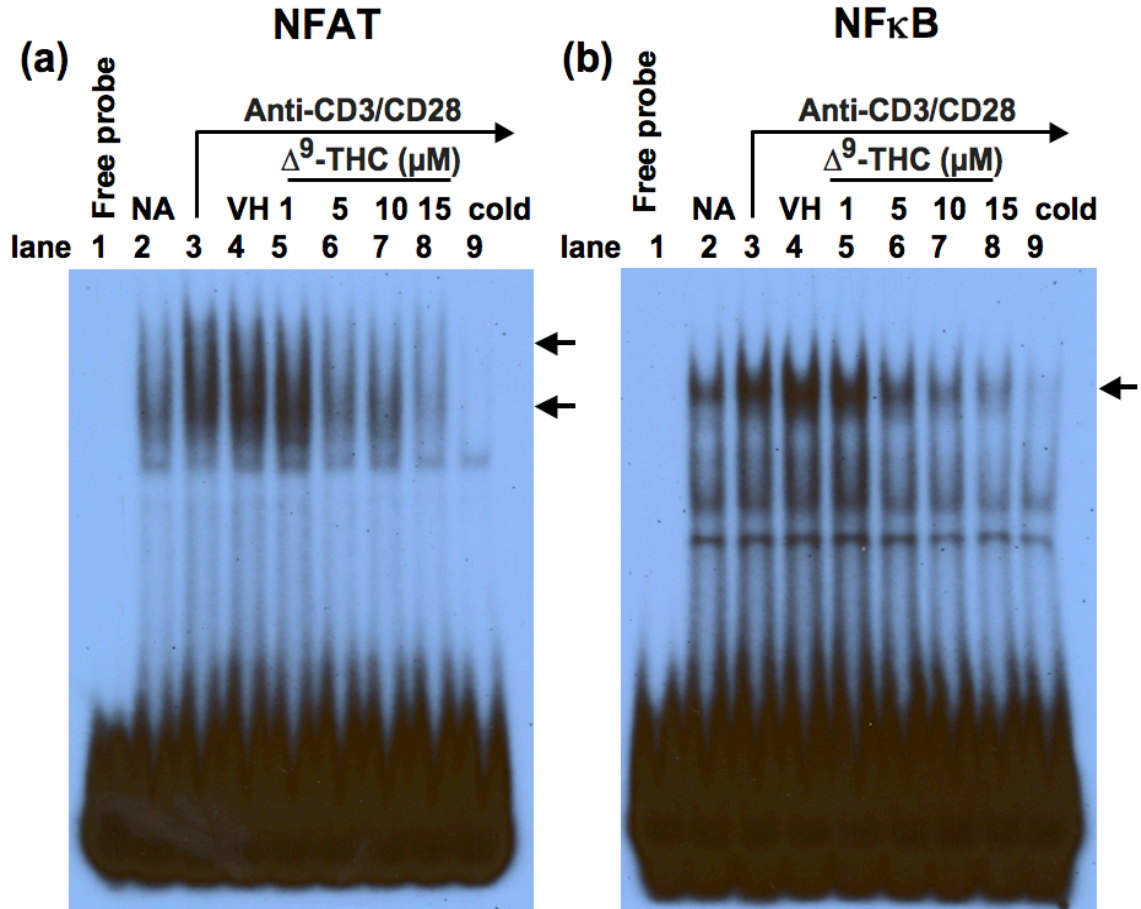


Figure 22. Δ^9 -THC impairs TCR-induced DNA binding activity of NFAT and NFκB in activated human T cells.

PBMC (2×10^7 cells) were treated with VH (lane 4) or Δ^9 -THC (1, 5, 10, and 15 μ M; 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 μ g) were incubated with oligonucleotide probes derived from human CD40L promoter containing either NFAT or NFκB 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 NFκB binding to the CD40L promoter. Arrows indicate the specific DNA-protein complexes. The data are representative of three independent experiments from different donors.

C. Δ^9 -THC does not affect 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 [59]). 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 [59]). To further elucidate the mechanism by which Δ^9 -THC suppresses NFAT-DNA binding activity, the effect of Δ^9 -THC pretreatment on the activity of GSK3 β in activated T cells was investigated. Human PBMCs were activated using anti-CD3/CD28 crosslinking. The peak level of pGSK3 β , which corresponds to its inactive state, was assessed by flow cytometry at indicated time points post activation. As shown in Figure 23, there was an increase in the amount of pGSK3 β in activated human CD4⁺ T cells as demonstrated by the increase of MFI, as early as 2 min, maximal at 5 min, and was declining back to the background level by 15 min. Pretreatment with Δ^9 -THC did not affect the level of pGSK3 β suggesting that Δ^9 -THC did not perturb anti-CD3/CD28-induced GSK3 β inactivation in human CD4⁺ T cells (Figure 24).

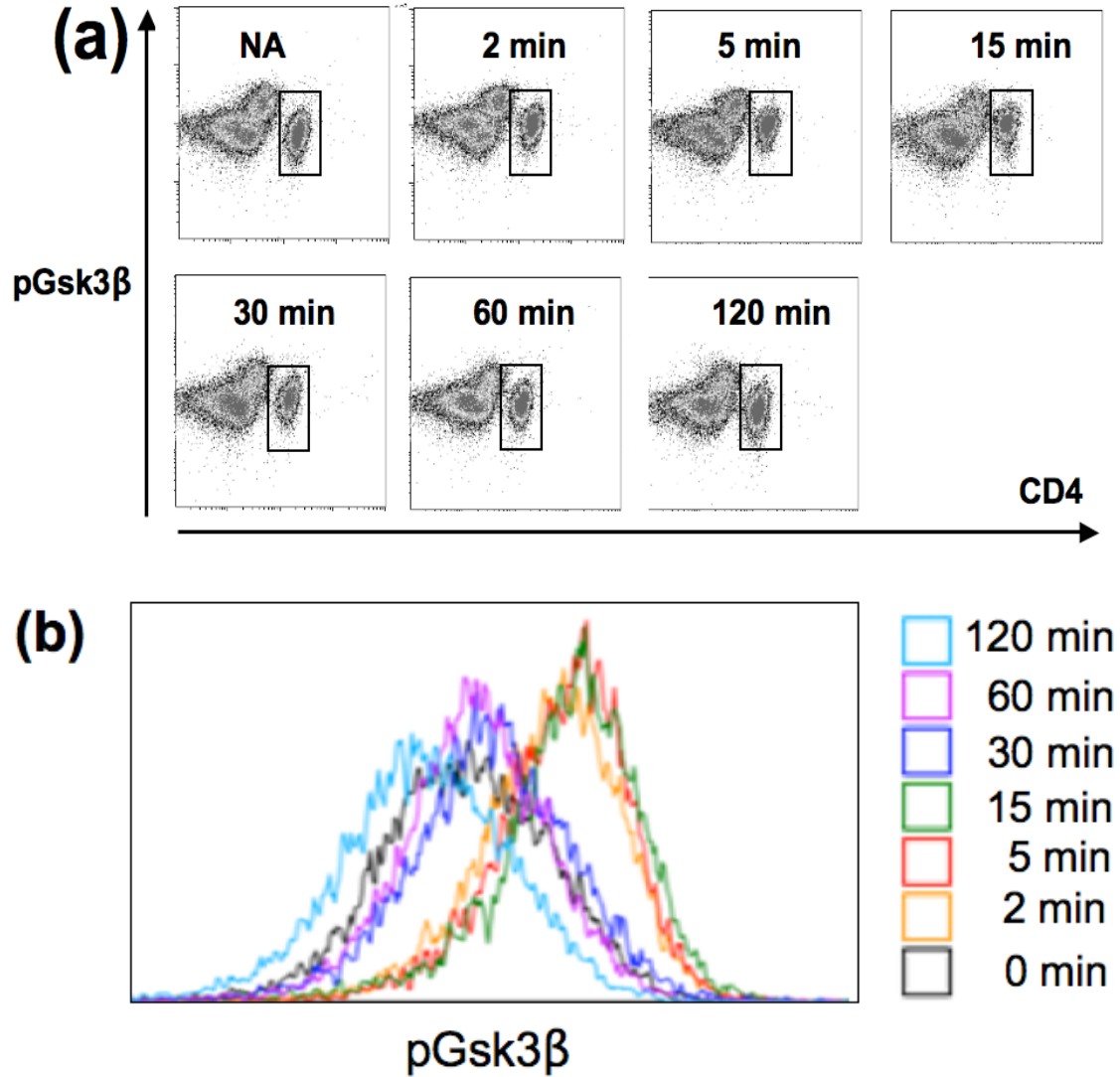


Figure 23. Peak time of anti-CD3/CD28-induced phosphorylation of GSK3β in activated human T cells.

PBMC (1×10^6 cells/ $100 \mu\text{L}$) were equilibrated at 37°C in 5% CO_2 for 3 h, followed by incubation with anti-CD3, anti-CD28, and IgG crosslinker on ice. T cell activation was initiated by transferring to 37°C water bath. Cells were fixed with 1.5% formaldehyde at indicated time points. Cells were permeabilized with ice-cold 100% methanol, followed by staining with anti-CD4 and anti-pGSK3β. The intracellular expression of pGSK3β in CD4⁺ T cells was assessed by flow cytometry. (a) Dot plot represents CD4⁺pGSK3β⁺ cell population at various time points (b) Histogram plot represents MFI of pGSK3β gated on CD4⁺ T cells at various time points. The data are representative of two independent experiments from different donors.

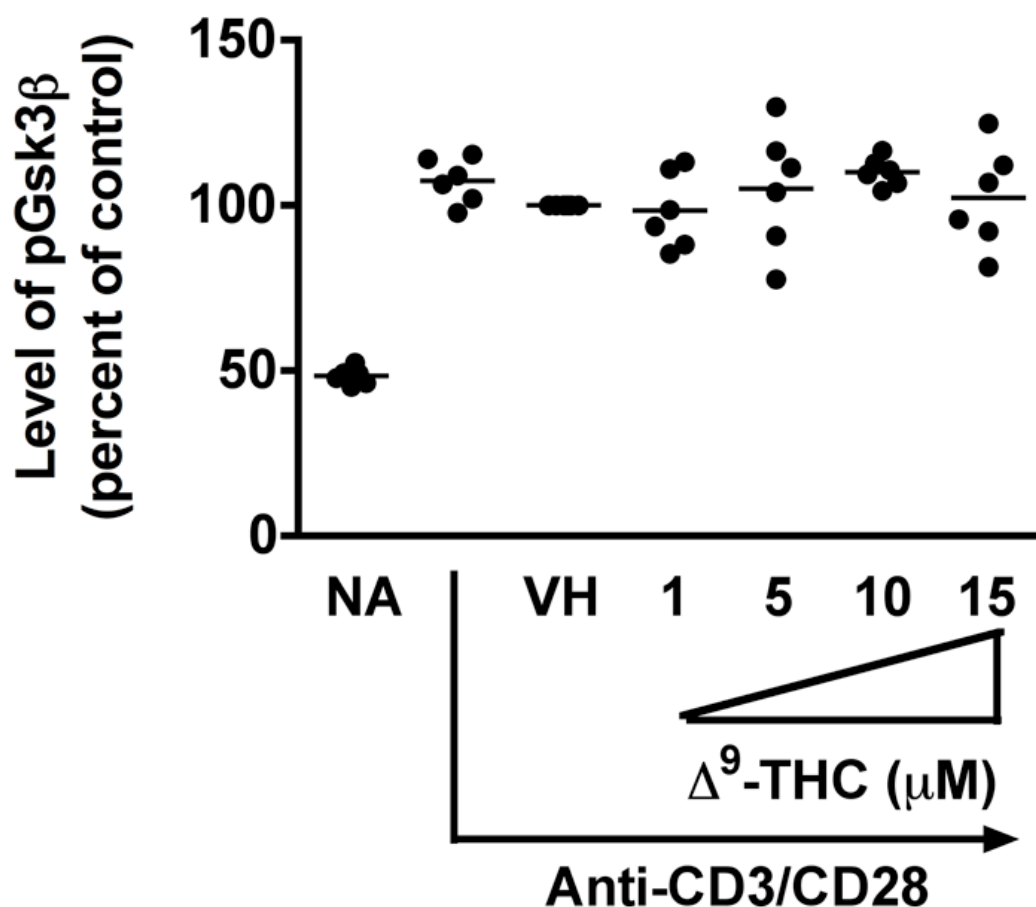


Figure 24. Effect of Δ^9 -THC on the activity of GSK3 β in activated human CD4⁺ T cells.

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 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. Each dot represents data from one individual donor.

D. Δ^9 -THC attenuates anti-CD3/CD28-induced elevated intracellular Ca^{2+} , but does not impair $\text{PLC}\gamma$ activation.

The inhibitory effect of Δ^9 -THC on the DNA binding activity of both NFAT and NF κ B strongly implicated altered intracellular Ca^{2+} regulation in the presence of Δ^9 -THC. To investigate the effects of Δ^9 -THC on anti-CD3/CD28-induced Ca^{2+} elevation in human CD4^+ T cells, naïve CD4^+ T cells were incubated with the Ca^{2+} indicator dyes fluo-3 and fura-red for 60 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 Ca^{2+} (Figure 25). Pretreatment with Δ^9 -THC resulted in a concentration-dependent attenuation of elevated Ca^{2+} following anti-CD3/CD28 crosslinking in activated human CD4^+ T cells with nearly complete loss of the response at 15 μM Δ^9 -THC (Figure 25). The increase in intracellular Ca^{2+} following TCR stimulation is mainly regulated by the activation of $\text{PLC}\gamma$ (reviewed in [50]). Furthermore, $\text{PLC}\gamma$ also controls NF κ B activation through the PKC θ pathway (reviewed in [334]). We next tested whether Δ^9 -THC affects the activation of $\text{PLC}\gamma$. The phosphorylation status of $\text{PLC}\gamma 1/2$ (p $\text{PLC}\gamma 1/2$), which corresponds to its active state, was determined by flow cytometry at 5 min post

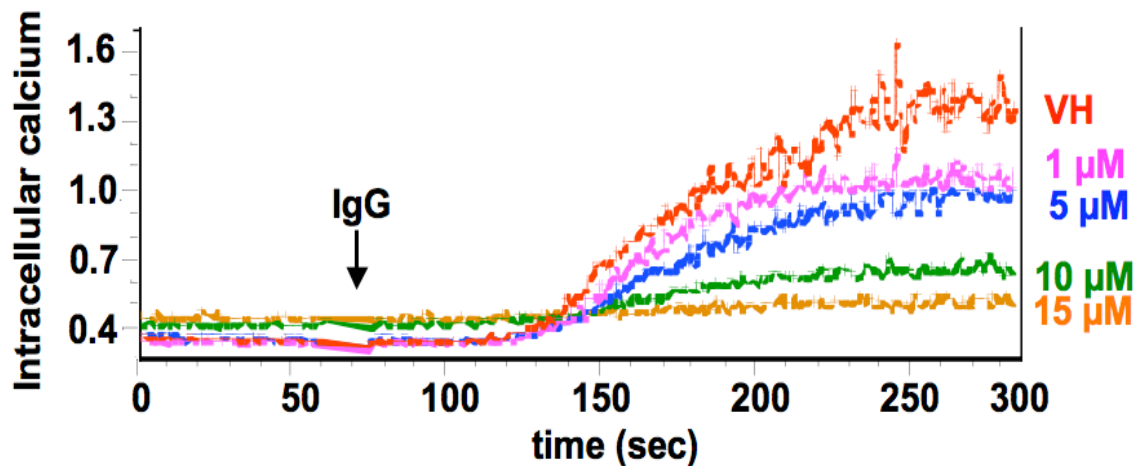


Figure 25. Δ^9 -THC suppressed TCR-induced elevation of intracellular Ca^{2+} in activated human CD4^+ T cells.

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.

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 anti-CD3/CD28-mediated increase of pPLC γ 1/2 in activated human CD4⁺ T cells (Figure 26). 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.

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

The 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 PMA/Io [333]. Along the same line, a study reported by Borner *et. al.* demonstrated that the T-cell inhibitory effect of Δ^9 -THC is due, in part, to the impairment of proximal TCR signaling cascades [308]. To test this hypothesis, the effect of Δ^9 -THC on proximal TCR signaling cascades was investigated. To identify the peak time induction, 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, were

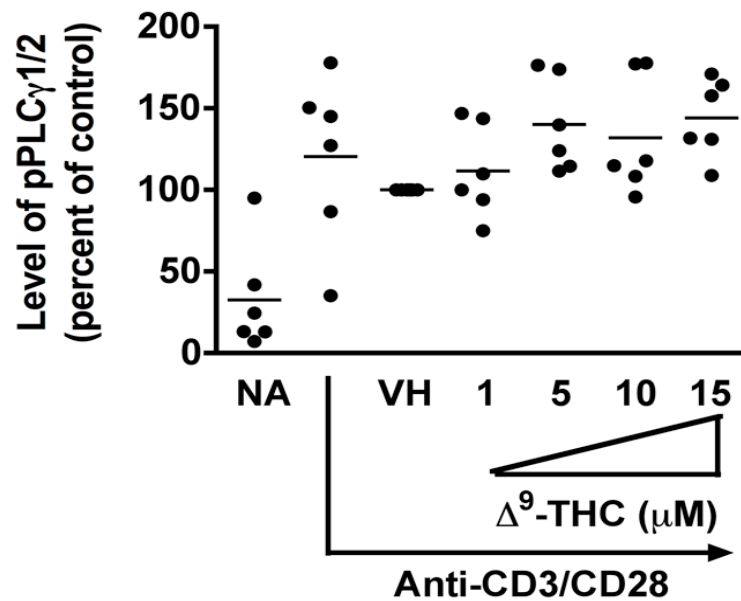


Figure 26. Effect of Δ^9 -THC on the activation of PLC γ in activated human CD4 $^+$ T cells.

PBMC (1×10^6 cells/ 100μ L) were equilibrated at 37°C in 5% CO_2 for 3 h, treated with Δ^9 -THC (1, 5, 10, and $15 \mu\text{M}$) 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 . 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. Each dot represents data from one individual donor.

measured by flow cytometry at the indicated time points post activation with anti-CD3/CD28 crosslinking. Upon activation, there was an increase in the amount of both pZAP70 (Figure 27) and pAkt (Figure 28) in activated human CD4⁺ T cells as early as 2 min, maximal at 5 min, and was declining to the background level by 15 min. 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 (Figure 29a and 29b, respectively). Interestingly, pretreatment with Δ^9 -THC increased the variability in the phosphorylation of ZAP70 among the human subjects (Figure 29a). Thus, a comparable mouse model was used to investigate the effect of Δ^9 -THC on the activation of ZAP70. We found that Δ^9 -THC did not affect the phosphorylation level of Zap70 in activated mouse CD4⁺ T cells (Figure 30). Collectively, these results demonstrate that Δ^9 -THC does not impair proximal TCR-associated signaling in activated human CD4⁺ T cells.

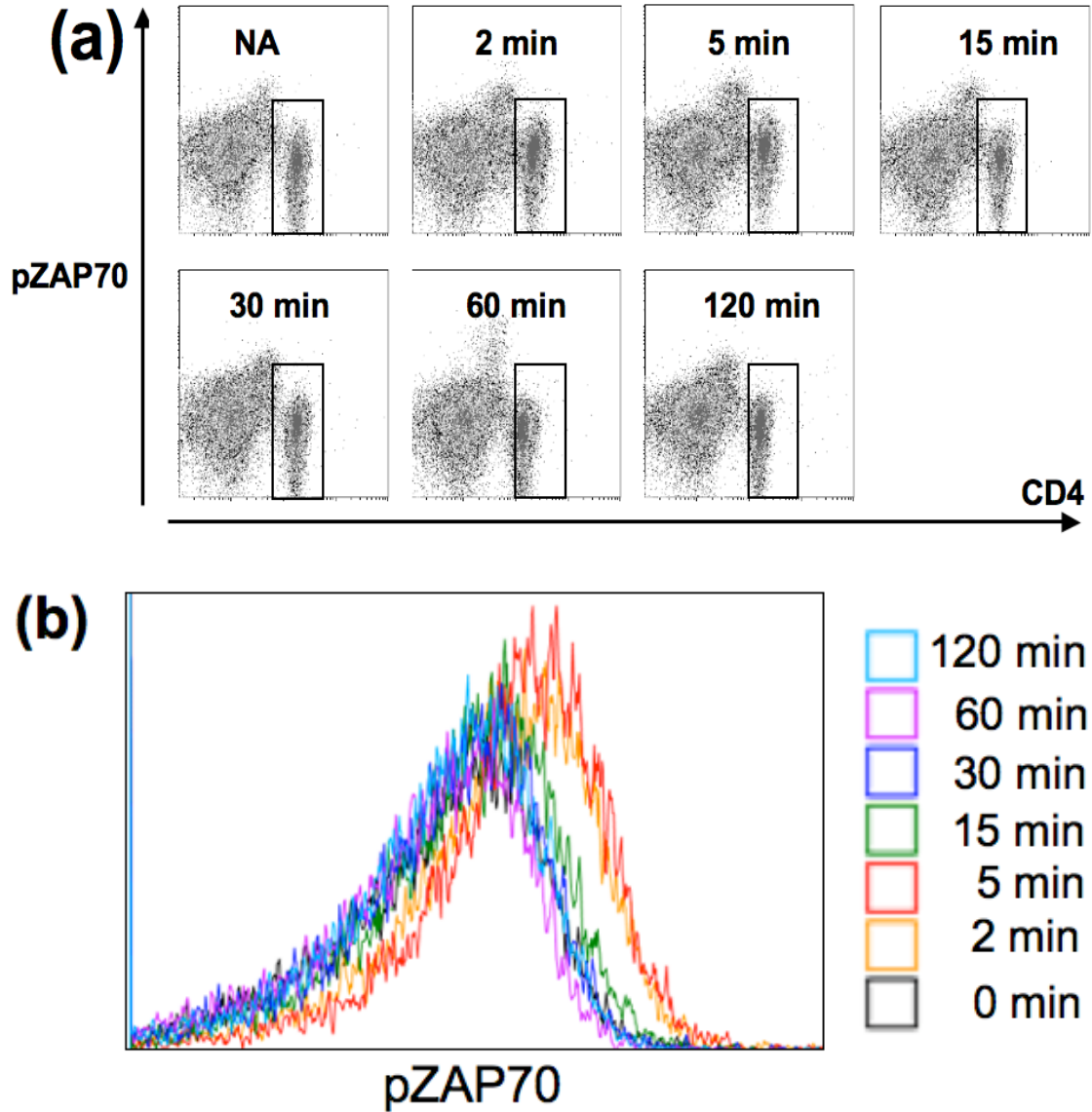


Figure 27. Peak time of anti-CD3/CD28-induced phosphorylation of ZAP70 in activated human T cells.

PBMC (1×10^6 cells/ $100 \mu\text{L}$) were equilibrated at 37°C in $5\% \text{CO}_2$ for 3 h, followed by incubation with anti-CD3, anti-CD28, and IgG crosslinker on ice. T cell activation was initiated by transferring to 37°C water bath. Cells were fixed with 1.5% paraformaldehyde at indicated time points. Cells were permeabilized with ice-cold 100% methanol, followed by staining with anti-CD4 and anti-pZAP70. The intracellular expression of pZAP70 in CD4⁺ T cells was assessed by flow cytometry. (a) Dot plot represents CD4⁺pZAP70⁺ cell population at various time points (b) Histogram plot represents MFI of pZAP70 gated on CD4⁺ T cells at various time points. The data are representative of two independent experiments from different donors.

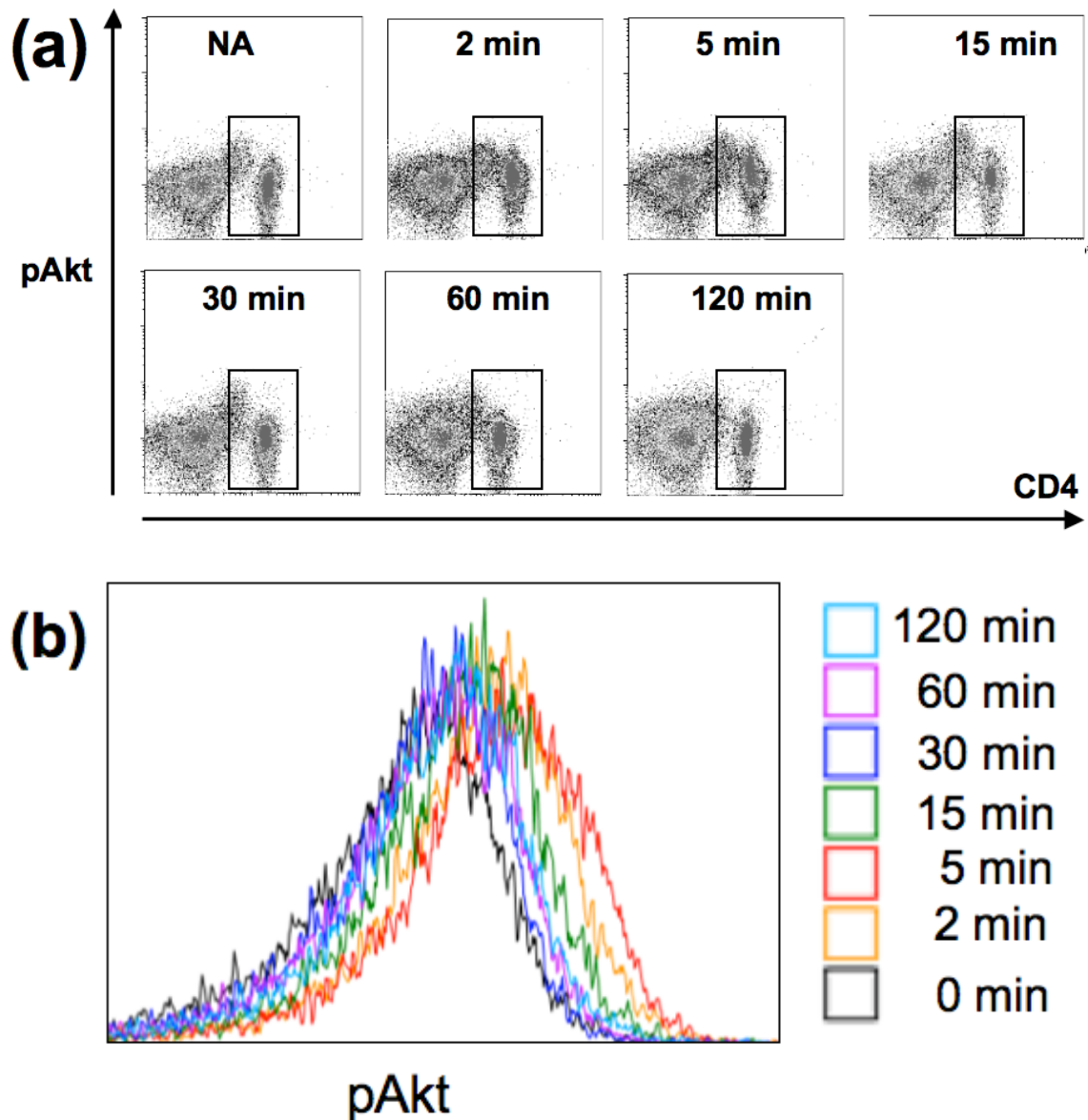


Figure 28. Peak time of anti-CD3/CD28-induced phosphorylation of Akt in activated human T cells.

PBMC (1×10^6 cells/100 μ L) were equilibrated at 37°C in 5% CO₂ for 3 h, followed by incubation with anti-CD3, anti-CD28, and IgG crosslinker on ice. T cell activation was initiated by transferring to 37°C water bath. Cells were fixed with 1.5% paraformaldehyde at indicated time points. Cells were permeabilized with ice-cold 100% methanol, followed by staining with anti-CD4 and anti-pAkt. The intracellular expression of pAkt in CD4⁺ T cells was assessed by flow cytometry. (a) Dot plot represents CD4⁺pAkt⁺ cell population at various time points (b) Histogram plot represents MFI of pAkt gated on CD4⁺ T cells at various time points. The data are representative of two independent experiments from different donors.

(a)

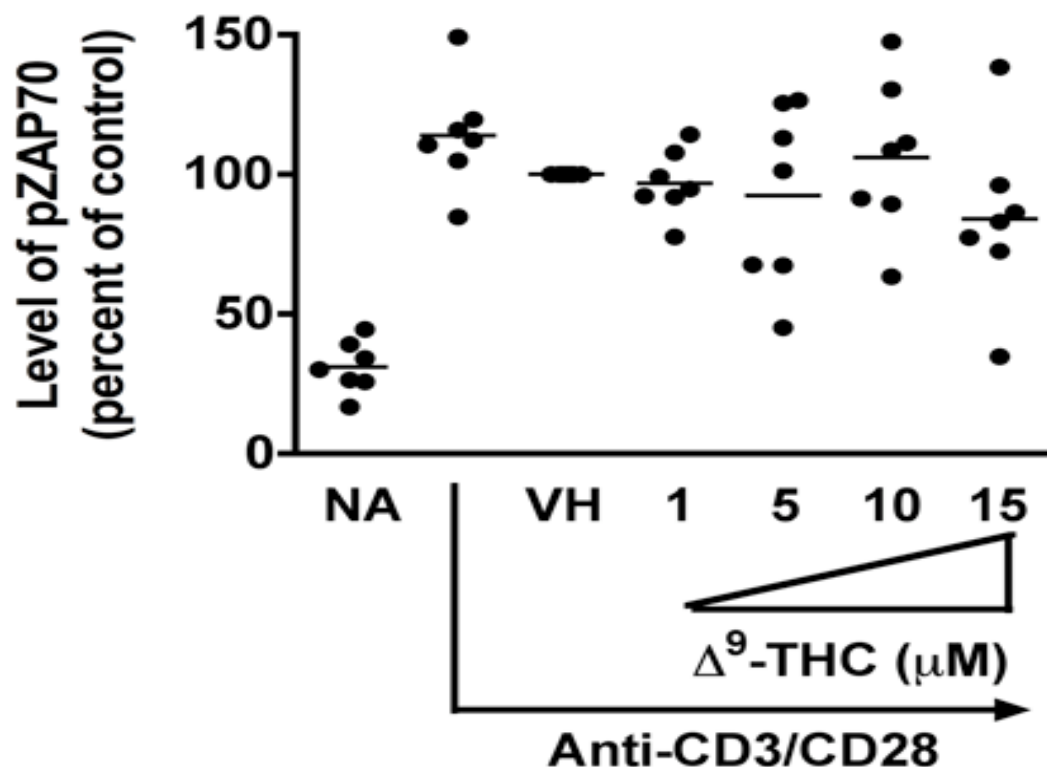
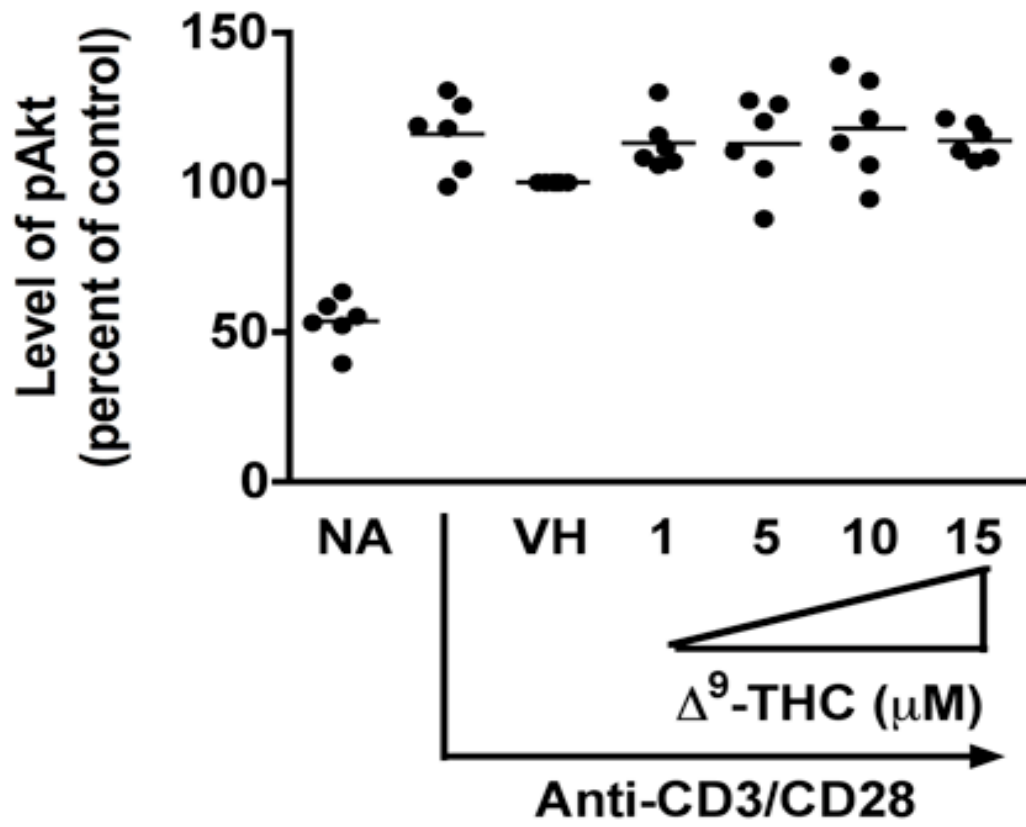


Figure 29. Effect of Δ^9 -THC on the proximal T cell receptor signaling molecules in activated human CD4⁺ T cells.

PBMC (1×10^6 cells/ 100μ 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, 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. (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. Each dot represents data from one individual donor.

Figure 29 (cont'd)

(b)



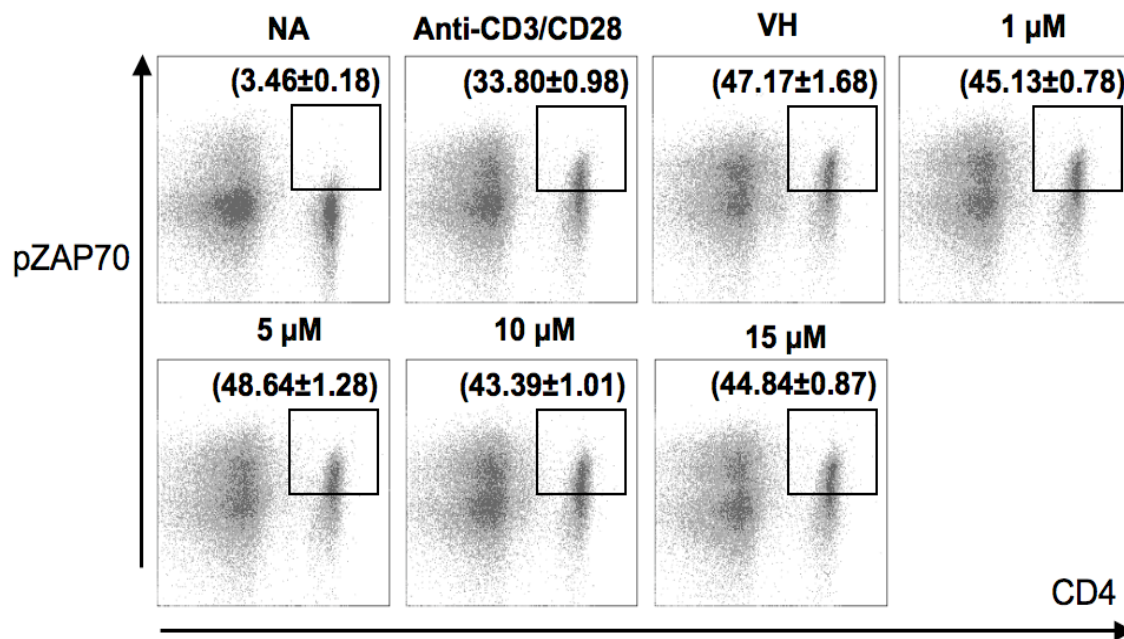


Figure 30. Effect of Δ^9 -THC on anti-CD3/CD28-induced phosphorylation of ZAP70 in activated mouse CD4⁺ T cells.

SPLC (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 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-pZAP70. The intracellular expression of pZAP70 in CD4⁺ T cells was assessed by flow cytometry. Dot plot represents the level of pZAP70 in CD4⁺ T cells.

III. Effect of Δ^9 -THC on CD40 plus cytokine-induced primary IgM response by HPB B cells.

A. Δ^9 -THC attenuates CD40L plus cytokine-induced primary IgM responses of HPB B cells.

This laboratory previously demonstrated a marked suppression of *in vitro* T cell-dependent antibody response by Δ^9 -THC in mouse was due in part to a direct effect on B cells [294]. Further, our lab established a polyclonal *in vitro* activation model of primary human B cell differentiation to generate primary antibody secreting cells, which mainly produce IgM [320]. Thus, the effect of Δ^9 -THC on the primary antibody response in humans was investigated. Naïve B cells were co-cultured with irradiated CD40L-L cells in the presence of recombinant cytokines IL-2, -6, and -10 (CD40L plus cytokine) and IgM secreting cells were enumerated by ELISPOT on day 7. The effect of Δ^9 -THC on IgM responses was assessed from 6 donors and the data are presented as percentage of control (VH-treated group). Pretreatment with Δ^9 -THC significantly decreased the percentage of IgM secreting cells induced by CD40L plus cytokine (Figure 31a), but not the total viable cells (Figure 31b). These results suggested that Δ^9 -THC-mediated suppression of primary IgM response in humans involves perturbation of differentiation of naïve B cells into plasma cells rather than direct cytotoxicity.

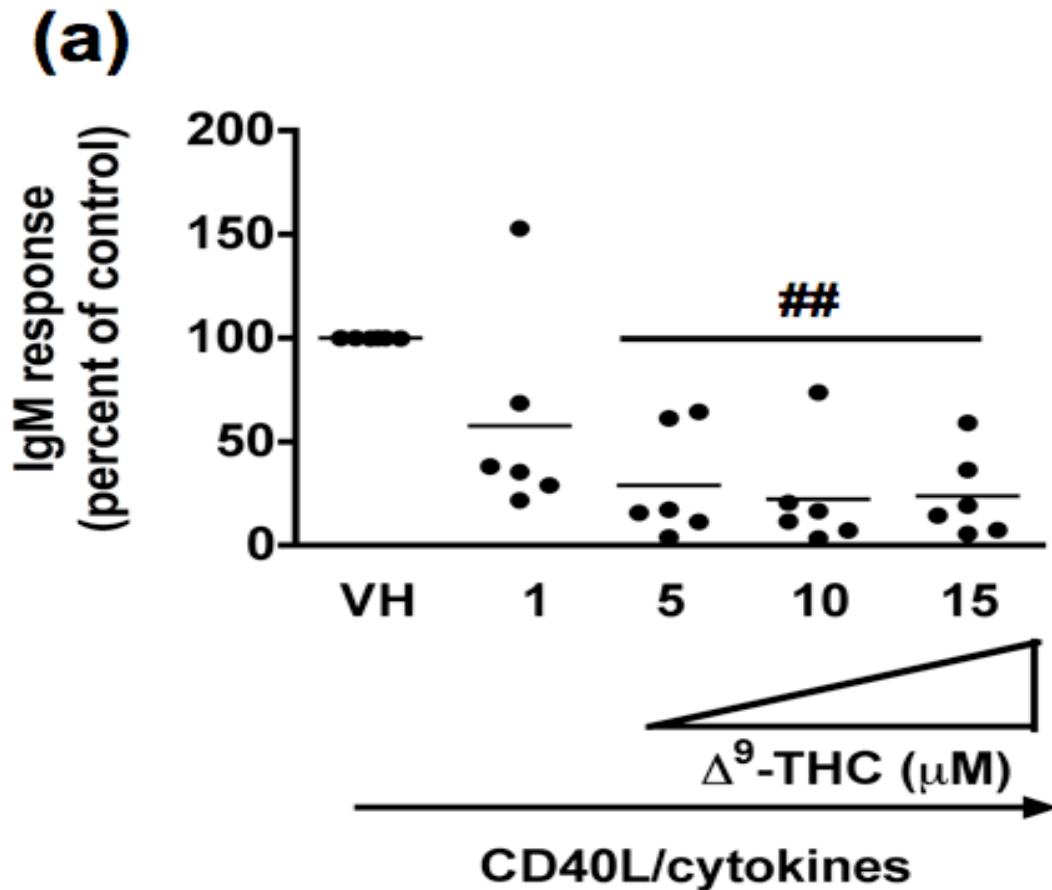
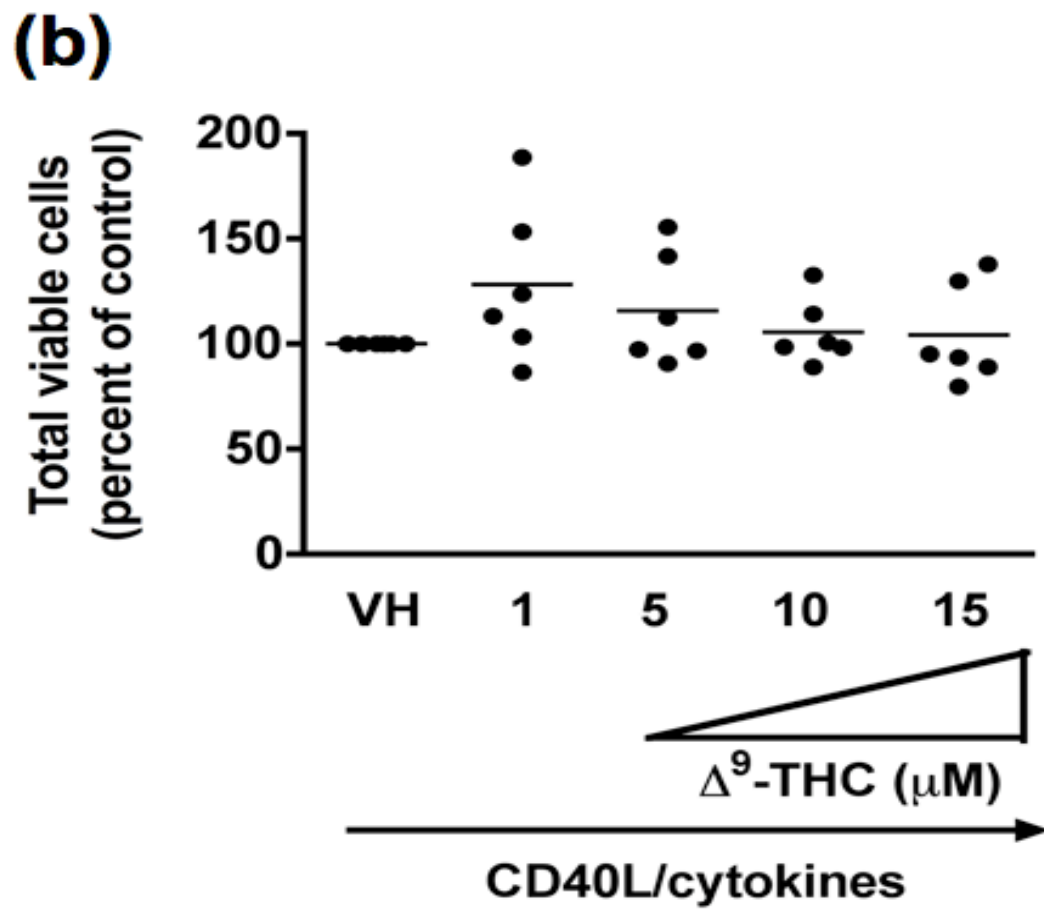


Figure 31. Effect of Δ^9 -THC on CD40L plus cytokine-induced IgM secreting cell response in humans.

HPB naïve B cells (1×10^6 cells) were treated with Δ^9 -THC at indicated concentrations or VH (0.02% DMSO) for 30 min and then cultured with irradiated CD40L-L cells (1.5×10^3 cells/well) in the presence of recombinant human IL-2 (10 U/mL), IL-6 (100 U/mL), and IL-10 (20 ng/mL). Cells were transferred to new culture plates without CD40L-L cells on Day 4 and cultured for additional 3 days. IgM secreting cells were enumerated by ELISPOT on day 7. The total viable cells were counted by Z1 Coulter Counter following pronase treatment. Data were normalized to the VH-treated group and presented as percent of control with at least three replicates per groups. (a) Scatter plot represents the number of IgM secreting cells. (b) Scatter plot represents the total viable cells. The ## indicates significant effect compared to VH-treated group at $p \leq 0.01$. Each dot represents data from one individual donor.

Figure 31 (cont'd)



B. Δ^9 -THC suppresses CD40L plus cytokine-induced surface expression of CD80, but not CD69, CD86, and ICAM1 in HPB B cells.

To identify which stages in plasma cell differentiation are modulated by Δ^9 -THC, we first examined whether Δ^9 -THC affects B cell activation by assessing surface expression of CD80, CD86, CD69, and ICAM1 on activated B cells by flow cytometry. Kinetic studies showed that surface expression of ICAM1 peaked on day 1, whereas peak expression of surface CD80 was on day 3 post activation (Figure 32a and 32b respectively). The magnitude of surface expression for both CD69 and CD86 by HPB B cells was highest on day 4 post activation [320]. Thus, the effect of Δ^9 -THC was investigated either on day 1 and/or day 3 post activation. Pretreatment with Δ^9 -THC did not affect the upregulation of ICAM1 (Fig 33a), CD69 (Fig 33b), and CD86 (Fig 33c), but significantly suppressed the upregulation of surface CD80 (Fig 33d). As the induction of surface CD80 by CD40 ligation in B cells is primarily regulated at the level of transcription [335,336], we next investigated whether Δ^9 -THC affects the steady-state level of *CD80* mRNA by RT-PCR. Pretreatment with Δ^9 -THC did not affect *CD80* mRNA expression induced by CD40 plus cytokine in activated HPB B cells (Fig 34). These data demonstrated that Δ^9 -THC impaired CD40L plus cytokine-induced B-cell activation, at least in part, through the downregulation of surface CD80.

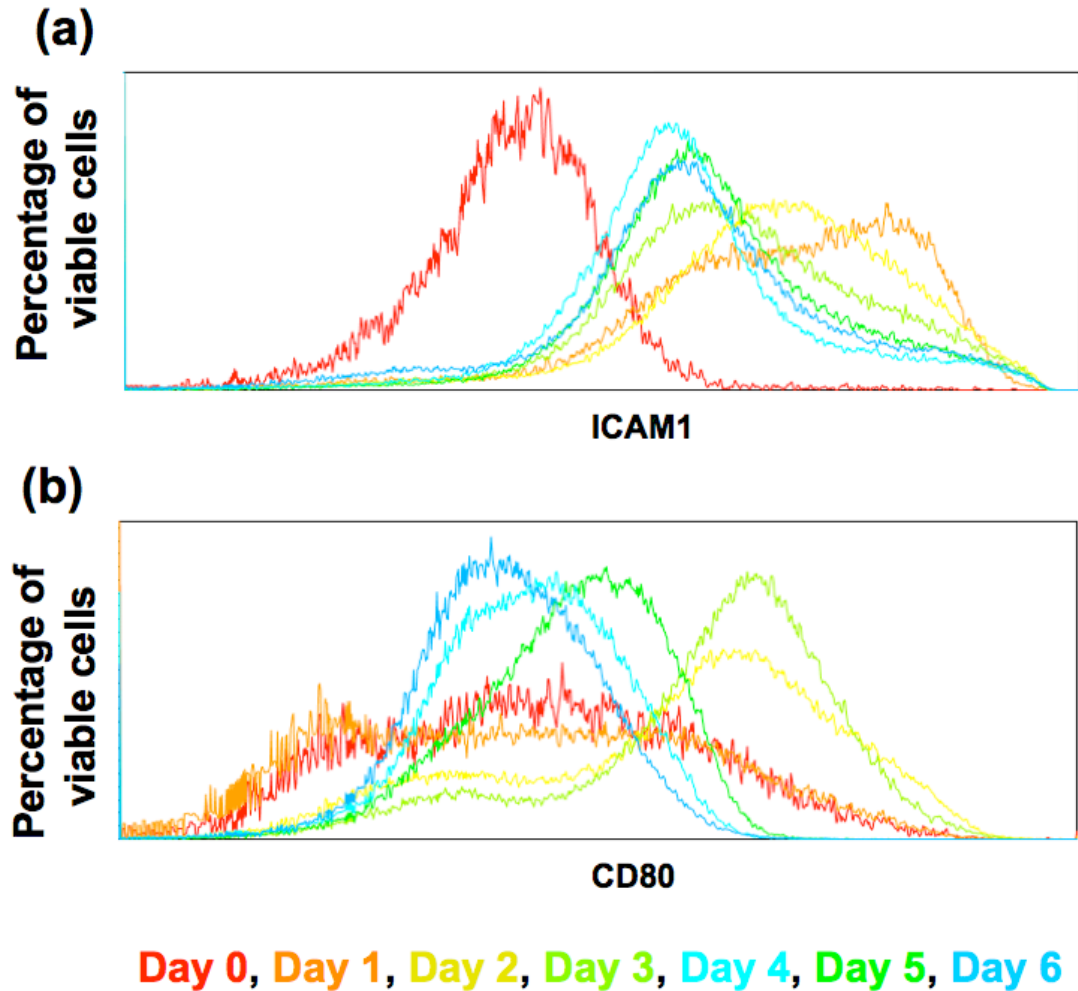


Figure 32. Peak time of CD40L plus cytokine-induced surface expression of ICAM1 and CD80 on HPB B cells.

HPB naïve B cells (1×10^6 cells) were cultured with irradiated CD40L-L cells (1.5×10^3 cells/well) in the presence of recombinant human IL-2 (10 U/mL), IL-6 (100 U/mL), and IL-10 (20 ng/mL). Cells were transferred to new culture plates without CD40L-L cells on Day 4 and cultured for additional 3 days. Cells were harvested everyday from day 0 to day 6. Dead cells were identified by staining with Live/Dead near-infrared Staining Kit, and are excluded. Surface expression of ICAM1 and CD80 induced by CD40L plus cytokine were assessed by flow cytometric analysis. (a) Histogram plot represents the MFI of ICAM1 gated on live population. (b) Histogram plot represents the MFI of CD80 gated on live population. Results represent two different donors with three replicates per treatment group.

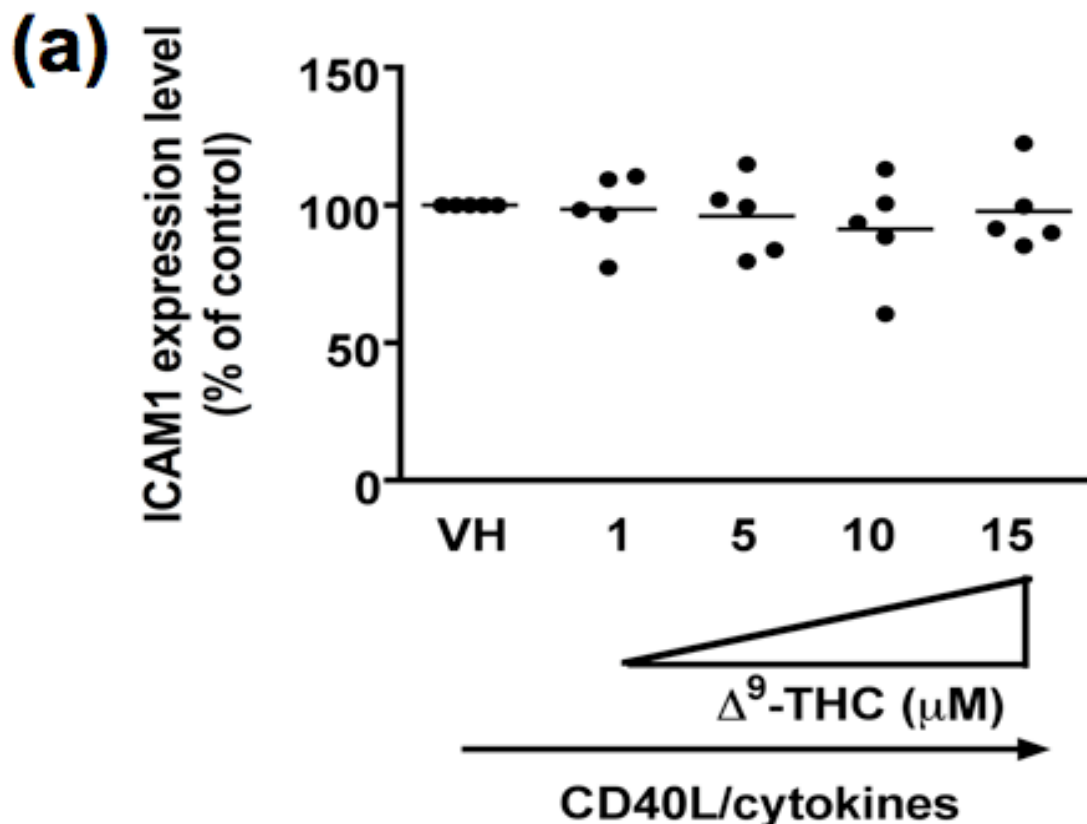


Figure 33. Effect of Δ^9 -THC on surface expression of activation markers in activated HPB B cells.

HPB naïve B cells (1×10^6 cells) were treated with Δ^9 -THC at indicated concentrations or VH (0.02% DMSO) for 30 min and then cultured with irradiated CD40L-L cells (1.5×10^3 cells/well) in the presence of recombinant human IL-2 (10 U/mL), IL-6 (100 U/mL), and IL-10 (20 ng/mL). Surface expression of B-cell activation markers induced by CD40L plus cytokine were assessed by flow cytometric analysis on day 1 for expression of ICAM1 (a) or day 3 for expression of CD69 (b), CD86 (c), and CD80 (d). Dead cells were identified by staining with Live/Dead near-infrared Staining Kit, and are excluded. Results depicted are mean fluorescent intensity of each surface marker. Data were normalized to the VH-treated group and presented as percent of control. The unstimulated B cells express each surface marker with the average less than 3%. The ## indicates significant effect compared to VH-treated group at $p \leq 0.01$. Each dot represents data from one individual donor.

Figure 33 (cont'd)

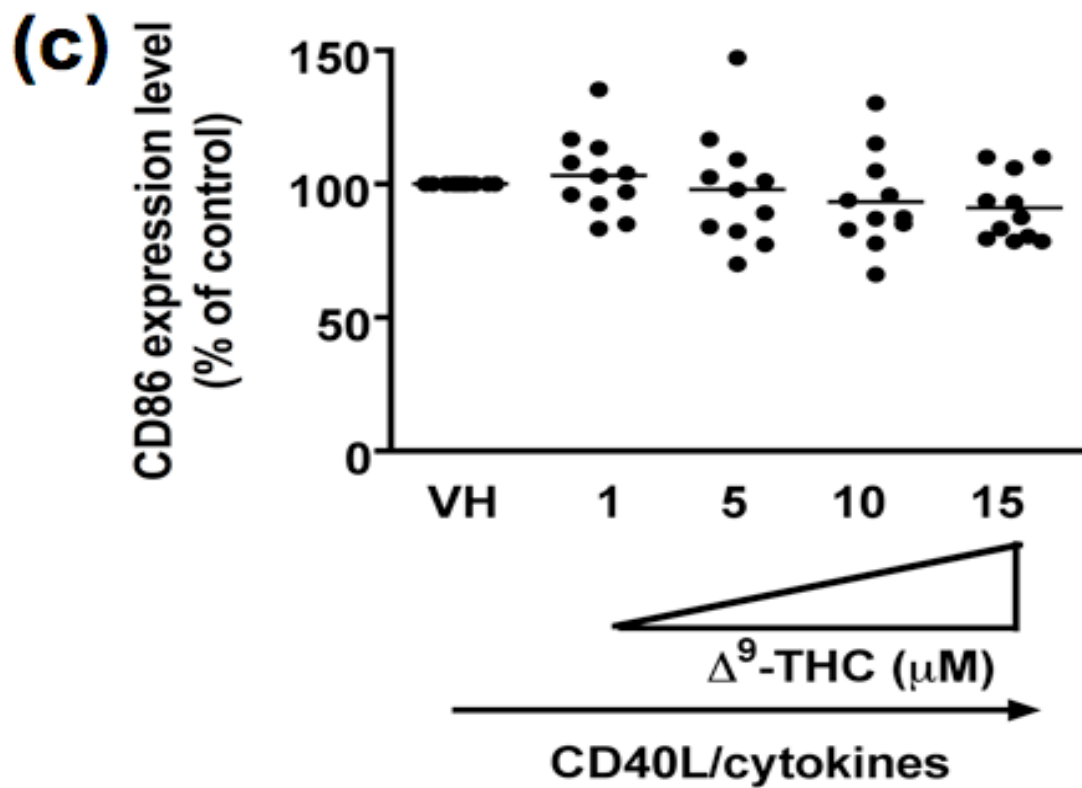
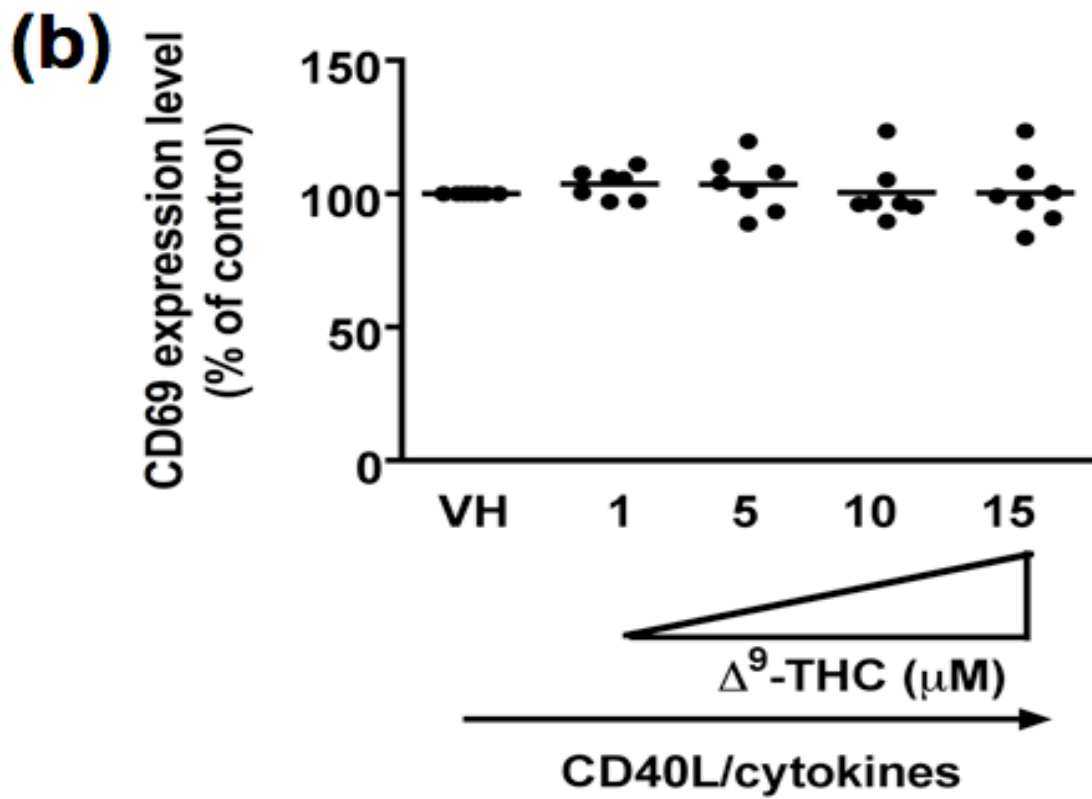
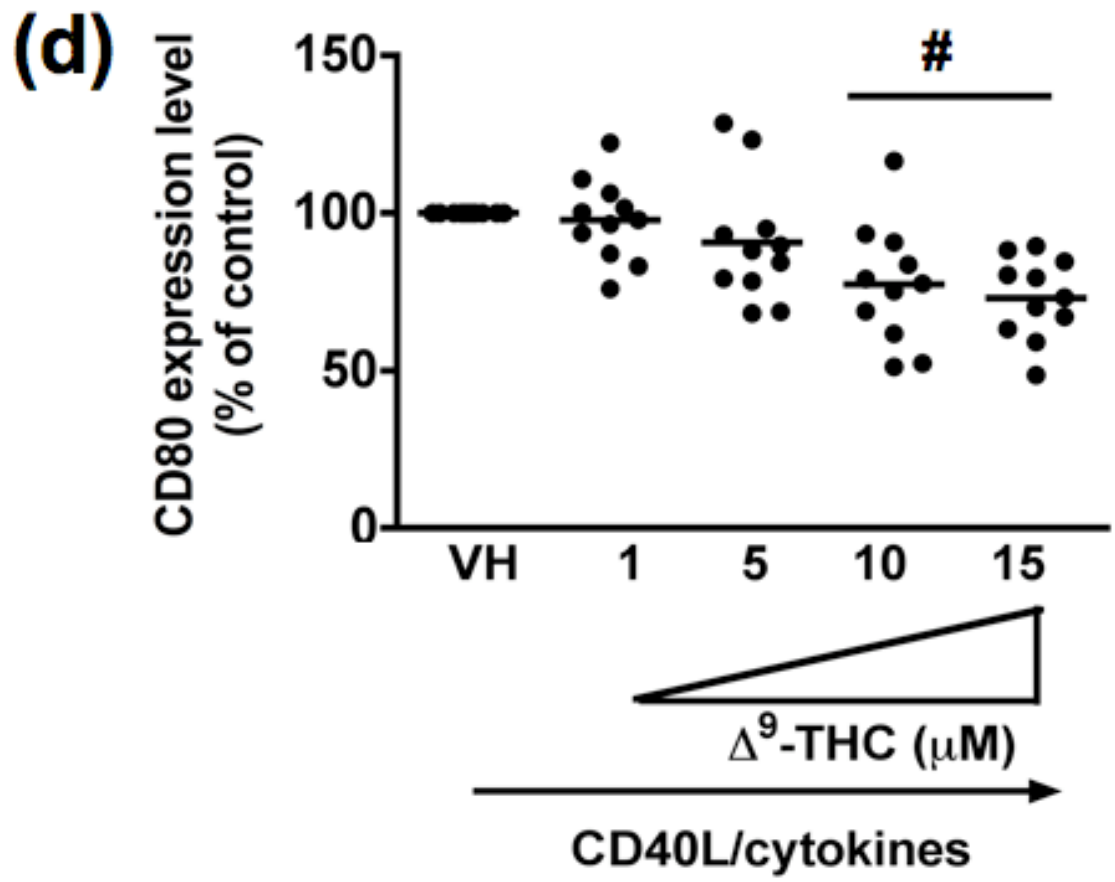


Figure 33 (cont'd)



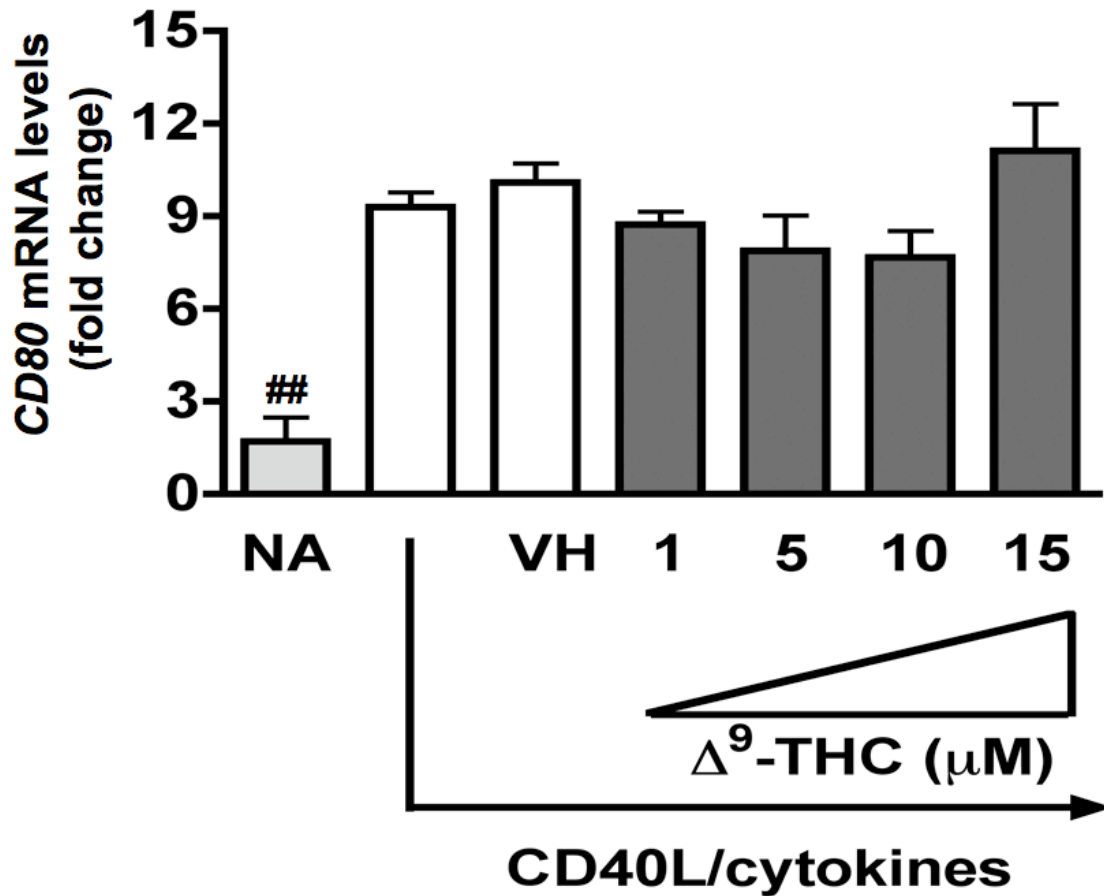


Figure 34 Effect of Δ^9 -THC on *CD80* mRNA levels in activated HPB B cells.

Human naïve B cells (1×10^6 cells) were treated with Δ^9 -THC at the indicated concentrations or VH (0.02% DMSO) for 30 min and then cultured with irradiated CD40L-L cells (1.5×10^3 cells/well) in the presence of recombinant human IL-2 (10 U/mL), IL-6 (100 U/mL), and IL-10 (20 ng/mL). Cells were harvested for RNA isolation on day 2. Steady-state *CD80* mRNA levels were determined by real-time PCR. The fold difference of *CD80* mRNA molecules relative to nonactivated resting cells (naïve, NA) was normalized using the endogenous reference, 18s rRNA. The ## indicates significant effect compared to VH-treated group at $p \leq 0.01$. Results represent three different donors with four replicates per treatment group.

C. Δ^9 -THC impairs CD40L plus cytokine-induced proliferation of HPB B cells.

Survival and proliferation of activated B cells are required and linked to plasma cell differentiation (reviewed in [337]). We therefore examined the effect of Δ^9 -THC on CD40L plus cytokine-induced proliferation of activated B cells by flow cytometry on day 7. Naïve B cells were labeled with CFSE dye before pretreatment with Δ^9 -THC. As shown in Figure 35, stimulation with CD40L plus cytokine induced B cells to undergo multiple divisions, as revealed by the dilution of CFSE signal. In general, Δ^9 -THC did not affect the number of cell divisions, but rather significantly decreased the number of cells that progressed to the latest division, thereby retaining B cells in earlier cell divisions (Table 1). These results suggest that attenuation of B-cell proliferation plays a role in Δ^9 -THC-mediated suppression of CD40 plus cytokine-induced IgM response in humans.

D. Δ^9 -THC suppresses CD40L plus cytokine-induced mRNA expression of *IGJ* in HPB B cells.

To investigate whether Δ^9 -THC perturbs the transcriptional regulatory network of terminally differentiated plasma cells, the steady-state mRNA expression of genes critical in plasmacytic differentiation including, but not limited to, *PRDM1* (encoding for Blimp-1), *PAX5*, and *IGJ* was assessed by RT-PCR on day 6 post activation, which is the peak time of induction [338]

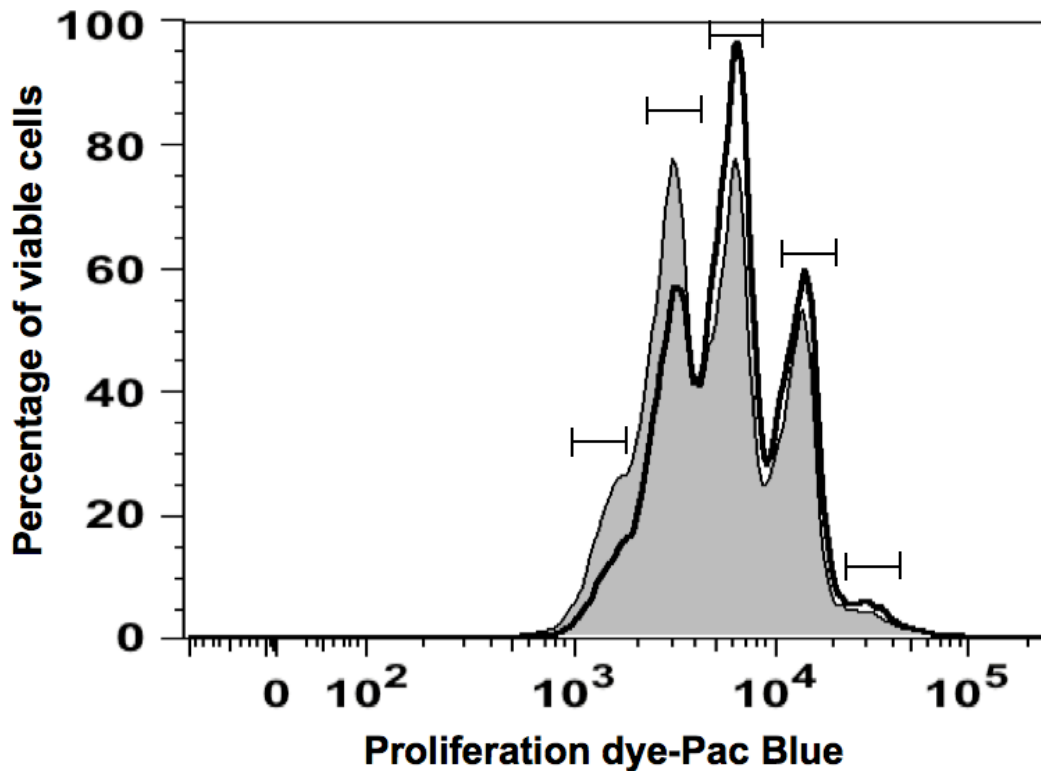


Figure 35. Δ^9 -THC impairs CD40L plus cytokine-induced proliferation of HPB B cells.

HPB naïve B cells (5×10^6 cells/mL) were labeled with the proliferation dye prior to treatment with Δ^9 -THC at indicated concentrations or VH (0.02% DMSO) for 30 min and then cultured with irradiated CD40L-L cells (1.5×10^3 cells/well) in the presence of recombinant human IL-2 (10 U/mL), IL-6 (100 U/mL), and IL-10 (20 ng/mL). Cells were transferred to new plates without CD40L-L cells on Day 4 and cultured for additional 3 days. Dead cells were excluded from the analysis using the Live/Dead Near-Infrared Dead Cell Staining Kit and flow cytometry analysis were performed on day 7. The concatenated data from three replicates per treatment groups were used. Histogram plot represents proliferation profile compared between groups treated with either VH (shaded) or 15 μ M Δ^9 -THC (open). Data are representative of 5 donors.

Table 1. Δ^9 -THC impairs CD40L plus cytokine-induced proliferation of HPB B cells.

Percentage of viable cells at each cell division upon treatment with Δ^9 -THC at indicated concentrations or VH (0.02% DMSO).

Groups	CD40L	VH	Δ^9 -THC (μ M)			
Cell division			1	5	10	15
0	1.9 $\pm$ 0.1	1.9 $\pm$ 0.1	2.2 $\pm$ 0.2	2.4 $\pm$ 0.1	2.4 $\pm$ 0.1	2.6 $\pm$ 0.2*
1	15.6 $\pm$ 0.9	15.0 $\pm$ 0.4	15.3 $\pm$ 0.4	17.0 $\pm$ 0.8	18.0 $\pm$ 0.3	17.8 $\pm$ 1.3
2	15.4 $\pm$ 0.6	12.3 $\pm$ 0.6	13.5 $\pm$ 0.7	14.6 $\pm$ 0.6	16.0 $\pm$ 0.3**	15.3 $\pm$ 1.0*
3	26.5 $\pm$ 0.7	24.3 $\pm$ 0.8	26.6 $\pm$ 0.6	28.9 $\pm$ 0.6**	30.9 $\pm$ 0.5**	30.1 $\pm$ 0.4**
4	24.3 $\pm$ 0.4	24.7 $\pm$ 0.3	23.8 $\pm$ 0.3	21.2 $\pm$ 0.2**	20.0 $\pm$ 0.6**	19.8 $\pm$ 0.7**

Pretreatment with Δ^9 -THC did not affect the upregulation of *PRDM1* mRNA levels (Figure 36) or the downregulation of *PAX5* mRNA levels (Figure 37), but significantly suppressed the upregulation of *IGJ* mRNA levels in activated B cells (Figure 38). These results demonstrated that attenuation of plasmacytic differentiation in human B cells by Δ^9 -THC is mediated, at least in part, through impairment of IgJ expression.

E. Δ^9 -THC suppresses CD40L plus cytokine-induced phosphorylation of STAT3, but not p65 NF κ B, in HPB B cells.

The mechanism by which normal human B cells proliferate to external signals is not entirely clear, but inhibition of NF κ B pathways has a profound effect on CD40-mediated B-cell proliferation and differentiation [98]. In addition, STAT signaling is involved in cell survival, proliferation and differentiation [reviewed in [116]]. Specifically, STAT3 induced by IL-6 promotes both B cell proliferation and differentiation into plasma cells [339]. In light of the above, we further investigated the effect of Δ^9 -THC on the immediate activation of p65 NF κ B and STAT3. Due to the dynamic changes in phosphorylation status, we selected an early time point after activation to increase sensitivity of detection, and therefore, the phosphorylation status of p65 NF κ B and STAT3 in activated HPB B cells was assessed by flow cytometry at 10 min post activation. The soluble recombinant human CD40L in combination with IL-2, -6, and -10 were utilized to promote rapid activation of the signaling pathway of interests. Stimulation of naïve B cells with

recombinant CD40L plus cytokine for 10 min induced phosphorylation of p65 and STAT3 (Figure 39a and 39b). Pretreatment with Δ^9 -THC did not alter the level of phosphorylated p65 (pp65) (Figure 39a), but significantly suppressed the level of phosphorylated STAT3 (pSTAT3) in a concentration-dependent manner (Figure 39b). These results demonstrated that STAT3 is one possible target of Δ^9 -THC.

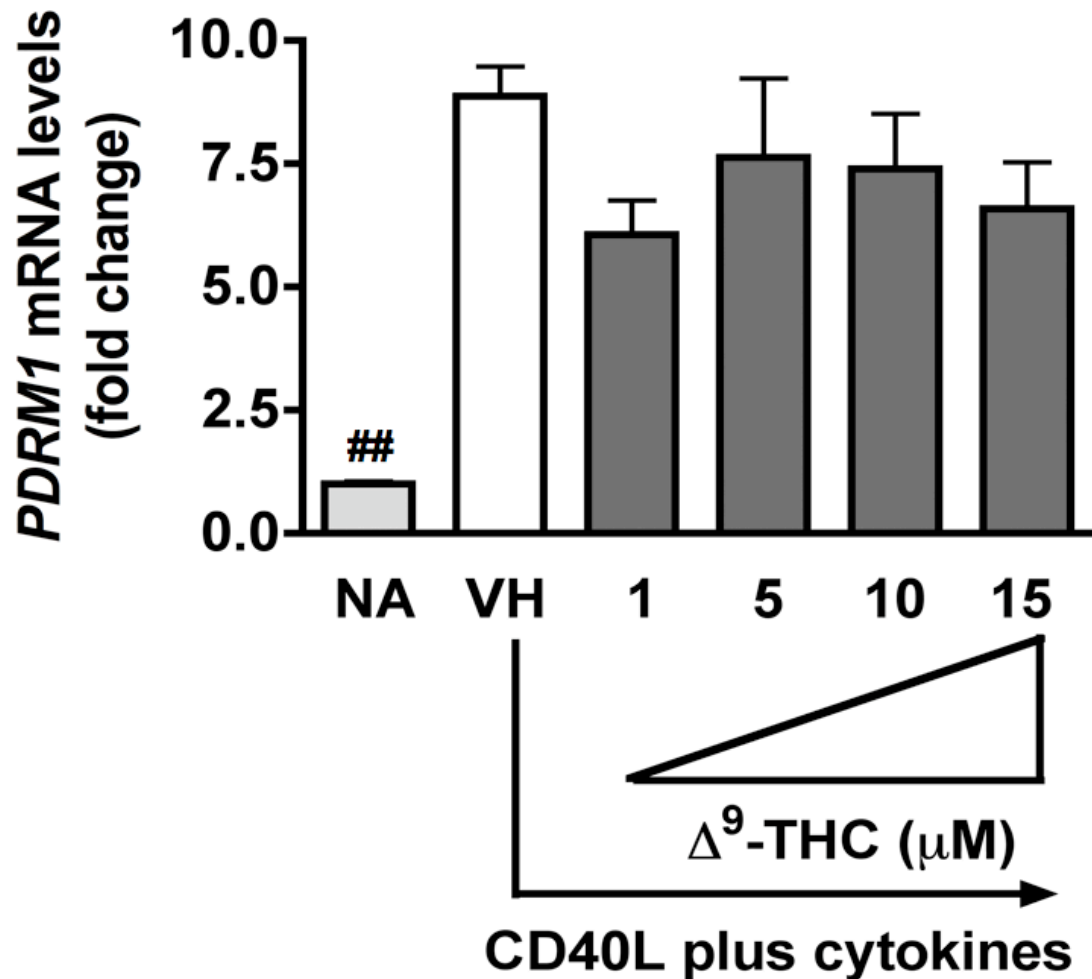


Figure 36. Effect of Δ^9 -THC on *PRDM1* mRNA levels in activated HPB B cells.

Human naïve B cells (1×10^6 cells) were treated with Δ^9 -THC at indicated concentrations or VH (0.02% DMSO) for 30 min and then cultured with irradiated CD40L-L cells (1.5×10^3 cells/well) in the presence of recombinant human IL-2 (10 U/mL), IL-6 (100 U/mL), and IL-10 (20 ng/mL). Cells were transferred to new plates without CD40L-L cells on Day 4 and cultured for 2 additional days. Steady-state *PRDM1* mRNA levels were determined by real-time PCR on day 6. The fold difference of *PRDM1* mRNA molecules relative to nonactivated resting cells (naïve, NA) was normalized using the endogenous reference, 18s rRNA. Results represent three different donors with four replicates per treatment group.

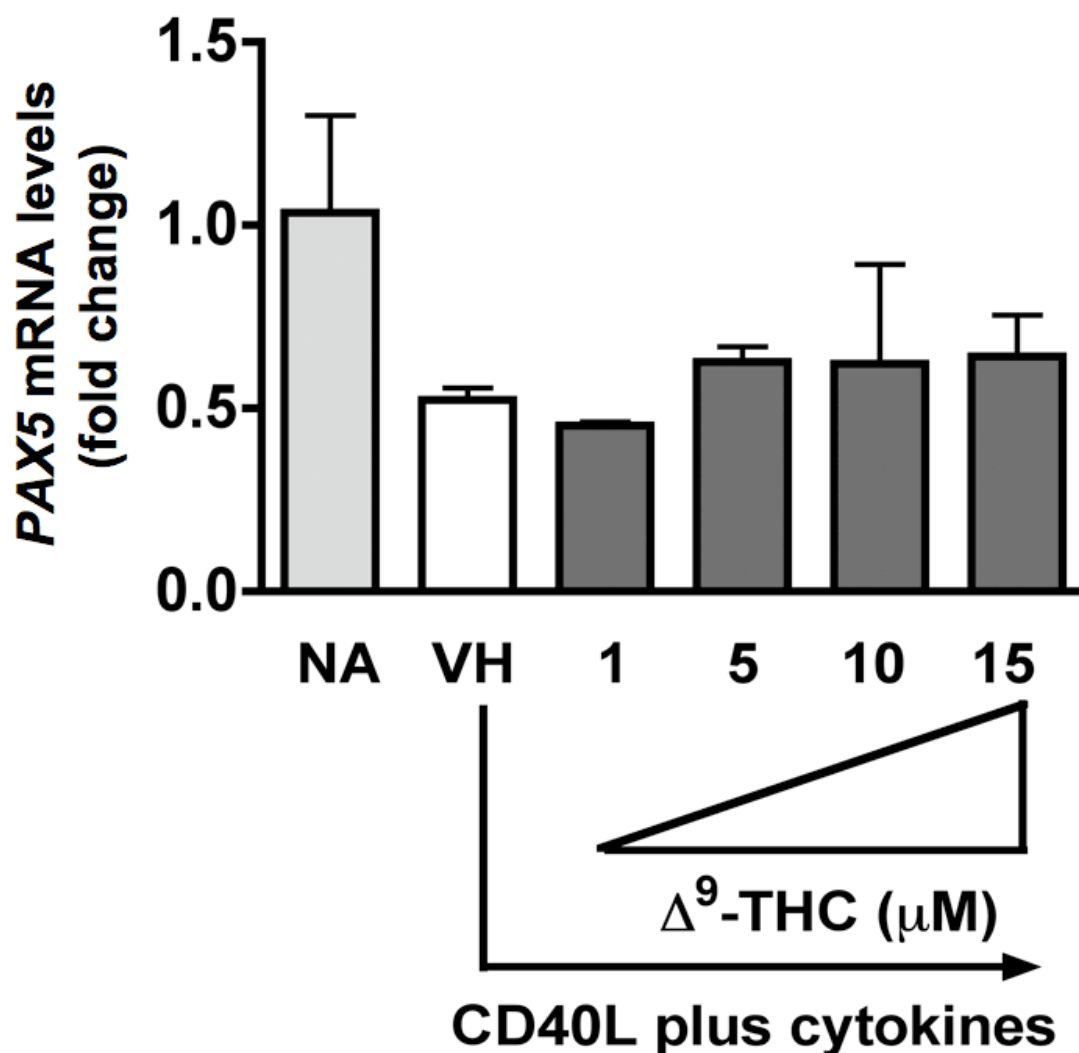


Figure 37. Effect of Δ^9 -THC on *PAX5* mRNA levels in activated HPB B cells

Human naïve B cells (1×10^6 cells) were treated with Δ^9 -THC at indicated concentrations or VH (0.02% DMSO) for 30 min and then cultured with irradiated CD40L-L cells (1.5×10^3 cells/well) in the presence of recombinant human IL-2 (10 U/mL), IL-6 (100 U/mL), and IL-10 (20 ng/mL). Cells were transferred to new plates without CD40L-L cells on Day 4 and cultured for 2 additional days. Steady-state *PAX5* mRNA levels were determined by real-time PCR on day 6. The fold difference of *PAX5* mRNA molecules relative to nonactivated resting cells (naïve, NA) was normalized using the endogenous reference, 18s rRNA. Results represent three different donors with four replicates per treatment group.

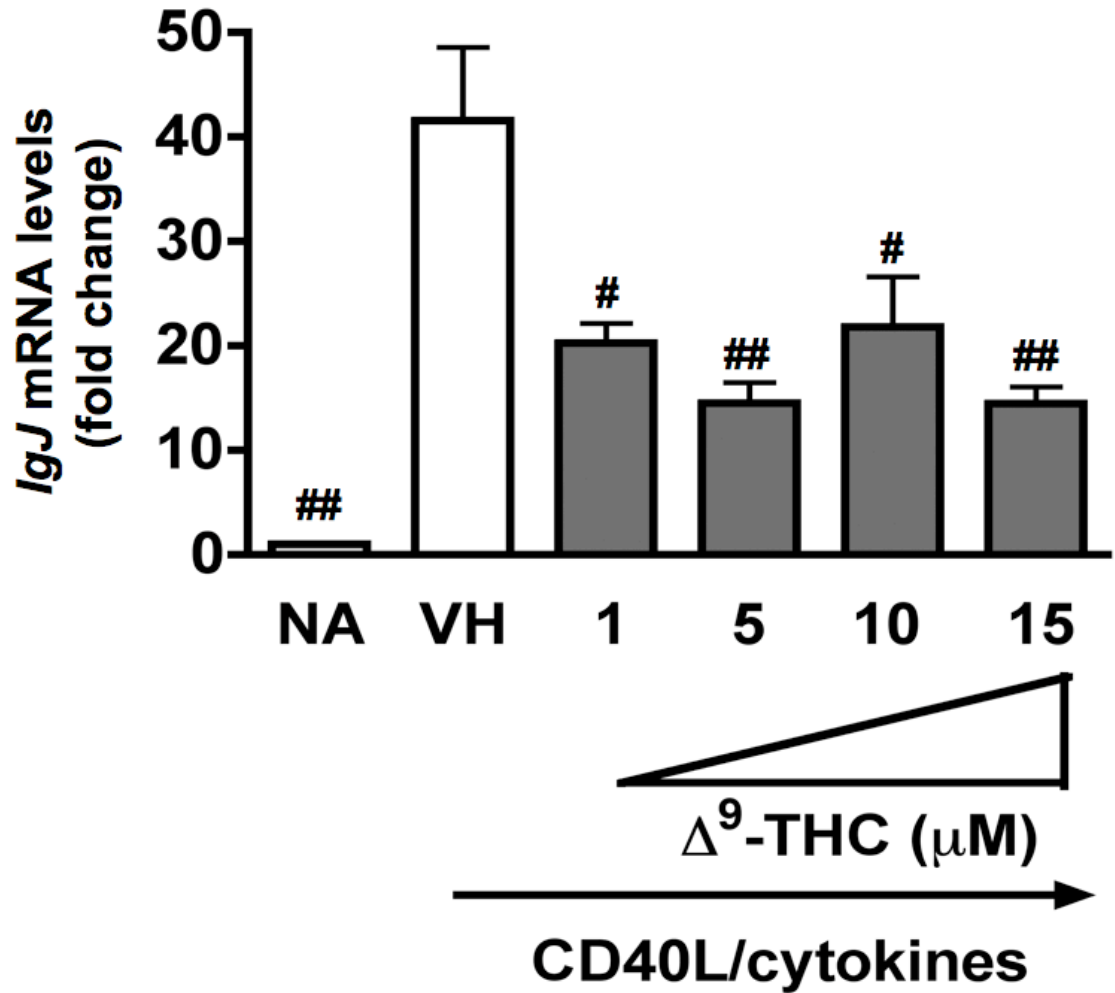


Figure 38. Δ^9 -THC suppresses CD40L plus cytokine-induced *IgJ* mRNA levels in activated HPB B cells.

Human naïve B cells (1×10^6 cells) were treated with Δ^9 -THC at indicated concentrations or VH (0.02% DMSO) for 30 min and then cultured with irradiated CD40L-L cells (1.5×10^3 cells/well) in the presence of recombinant human IL-2 (10 U/mL), IL-6 (100 U/mL), and IL-10 (20 ng/mL). Cells were transferred to new plates without CD40L-L cells on Day 4 and cultured for 2 additional days. Steady-state *IgJ* mRNA levels were determined by real-time PCR on day 6. The fold difference of *IgJ* mRNA molecules relative to nonactivated resting cells (naïve, NA) was normalized using the endogenous reference, 18s rRNA. The # or ## indicates significant effect compared to VH-treated group at $p \leq 0.05$ or ≤ 0.01 , respectively. Results represent three different donors with four replicates per treatment group.

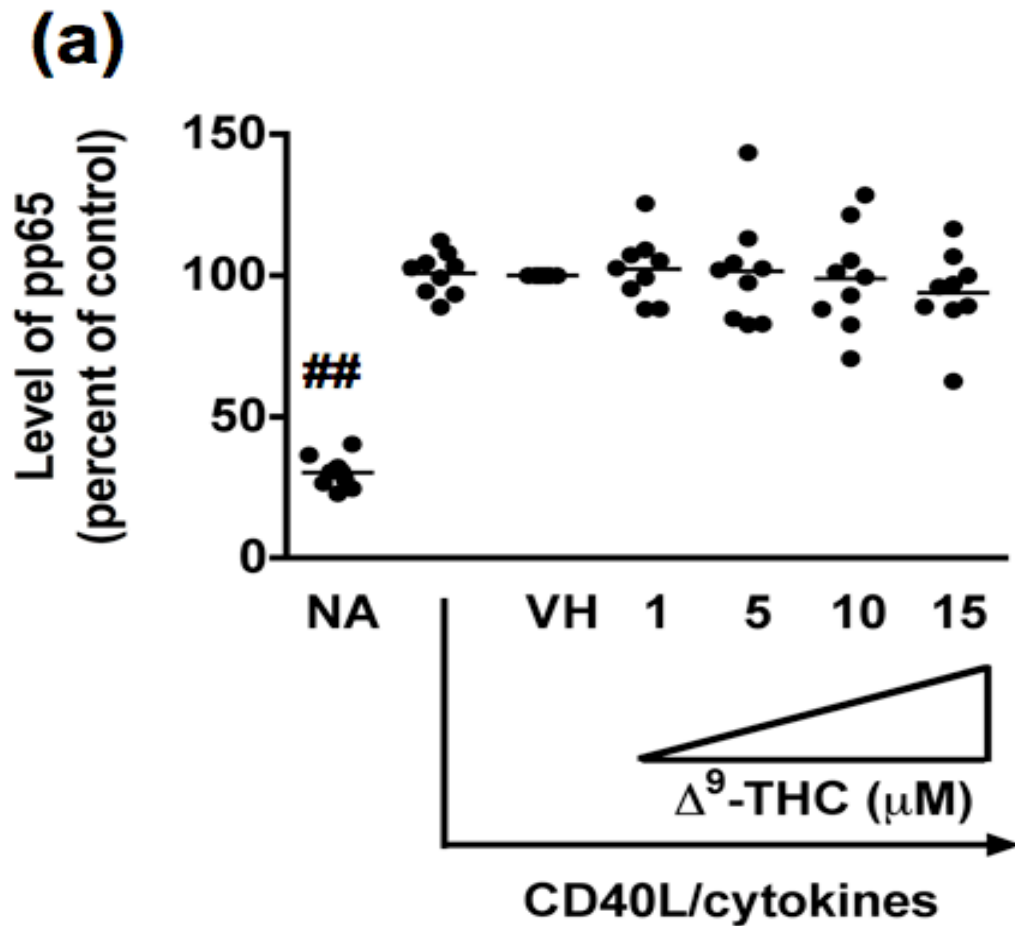
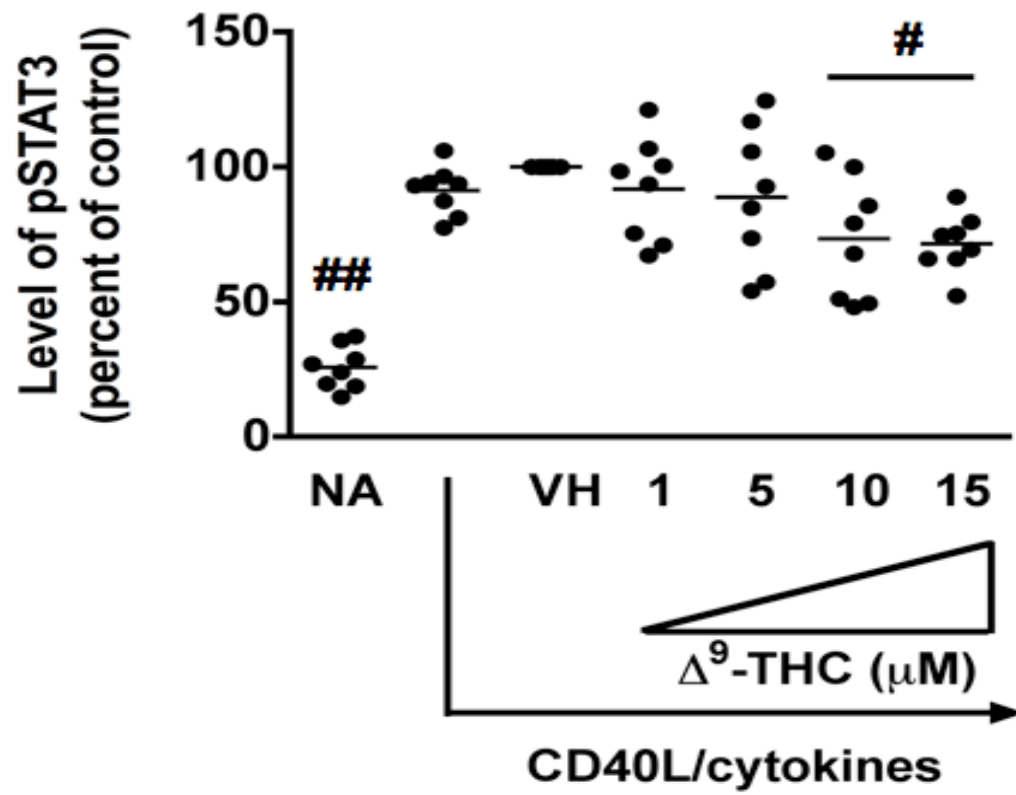


Figure 39. Effect of Δ^9 -THC on the phosphorylation of p65 NF κ B and STAT3 in activated HPB B cells.

HPB naïve B cells (1×10^6 cells) were equilibrated at 37°C in 5% CO₂ for 3 h prior treated with Δ^9 -THC at indicated concentrations or VH (0.02% DMSO) for 30 min. B cells were activated with recombinant human CD40L (200 nM), human IL-2 (10 U/mL), IL-6 (100 U/mL), and IL-10 (20 ng/mL) for 10 min at 37°C. Cells were fixed with 1.5% paraformaldehyde and permeabilized with ice-cold 100% methanol. The intracellular expression of pSTAT3 and pp65 NF κ B was assessed by phosflow cytometry. (a) Scatter plot represents the level of pp65 NF κ B. (b) Scatter plot represents the level of pSTAT3. Results depicted are mean fluorescent intensity of each surface marker. Data were normalized to the VH-treated group and presented as percent of control. The # or ## indicates significant effect compared to VH-treated group at $p \leq 0.05$ or ≤ 0.01 . Each dot represents data from one individual donor.

Figure 39 (cont'd)

(b)



DISCUSSION

This dissertation research aimed to investigate the role of CD40-CD40L interaction in Δ^9 -THC-mediated suppression of the T cell-dependent IgM response. Toward this end, the effect of Δ^9 -THC on both T and B cells were determined. The first section focused on characterizing the effect of Δ^9 -THC on the upregulation of CD40L expression in activated mouse and human T cells. The second section focused on how Δ^9 -THC suppresses CD40L-induced primary antibody response in primary human B cells.

I. Effect of Δ^9 -THC on the upregulation of CD40L on activated CD4⁺ T cells

The strength and duration of CD40 signaling is crucial in initiating adaptive immune responses mainly involved in inflammation and is controlled by the presence of its cognate ligand, CD40L, which is mainly expressed on the surface of activated CD4⁺ T cells. Δ^9 -THC impairs multiple aspects of T cell action, including activation, accessory, and effector functions [294,303]. Thus, the influence of Δ^9 -THC on the upregulation of CD40L on activated mouse CD4⁺ T cells was initially investigated, followed by extensive characterization of critical molecular events that are involved in primary human T cells.

In studies investigating the effect of Δ^9 -THC on the upregulation of surface CD40L on activated mouse CD4⁺ T cells, two modes of *in vitro* T cells activation were used. In concordance with prior reports, similar CD40L expression kinetics were observed with splenic CD4⁺ T cells activated by either PMA/Io or anti-CD3/CD28 (reviewed in [69]). It is noteworthy that while we observed a similar kinetic profile, the magnitude of surface CD40L expression induced by both stimuli under our experimental condition was modest compared to published reports and could be due to several factors including differences in culture conditions, mouse strain, and species (human versus mouse). Another possibility that might contribute to the modest CD40L induction is the internalization of CD40L after binding to its receptor, CD40, on B cells [75]. Although this possibility seems plausible since we utilized splenocytes, which are heterogeneous and contain B cells, we excluded this possibility, as T cell enrichment did not increase the magnitude of surface CD40L expression. We also found that the kinetics of *CD40L* mRNA upregulation induced by these two stimuli were quite different. PMA/Io induced steady-state *CD40L* mRNA levels rapidly, but transiently, while steady-state *CD40L* mRNA levels induced by anti-CD3/CD28 were slow, but sustained. These differences might be explained by the observation that the CD28 costimulatory signal promoted mRNA stability for many T cell-derived cytokines [340]. However, *CD40L* mRNA is still very unstable, with a $t_{1/2}$ of approximately 23 min in mouse [341].

We show here that Δ^9 -THC distinctly modulated the upregulation of surface CD40L induced by different T cell activation stimuli. Δ^9 -THC suppressed the upregulation of both surface CD40L expression and steady-state *CD40L* mRNA levels induced by anti-CD3/CD28 in a concentration-dependent manner. Δ^9 -THC did not affect the upregulation of surface CD40L induced by PMA/Io. This is in contrast to our previous reports showing that Δ^9 -THC suppressed PMA/Io-induced cytokines (such as IL-2 and IFN γ), and co-stimulatory molecules (such as ICOS) [294,303]. Unlike other proteins that are induced by T cell activation, the expression of CD40L on activated T cells largely depends on an increase in $[Ca^{2+}]_i$ through the activation of calcineurin and Ca^{2+} /Calmodulin kinase Type IV [327,342]. This correlates well with numerous reports showing that NFAT, the activation of which requires calcineurin, is a critical transcription factor in regulating CD40L expression [323,343,344]. The importance of NFAT is also evidenced by a marked decrease in CD40L expression in the presence of CsA, an immunosuppressive drug that blocks NFAT activation and nuclear translocation [325]. As stated previously, our laboratory has demonstrated marked suppression by Δ^9 -THC of NFAT activation [303] and leads us to speculate that impaired NFAT likely also contributes to suppression of CD40L by Δ^9 -THC. It is also noteworthy that the downregulation of surface CD4, a co-

receptor on helper T cells, after PMA/Io treatment as observed here is a common phenomenon [345-348], but did not affect the upregulation of CD40L.

Based on the fact that PMA/Io activates T cells by bypassing the proximal TCR signaling, it is possible that Δ^9 -THC might target one or more signaling molecules between the T cell receptor and the activation of PKC- θ and/or induction of intracellular calcium. This is in accordance with a study demonstrating that Δ^9 -THC suppressed the activation of proximal TCR signaling components, partly due to the stabilization of Lck, the kinase responsible for initiating TCR signaling, when present in its inactive form [308]. Borner and coworkers also demonstrated the involvement of CB1 and CB2 in Δ^9 -THC-mediated impairment of proximal TCR signaling, as evidenced by reversion of the suppressive effect of Δ^9 -THC, in the presence of CB1 and CB2 antagonists. This is in contrast to the results reported here, in which the suppressive effect of Δ^9 -THC on anti-CD3/CD28-induced CD40L expression occurred in the presence and absence of CB1 and CB2 in mice. It is noteworthy that Borner and coworkers studied the involvement of CB1 and CB2 by using IL-4 stimulated Jurkat or primary human T cells to induce CB1 expression (prestimulated cells) together with CB1 and CB2 selective antagonists (AM281 and AM630, respectively). In contrast, in our experiments, splenocytes from CB1/CB2 null mice (CB1^{-/-}CB2^{-/-} mice) were used eliminating the need for cannabinoid receptor antagonists, which are known to have inverse agonist activity as well as off-target effects,

particularly in case of AM281 [349]. These differences in experimental conditions might account for the discrepancy between our findings and that of Borner and coworkers. At this time, we cannot exclude the possibility that cannabinoids target various orphan G protein-coupled receptors (e.g. GPR55 and GPR119), nuclear hormone receptors (e.g. the PPAR α and γ), or ion channels (e.g. the TRPV1 and 2, and the TRPC1 (reviewed in [349,350])). However, we have ruled out the possible involvement of GR in suppression of CD40L by Δ^9 -THC. Finally, although less likely, the CB1/CB2-independent mechanism may also be explained by the fact that Δ^9 -THC, which is very lipophilic, preferentially aligns its hydrophobic portion parallel with the membrane phospholipid chain (reviewed in [351]). This perpendicular orientation results in alteration of plasma membrane's thermodynamic properties [352] and may disrupt the migration and interaction of TCR/co-receptors in the plasma membrane during activation. Thus, it is possible that Δ^9 -THC may exert its suppressive effect by interfering the formation of TCR microcluster, which has been shown to be important in T cell activation [reviewed in (Yukosuka and Saito, 2010)].

In studies characterizing the involved mechanism in human, we first investigated whether Δ^9 -THC suppresses CD40L expression by activated primary human CD4⁺ T cells. Consistent with our previous study demonstrating the suppression of CD40L upregulation by Δ^9 -THC in mouse splenic T cells [333],

Δ^9 -THC attenuated anti-CD3/CD28-induced CD40L expression in human T cells at both the protein and mRNA level. It is notable that the kinetics of CD40L upregulation in human that we observed here is different from our observations in mouse T cells and several previous reports, in which the peak induction was early, approximately 6-8 h post stimulation [324-327,333]. Although the rapid induction of CD40L on 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 [71-74]. Here we report that the peak time of induction of surface CD40L expression on activated human CD4⁺ T cells was 48 h post activation with anti-CD3/CD28. This is in accordance with reports showing that the second peak occurred at 48 h. However we did not observe the 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.* 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 [73,74].

Mechanistically, we demonstrate that Δ^9 -THC impaired the DNA-binding activity of both NFAT and NF κ B, two critical transcription factors involved in regulating CD40L expression [76,78-80]. 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) [323,353]. Δ^9 -THC-mediated suppression of NFAT-DNA complexes observed here was consistent with our previous mouse study in which we demonstrated that the mechanism of T cell suppression by Δ^9 -THC involves, at least in part, the impairment of NFAT activity [303]. In addition, CBN, another immunomodulatory plant-derived cannabinoid compound, 2-AG, an endogenous cannabinoid, and 15-deoxy- $\Delta^{12,14}$ -PGJ₂-glycerol ester, a putative metabolite of 2-AG, were all found to inhibit the DNA-binding activity of NFAT in activated T cells [354-357]. 2-AG also inhibited the nuclear translocation of both NFATc1 and NFATc2 [358]. In order to characterize the molecular target responsible for Δ^9 -THC-mediated suppression of NFAT, the possible involvement of GSK3 β , a kinase regulating NFAT activation, was investigated, but was found not to be affected by Δ^9 -THC. These results clearly demonstrated that GSK3 β is not involved in cannabinoids-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, which is consistent with a report by Shena *et. al.*, who also identified p65 containing NF κ B-DNA binding complexes in the absence of p50 [80]. Again, these results are consistent with another study from our laboratory demonstrating that CBN primarily suppressed the DNA binding of p65 and c-Rel in mouse T cells [311]. Overall, these results demonstrate that suppression of NFAT and

NF κ B DNA binding activity are common mechanisms underlying the impairment of T cell activity by cannabinoids.

Part of the mechanism by which NFAT and NF κ B DNA binding activity are suppressed by Δ^9 -THC involves dysregulation of Ca^{2+} since we demonstrate for the first time that Δ^9 -THC significantly impaired anti-CD3/CD28-induced Ca^{2+} elevation in activated primary human CD4^+ T cells. This observation is somewhat in contrast to our prior finding that immunomodulatory cannabinoids, Δ^9 -THC, CBN, and HU-210 robustly increased the influx of extracellular Ca^{2+} in resting T cells [239,240]. It is noteworthy that although this study investigated the effect of Δ^9 -THC on Ca^{2+} signaling in response to anti-CD3/CD28 activation, pretreatment with Δ^9 -THC and/or preincubation with anti-CD3 and anti-CD28 in the absence of the IgG crosslinker did not increase $[\text{Ca}^{2+}]_i$. 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 Δ^9 -THC on Ca^{2+} elevation. However, our results are consistent with a report demonstrating that pretreatment with Δ^9 -THC suppressed the increase of $[\text{Ca}^{2+}]_i$ induced by concanavalin A in mouse thymocytes [359]. The exact mechanism by which Δ^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 Δ^9 -THC did not affect anti-CD3/CD28-induced phosphorylation of PLC γ 1/2, the active forms of PLC γ that generate IP₃ to release Ca²⁺ from intracellular stores. To date, the activation of PLC γ is the major pathway responsible for the IP₃ production; therefore it is unlikely that Δ^9 -THC affects IP₃ production without changes in the activation of PLC γ . In contrast, if Δ^9 -THC altered the capacity of IP₃ receptors to bind IP₃, a similar profile of activity could be observed. Another possibility is that Δ^9 -THC may affect distal steps in receptor-mediated Ca²⁺ mobilization. Indeed, not only Ca²⁺ channels, but voltage- and Ca²⁺-dependent potassium channels, play critical roles in promoting the sustained increase of [Ca²⁺]_i (reviewed in [360]). Therefore, Δ^9 -THC might directly or indirectly target one of the aforementioned ion channels in T cells. Additional studies are required to decipher the detailed molecular mechanisms of Ca²⁺ regulation by Δ^9 -THC in T cells.

In conjunction with the absence of an inhibitory effect of Δ^9 -THC on phosphorylation of PLC γ 1/2, Δ^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 clearly demonstrated that Δ^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 Δ^9 -THC suppressed TCR-induced phosphorylation of ZAP70 [308]. 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 PLC γ upon TCR stimulation [361]. In addition, here we demonstrated that 30 min pretreatment with Δ^9 -THC significantly suppressed TCR-induced CD40L expression; whereas Borner *et al.* did not observe any effect of Δ^9 -THC in any measurement unless the cells were pretreated with Δ^9 -THC for 2 h. Differences in clones and concentration of anti-CD3 and anti-CD28, as well as the differences in concentration of Δ^9 -THC, might also account for the differential effect of Δ^9 -THC on proximal TCR signaling [308].

II. Effect of Δ^9 -THC on CD40L plus cytokine-induced primary antibody response in primary human B cells.

This section focused primarily on the effect of Δ^9 -THC on primary antibody responses, the effector functions of B cells. We demonstrated here that Δ^9 -THC

significantly attenuated the primary antibody response by HPB B cells stimulated with CD40L plus cytokine. This finding is contrast to previous reports describing B cell is not a sensitive target for Δ^9 -THC-mediated suppression of IgM response against sRBC as Δ^9 -THC did not alter the number of IgM antibody forming cells induced either by LPS, the polyclonal B cell activator, both *in vivo* [298] and *in vitro* [294]. However, our result is consistent with a previous study using mouse splenic B cells in which treatment with Δ^9 -THC decreased the numbers of IgM secreting cells induced by CD40L plus cytokine [294]. These results clearly demonstrate that the B cell is a direct cellular target of Δ^9 -THC in the suppression of T-cell dependent antibody response in both mouse and humans. Furthermore, we extended our initial investigation of direct Δ^9 -THC effects on B cells to elucidate key molecular events that contribute to the mechanism by which Δ^9 -THC suppressed B cell differentiation.

We demonstrated that Δ^9 -THC selectively alters the initial activation of B cells as evidenced by significant suppression of surface CD80 expression induced by CD40L plus cytokine. However, the expression of other B cell surface molecules, CD69, CD86, or ICAM1, remained unaffected upon treatment with Δ^9 -THC. These aforementioned molecules play critical roles in the T cell-B cell interaction, especially CD80 and CD86 [reviewed in [362]]. Both CD80 and CD86 serve as ligands for CD28 or CTLA4 expressed on activated CD4⁺ T cells,

thereby increasing or decreasing, respectively, the T cell activation signal [363]. Therefore, the attenuation of surface CD80 expression on activated B cells by Δ^9 -THC could play an important role in the initial phase of plasma cell differentiation by disrupting the antigen presentation capacity of B cells. A recent study suggested that CD80 plays important role in formation and/or maintenance of long-live plasma cells, in which the antibody production from long-live plasma cells, but not plasmablasts, was impaired in CD80-deficient mice [364]. Therefore, it is possible that the selective inhibitory effect of Δ^9 -THC on the upregulation of CD80 may impair the production of high-affinity long-lived plasma cells and memory B cells. The exact mechanism(s) by which Δ^9 -THC mediates suppression of surface CD80 expression is still unknown and warrant further investigation, but our observation that Δ^9 -THC did not affect the steady-state level of *CD80* mRNA suggests that Δ^9 -THC regulates the expression of surface CD80 at the post-transcriptional level. One possibility is Δ^9 -THC increases internalization and degradation of surface CD80. Although regulation of CD80 degradation is not understood, the ubiquitin-dependent degradation of surface CD86 has been shown to be mediated, in part, by ubiquitin ligase membrane-associated RING-CH-1 (MARCH1) [365]. Although overexpression of MARCH1 did not affect the surface expression of CD80 [365], the possibility that Δ^9 -THC

may facilitate the internalization and/or degradation of CD80 by altering a yet-unknown ubiquitin ligase cannot be ruled out.

Our finding that Δ^9 -THC impaired the ability of activated B cells to divide correlates well with the suppressive effect on the generation of IgM secreting cells since differentiation into Ig-secreting cells is also linked to cell division [366,367]. It is noteworthy that the anti-proliferative effect of Δ^9 -THC on activated B cells, at least in this model, did not affect the number of total viable cells. Although Δ^9 -THC and other cannabinoids have been shown to induce apoptosis in immune cells (reviewed in [368]), we did not find any evidence of cell loss. Next we investigated whether Δ^9 -THC impaired the transcriptional regulatory network involved in plasmacytic differentiation and demonstrated that Δ^9 -THC significantly suppressed *IGJ* mRNA expression, but did not alter the mRNA level of *PRDM1*, “the master regulator” of plasma cell differentiation, or *PAX5*, one of critical transcription factors in maintaining B cell identity (reviewed in [3]). Taken together, these results suggested that suppression by Δ^9 -THC of the primary IgM response in human primary B cells is, at least in part, mediated through the impairment of IgJ expression.

At the molecular level we demonstrated that treatment with Δ^9 -THC attenuated the phosphorylation of STAT3, but not p65 NF κ B, in response to recombinant CD40L and cytokine. It is well established that CD40 signals mainly

through NF κ B, whereas cytokines signal mainly through STAT pathways; in particular IL-10 induces phosphorylation of STAT3 [134]. STAT3 is necessary for the generation of plasma cells by promoting B cell survival *in vitro* [151,369] and increasing the upregulation of Blimp1 [149]. Further, mutations of STAT3 resulted in a reduction of DNA-binding activity and was associated with decreasing the induction of Blimp1 in response to recombinant CD40L plus IL-10 or IL-21 [161]. Taken together, this finding suggests that IL-10-mediated induction of STAT3 could be another putative target of suppression by Δ^9 -THC and likely contributes to Δ^9 -THC-mediated impairment of IgM response in humans.

The inability of Δ^9 -THC to modulate the phosphorylation of p65 NF κ B is in contrast to previous studies from our laboratory demonstrating as mentioned earlier, that CBN suppressed the DNA binding of p65 and c-Rel in mouse T cells [311] and our finding here that Δ^9 -THC suppresses the DNA-binding activity of NF κ B in activated human T cells. These findings suggest that Δ^9 -THC exhibits stimulation- and/or cell type-specific selectivity in NF κ B inhibition. It is noteworthy that NF κ B is one of the critical transcription factor involved in the regulation of genes encoding for CD69, CD80, CD86, and ICAM1 [100,370-372]. Therefore, the lack of an effect of Δ^9 -THC on CD40-mediated phosphorylation of

p65 NF κ B supports our findings that Δ^9 -THC did not affect cell surface expression of CD86, CD69 and ICAM1 or gene transcription of *CD80*.

III. Concluding remarks

The studies reported in this dissertation demonstrated that suppression of primary IgM response in humans by Δ^9 -THC involves the perturbation of CD40-CD40L interactions, in particular the upregulation of CD40L in activated CD4⁺ T cells. Moreover, these studies show that the mode of T cell activation leading to the induction of surface CD40L plays an important role in determining the magnitude of sensitivity to Δ^9 -THC. While Δ^9 -THC impaired CD40L expression induced by anti-CD3/CD28, it did not appear to alter the PMA/Io-induced CD40L expression in mouse splenic CD4⁺ T cells. These findings suggested that Δ^9 -THC targets proximal TCR-associated signaling. However, the demonstration that Δ^9 -THC did not interrupt proximal TCR signaling events (e.g. tyrosine phosphorylation of ZAP70, Akt, and PLC γ 1/2) in primary human CD4⁺ T cells suggested that the aforementioned membrane-proximal effectors were not targeted by Δ^9 -THC, at least in human primary T cells.

The precise role of CB1 and/or CB2 in cannabinoid-mediated immune modulation remains elusive. Both receptor-dependent and receptor-independent mechanisms are involved depending on cell type and the response being

measured [294,309,373], suggesting the existence of additional receptors beyond CB1/CB2 (reviewed in [350]). With the demonstration that Δ^9 -THC attenuated anti-CD3/CD28-induced CD40L expression in a CB1/CB2 receptor independent manner, another potential target, GR, was examined. In fact, dexamethasone, a potent GR agonist, suppresses T-cell dependent humoral immune responses, shares structural similarity with Δ^9 -THC and also suppresses anti-CD3/CD28-induced CD40L expression in mouse splenic CD4⁺ T cells [374,375]. However, none of the measurements used in this present study were able to demonstrate the potential interaction between Δ^9 -THC and GR. Collectively, suppression of anti-CD3/CD28-induced CD40L expression in mouse splenic CD4⁺ T cells by Δ^9 -THC likely occurs independently of CB1, CB2 and GR.

Δ^9 -THC was shown to impair the DNA binding activity of both NFAT and NF κ B to their response elements derived from CD40L promoter, thereby attenuating the transcription of *CD40L* gene and resulting in reduction of steady-state *CD40L* mRNA levels in activated human primary T cells. The inhibitory effects of Δ^9 -THC on NFAT are likely mediated through impairment of T cell activation, rather than retention of inactive cytoplasmic NFAT, as evidenced by a lack of change in the phosphorylation status of GSK3 β , a NFAT kinase that regulates cytoplasmic-nuclear localization (reviewed in [59]). Importantly, Δ^9 -

THC impaired anti-CD3/CD28-induced Ca^{2+} elevation, the major mechanism involved in NFAT activation, and to a certain extent is also involved in NF κ B activation (reviewed in [31]). These findings identify perturbation of the calcium-NFAT and NF κ B signaling cascade as a key event in the mode of action by which Δ^9 -THC exerts a suppressive effect on human T cell function. Future studies are needed to elucidate the exact mechanism by which Δ^9 -THC exerts its suppressive effect on anti-CD3/CD28-induced Ca^{2+} elevation in primary human CD4^+ T cells.

In addition to suppression of CD40L upregulation on activated CD4^+ T cells, Δ^9 -THC attenuated B-cell function involved in the generation of IgM secreting cells in humans. Δ^9 -THC was found to impair the initial activation stage of B cells as demonstrated by significant suppression of surface CD80 expression induced by CD40L plus cytokine. Further, Δ^9 -THC did not affect the steady-state levels of *CD80* mRNA, suggesting that suppression of surface CD80 expression by Δ^9 -THC regulates the expression of CD80 at the post-transcriptional level. Importantly, no change in the expression of CD69, CD86, and ICAM1 was observed in the presence of Δ^9 -THC. These results suggest that Δ^9 -THC did not affect the common transcription factors regulating expression of

the genes encoding for all aforementioned molecules. Indeed, this notion is well supported by the demonstration that Δ^9 -THC did not perturb CD40-induced activation of NF κ B, as evidenced by the absence of alterations in the phosphorylation status of p65 NF κ B in activated human B cells. An avenue for future research in this area would be to investigate the biological consequence of Δ^9 -THC-mediated specific suppression of surface CD80 on T cell activation, as CD80 is one of co-stimulatory molecules that can provide either positive or negative signals to T cells during T cell-B cell interaction [363].

Proliferation of activated human B cells also attenuated by Δ^9 -THC. Further, Δ^9 -THC was shown to impair transcription of the genes involved in plasmacytic differentiation, as evidenced by decreased mRNA levels of *IGJ*. With the demonstration that Δ^9 -THC impairs B cell function, the immediate downstream signaling molecules in B cell activation were examined. These studies showed that Δ^9 -THC attenuated the phosphorylation of STAT3 but not p65 NF κ B. Collectively, these findings demonstrated that suppression by Δ^9 -THC of the IgM response by human primary B cells is mediated, at least in part, through impairment of STAT3 activation, proliferation and plasmacytic differentiation. Future studies are required to 1) elucidate the molecular mechanism underlying suppression of *IGJ* mRNA, which may be mediated

through alterations in STAT5 [376] and 2) investigate how suppression of STAT3 activation may lead to impaired B cell proliferation and plasmacytic differentiation.

In summary, this dissertation research advances the current state of the science in this area in several ways. First, the demonstration that Δ^9 -THC attenuates CD40L expression on T cells is a novel observation, and potentially one of the key events in the mechanism by which Δ^9 -THC suppresses T-cell dependent humoral immune response in humans. Second, the demonstration that suppression of CD40L by Δ^9 -THC occurs independently of CB1 and CB2 emphasizes that cannabinoid receptors are not involved in all aspects of immune modulation by cannabinoid compounds. Further, this study is the first to show that there is no potential interaction between Δ^9 -THC and GR. This finding is also significant as it rules out the possible involvement of GR in the immune suppression mediated by Δ^9 -THC. Third, the demonstration that Δ^9 -THC impaired Ca^{2+} elevation in activated human T cells resulting in suppression of NFAT-and $\text{NF}\kappa\text{B}$ -DNA binding activity is also important. This finding not only supports the notion that immediate perturbation of intracellular Ca^{2+} levels by cannabinoids, including Δ^9 -THC, is central to the initiation of signaling cascades responsible for impairment of T cell function, but also challenges previous findings demonstrating that Δ^9 -THC and CBD enhanced Ca^{2+} elevation in both

resting and PMA/Io-stimulating lymphocytes [239,240,299]. Fourth, dysregulation of CD40 plus cytokine-induced B-cell activation, proliferation and differentiation into IgM secreting cells by Δ^9 -THC further provides mechanistic insights by which Δ^9 -THC suppresses T-cell dependent humoral immune response in humans. Finally, we provided the first conclusive evidence that Δ^9 -THC selectively suppresses surface expression of CD80 in activated human B cells, which may provide a new therapeutic strategy for diseases mediated by excess CD80 activation, such as Minimal Change Disease, the most common nephrotic syndrome in children (reviewed in [377]). Additionally, the novel identification that inactivation of STAT3, but not p65 NF κ B, serves as one possible target of Δ^9 -THC in activated human primary B cells and underscores the direct effect of Δ^9 -THC on intrinsic signaling in B cells. The differential effect of Δ^9 -THC on NF κ B signaling also emphasizes the multi-faceted mechanisms involved in immune suppression by Δ^9 -THC, which most likely depend on cell types. Figure 40 summarized the possible mechanisms involved in suppression of T cell-dependent humoral immune response by Δ^9 -THC. Despite the predominant role of CD40-CD40L interaction in the T cell-dependent humoral immune response, the aberrant CD40L expression on activated T cells was demonstrated in the pathogenesis of many autoimmune and inflammatory diseases (reviewed in [378]). In addition, constitutively activated STAT3 was

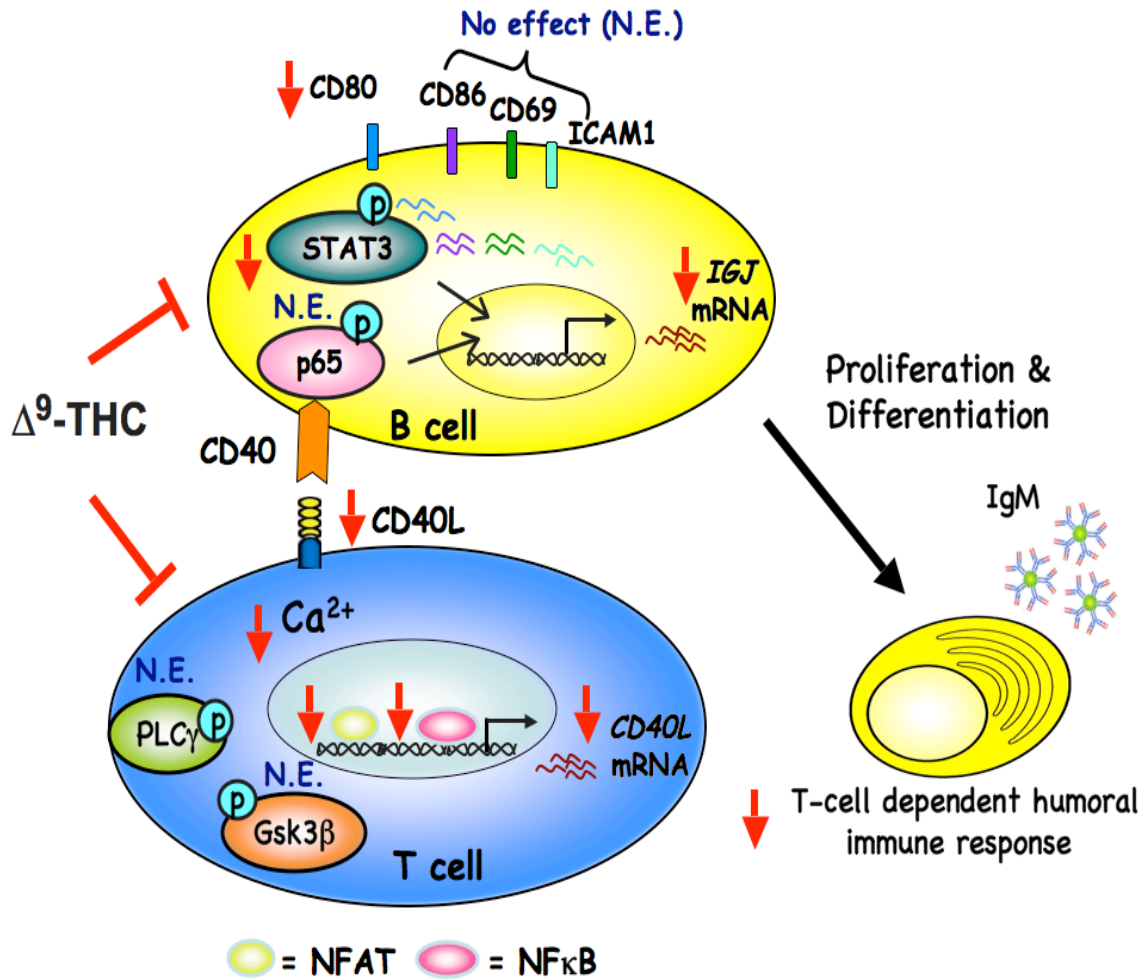


Figure 40. Schematic diagram summarizing the possible mechanisms involved in suppression of T cell-dependent humoral immune response by Δ^9 -THC.

Δ^9 -THC suppressed both T cells and B cells, which are the major players in T-cell dependent humoral immune response. The suppression of CD40L expression on activated CD4^+ T cells by Δ^9 -THC is mediated at least by perturbation of the calcium-NFAT and NF κ B signaling cascade without affecting the activation of proximal kinases in TCR signaling. The suppression of B cell function by Δ^9 -THC is mediated at least by impairment of STAT3 activation without affecting the activation of p65 NF κ B subunit.

shown to promote cell proliferation and survival of the activated B-cell subtype of diffuse large B-cell lymphoma [379]. Hence, these critical observations may be of practical importance in future efforts to develop effective and safer therapeutic strategies in the treatment of inflammatory related and/or autoimmune diseases as well as cancer, especially for B-cell lymphomas. Collectively, an understanding of the mechanisms by which Δ^9 -THC, and structurally-related cannabinoids, affect T and B cell function might allow for the development of cannabinoid-based therapeutic strategies and also provide important information when weighing benefit to risk in immunocompromised patients such as those undergoing cancer chemotherapy or suffering from HIV/AIDS.

APPENDICES

APPENDIX A. Antibodies

Table 2. List of antibodies used in this dissertation research.

<u>Target</u>	<u>Fluoro- phore</u>	<u>Clone</u>	<u>Host</u>	<u>Reactivity</u>	<u>Supplier</u>
CD4	PerCP-Cy5.5	OKT4	Mouse	Human	Biolegend
CD4	APC	RPA-T4	Mouse	Human	BD Biosciences
CD4	FITC	OKT4	Hamster	Human	BD Biosciences
CD4	V450	RPA-T4	Mouse	Human	BD Biosciences
CD4	V450	RM4-5	Rat	Mouse	BD Biosciences
CD16/32	None	2.4G2	Rat	Mouse	BD Biosciences
CD40L	PE	24-31	Mouse	Human	Biolegend
CD40L	APC	RM1	Hamster	Mouse	eBiosciences
CD69	PE/Cy7	FN 50	Mouse	Human	Biolegend
CD80	PE/Cy5	2D10	Mouse	Human	Biolegend
CD86	PE	IT2.2	Mouse	Human	Biolegend
ICAM1 (CD54)	APC	HCD54	Mouse	Human	Biolegend
Akt (pS473)	PE	N/A	Mouse	Human	BD Biosciences
GSK3 β (pS9)	FITC	N/A	Mouse	Human	R&D
p65 NF κ B (pS529)	FITC	K10-895.12.50	Mouse	Human	BD Biosciences
PLC- γ 2 (pY759)	PE	K86-689.37	Mouse	Human	BD Biosciences

Table 2 (cont'd)

STAT3 (pY705)	AF647	4/P-STAT3	Mouse	Human	BD Biosciences
ZAP70 (pY319)/Syk (pY352)	AF647	N/A	Mouse	Mouse /Human	BD Biosciences

APPENDIX B. TaqMan Primers

Table 3. List of Tagman primers used in this dissertation research.

<u>Gene</u>	<u>Catalog number</u>	<u>RefSeq</u>
18s	4319413E	X03205.1
Human CD40L	Hs99999102_m1	NM_000074.2
Human CD80	Hs00175478_m1	NM_005191.3
Human IgJ	Hs00376160_m1	NM_144646.3
Human PAX5	Hs00277134_m1	NM_016743.1
Human PRDM1	Hs00153357_m1	NM_001189.

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