Δ^9-TETRAHYDROCANNABINOL-MEDIATED SUPPRESSION OF THE INTERFERON-α (IFNα) RESPONSE BY PLASMACYTOID DENDRITIC CELLS AND IFNα-MEDIATED ACTIVATION OF T CELLS IN HEALTHY AND HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTED HUMAN SUBJECTS

By

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ABSTRACT

Δ^9-TETRAHYDROCANNABINOL-MEDIATED SUPPRESSION OF THE INTERFERON-α (IFNα) RESPONSE BY PLASMACYTOID DENDRITIC CELLS AND IFNα-MEDIATED ACTIVATION OF T CELLS IN HEALTHY AND HUMAN IMMUNODEFICIENCY VIRUS (HIV) INFECTED HUMAN SUBJECTS

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Δ^9-tetrahydrocannabinol (THC) is the primary psychoactive cannabinoid congener in *Cannabis sativa* and is a well characterized modulator of immune activation. In murine models, treatment with THC can exacerbate viral and bacterial infection, in part, by suppression of the inflammatory cytokine response. One of the key classes of cytokines suppressed by THC is type I interferons (IFN), a group of cytokines consisting of IFNα and IFNβ. The primary source of IFNα during acute antiviral immune responses is the Plasmacytoid Dendritic Cell (pDC), which can secrete 1000-fold more IFNα than other circulating peripheral blood mononuclear cells (PBMC). Paradoxically, patients infected with human immunodeficiency virus (HIV), a chronic viral infection that causes immunodeficiency via infection and depletion of $CD4^+$ T cells, have fewer circulating pDC with a reduced capacity to secrete IFNα. Furthermore, circulating pDC number has been correlated with $CD4^+$ T cell number and treatment with IFNα can reduce HIV-mediated $CD4^+$ T cell depletion. Conversely, hyperactivation of pDC is associated with T cell exhaustion and is implicated in HIV-associated neurocognitive disorders (HAND). Interestingly, many HIV patients utilize medicinal cannabinoids to combat the effects of chronic HIV infection. The focus of this project was to determine if IFNα-mediated stimulation of T-cells can be suppressed by THC by testing the following hypothesis: THC will suppress TLR-9-dependent activation of pDC, subsequent efficacy of pDC-mediated

T cell activation, and CD8$^+$ T cell-mediated activation of astrocytes. These studies revealed that CpG-ODN-induced IFNα secretion and expression of CD83, a costimulatory molecule, by pDC is suppressed by THC in a concentration dependent manner. Furthermore, key intracellular signaling events required for inflammatory cytokine secretion by pDC were suppressed by treatment with THC and CBR2-specific agonists in pDC from healthy donors. Additionally, pDC from HIV+ donors were more sensitive to THC-mediated suppression than pDC from healthy donors. Treatment with THC also inhibited IFNα-mediated activation of CD4$^+$ and CD8$^+$ T cells from healthy and HIV+ donors. Specifically, treatment with THC diminished IFNα-induced IL-7R expression, cognate signaling, and subsequent proliferation. Interestingly, and in contrast to the results in pDC, T cells from HIV+ donors were less sensitive to the suppressive effects of THC. Finally, stimulation by CD3/CD28/IFNα induced the secretion of IFNγ and TNFα by CD8$^+$ T cells from healthy donors. Further, IFNγ and TNFα induced secretion of inflammatory cytokines by U251 astrocytes. Coculture of CD8$^+$ T cells with U251 astrocytes and direct stimulation of U251 astrocytes with recombinant TNFα and IFNγ revealed that treatment with THC reduced both the activation and secretion of cytokines from CD8$^+$ T cells and the subsequent cytokine-mediated stimulation of the U251 astrocytes. Collectively, these studies have provided evidence for the use of cannabinoids in ablating the type of neuroimmune interactions which can lead to HAND by demonstrating that THC can suppress the activation of pDC, and subsequent activation of T cells and astrocytes.

To my Mother: you raised four kids by yourself and never asked for anything. When life tried to shove you down, you fought back. The love you gave and the determination you showed have stayed with all your children into adulthood. I remember once saying, “This is hard,” when referring to some elementary school homework. You said, “That’s not hard. You got the easy part. All you have to do is learn it. The people that discovered it had the hard part.”

Looks like I’m doing the hard part now, mom.

Thank you for everything.

To Megayn, you have been my inspiration and driving force for so long, I don’t even remember what life without you is like. I wouldn’t call it life at all. My desire to impress you has driven everything I have accomplished and become. I don’t know what the future holds for us or where our lives will lead, but if we stay in each other’s lives – Nothing Else Matters.

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I would also like to acknowledge my committee members, Dr. Cheryl Rockwell, Dr. Keith Lookingland, and Dr. Andrea Amalfitano, for their time, suggestions, and discussions both in and out of my committee meetings. I would like to extend my gratitude to them for not only challenging me, but also being a fair and friendly committee. I've heard horror stories from some students about their experiences during committee meetings. In this respect, I was lucky. I never prepared for a committee meeting with trepidation or stood before them as a deserter feels before his firing squad. I knew they only cared for my development as a scientist and for the progress of my project. I wish every student's committee could be like mine.

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LIST OF ABBREVIATIONS

AF	Alexa Flour®
Akt	Protein kinase B
ANOVA	Analysis of variance
APC	Allophycocyanin
BSA	Bovine serum albumin
BV	Brilliant violet™
cAMP	cyclic-adenosine monophosphate
CB1	Cannabinoid Receptor 1 protein
CNR1	Cannabinoid Receptor 1 gene
CB1	Cannabinoid Receptor 2 protein
CBR2	Cannabinoid Receptor 2 gene
CBD	Cannabidiol
CD	Cluster of differentiation
CpG-ODN	Cytosine-phosphate-guanine oligodeoxynucleotides
CTL	Cytotoxic T lymphocyte (CD8$^+$ T cell)
Cy5.5/7	Cyanin5.5/7
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FAS	First apoptosis signal
FASL	FAS-ligand
FITC	Fluorescein isothiocyanate
HIV	human immunodeficiency virus
IFNα	Interferon α

IKK	Inhibitor of nuclear factor κ-B kinase
IL	Interleukin
ISRE	Interferon sensitive response element
IRAK	Interleukin receptor associated kinase
IRF7	Interferon response factor 7
JAK	Janus family kinase
JNK	c-Jun N terminal kinase
MAPK	mitogen activated protein kinases
MFI	mean fluorescence intensity
mTOR	mammalian target of Rapamycin
mRNA	messenger ribonucleic acid
NFκB	Nuclear factor κ B
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PerCP	Peridinin chlorophyll protein complex
OPN	Osteopontin
pDC	Plasmacytoid dendritic cells
PE	Phycoerythrin
PI3K	Phosphoinositide 3-kinase
PLCγ	Phospholipase C γ
STAT	Signal transducer and activator of transcription
TANK	TRAF family member-associated NF-κ-B activator
TBK1	TANK binding kinase
THC	Δ^9-tetrahydrocannabinol
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TCR	T cell receptor
Th	T helper cell (CD4$^+$ T Cell)
TyK	Tyrosine kinase
VC	Vehicle control

LITERATURE REVIEW

I. Cannabinoids and the endocannabinoid system

A. Cannabis legality and medicinal cannabinoids

The federal government identifies marijuana and THC as a schedule 1 drug by the standards of the Drug Enforcement Agency (DEA). A schedule 1 designation is, according the Controlled Substances Act (I.B.812) assigned to: “drugs, substances, or chemicals [which] are with no currently accepted medical use and a high potential for abuse.”

Despite this designation, the pharmacological potential of cannabinoids was recorded in China around 2000 BCE, where medicinal teas were prescribed for conditions like rheumatism [1]. Likewise, many cultures have utilized *Cannabis sativa* for both medicinal and religious purposes [2, 3]. Within the United States, marijuana was prescribed for a variety of medicinal applications during the mid-19th century. It wasn’t until the Marihuana Act of 1937, and the removal of marijuana from the American pharmacopeia in 1941, that marijuana was officially viewed as a drug of abuse [4]. In 1970, the Comprehensive Drug Abuse Prevention and Control Act, now called the “Controlled Substances Act”, established the “schedule 1” designation. This law officially made possession and use of marijuana a federal offense [5].

In 1996, California was the first state to pass a law protecting the use of medicinal marijuana [6]. While many view this event as a turning point for the acceptance of medicinal marijuana, decriminalizing of marijuana began in 1973, only 3 years after the Controlled Substances Act [7]. As of 2018, 26 of the 50 states in the United States of America have legalized medical marijuana and 8 of those states have legalized recreational marijuana. Likewise, 13 additional states have either decriminalized marijuana or restricted the THC content of medicinal marijuana [8] (Figure 1).

Marijuana is now accepted for use in a variety of conditions. Most notably, patients suffering from the effects of chemotherapy and HIV infection utilize medicinal cannabinoids for the remediation of nausea (antiemetic) [9], stimulation of hunger (orexigenic) [10], and relief of pain (analgesic) [11]. Cannabinoid therapies have also been used to treat refractory epilepsy [12], rheumatoid arthritis [13, 14], glaucoma [15], and, most controversially, cannabinoids have been suggested for the treatment of social withdrawal in people with autism [16, 17]. Like every drug, marijuana has side effects and consequences of use. Specifically, use of marijuana can cause paranoia [18], psychosis [19], hypothermia [20], and memory deficiencies [18, 21]. The most concerning side effect of marijuana use is the reduced executive processing capacity of people exposed to marijuana during adolescence [22]. As it stands, marijuana is a potent, legally nebulous, drug with a variety of potential applications and side effects.

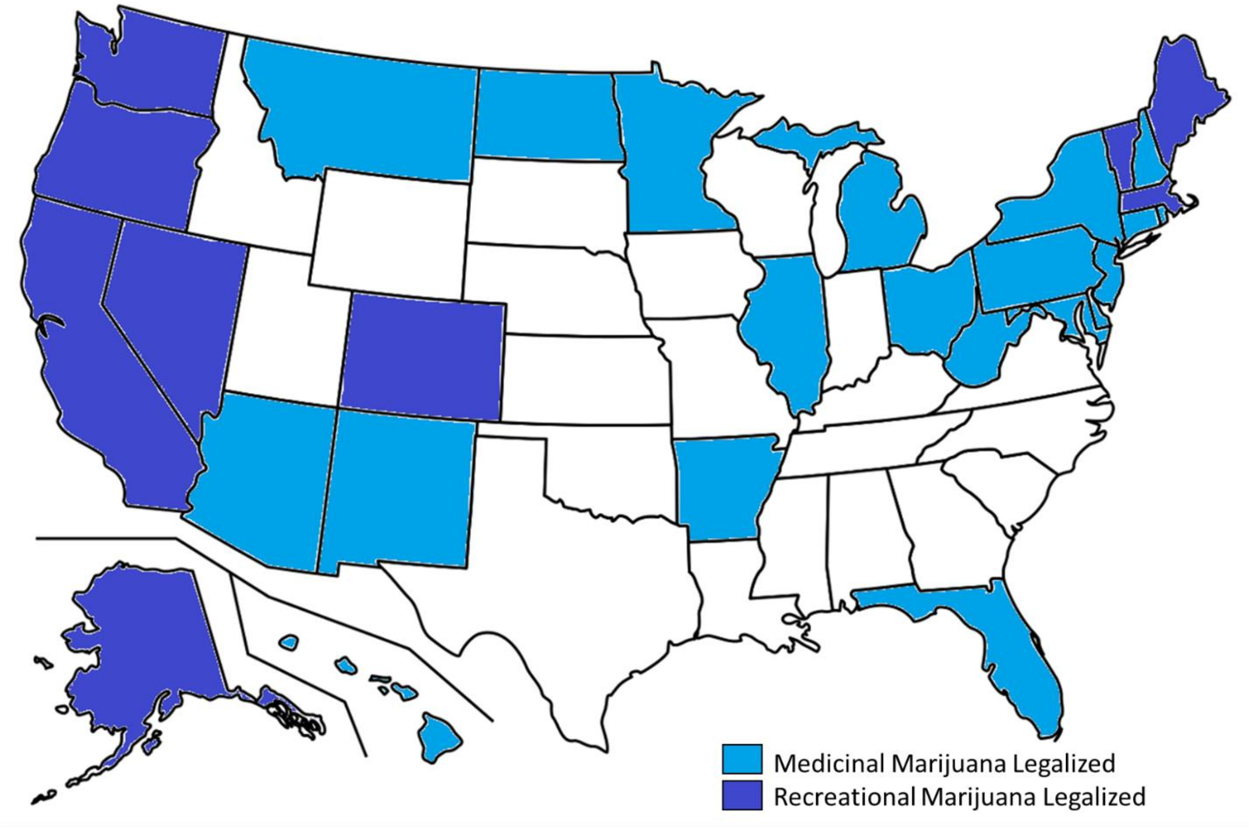

Figure 1. Marijuana legalization status by state. Map of the United States indicating states with legalized medicinal and recreational marijuana.

B. Discovery and types of cannabinoids

The idea that specific compounds were responsible for the pharmacological activity of *C. sativa* was first suggested in the early 20th century. These compounds would later be termed “cannabinoids” as they were first derived from plants in the genera *Cannabis* [23]. It wasn’t until the 1940’s that the first, and most studied, cannabinoids were experimentally tested [23]. These cannabinoids included: cannabinol (CBN), cannabidiol (CBD), and Δ^9-tetretrahydrocannabinol (THC). While the aforementioned cannabinoids are the most widely investigated, over 60 *C. sativa*-derived cannabinoids have been identified [23-25].

Today, the family of cannabinoids and cannabinoid-like compounds compose three major groups; 1) phytocannabinoids – cannabinoids derived from plant material, sometimes called “classical cannabinoids” [26], 2) synthetic cannabinoids – lab-derived compounds which bind to the cannabinoid receptors (see next section) but are not found from natural sources [27], and 3) endogenous cannabinoids (endocannabinoids) – arachidonic acid metabolites known to bind the canonical cannabinoid receptors[28].

The naming of the compounds and the receptors to which they bind, the cannabinoid receptors, is cyclical. As mentioned above, classical cannabinoids (now called “phytocannabinoids”) were so named because they were derived from plants of the *Cannabis* genus. The G-coupled protein receptors which were bound by these compounds were named “cannabinoid receptors” [23, 29]. However, endocannabinoids and synthetic cannabinoids are so named because they bind the cannabinoid receptors.

Another class of “Phytocannabinoids” have been identified in the plant genera *Echinacea*[30]. Non-*Cannabis*-derived cannabinoids differ from *Cannabis*-derived cannabinoids in that non-*Cannabis*-derived cannabinoids are typically alkamides [30] while *Cannabis*-derived cannabinoids are aromatic terpenoids [25, 26]. Though different in structure, most phytocannabinoids share key characteristics, specifically: 1) they are not water soluble; 2) they bind one or both of the canonical cannabinoids receptors (i.e. CB1 and CB2 – more below); and 3) they are metabolized by cytochrome P450-2C9 [31]. New plant-derived compounds are being discovered every year and current research may expand the family of accepted phytocannabinoids in the years to come [32, 33].

Synthetic cannabinoids are typically based on the structure of *Cannabis*-derived or endogenous cannabinoids. There are several families of synthetic cannabinoids with the following prefixes: 1) JWH – compounds generated by the work of John W. Huffman, PhD; 2) AM – compounds generated by Alexandros Makriyannis, PhD; 3) HU – compounds derived by the work of Raphael Mechoulam, PhD at the Hebrew University of Jerusalem; 4) CP – compounds generated by Pfizer®; and WIN – compounds produced by Sterling-Winthrop, Inc. While these synthetic cannabinoids are the most studied, new compounds continue to be synthesized. Specifically, at the time of writing this, a new family of indazole-based cannabinoids with the suffix -NACA (e.g. APINACA, N-(1-adamantyl)-1-pentyl-1H-indazole-3-carboxamide) have been developed [34]. Regardless of their designation, synthetic cannabinoids bind one, or both, of the canonical cannabinoid receptors [35]. These compounds can either be full agonists, compounds which bind and activate the target receptors fully, or partial agonists, compounds which bind to the cannabinoids receptors but do not illicit a full response [35].

Endocannabinoids are the so called “natural” cannabinoids of the body and were discovered in the early 1990’s [28]. Derived from arachidonic acid, the two most studied endocannabinoids are 2-Arachidonoylglycerol (2-AG) and anandamide (AEA). Both 2-AG and AEA have been well characterized in neuronal tissues [36, 37]. Unlike the *Cannabis*-derived phytocannabinoids, which can have a half-life of 20 hours to 4 days in heavy marijuana users [38], 2-AG and AEA have short half-lives of 5 and 10 minutes, respectively. *In vivo,* 2-AG and AEA are rapidly degraded by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase [39, 40]. Contrary to some popular opinions, endocannabinoids are not only “feel good” chemicals in the body, these compounds play key roles in several physiological processes. Specifically, synthesis of 2-AG is required for the production of prostaglandin-E2, which is needed to generate a fever response [41]. Furthermore, both 2-AG and AEA act as retrograde neurotransmitters [42, 43], which are compounds secreted from a post-synaptic cell and on to the pre-synaptic cell in a neuronal synapse. Retrograde neurotransmitters typically act to dampen the release of neurotransmitters, like glutamate [44].

C. Cannabinoid receptors and the endocannabinoid system

The first canonical cannabinoid receptor, Cannabinoid receptor 1 (CB1), was discovered in rat neuronal cells in 1990 and was closely followed by the naming of the second canonical cannabinoid receptor, (CB2), in 1993 [23] (Figure 2). The genes for CB1 and CB2 are similarly named CNR1 and CNR2, respectively. Since the discovery of the cannabinoid receptors, much has been learned about their function. Both CB1 and CB2 are Gi/Go-protein coupled receptors [45] that affect cell function by suppressing the activity of adenylate cyclase [46], ERK/MAPK [47], PLCγ [48], and the PI3K-AKT-mTOR pathways [49, 50]. Signaling through the cannabinoid receptors also perturbs Ca^{+2} currents by simultaneously blocking N and P/Q calcium channels [51]. The effects on calcium channels is largely mediated through reduced cAMP formation following the inhibition of adenylate cyclase and the release of intracellular calcium stores [52]. This perturbation of Ca^{+2} likely plays a role in the cannabinoid-dependent suppression of mTORC1 pathways by the activity of calmodulin [53].

The relative distribution of the cannabinoid receptors underlies their respective physiological effects. CB1 is highly expressed in cells of the CNS where the highest density of receptor expression is on the presynaptic terminal of the neuronal synapse [54]. CB1 plays a role in modulating the release of neurotransmitters from presynaptic cells when it is bound by endocannabinoids secreted from the post synaptic cell. Binding of the CB1 by cannabinoids closes the N, P/Q, and L-type calcium channels thus inhibiting calcium flux into the cell. The loss of calcium reduces synaptobrevin-synaptotagmin association and SNARE complex formation, thereby reducing neurotransmitter release from the presynaptic cell [55]. This type of action by cannabinoids likely underlies the anti-convulsant effects of cannabinoids [56].

CB2 is found primarily in the cells of the immune system and the levels of expression vary between cell types, such that: B cells > Natural killer (NK) cells > neutrophils > CD8$^+$ T cells > monocytes > CD4$^+$ T cells [23, 57, 58]. In the immune system, signaling through CB2 leads to modulation of immune responses. In most cases, cannabinoids elicit anti-inflammatory and immunosuppressive effects such that the release of inflammatory cytokines, proliferation, and cell-mediated cytotoxicity is suppressed by cannabinoid treatment [58-61]. While some processes of immune cell function are suppressed by cannabinoids, others are enhanced by cannabinoids. Specifically, the endogenous cannabinoid 2-AG can induce chemotaxis in some immune cells [62].

While the distribution of the cannabinoid receptors is generally considered to be "CB1 in the CNS" and "CB2 in the periphery", there are notable exceptions. For example, microglia, the resident macrophage of the CNS, have detectable levels of CB2 [63]. Likewise, stimulation of the immune cells with inflammatory cytokines causes elevated expression of CB1 [64]. Furthermore, tissues such as the testes [65] and osteoclasts[66], a specialized type of macrophage, also express cannabinoid receptors. Lastly, while CB1 and CB2 are the canonical cannabinoid receptors, there are known orphan receptors which may also have physiological roles. Specifically, transient receptor potential cation channel subfamily V member 1 (TRPV1) [67], Vanilloid receptor 1 (VR1) [68], and three other known G-protein coupled receptors (GPR), GPR55 [69], GPR 18 [70], and GPR 19 [71] are all considered "orphan cannabinoid receptors".

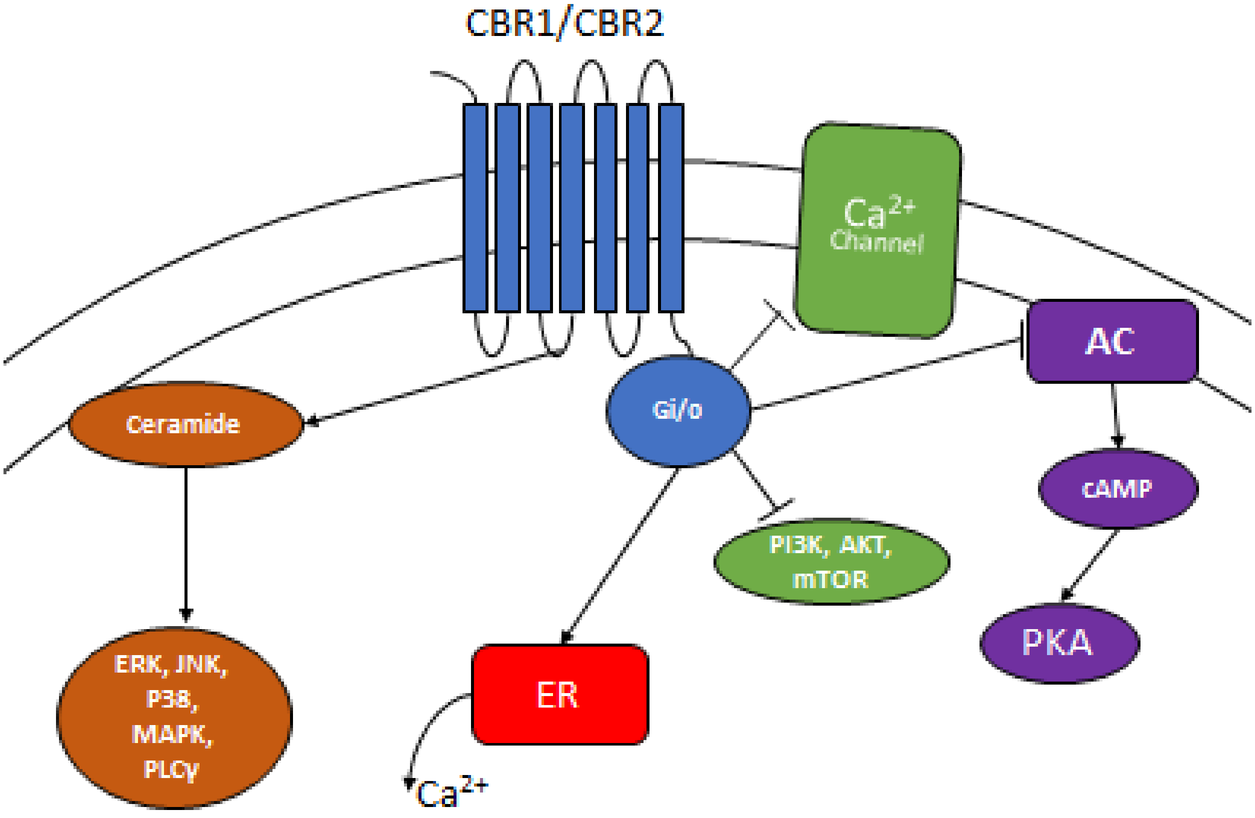

Figure 2. Cannabinoid receptor 1 (CB1) and CB2 shared signaling events. The cannabinoid receptors are 7-transmembrane subunit G-coupled protein receptors which share common signaling pathways via the activation of Gi/o.

II. Δ^9-Tetrahydrocannabinol (THC) and CB2-selective agonists (JWH-133 and JWH-015)

A. Pharmacokinetics of THC

Δ^9-tetrahydrocannabinol (THC) is the primary psychoactive phytocannabinoid in *Cannabis sativa* and is the central compound being tested through these studies. THC is typically inhaled as either a smoke or vapor, or administered orally [72]. Both the route of administration and the percent of THC in marijuana can alter the rate of absorption and peak plasma concentration considerably [73]. Specifically, THC absorption through inhalation is rapid, with plasma concentration reaching a peak (100-200 ng/ml) within the first 10 minutes of smoking or “vaping” [73, 74]. Absorption of THC following oral administration is slower, taking over an hour to reach peak plasma concentration and the peak concentration is typically lower, approximately 3 ng/ml. Much of this loss during oral consumption is likely due to the first pass effect [73, 74]. In addition, THC is highly lipophilic and readily distributes from the blood into adipose tissues. This lipophilicity results in a large volume of distribution (~1.4 L/kg body weight) [75]

As previously mentioned, THC is a phytocannabinoid and is metabolized via CyP-2C9 in the liver [76-78]. The major metabolite of THC is 11-hydroxy-THC which is further oxidized to 11-nor-9-carboxy-THC (THC-COOH) before being excreted in the feces (biliary excretion) or urine as glucuronic acids or free metabolites [79, 80].

B. Effects of THC on the CNS

THC is a partial agonist for both canonical cannabinoid receptors and binds with comparable affinity (CB1, Ki=10nM; CB2, Ki=24nM)[24]. Therefore, the following sections will focus on the effects of THC through CB1 and CB2. However, it is noteworthy that some of the effects of THC are not mediated through either of the canonical cannabinoids receptors [57], indicating the promiscuity of THC and the potential for activity through other modes of action including via the orphan cannabinoid receptors (mentioned above).

In the CNS, treatment with THC typically reduces the release of neurotransmitters, but can enhance the release of excitatory neurotransmitters while reducing the release of inhibitory neurotransmitters [81]. Specifically, release of dopamine has been reported by THC, but the amount of dopamine released is considered insignificant [82]. This relatively minor increase in dopamine by administration of THC likely underlies the relatively low threat of addiction with chronic use. Regardless of the low risk of addiction, THC does induce anti-nociception, hypomotility, hypothermia, and catalepsy in mouse models [83]. In humans, use of marijuana and THC causes impairment of learning and memory, which is especially prominent and persistent when *Cannabis* is used during adolescence [21]. Lastly, THC can prevent chemotherapy-induced nausea and vomiting by direct and indirect activation of 5-HT, serotonin, receptors in the raphe nucleus and terminal forebrain [84]. Many of the deleterious effects of marijuana use are associated with their actions on the CNS and PNS. Specifically, impaired cognitive function, tachycardia, hypotension, and supine hypertension in humans [74, 85-87].

C. THC-mediated exacerbation of infections

Plant-derived THC, or the synthetic form of THC, marinol (dronabinol), is a well characterized immune modulator[88-90]. In mouse models of herpes simplex virus Type II[91, 92], *Listeria monocytogenes*[92], and influenza virus Type A [59, 93], THC administration exacerbated disease progression. While THC has been shown to have suppressive effects on the function of many different immune cell populations, THC-mediated suppression of interferon secretion was demonstrated in all the aforementioned models of disease [61]. Though the effects of THC on the immune system are still being elucidated, the suppression of interferon (Type I and II) responses by THC are a key mechanism by which viral infections are potentiated. THC can also suppress T cell response to viral infections [94, 95], including HIV[96]. Furthermore, pDC secretion of IFNα is acutely sensitive to THC-mediated suppression and pDC from HIV+ donors are more sensitive to THC-mediated suppression than pDC from healthy donors [97].

D. HIV patients' use of medicinal cannabinoids

In 2015, the Centers for Disease Control and Prevention (CDC) estimated 1.2 million people were infected with HIV in the United States and 36.9 million are infected globally. Anti-retroviral therapy (ART) is the primary therapy for HIV patients in the United States and has been since the mid 1990's [98]. While effective, ART therapy can also induce nausea and reduce appetite [99]. Furthermore, HIV infection, even when properly controlled by ART, is associated with physical wasting [100, 101] and anxiety [102, 103], both of which can have deleterious effects on host immune responses. The effects of both HIV infection and ART has led to a significant number of HIV patients utilizing cannabinoid-based therapies such as medical marijuana (mixtures of *Cannabis sativa* and *Cannabis indica*) and dronabinol (marinol) [104-106].

Currently, the utilization of cannabinoid-based therapies in the HIV+ population is controversial. Specifically, cannabinoid use reduces the concentration of circulating anti-retroviral drugs, yet these studies indicated little effect of cannabinoids on retroviral therapy efficacy or immune cell function [105, 107]. Furthermore, it is difficult to distinguish between the direct effects of the cannabinoids on leukocyte function and possible confounders in these studies.

As previously mentioned, THC and the chemically identical synthetic cannabinoid dronabinol (*e.g.* marinol) are potent immunosuppressive compounds [88, 90]. It is well established that THC can suppress T cell responses to viral infections [94, 95], including HIV[96]. Additionally, pDC secretion of IFNα is acutely sensitive to THC-mediated suppression and pDC from HIV patients are more sensitive to THC-mediated suppression than pDC from healthy donors [97]. This is significant since both IFNα [108] and pDC function [109-112] have been correlated with T cell health in HIV patients. To date, the immunological consequences of THC use in HIV patients has not been elucidated.

E. Potential CB2 targeted therapies for autoimmune disorders

IFNα and TNFα promote a robust response by the host immune system [113] and collaboratively enhance tumor cell apoptosis [114]. However, inappropriate activation of pDC, a specialized type of innate immune cell described in greater detail below [115-117], and sustained levels of both IFNα [118] and TNFα [119] can become maladaptive in autoimmune conditions. Lupus erythematosus develops as necrotic and apoptotic host cells die and pDC respond to host genomic material [116, 120-123]. Furthermore, exaggerated responses by pDC can exacerbate a disease state. Specifically, prolonged activation of pDC may expediate T cell exhaustion in women infected with HIV [124] and chronic activation of pDC may play a role in mediating monocyte activation, a contributing factor to the development of HIV-associated neurocognitive disorders (HAND) [125-127]. Interestingly, both the secretion of IFNα from pDC [97] and subsequent IFNα-mediated stimulation of monocytes [125] can be suppressed by treatment with cannabinoids.

Suggestions for *C. sativa* use in the remediation of inflammatory conditions is well documented [128, 129]. However, as indicated above, *C. sativa* contains THC, a powerful psychotropic compound [22, 129-131] which is strictly regulated by the DEA and still considered a schedule 1 drug, despite state legalization status, due to the possibility for addiction formation and consequence of use on health. Thus, utilization of *Cannabis* can cause legal problems for patients [5].

As indicated above, THC acts by binding to the two canonical cannabinoid receptors, CB1 and CB2. The binding of THC, or any cannabinoid compound, to CB1 is responsible for inducing the aforementioned psychotropic effects [132]. For this reason, CB1-selective agonists are strictly controlled by the DEA [133]. However, CB2 binding by THC likely mediates the anti-inflammatory and immuno-suppressive effects of THC in both *in vivo* and *in vitro* models. Furthermore, many CB2-selective agonists (synthetic cannabinoids) have been developed. Specifically, JWH-015 and JWH-133 have been implicated as having therapeutic potential in inflammatory conditions [61, 134-136].

F. JWH-015 and JWH-133

John W Huffman, PhD, developed over 450 cannabinoid compounds with the explicit purpose of investigating cannabinoids in the treatment in multiple sclerosis and HIV. Many of these compounds are structurally similar to THC (Figure 3A), but key alterations to their chemical structure have resulted in altered specificity for CB1 and CB2. The compound (2-methyl-1-propyl-1H-indol-3-yl)-1-naphthalenyl-methanone, or JWH-015 (Figure 3B), was one of the first CB2-selective agonists developed by Dr. Huffman's group and has a 28:1 affinity differential for CB2 (K_i = 13.8nM) to CB1 (K_i = 383nM). The compound 3-(1,1-dimethylbutyl)-6aR,7,10,10aT-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran, or JWH-133 (Figure 3C), is a potent CB2-full agonist with a 200:1 affinity differential for CB2 (K_i = 3.4nM) compared to CB1 (K_i = 677 nM). JWH-015 has shown therapeutic potential in reducing inflammation in murine models of multiple sclerosis and suppresses T cell infiltration of the spinal cord [63, 137]. Similarly, JWH-133 has been used extensively in the testing of various neuroinflammatory models including Alzheimer's disease and microglia-mediated neurotoxicity [138-142]. In addition, JWH-133 has been indicated as a possible therapeutic for peripheral inflammatory conditions [143-145]. For the studies presented in this dissertation, only synthetic cannabinoids in the "JWH" family of compounds were used. Specifically, these compounds were chosen due to: 1) the available information in the literature; 2) the characterization of these compounds in different models; 3) selectivity for CB2; and 4) the similarity of the compounds to THC on the metrics of a) structure, b) size, and c) solubility.

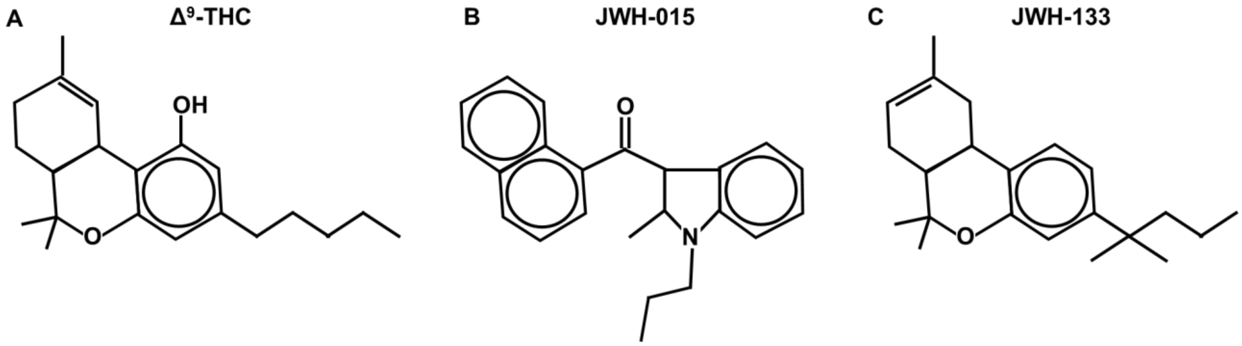

Figure 3. Structure of THC and the CB2-selective agonists, JWH-015 and JWH-133. Molecular structure of: A) THC, B) JWH-015 and C) JWH-133 oriented to demonstrate the similarity and differences between their respective structures. THC, JWH-015, JWH-133 are highly lipophilic and close in molecular weight, specifically: THC = 314.57g/mol; JWH-015 = 327.43; and JWH-133 = 312.49g/mol. THC, JWH-015, and JWH-133 are also have comparable solubility in EtOH, specifically: THC: = 20 mg/ml (~64mM); JWH-015 = 10 mg/ml (~32mM); JWH-133 = 20 mg/ml (~64mM). For the experiments presented in this dissertation, all cannabinoid stocks were prepared at 32mM in 200 proof ethanol.

III. The immune system

A. General overview of innate and adaptive immune responses

The immune system is a decentralized organ and the cells of the immune system are distributed throughout nearly all the other tissues of a host organism [146, 147]. The immune system serves to repair damaged tissue and protect the host from infection by pathogens such as parasites, fungi, bacteria, and viruses [148]. While not considered cellular members of the immune system, the first layers of protection are barriers like the skin and physiological conditions such as the low pH of the stomach and urogenital tract in women [149]. The trachea and bronchi of the pulmonary tract also have mucus and cilia that form the mucociliary escalator which serves as a significant barrier to infection [150]. These are efficient barriers and serve to protect the host from most infectious agents. However, all barriers can be compromised by injury or tenacious pathogens.

All the cells of the immune system are derived from hematopoietic stem cells which reside in bone marrow [151]. Hematopoietic stem cells differentiate into mature immune cells via a process called hematopoiesis. Most immune cells can be grouped into two major classes: 1) lymphoid cells, which derive from a common lymphoid progenitor cell and include NK cells, B cells, and T cells; and 2) myeloid cells, which derive from a common myeloid progenitor cell and include monocytes (immature macrophages), dendritic cells, granulocytes (basophils, eosinophils, mast cells, and polymorphonuclear cells), and megakaryocytes [152] (Figure 4). Furthermore, with the exception of NK cells, lymphoid cells are principally responsible for the adaptive immune response while the cell-mediated component of innate immune responses are largely handled by myeloid cells and NK cells. Lastly, while most cells of the immune system can be classified as either lymphoid or myeloid, it should be noted that some cell types, like the pDC (described below), contain characteristics of both cell lineages and there is contention over their origin [153].

The innate immune response is a non-specific arm of the host immune response. For example, the complement proteins, a set of liver derived peptides, can aid in the removal of pathogens by sequestration of erythrocytes, as a source of iron for pathogens, in the spleen and by forming a pore in pathogens and causing direct lysis or by opsonization [154].

Another way innate immune cells identify pathogens is through recognition of pathogen associated molecular patterns (PAMPs) via receptors both on and in immune cells. The Toll-like receptors (TLR) (described in more detail below) are the most well characterized family of pathogen recognition receptors (PRR). Once activated by a PAMP, innate immune cells respond by secretion of inflammatory and chemotactic cytokines which signal to other host cells that there is an infection. For example, responses to IFNα include the upregulation of nuclease and anti-viral machinery and recruitment of immune cells to the site of infection [155]. Dendritic cells displaying antigens of the infectious agent will then migrate into the lymphatic ducts and migrate to lymph nodes, where they will act as antigen presenting cells (described in more detail below) [147, 156].

Unlike the innate immune response, which is broadly directed against PAMPs, the cells of the adaptive immune response are highly specific to antibody generating molecules called “antigens”. The adaptive immune response begins when a dendritic cell, or other antigen presenting cell, presents an antigen to a helper T cell [147] (described in more detail below). Antigens are presented by dendritic cells and B cells on surface proteins called the major-histocompatibility complex (MHC). These molecules can be identified as either MHC class I, which is present on all nucleated host cells, or MHC class II, which are present on antigen presenting cells like dendritic cells and B cells [157]. MHC class I molecules typically present antigens from within a host cell, such as those generated during viral infection while MHC class II present antigens originating outside of the cell, such as from phagocytized bacteria [157]. In humans, the MHC molecules are called the human leukocyte antigen (HLA) and certain HLA haplotypes, such as B35, are associated with elevated risk of disease states, including susceptibility to infection by HIV [158, 159].

Once stimulated, an antigen presenting cell will present an antigen in the context of an MHC molecule (described below) to T cells [160]. Once activated, a T helper cell (CD4$^+$) will then direct the activation, function, and maturation of other immune cells including B cells [161] and CD8$^+$ (cytotoxic) T cell response (described below) [162]. It is for this reason that CD4$^+$ T cells are central to the host adaptive immune response and why conditions that affect CD4$^+$ T cells, such as some autoimmune disorders and infection by HIV, can be so devastating.

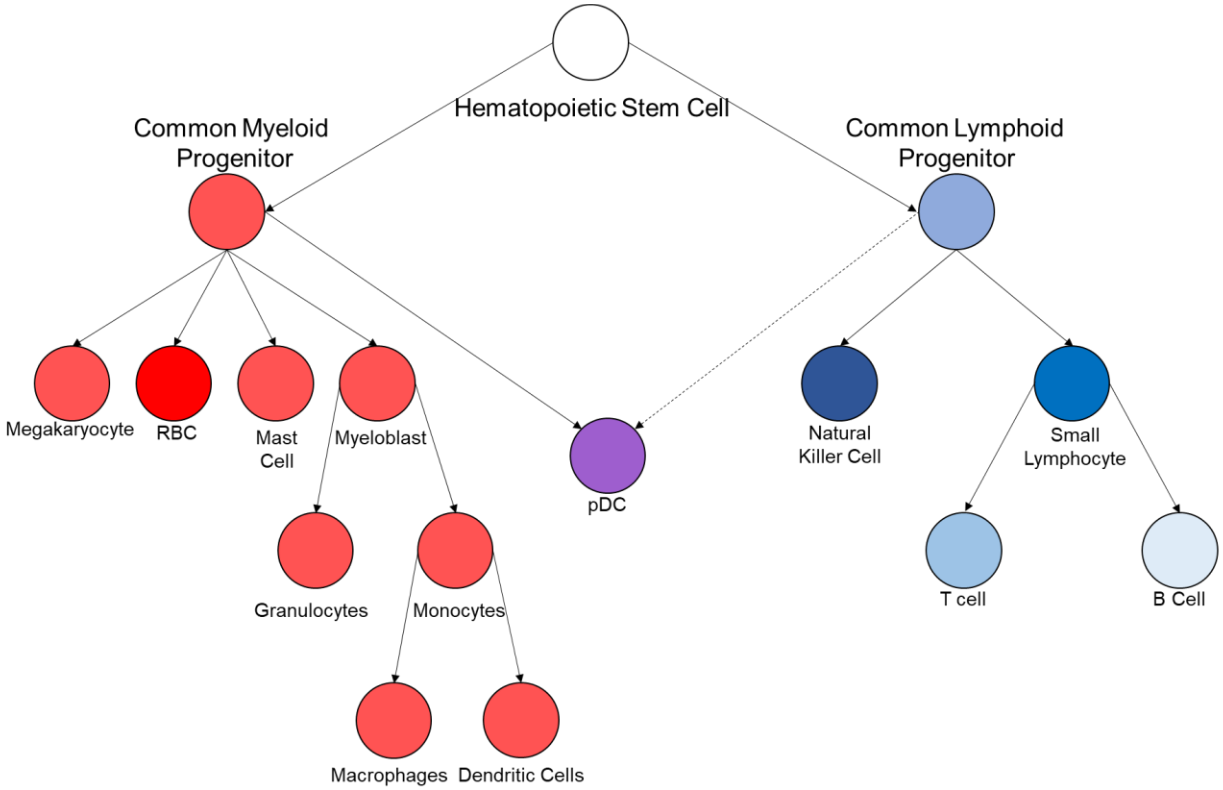

Figure 4. Hematopoiesis. Immune cells develop from a common hematopoietic stem cells, then become committed to either the myeloid or lymphoid lineage. The fate of cells as they develop is largely dependent upon cytokines secreted from other immune cells and stromal cells in the bone marrow.

B. Toll like receptors (TLR) and TLR9 activation

As indicated above, Toll like receptors (TLR) are a family of receptors that bind to macromolecules derived from pathogens PAMPs' [155], which can be carbohydrates (e.g. β-glucan, lipopolysaccharides), proteins (e.g. flagellin), lipids (e.g. monophospholipid-a), viral RNA, or DNA [155]. While each TLR plays a role in initiating the innate immune response, TLR's that recognize non-host extra-nuclear genomic material, consistent with viral infection, strongly stimulate pDC. Specifically, TLR activation through binding of endosomal TLR3 (ssRNA), TLR7/8 (dsRNA), and TLR9 (unmethylated DNA) illicit a strong IFNα response by pDC [112, 117, 163-166].

Ligation of TLR9 is a particularly potent inducer of IFNα secretion by pDC [167]. TLR9 is located in endosomes and can be activated by unmethylated Cytosine-phosphate-guanine (CpG) oligodeoxynucleotides (ODN) [168]. There are three distinct types of CpG-ODN classes (A-C) which are categorized by the presence of palindromic sequences, size, phosphorothiolated backbones, and combinations of these features [169, 170]. Type A CpG contains both a central palindromic sequence and a modified 3' tail which enables the formation of larger structures. These structures will remain within TLR9-containing endosomes enabling strong TLR9 stimulation [171]. Of the Type A CpG-ODN, 2216 drives potent IFNα secretion by human pDC [97].

TLR9 shares signaling pathways with TLR7 and 8. Specifically, signaling from the TLRs is mediated through MyD88, which can stimulate multiple IRAK/TRAF signaling pathways [172-174]. In pDC, stimulation of TLR9 leads to significant secretion of IFNα via phosphorylation and subsequent nuclear translocation of interferon response factor 7 (IRF7) [175]. Classically, IRF7 is phosphorylated by TRAF6 following TLR9 ligation by unmethylated DNA. However, TLR9 activation also causes the activation of TRAF3 [176] which can phosphorylate TANK-binding kinase 1 (TBK1)[173]. TBK1 is most associated with TLR3-mediated [177] induction of IFNα [178], and does so via phosphorylation of IRF7 and IRF3 [179]. Furthermore, phosphoinositide-3-kinase (PI3K), a key kinase involved in many cell processes, including mTOR-AKT signaling, is also critical for IRF7 phosphorylation and IFNα responses [180]. This redundancy of IRF7 phosphorylation, combined with the elevated levels of IRF7 expression in pDC, likely underpin the characteristic strong IFNα response by pDC.

C. Plasmacytoid dendritic cells (pDC)

pDC compose a minor population (0.2-0.5%) of circulating PBMC but play a crucial role in bridging the innate and adaptive antiviral immune response [115, 163, 164, 166]. As mentioned, pDC respond to viral threats by sensing viral-derived genomic material through TLR3 (dsRNA), 7 & 8 (ssRNA), and 9 (unmethylated DNA) [115]. Following stimulation, pDC produce up to 1000-fold more IFNα than other leukocytes in response to stimulation with viral-type TLR-ligands [181, 182]. Secretion of IFNα by pDC stimulate many immune cells including NK cells [183, 184], B cells [166], and T cells [184, 185] (Figure 5).

The capacity of pDC to secrete IFNα has been well documented, but they are can also express costimulatory factors, like CD83 [97], and secrete tumor-necrosis factor A (TNFα) [186]. TNFα is part of the acute phase response and has widespread effects during viral and bacterial infection [187, 188]. For example, TNFα can enhance dendritic cell function [189], pattern T cell responses [190], and promotes clearance of virally infected and cancerous host cells [191]. The induction of TNFα is likely mediated through activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) [192]. In pDC, NFκB signaling can be achieved through activation of IKKγ (NFκB essential modulator – NEMO) by phosphorylation via TRAF6 which enables the phosphorylation of IKKα/β and subsequent release/activation of NFκB [193]. NFκB activation can drive the secretion of cytokines and pDC maturation. Like IFNα, TNFα plays a key role in the immediate/early response to viral pathogens [194].

Somewhat paradoxically, pDC number and function is suppressed in association with certain types of viral infections including hepatitis C virus (HCV) and HIV [195, 196]. In a rhesus macaque model of HIV infection, using Simian Immunodeficiency Virus (SIV), the number of circulating pDC is reduced during the acute stage of SIV infection as pDC migrate to the gut [197]. In both HIV and SIV infection, gut lymphoid tissue is a key site of viral replication and, therefore, a target for pDC recruitment. However, pDC may be susceptible to productive HIV infection [198]. Infection by HIV may perturb pDC function resulting in reduced secretion of IFNα [111]. This reduced capacity for IFNα secretion during infection may hinder host responses. This is evidenced by administration of IFNα resulting in protection against HIV-mediated CD4$^+$ T cell depletion in a humanized mouse model [108], and lead to an inability to appropriately control the infection [199]. HIV infected pDC may also directly facilitate the infection of CD4$^+$ T cells during the acute phase of HIV infection by becoming productively infected by HIV and passing HIV virions to CD4$^+$ T cells during close association [200]. Furthermore, the loss of pDC in circulation is correlated with an increase in HIV viral serum titer such that fewer circulating pDC translated into a deficiency in antiviral response [201].

IFNα and TNFα both promote a robust response by the host immune system [113] and collaboratively enhance tumor cell apoptosis [114]. However, inappropriate activation of pDC [115-117] and sustained levels of both IFNα [118] and TNFα [119] can become maladaptive in autoimmune conditions and during chronic HIV infection. Lupus erythematosus develops as necrotic and apoptotic host cells die and pDC respond to host genomic material through the TLR-9 pathway [116, 120-123]. Furthermore, exaggerated, yet appropriate, responses by pDC can exacerbate a disease state. Specifically, elevated activation of pDC may expediate T cell exhaustion in women infected with HIV [124] and chronic activation of pDC may play a role in mediating monocyte activation, a contributing factor to the development of HIV-associated neurocognitive disorders (HAND) [125-127]. Collectively, the health and function of pDC have broad implications for HIV+ patients as loss of pDC function could exacerbate susceptibility to opportunistic viral infection while hyperactivation could be pathological.

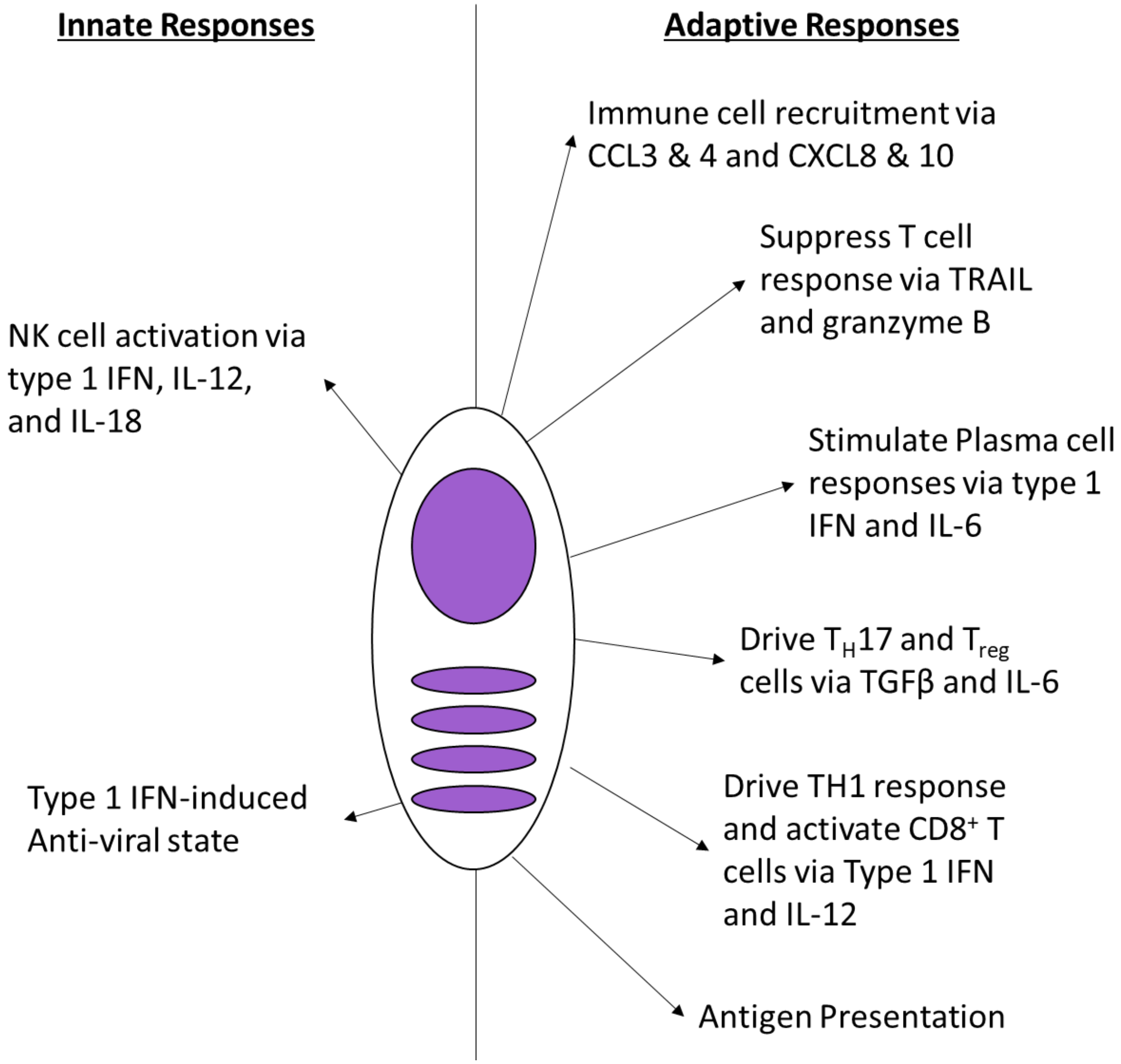

Figure 5. pDC bridge the innate and adaptive immune responses. pDC play a key role that bridges both innate and adaptive immune cell responses by recognizing viral threats and stimulating adaptive cells through robust secretion of cytokines.

D. Signaling through the type I Interferon α receptor (IFNAR)

The Type 1 interferon receptor (IFNAR) is composed of two subunits, IFNAR1 and IFNAR2 [202], which contain the tyrosine kinase 2 (TyK2) [203] and Janus kinase 1 (JAK1) [204]. IFNAR1 is considered the low affinity subunit while IFNAR2 is the high-affinity subunit for IFNα and IFNβ with a 1:2 binding affinity differential (IFNα:IFNβ, 220nm:100nm) [205-207]. Upon binding of IFNα/β, multiple signaling cascades are activated including: phosphoitenisol-3-kinase (PI3K) [208], Akt [209], MAPK [210], and JAK-STAT signaling [211, 212]. In the JAK-STAT signaling cascade, STAT1 and STAT2 are rapidly phosphorylated by JAK1 and TYK2 [212]. Though the primary signaling of IFNAR is mediated through STAT1, STAT2 is phosphorylated first and plays a role in potentiating strong STAT1 phosphorylation [211]. The phosphorylated STAT1 dimers can form homodimers or heterodimers with pSTAT2 [212]. Upon forming a pSTAT1-pSTAT2 heterodimer, interferon response factor 9 (IRF9) will bind to the pSTAT-pSTAT2 dimer and form interferon-stimulated gene factor 3 (ISGF3) [213]. ISGF3 can translocate to the nucleus and bind to interferon sensitive response elements (ISRE) on the promoter regions of key genes [214], including cytokine receptors [215].

E. T cells – a general overview

T cells are lymphocytes and participate in the adaptive immune response. Derived from common-lymphoid progenitors, T cells mature in the thymus where they are educated by resident dendritic cells by way of both positive and negative selection [216]. Specifically, T cells should be able to identify host MHC molecules (positive selection) while not responding to host antigens (negative selection) [217]. Classical T cells are identified as $CD3^+$ (T cell receptor costimulatory protein) leukocytes and then subdivided as $CD4^+$ (T helper – Th) or $CD8^+$ (Killer T cell or Cytotoxic T lymphocyte – CTL) [218].

F. Role of $CD4^+$ T cells

During an immune response, professional antigen presenting cells (APC), present pathogen-derived antigens in the context of MHC II to T-cell receptors (TCR) on antigen-specific T helper (Th) cells [219]. Th cells express a MHC class recognition molecule, CD4, which enables proper recognition of MHC II on APC [219]. $CD4^+$ T cells are central to host adaptive immunity and coordinate the stimulation of B cells and $CD8^+$ (cytotoxic) T cells [220].

If an APC has been stimulated by ligation through a PRR, like a TLR, in conjunction with antigen, the APC will express costimulatory molecules, such as CD80 (B7.1) and CD86 (B7.2) [221], and secrete cytokines[222]. Antigen presenting cells and helper T cell will then form an immunological synapse where the antigen being presented by the MHC molecule is bound by the T cell receptor on the T cell (Figure 6). The cytokines secreted by the antigen presenting cell will assist in patterning T cell responses [223]. This stimulation of $CD4^+$ T cells by 1) antigen, 2) co-stimulatory molecules and 3) cytokines compose the “three signal hypothesis” of T cell stimulation.

Stimulated $CD4^+$ T cells will interact with B cells presenting the antigen which they recognize [161]. These $CD4^+$ T cells express CD40L, a ligand for CD40 expressed on B cells. Similar to the three-signal hypothesis in T cells, B cells are stimulated by antigen specific T cells, co-stimulatory receptor (CD40/CD40L), and cytokines secreted by the $CD4^+$ T cell [224].

The stimulation of B cells by CD4$^+$ T cells is characteristic of a Type 2 T helper cell response, otherwise known as a TH2 response [225]. The TH2 response along with the TH1 response, characterized by the stimulation of CD8$^+$ cells (described below) and macrophages, compose the classical arms of T helper cell function [226]. However, there are other subdivisions of T helper cells including: TH9, so named due to the secretion of IL-9 during helminth infections [227]; TH17, characterized by T cell secretion of IL-17, an inflammatory cytokine [228]; and TH22, which is common in bacterial infections and identified by IL-22 secreting T cells [229].

In addition to their role in activating the adaptive immune responses, CD4$^+$ T cells also play a key role in mitigating immune responses. Specifically, FOXp3$^+$ CD4$^+$ T cells are called “regulatory” T cells (T-reg) and serve to reduce the intensity of immune responses. Specifically, these cells can be divided into thymus derived (tT-reg) and peripherally-derived (pT-reg). These cells suppress immune function by secretion of IL-10, IL-35, and transforming growth factor Beta (TGFβ) [230]. They can also induce apoptosis in effector T cells, through secretion of granzyme B [231], and can interfere with CD28 stimulation of T cells through the interaction with CD80/86 via expression of CTLA4 [232]. Much of this regulatory action is facilitated through an IL-2 feedback loop such that activated T cells secrete IL-2 [233], which stimulates T-reg and induces their immunosuppressive action.

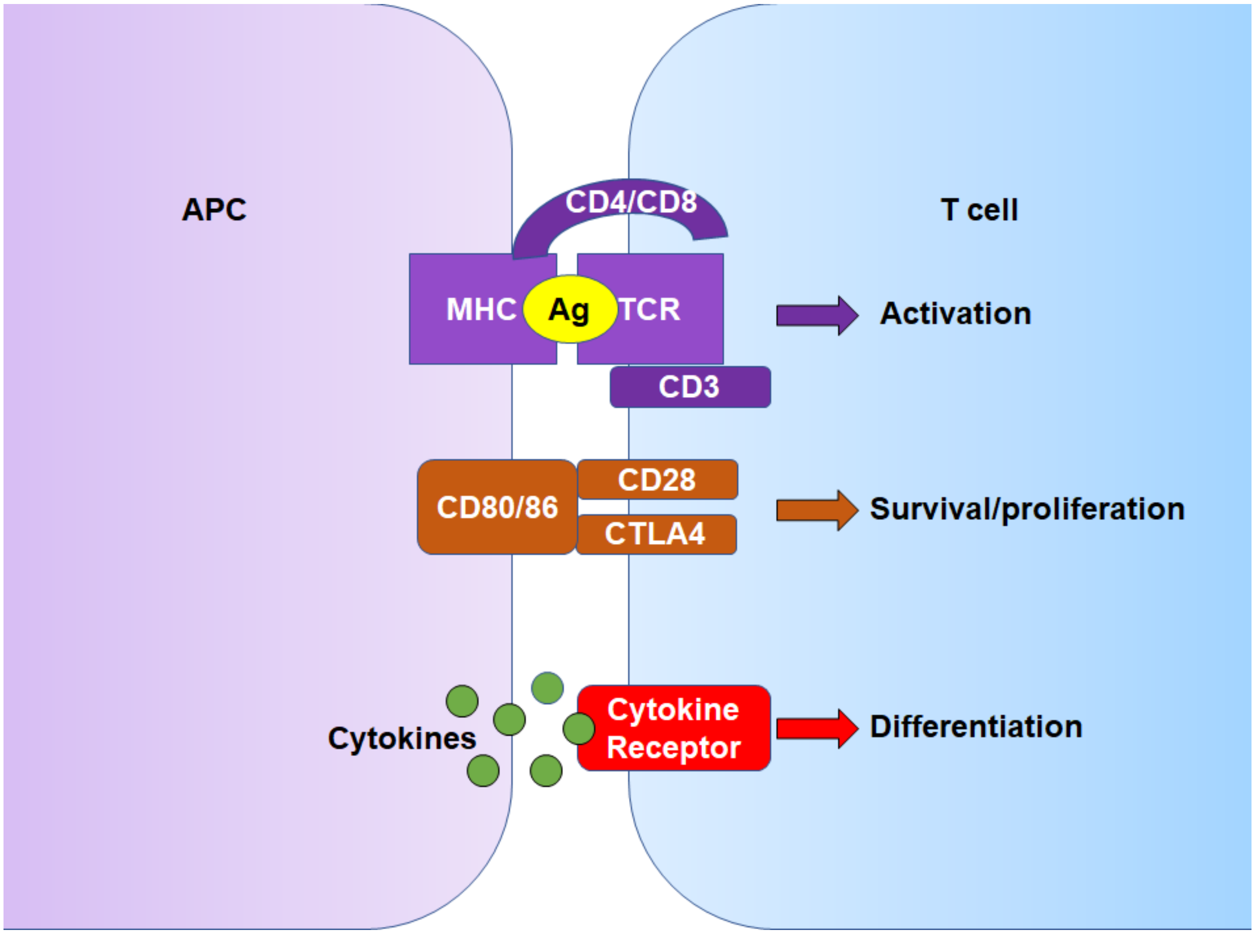

Figure 6. Immunological synapse during antigen presentation to T cells. T cells must be stimulated by antigen (Ag) in the context of a MHC molecule expressed on the surface of an antigen presenting cell (APC). To become fully differentiated and activated, T cells require three points of stimulation, so called the “three signal hypothesis” which include: 1) Antigen presented in an MHC, 2) costimulatory molecules, and 3) Cytokines.

G. Role of CD8$^+$ T cells

CD8$^+$ T cells are also called “killer T cells” or “cytotoxic T lymphocytes” (CTL) upon activation [234]. Unlike the CD4$^+$ T cells, which direct adaptive immune response, CD8$^+$ cells directly act upon host cells to prevent further spread of viral pathogens and remove altered or damaged host cells, such as cancerous cells [235].

CD8$^+$ T cells become activated through presentation of antigens in the context of MHCI [236]. While dendritic cells can cross-present antigens between MHCII and MHCI [237, 238], antigens presented on MHCI are typically intracellular proteins like those found during viral infection or altered self-antigens. Just like the CD4$^+$ Th cells, CD8$^+$ cells must be activated through recognition of the antigen presented within MHCI via their TCR while their CD8 also binds to the MHCI. The CD8$^+$ T cells will then be stimulated through costimulatory molecules, like CD80/86, and cytokines [236].

To achieve optimal stimulation of CD8$^+$ cells and to induce maturation of CD8$^+$ T cells into long lived memory cells, antigen presenting cells must be “licensed” by activated CD4$^+$ T cells. To achieve “licensing”, APC are stimulated through costimulatory receptor binding to the CD4$^+$ T cell (CD40/CD40L and B7.1/2/CD28) and cytokine stimulation [162, 239] (Figure 7). Furthermore, CD4$^+$ T cells can support CD8$^+$ T cell activation and activated populations through secretion of cytokines like IL-2 and IFNγ [240-242].

Once activated, CD8$^+$ T cells will proliferate and differentiate into memory and effector cells, just like CD4$^+$ T cells. In addition to cytolytic functions, CD8$^+$ cells can secrete cytokines including IFNγ [243] and TNFα [244]. These cytokines alone, or in combination, have noted inflammatory [245, 246] and anti-tumor activity [191, 247]. CD8$^+$ T cells can also release cytolytic granules which contain perforin and granzymes [248, 249]. Once the perforins form holes in the membrane of the target cell, the granzymes enter the cell and, through cleavage of proteins at serine residues, induce apoptosis [234]. Likewise, CD8$^+$ T cells can directly induce apoptosis via expression of FAS-ligand (FAS-L), a member of the TNFα family of receptors [250]. FAS-L on CD8$^+$ T cells can bind to FAS receptor (FAS-R) [251] on target host cells. Upon binding, the FAS will aggregate on the cell membrane to form the death-inducing signaling complex (DISC), causing activation of FAS-associated death domain (FADD) and caspase-8 [252]. Regardless of the mechanism, the typical result of CD8$^+$ T cell activation is the death of target cells. These target cells, likely infected with a virus or that are cancerous, will then be removed along with the threat of further spread of the virus or malignant cells.

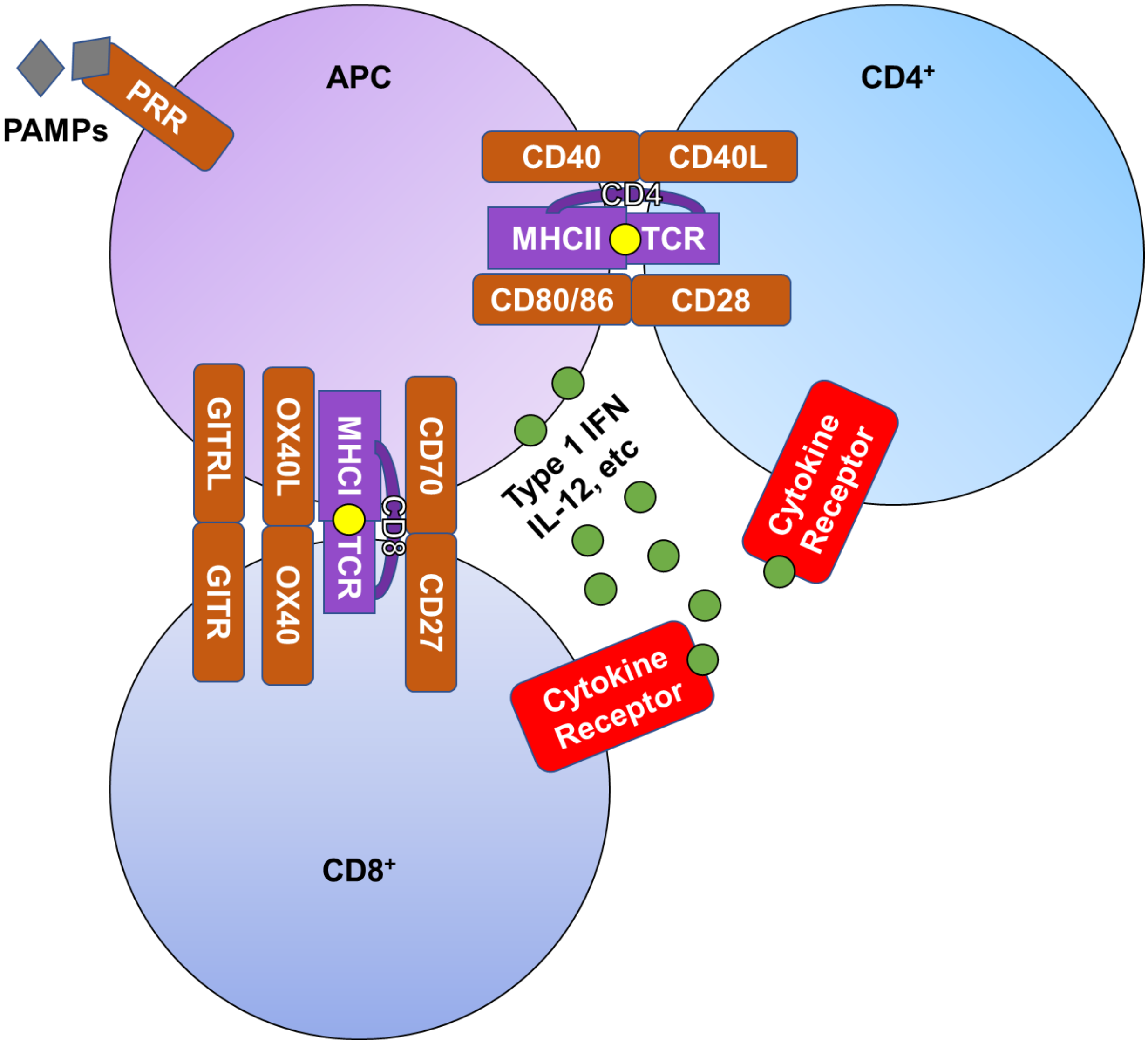

Figure 7. Dendritic cell licensing by CD4$^+$ T cells. Dendritic cell licensing by CD4$^+$ T cells enables optimum CD8$^+$ T cell activation which is achieved during the presentation of antigen to CD4$^+$ T cells. CD40 expressed on the antigen presenting cell interacts with CD40 ligand (CD40L) expressed on the CD4$^+$ T cell during antigen presentation. This interaction facilitates reciprocal stimulation of the APC by CD4$^+$ T cells as part of the licensing process. Interactions of Glucocorticoid-induced tumor necrosis factor receptor family-related receptor (GITR), CD27, and OX40 expressed on CD8$^+$ T cells with GITR ligand (GITRL), CD70, and OX40L, respectively, expressed on the APC facilitate CD8$^+$ T cell response and lasting memory cell formation. Lastly, cytokines secreted by the APC and the CD4$^+$ T cell further stimulate the CD8$^+$ T cell and pattern the effector cell functions.

IV. T cells during HIV infection

A. CD4$^+$ T cell depletion during HIV infection

CD4$^+$ T cell leukocytopenia is a hallmark of HIV infection, with CD4$^+$ T cells being the primary target for HIV [253, 254]. However, while the infection of CD4$^+$ T cells by HIV can directly cause cell death, the majority of HIV-related CD4$^+$ T cell loss during HIV infection is due to cell-mediated killing via CD8$^+$ T cells [255], NK cells [256], and pDC [257-260] in the gastrointestinal tract [261]. Upon infection by HIV, CD4$^+$ T cells will express HIV antigens in their MHC I, making them targets for CD8$^+$ T cell-mediated killing[255]. Conversely, HIV can inhibit the presentation of MHC I molecules to the surface of virally infected CD4$^+$ T cells. This lack of MHC I can lead to the "missing self" hypothesis of NK cell recognition and targeted cell killing [262] or protect virally infected cells [256]. Furthermore, NK cells may also deplete HIV-infected CD4$^+$ T cells via antibody-dependent cell-mediated cytotoxicity (ADCC) [263]. Lastly, pDC can express tumor-necrosis apoptosis inducing ligand (TRAIL), which induces apoptosis via TNF-family death receptors, and may also contribute to CD4$^+$ T cell depletion during HIV infection [259, 260]. This perturbation of CD4$^+$ T cells causes a loss of adaptive immune responses including loss of cytotoxic T lymphocyte [264] and B cell functions[265], culminating in acquired immune deficiency syndrome (AIDS) [266].

B. Antiretroviral therapy and HIV infection in 2018

Since the early to mid-1990's, the standard of care following HIV diagnosis is anti-retroviral therapy (ART) [267, 268]. Beginning with azidothymidine (AZT), the first drug available for use in HIV patients in 1987 [269], the number of anti-retroviral therapeutics has now greatly expanded (Figure 8).

According to the NIH, there are, at the time of writing this, 40 FDA approved antiretroviral therapeutic treatments across seven classes of drugs and combination therapies, including: nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, protease inhibitors, fusion inhibitors, entry inhibitors, HIV integrase strand transfer inhibitors, and multi-class combination drugs. Regardless of the drugs or the specific cocktail, ART facilitates $CD4^+$ T cell restoration and, by extension, restoration of normal $CD8^+$ T cell populations [270] by suppressing viral replication and spread. Due to the efficacy of modern ART, HIV infected patients have life expectancies comparable to non-HIV infected individuals [271]. However, some HIV patients continue to have health complications and T cell deficiencies despite successful ART therapy [272].

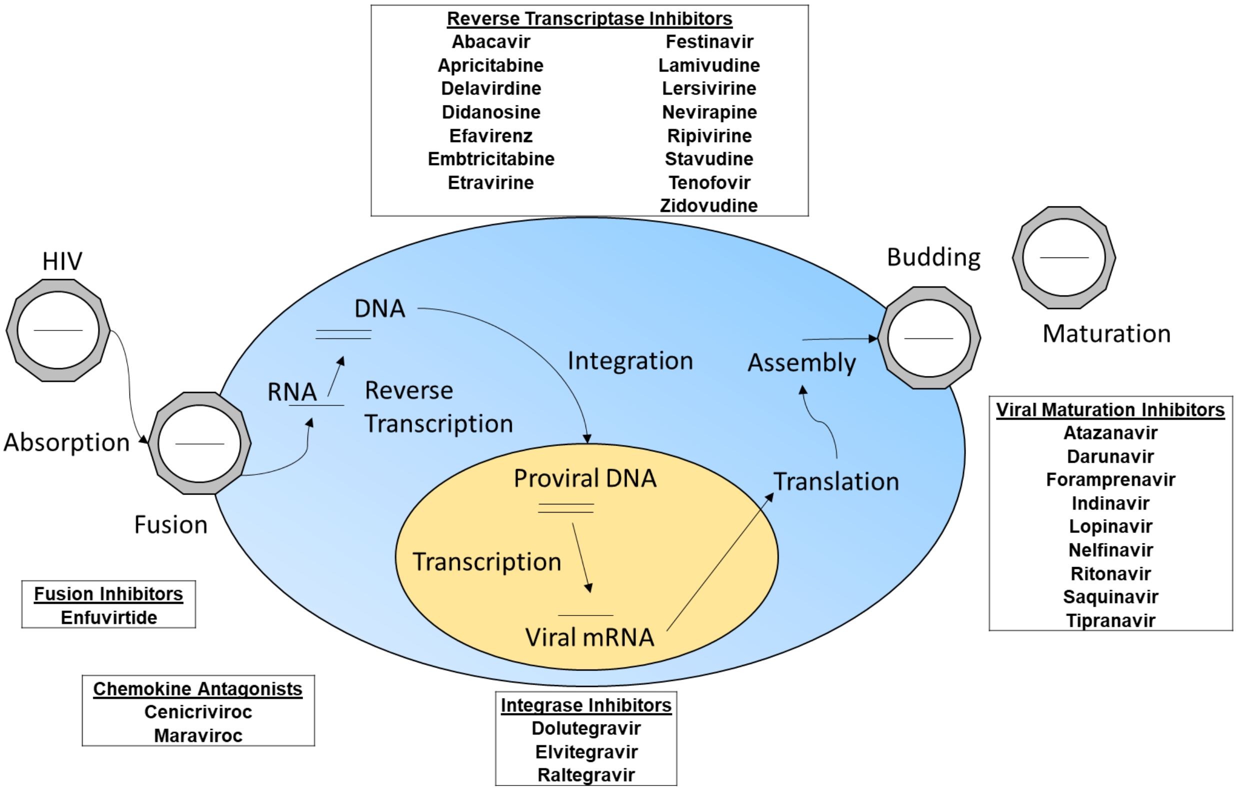

Figure 8: Approved antiretroviral drugs for the use in treating HIV infection. Antiretroviral drugs target many different points in the HIV lifecycle including: fusion inhibitors, chemokine antagonists, integrase inhibitors, reverse transcriptase inhibitors, and inhibitors of budding and virion maturation. Not shown here is Cobicistat, approved 2014, which is a pharmacokinetic enhancer of an established ART regimen.

C. T cell exhaustion and IL-7R deficiency

T cells are considered exhausted when they lose their ability to respond, including the loss of proliferative potential [273-275]. Exhausted T cells express high levels of inhibitory molecules including: PD-1, which contains immunoreceptor tyrosine inhibitory domains (ITIMs) [276]; CTLA-4 , which interferes with CD28 binding to CD80 and CD86 (B7.1 and B7.2)[277], and LAG3, which inhibits CD4-MHCII binding [278]. Furthermore, exhausted T cells also demonstrate a loss of IL-7 receptor (IL-7R) on their surface [279].

T cell exhaustion can arise through chronic exposure to antigens and inflammatory cytokines [273, 274]. In HIV infection, hyper activation of pDC in women during the early phase of infection is associated with faster depletion of T cells by elevated secretion of IFNα and subsequent activation of T cells leading to exhaustion [112, 280, 281]. Furthermore, chronic exposure to HIV antigens and loss of gut lumen integrity during chronic HIV infection, termed “leaky gut syndrome”, may also lead to HIV-related T cell exhaustion in patients successfully treated with antiretroviral therapy [282, 283].

Reduced expression of IL-7R on T cells in HIV patients with low $CD4^+$ T cell nadir has been documented by several groups [284-286]. IL-7 is a crucial cytokine for T cell health as it drives both differentiation and peripheral maintenance of T cells [287]. Likewise, IL-7 can enhance expansion of T cells from HIV+ donors [288-290] . Clinically, the use of IL-7 in HIV patients reversed T cell leukocytopenia and restored gut lumen integrity (*e.g.* Leaky-gut syndrome) [291]. IL-7R expression is tightly regulated and there may be more to the observed deficiencies in IL-7R expression in HIV patients. Specifically, the IL-7Rα gene promoter contains a Type-1-interferon inducible promoter region, also called an interferon sensitive responsible element (ISRE) [215], discussed above.

D. IL-7 receptor signaling and STAT5

The IL-7R is composed of 2 chains, the common IL-2R gamma-chain and the IL-7-specific alpha-chain (IL-7Rα) which contain JAK3 and JAK1, respectively, on their intercellular domains [292]. The IL-7Rα subunit can be induced by several factors, including type 1 interferons discussed above [215].

Once bound by IL-7, signaling through the IL-7R can be categorized as either anti-apoptotic or mitogenic [293] (Figure 9). JAK-dependent phosphorylation of the PI3K pathway is considered anti-apoptotic as activated PI3K will phosphorylate AKT[294], which can suppress activation of Bad, a pro-apoptotic protein [295]. The mitogenic arm of IL-7 signaling is composed of JAK-STAT (STAT1, STAT3, and STAT5) [293] signaling, JAK phosphorylation of insulin receptor substrate 1 (IRS1) [296], and phosphorylation of SH2 domain-containing transforming protein C1 (SHC1) [297]. Phosphorylation of STAT5 is known to induce a potent proliferative response and is required to maintain appropriate $CD8^+$ T cell effector function [298, 299]. STAT5 is encoded by STAT5a and STAT5b which both bind to similar DNA core motifs, although there are some differences in their DNA binding preference [300, 301]. Lastly, activation of STAT5 through various receptors, including IL-7, is critical in the development, function, and maintenance of T cell populations especially during chronic infection with HIV [218, 286-289, 291, 302].

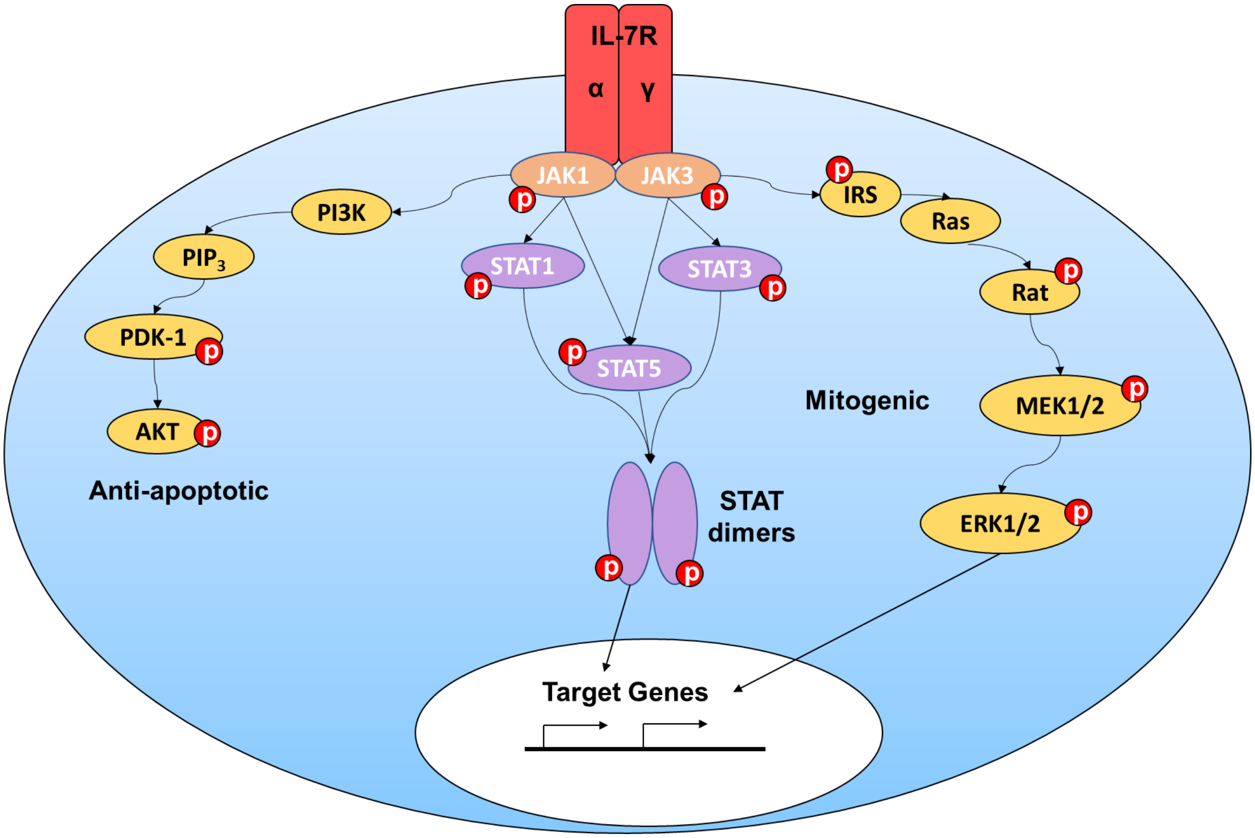

Figure 9. IL-7 receptor signaling. IL-7 Receptor signaling involves multiple phosphorylation events which promote cell division (mitogenic) and prevent cell death (anti-apoptotic). Signaling through STAT proteins drives target gene transcription including growth factors and other cytokine receptors, like IL-2Rβ.

E. Role of IFNα in T cells during HIV infection

Type 1 interferons are a class of anti-viral cytokines composed of IFNα and IFNβ [303]. As previously mentioned, pDC are the primary IFNα secreting leukocyte [164] and have a direct influence on T cell health during HIV infection. Circulating pDC and $CD4^+$ T cell numbers are positively correlated [201] and chronic infection with HIV results in the reduction of pDC number and function [110]. IFNα also inhibits HIV expansion [304] and provides protection for $CD4^+$ T cells from HIV-mediated depletion in a humanized mouse model [108], implicating a link between pDC function and T cell health. Furthermore, pDC promote T cell activation and protection against certain viral infections when using a Fc-fused IL-7 [305] which is likely due to the inherent synergy of IFNα-induced expression of IL-7R [215] increasing the receptivity of T cells to stimulation by IL-7.

However, as previously mentioned, elevated levels of IFNα during the early phase of infection has been associated with faster progression of HIV infection to AIDS in women through activation of T cells leading to T cell depletion and exhaustion [109, 112, 196, 258]. Likewise, monocytes in HIV infected people have an IFNα gene signature and this phenotype is associated with elevated inflammation [306]. Specifically, IFNα induces classical monocytes to transition into $CD16^+$, “inflammatory” monocytes which have been implicated in several inflammatory conditions such as arthritis, lupus erythematosus, and HIV-associated neuroinflammation [125, 307].

F. HIV-associated neurocognitive disorders (HAND)

With the advent of anti-retroviral therapy in the mid 1990's and the numerous potent anti-viral drugs that have been developed since, infection with HIV has turned into a chronic, but manageable, infection. However, while neurocognitive disorders have always been part of HIV pathology, markers such as $CD4^+$ T cell number and viral burden are no longer good indicators of increased risk of neurocognitive impairment [308-310]. Instead, peripheral markers of inflammation and cardiovascular disease have been associated with elevated risk of developing neurocognitive impairment [308].

Diagnosis of HIV-associated neurocognitive disorders (HAND) can be difficult due to the variances in presentation. HAND is largely dependent upon deficiencies in executive functions, motor skills, and behavioral patterns. Therefore, HAND is divided into symptomatic and asymptomatic cognitive impairment [126, 308-310]. Patients suffering from asymptomatic cognitive impairments don't report any symptoms but display deficiencies when required to perform neurocognitive tests [310]. Symptomatic cognitive impairment is further divided into two forms, 1) mild cognitive disorder and 2) HIV-associated dementia (HAD) [311, 312]. Patients suffering from mild/minor cognitive disorder have noticeable deficiencies to their cognitive function or changes in their behavior or motor skills [313]. However, while these patients present with symptoms, they are largely independent and functional. The most severe form of symptomatic HAND is HAD, which presents as noticeable and severe deficits in executive functions, memory deficiencies, and confusion [314].

The initiating event of HAND is not known, but there are two prevailing theories: 1) neural-HIV infection and 2) the "Trojan Horse" hypothesis. The neural-HIV infection hypothesis asserts that during the acute phase of HIV infection, HIV crosses the blood brain barrier and establishes infection of the glial cells in the central nervous system [315]. In particular, microglia, the resident macrophage of the central nervous system, are susceptible to productive infection by HIV [316, 317]. The “Trojan Horse” hypothesis states that immune cells carrying HIV virus migrate across the blood brain barrier and establish neural HIV infection. Monocytes, a phagocytic myeloid cell, have been implicated in this hypothesis as monocytes can be infected by HIV and can carry pathogens across the blood brain barrier where productive infection is established [318]. Evidence for either theory is difficult as brain samples are sourced from deceased patients which show a high degree of infiltration by $CD14^+$ $CD16^+$ “inflammatory” monocytes [319]. Likely, the explanation is a mix of both theories. Regardless of the mechanism, peripheral activation of immune cells is thought to contribute to the development of the neuroinflammation associated with HAND.

G. $CD8^+$ T cell involvement in HIV-associated neuroinflammation

HIV associated neuroinflammation progresses similar to other types of immune cell mediated processes. Namely, the myeloid (innate) cells will infiltrate the tissue first and secrete inflammatory factors [320]. These inflammatory factors include cytokines, such as TNFα, monocyte chemotactic protein 1 (MCP1) [321], and IL-1β [322], and even damage associated molecular pattern (DAMP) proteins like high-mobility group box 1 (HMGB1) [323]. These factors can then induce glial cell activation and secretion of additional inflammatory and chemotactic factors [324]. In particular, interferon-γ induced protein-10 (IP-10) [125, 325], also known as CXCL10, serves as a chemotactic factor for many immune cells, including monocytes and T cells [326].

$CD8^+$ cells play a key role in neuroinflammation, but $CD8^+$ T cells can have various effector functions which can have both deleterious and protective effects during neuroinflammation [327]. $CD8^+$ T cells are potent secretors of IFNγ. IFNγ induces glial cell secretion of IP-10 which promotes further infiltration by leukocytes, thereby exacerbating inflammation [328]. Furthermore, IFNγ stimulates microglia to secrete TNFα [329] and induces the production of reactive oxygen species(ROS) [330, 331]. Microglial-derived ROS can cause oxidative stress on other glial cells and be neurotoxic with prolonged exposure [332]. TNFα, either derived from myeloid cells, T cells, or microglia, causes dysregulation in another type of glial cell, the astrocyte (described below) [333, 334]. In addition to IFNγ, $CD8^+$ T cells can also release granules in response to antigen recognition. Degranulation may be neuroprotective in that targeted apoptosis of infected cells removes the viral threat [255, 335]. IFNα can increase the number of both $IFN\gamma^+$ [336] and cytolytic $CD8^+$ T cells [337], leaving the role of IFNα in this response unclear.

V. IFNγ and neuroinflammation

A. IFNγ receptor, signaling, and cannabinoids in neuroinflammation

Like IFNAR, IFNγR is composed of two heterodimeric subunits termed IFNGR1 and IFNGR2. However, unlike IFNAR there are 4 chains which compose the IFNGR, two IFNGR1 and two IFNGR2. IFNGR1 is first bound by IFNγ which then causes rapid dimerization of IFNGR1 chains. The dimerization of IFNGR1 enables recruitment of IFNGR2 which enables high affinity binding of IFNγ to the receptor. Each of the IFNGR1 and IFNGR2 chains contains JAK1 or JAK2, respectively [338]. Upon binding by IFNγ, JAK1 and JAK2 phosphorylate STAT1 [339]. pSTAT1 can then dimerize and translocate to the nucleus where it will drive the induction of various genes [204]. These genes typically augment immune response and include IP-10 [340] and inducible nitric oxide synthase (iNOS) [341-343].

The connection between IFNγ-mediated neuroinflammation and cannabinoids has been previously studied. The findings from these studies suggest that cannabinoids, either endogenous or therapeutic, could play a role in diminishing the effects of IFNγ-induced neuronal injury during inflammatory processes [344]. Specifically, the effect of IFNγ on microglia can be suppressed by CB2 agonist JWH-015 [63] Likewise, previous research has suggested cannabinoid compounds may be neuroprotective in other models of neuroinflammation [345] including ischemic stroke [346].

B. Astrocytes

Astrocytes are glial cells which play a key role in neuroinflammatory processes [324, 325, 347]. These cells compose between 20-40% of the glial cells in the brain and play a key role in maintaining the blood brain barrier [348, 349]. Astrocytes also play a key supportive role for neuronal function by glutamate recycling, a process by which the levels of glutamate, an excitatory neurotransmitter released by neurons, is maintained, partially, via reuptake of glutamate by astrocytes [350].

During inflammatory processes, astrocytes can become stimulated to secrete inflammatory and chemotactic cytokines by leukocytes, specifically: MCP-1, IL-6, and IP-10 [351]. Furthermore, astrocytes can be directly stimulated by IL-6, TNFα, and IFNγ [352, 353] to express FAS and FAS-L.

While the direct secretion of inflammatory and chemotactic cytokines can augment the type of leukocyte-dependent neuroinflammation associated with HAND, the perturbed function of astrocytes can also lead to neuronal toxicity. As indicated above, TNFα can augment the secretion of inflammatory cytokines by astrocytes. However, TNFα can also cause a reduced capacity for astrocytes to uptake glutamate [333, 334]. The consequence of excess glutamate is excitotoxicity, whereby N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are over-stimulated by the presence of excess glutamate [354-357]. The over-activation of these receptors leads to elevated levels of calcium. This excess intracellular calcium causes the activation of numerous enzymes, including: phospholipases [358], endonucleases, and caspases [359] which can culminate in cell death. Neuronal death due to excitotoxicity is directly tied to the cognitive deficits seen in traumatic brain injury and multiple sclerosis [356, 357, 360, 361]. Conversely, it should be noted that IFNγ can also induce microglia to uptake glutamate, but the impact to overall glutamate levels is unclear [362].

Collectively, the activation of astrocytes by IFNγ secreting T cells, in the context of HIV-associated neuroinflammation, can augment inflammatory processes, exacerbate inflammation, and likely plays a role in the neuronal death and subsequent cognitive decline in conditions like HAND.

VI. Rationale and goal of these studies

Immune modulation by cannabinoids has been investigated in the Kaminski lab using both *in vivo* mouse models and *in vitro* mouse and human models [363-365]. Furthermore, the suppression of interferon secretion by cannabinoids is well established in the literature [61]. However, the direct effects of phyto-cannabinoid treatment on the function of pDC, the primary source of Type 1 interferon, was unknown. Furthermore, pDC secretion of IFNα during HIV infection is associated with the maintenance of T cell number and function, but also T cell exhaustion. IFNα can also drive CD8$^+$ T cells towards secreting IFNγ and TNFα. As described above, the secretion of IFNγ and TNFα by CD8$^+$ T cells can contribute to the development of neuroinflammation, at least in part, through the stimulation of astrocytes. Lastly, while the effects of cannabinoids on IFNγ-induced neuroinflammation have been studied in murine models, testing the effects of cannabinoid-mediated modulation of human CD8$^+$ T cell-induced activation of astrocytes had not been characterized *in vitro*.

Due to the relationship between pDC function, T cell activation, and astrocyte stimulation by CD8$^+$ T cells, and the unknown effects of THC on this system, the following hypothesis was developed and tested:

"THC suppresses TLR-9-dependent activation of pDC, the subsequent efficacy of pDC-mediated T cell activation, and CD8$^+$ T cell-mediated activation of astrocytes."

To test this hypothesis, two distinct areas of investigation were developed: 1) THC-mediated modulation of pDC activation and 2) THC-mediated modulation of T cell activation by IFNα and subsequent CD8$^+$ T cell-mediated activation of astrocytes. Each area of investigation is composed of 2 specific aims (SA).

Area of investigation # 1: THC-mediated modulation of pDC activation

Very little was known about cannabinoid-mediated modulation of pDC function during the planning of these studies. Furthermore, there was no information in the literature concerning changes in the sensitivity of leukocytes to modulation by cannabinoids during HIV infection. Therefore, the objective of this first area of investigation was to characterize the effect of THC treatment on pDC activation comparing pDC from healthy and HIV+ donors.

SA1: Determine whether THC impairs pDC-derived IFNα production in healthy verses HIV+ donors

SA1 focused on characterizing the effect of THC treatment on the TLR-mediated activation of pDC from healthy and HIV+ donors. Initially, pDC from healthy donors were stimulated by various TLR agonists and the induction of CD83 and secretion of IFNα was compared between modes of activation. The expression of cannabinoid receptor mRNA levels was compared in PBMC from healthy and HIV+ donors as was THC-mediated suppression of TLR-9-induced activation by CpG. Furthermore, THC-mediated suppression of IRF7 phosphorylation was compared between healthy and HIV+ donors. Lastly, the effect of THC treatment on TLR-7/8 activation, a mimic for stimulation of pDC by HIV, was performed in pDC from healthy donors.

SA2. Elucidate the intracellular mechanism for CB2-mediated suppression of IFNα and TNFα secretion by pDC

SA2 focused on elucidating the molecular mechanisms by which THC impaired pDC response to CpG by determining the role of CB2 in that suppression. Specifically, THC-mediated reduction of CpG-induced IFNα and TNFα responses was compared to suppression by the CB2-selective agonists, JWH-015 and JWH-133. Differences were determined by measuring the phosphorylation events of key intracellular signaling proteins related to the induction of both cytokines.

Area of investigation # 2: Modulation of IFNα-mediated activation of T cells and subsequent $CD8^+$ T cell-induced stimulation of astrocytes by THC

The studies for the second area of investigation were undertaken to address the conflicting information regarding the role of pDC activation and T cell health during HIV infection. Specifically, the role of IFNα and IL-7 on T cell proliferation was investigated as was the effect of THC treatment on IFNα and IL-7 stimulation of T cells. Furthermore, the effect of treatment with THC on the role of IFNα-mediated activation of $CD8^+$ T cells and subsequent activation of astrocytes was investigated using a novel *in vitro* co-culture system.

SA3: Determine the consequences of IFNα activation and suppression by THC on $CD4^+$/$CD8^+$ T cells from healthy and HIV+ donors

SA3 focused on comparing the effects of THC-mediated modulation of IFNα-induced activation of T cells from healthy and HIV+ donors. Specifically, IFNα-mediated phosphorylation of STAT1 was compared between T cell subtypes and between healthy and HIV+ donors. Lastly, the effect of THC on IFNα-induced IL-7R expression, cognate signaling, and IFNα/IL-7-mediated augmentation of T cell proliferation was investigated.

SA4: Evaluate the effects of THC treatment on CD8$^+$ T cell-mediated activation of astrocytes (U251)

In SA4, the effects of THC treatment on IFNα-stimulated CD8$^+$ T cell effector function and CD8$^+$ T cell-mediated activation of astrocytes (U251) was investigated. This series of experiments compared the effect of THC treatment on naïve CD8$^+$ T cells versus THC treatment on differentiated CD8$^+$ effector T cells. Further, the effect of THC on TNFα and IFNγ-mediated stimulation of astrocytes was tested. Finally, the effect of THC on CD8$^+$ T cell-driven activation of astrocytes was directly investigated by using an *in vitro* co-culture system.

MATERIALS AND METHODS

I. General techniques

A. Peripheral blood mononuclear cell (PBMC) isolation and cell identification: Leukocyte packs were purchased from the Gulf Coast Regional Blood Center (Houston, TX). Blood was diluted 1:1 with Hanks Balanced Salt Solution from Gibco™ (Grand Island, NY) and layered on 15 ml Ficoll Paque Plus (GE Healthcare Life Sciences, Pittsburgh, PA) in SepMate 50mL conical tubes by StemCell Technologies (Vancouver, BC, Canada). Leukocytes were centrifuged at 1300 x g for 25 min at 4ºC. The leukocyte layer was re-suspended in RPMI Media from Gibco™ containing 5% Human AB Serum (Sigma-Aldrich, St. Louis, MO), 1% Penicillin-Streptomycin (Gibco™), and 0.035% β-mercaptoethanol. pDC were identified using mouse anti-human antibodies by Miltenyi Biotec GmgH© (Bergisch Gladbach, Germany) as $CD303^+$ $CD123^+$ cells.

B. HIV+ donor recruitment and data management. HIV+ donors voluntarily enrolled via the Mid-Michigan HIV consortium (MMHC) under the Institutional Review Board (IRB)-approved protocol (IRB # 11-202) and into the MMHC Registry. HIV+ donors were males between the ages of 31 and 71, with an average age of 54.4 years, had $CD4^+$ counts above 500ct/ml of blood, had CD4:CD8 ratios >1, did not use medicinal cannabinoids, had HIV viral burdens below the detectable limit (<5 HIV mRNA copies/ml of blood), were not co-infected with any strain of hepatis, and were recruited from clinics attended by Dr. Peter Gulick. The status of medicinal cannabinoid use was determined by self-reporting and verified via plasma detection of THC metabolites using a THC ELISA Forensic Kit

(Neogen Corporation, Lansing, Michigan, USA). HIV+ donors received the standard of care and were not asked to change any lifestyle habits to participate. All subjects, questionnaires, and abstracted medical record data of the MMHC are managed using the Research Electronic Data Capture (REDCap) (Vanderbilt University), which supports 21 Code of Federal Regulations (CFR) Part 11 compliance for clinical research and trials data and HIPAA guidelines.

C. Surface staining protocol: PBMC, purified leukocytes, or astrocytes were all surface stained with the same procedure. Cells were first washed with FACS buffer and then stained with antibodies according to manufacturer's suggested concentrations by incubating at 4°C for 10 minutes. Samples were stained in 100µL contained either TruStain FX Fc blocking agent by BioLegend (San Diego, CA), Fc blocking reagent by Miltenyi Biotec (Bergisch Gladbach, Germany), or 5% Human Ab serum (Sigma Aldrich). Following incubation, cells were washed three times with FACS buffer. Cells were fixed using 100 µl/sample of fixation buffer by BD Biosciences (San Jose, CA) for 15 min at 4°C. Cells were then washed once more with FACS buffer before being stored in FACS buffer until they were read by flow cytometry or utilized for intracellular staining.

D. Intracellular staining protocol: PBMC, isolated leukocytes, and astrocytes were intracellularly stained following the same protocol. Fixed cells were washed 3 times with 1X PermWash™ buffer from BD biosciences prepared according to manufacturer's directions. Cells were stained in 100µL of staining buffer containing antibodies for the specific target (e.g. IFNγ, TNFα, IP-10, etc.) and 7% human Ab serum. Cells were incubated with the appropriate staining cocktail for 30 min at 4°C then washed 3 times with 1X PermWash. Cells were washed once more in FACS buffer before being resuspended in FACS buffer for cytometric analysis.

E. LegendPlex™: LegendPlex cytometric bead array was used to measure the secretion of cytokines from pDC (TNFα and IFNα) and CD8$^+$ T cells (IL-2, IL-4, IL-10, IL-6, IL-17A, TNFα, sFAS, sFASL, IFNγ, Granzyme A, Granzyme B, Perforin, and Granulysin). In either case, the protocol was performed per the manufacturer's directions. Briefly, detection beads were sonicated and incubated with media from purified cells (either pDC or CD8$^+$ T cells). Beads were bound to target cytokine, washed, then detection antibodies, and then cytokine concentrations determined through flow cytometric analysis and a standard curve. The BD canto II was used for data acquisition and accompanying LegendPlex Software was used for analysis.

F. Cannabinoids: Δ^9-Tetrahydrocannabinol (THC) was supplied by the National Institute of Drug Abuse (NIDA) prediluted in 100% ethanol while cannabidiol (CBD) was supplied as a neat powder. JWH-015 and JWH-133 were purchased from Cayman Chemicals (Ann Arbor, MI) as either neat powder or diluted in methyl acetate, respectively. For the JWH-133, methyl acetate was removed by evaporation using nitrogen gas. CBD, JWH-015, and JWH-133 were diluted in 100% ethanol at a concentration of 32mM to match the concentration for THC and solubility of JWH-015. THC and CBD were stored at -80^{o}C and the JWH compounds were stored at -20^{o}C, per manufacturer's directions.

II. Techniques for plasmacytoid dendritic cells (pDC)

A. Identification of pDC from PBMC*:* PBMC were isolated as indicated in the general methods section. To identify pDC, anti-human CD303 and CD123 antibodies from Miltenyi Biotec were used and the surface staining procedure from the general methods section was used to stain the pDC.

B. pDC purification by magnetic activated cell sorting (MACS*)*: pDC were isolated by negative selection using MACs isolation kits from Miltenyi Biotec© per the manufacturer's instructions. Briefly, PBMC cell concentrations were determined using a Coulter Cell Counter and the appropriate volume of non-pDC antibody cocktail was incubated with PBMC followed by washing and incubation with magnetic beads. Labeled PBMCs were then passed through a MACS depletion column affixed to a MACS magnet with unstimulated pDC being collected in the flow through. The number of PBMCs in a single leukocyte pack range from 3.0 – 11 x 10^8 total PBMC with an average of 6 x 10^8 total PBMC and 0.9 – 1 x 10^6 pDC per leukocyte pack containing 6 x 10^8 total PBMC when accounting for isolation efficiency.

C. Gene expression analysis: RNA was isolated using Qiagen© RNeasy™ kits (Germantown, MD) per the manufacturer's instructions. Briefly, cells were lysed using lysing buffer containing β-mercaptoethanol and stored at -20°C. Lysates were then purified and treated with DNase from Promega© ST Total RNA Isolation Kit™ (Madison, WI). RNA concentrations were determined by Nanodrop™ (Thermo-Fisher Scientific, Waltham, MA). RT-PCR was performed using High Capacity cDNA RT-PCR kit by Applied Biosystems™ (Foster City, CA). cDNA was frozen at -20°C. Gene analysis was determined by Real Time Quantitative PCR (Qt-PCR) using TaqMan™ probes for CNR1

(Hs00275634_m1) and CNR2 (Hs00275635_m1) by Life Technologies™ (Compendia Bioscience, Ann Arbor, MI) with 18sRNA as a loading control.

D. Treatment with cannabinoids or vehicle control and cell stimulation: (6aR,10aR)-delta-9-tetrahydrocannabinol (Δ^9-Tetrahydrocannabinol or THC) was supplied by the National Institute of Drug Abuse (NIDA) and 3-(1,1-dimethylburyl)-6aR,7,10,10aR-tetrahydro-6-6-9-trimethyl-6H-dibenzo[b,d]pyran (JWH-133) was purchased from Cayman Chemicals (Ann Arbor, MI). PBMCs were treated with either THC, JWH-133, or Vehicle control (VC - 0.026% Ethanol). The appropriate concentrations were prepared in Complete-RPMI to a final EtOH concentration of 0.026% for VC, THC, and JWH-133. The prepared cell suspensions and appropriate treatments were added to flat bottom 96 well tissue culture plates and incubated at 37^{o}C and 5% CO_2 for 30 min. Cells were stimulated with CpG-ODN Type A 2216 (15 µg/ml) (InvivoGen©, San Diego, CA) following treatment with cannabinoids.

E. IFNα capture assay: Secretion of IFNα was determined using the IFNα Capture Assay by Miltenyi Biotec per the manufacturer’s directions. Treated cells were bound with IFNα capture reagent and placed into warm media and incubated under continuous motion for 30 min. Cells were then washed and incubated with IFNα detection antibody. Cells were fixed using CytoFix™ buffer by BD Biosciences (San Jose, CA) and IFNα secretion by pDC was determined by flow cytometry.

F. Phosphoprotein detection: Treated PBMCs were washed and pDC were stained as described. pIRF7, pTBK1, and pIKKγ levels were determined using Phosflow™ antibodies and the harsh detergent method by BD Biosciences©. In brief, cells were fixed using BD cytofix buffer for 10 min at 37^{o}C then permeabilized using 1x of perm buffer

IV™, stained for 1 hr under continuous motion using FACS buffer and 5% Human AB serum, washed 3X with 0.5x perm buffer, and analyzed by flow cytometry.

G. IFNA2 and TNFA2 gene expression by PrimeFlow™: PrimeFlow™ RNA assay (eBiosciences©, San Diego, CA) was performed per manufacturer's directions (Figure 10). Treated PBMCs were fixed, permeabilized, and bound with either the IFNA2 (NM_000605) probe alone or both the IFNA2 probe and the TNFA2 (NM_000594) probe in unison. The mRNA signal was then amplified and detected using either Alexa Fluor 647 or the Alexa Fluor 488 (Thermo-Fisher Scientific, Waltham, MA) labeled probes. Relative gene expression was determined via flow cytometry.

H. Intracellular detection of IFNα and TNFα: IFNα$^+$ and TNFα$^+$ pDC were determined by intracellular staining with antibodies by BioLegend. In brief, harvested cells were stained were stained for CD303 and CD123 as indicated above and fixed using CytoFix™ buffer by BD Biosciences (San Jose, CA). Fixed cells were permeabilized using PermWash™ buffer (BD Biosciences) by washing with 1X PermWash™ and preparing IFNα/TNFα master mix in PermWash™ buffer with 7% Human Ab serum to reduce non-specific staining. Cells were stained for 30 min at 4^{o}C, washed with PermWash™, resuspended in FACS. IFNα$^+$ and TNFα$^+$ pDC were determined by flow cytometric analysis.

I. Data analysis. GraphPad© Prism 5.0™ was used for statistical analysis. Where appropriate, samples were normalized to 0µM THC + CpG, which was considered 100% maximum response for each individual donor and the appropriate statistical test was performed (see Figures).

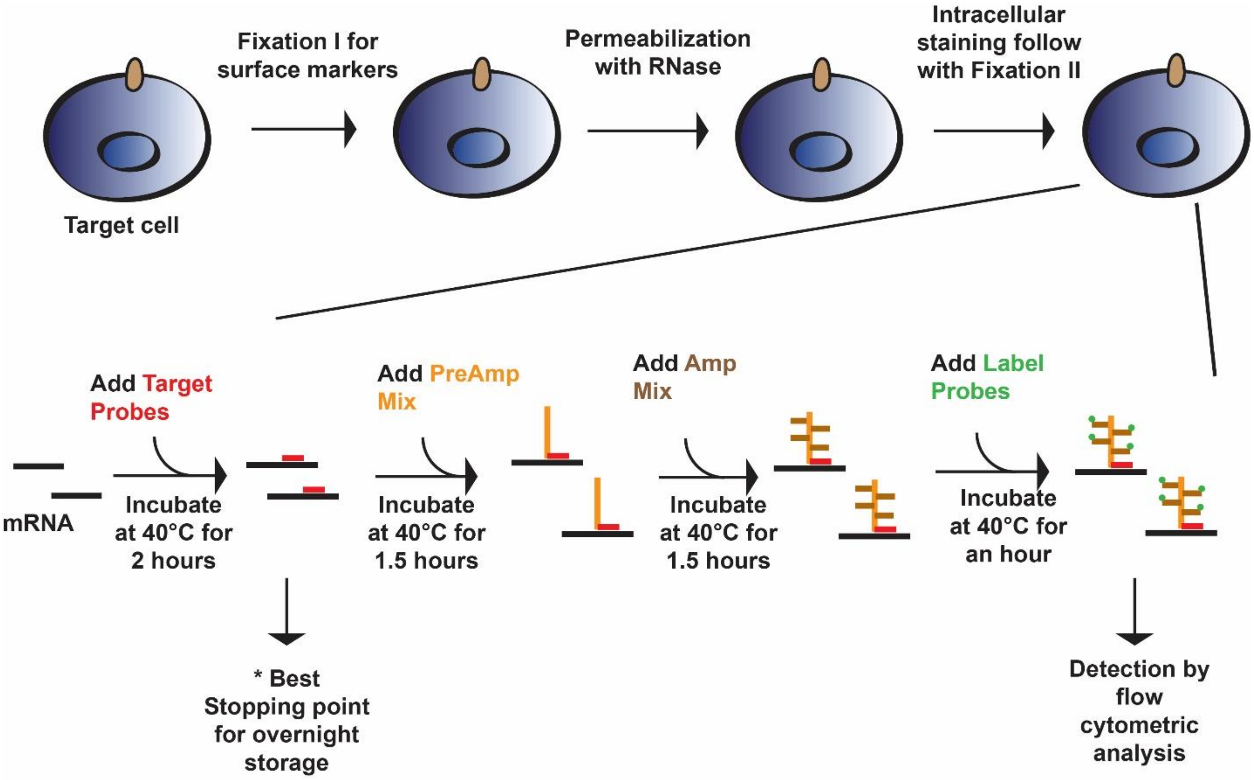

Figure 10. Overview of the standard PrimeFlow™ assay protocol. Cells are first fixed with fixation buffer I, stained for surface markers, then permeabilized and treated with RNase inhibitors. Cells are then stained for intracellular markers and further fixed with fixation buffer II. To amplify the target mRNA, target probes are hybridized to the mRNA of interest. A preamplifier (PreAmp) oligonucleotide is then bound to the target probe/mRNA dimer followed by the binding of amplification (Amp) probes. Finally, fluorescent dye labeled probes are bound to the Amp probes and detected through flow cytometry [366].

III. Techniques for T Cells

A. T cell identification: PBMC were isolated from leukocyte packs or HIV+ Donors. The procedure of PBMC isolation and handling of HIV+ donor information proceeded as indicated under “General Techniques”. T cells were identified using mouse anti-human antibodies by BioLegend® (San Diego, CA) as CD3$^+$ cells. Helper T cells and CTL were identified as either CD4$^+$ or CD8$^+$ respectively. Memory and non-memory T cells were identified by the expression of CD45RO such that memory cells were identified as CD45RO$^+$ and non-memory cells were identified as CD45RO$^-$.

B. T cell purification by MACS: Either pan T cells or naïve CD8$^+$ T cells were isolated by way of negative selection using MACs Pan-T cell isolation kits from Miltenyi Biotec (Bergisch Gladbach, Germany) or the naïve CD8+ T cell isolation kit from BioLegend (San Diego, CA) in accordance with the manufacturer’s instructions. In short, following PBMC isolation, the cell concentration was determined using a Coulter Cell-Counter (Beckman-Coulter Inc, Brea, CA) and the appropriate volume of non-T cell antibody cocktail was incubated with PBMC followed by washing with MACS buffer (1 X phosphate buffered saline (PBS), 0.5% bovine serum albumin (BSA), and 2 mM EDTA) and incubation with magnetic beads. Labeled PBMCs were then passed through a MACS depletion column that was affixed to a MACS magnet. T cells were collected in the flow through. BioLegend protocol, for the isolation of CD8$^+$ T cells, does not require a column for separation. Instead, bead-bound cells were incubated in a MojoSort™ magnet in a polypropylene, round bottom tissue culture tube and incubated for 5 min. CD8$^+$ T cells were then poured off and used for various assays.

C. Treatment with cannabinoids or vehicle control: Δ^9-Tetrahydrocannabinol (THC) was supplied by the National Institute of Drug Abuse (NIDA). Purified T cells or whole PBMCs were treated with either THC or Vehicle control (VC - 0.026% Ethanol) prepared in C-RPMI. The prepared cell suspension and appropriate treatment were added to flat bottom 96 well tissue culture plates. Cells were incubated at 37^oC and 5% CO_2 for 30 min before being stimulated (below).

D. Stimulation of T cells: Following treatment with THC or VC, PBMC or isolated T cells were stimulated as follows: 1) to measure the phosphorylation of STAT1, cells were stimulated with 100U/ml of universal IFNα for (PBL Assay Science, Piscataway, NJ) 30 min before harvesting for phospho-protein detection (below), 2) to measure IFNα-induced IL-7Ra mRNA and protein expression in isolated T cells or PBMC, respectively, the cells were treated with 100U/ml of IFNα for 48 hr before harvesting and measurement of the respective endpoints (below); 3) IL-7 induced phosphorylation of STAT5 on day 0 was measured by stimulating VC treated T cells with 10 ng/ml of IL-7 for 15 minutes before harvesting for phospho-protein detection (below); 4) to measure IFNα-induced augmentation of IL-7-dependent STAT5 phosphorylation, cells were treated with 100U/ml of IFNα for 48 hr and then stimulated with IL-7 for 15 minutes before harvesting for phospho-protein detection (below); and 5) for measuring IL-7-augmented proliferation of T cells (below), cells were stimulated with 100U/ml of IFNα, 2.5ug/ml mouse anti-human CD3 antibody (BioLegend), and 2.5ug/ml mouse anti-human CD28 antibody for 48 hours followed by stimulation with 10ng/ml of IL-7 or vehicle control (sterile, endotoxin free water from Invivogen, San Diego, CA) at which point cells were incubated for another 48 hr before harvesting.

E. Gene expression analysis: RNA was isolated using RNeasy® kits (Qiagen, Hilden, Germany) per the manufacturer's instructions. Briefly, cells were lysed using lysing buffer containing β-mercaptoethanol and stored at -20°C. Lysates were then purified and treated with DNase from Promega's ST Total RNA Isolation Kit (Madison, WI). RNA concentrations were determined by Nanodrop (Thermo-Fisher Scientific, Waltham, MA). RT-PCR was performed using High Capacity cDNA RT-PCR kit by Applied Biosystems (Foster City, CA). cDNA was stored at -80°C. Gene analysis was determined by Real Time Quantitative PCR (Qt-PCR) using TaqMan probes for IL7RA (Hs00902334_m1) by Life Technologies (Compendia Bioscience, Ann Arbor, MI) with 18sRNA as the loading control.

F. Phosphoprotein and IL-7Rα detection: Treated PBMCs were washed and T cells were stained as described (above). pSTAT1 and pSTAT5 levels were determined using Phosflow™ antibodies and the harsh detergent method by BD biosciences (San Jose, CA). In brief, cells were fixed using BD cytofix buffer for 10 minutes at 37°C, permeabilized with 1x of Phosflow perm buffer IV, stained for 1 hour under continuous motion in FACS buffer (1X PBS, 1%, BSA, and 0.1% sodium azide) containing 7% Human AB serum, washed once with 0.5x Phosflow perm buffer, washed twice with general FACS buffer, and then immediately analyzed by flow cytometry. IL-7Ra surface expression was determined by surface staining with mouse anti-human antibodies (BioLegend).

G. Detection of T cell proliferation: Prior to activation (above) PBMC were treated with violet CellTrace™ dye by Thermo-Fisher per the manufacturer's directions. In brief, the violet dye was resuspended in DMSO which was then diluted in warm, sterile, PBS such that 1ml of the stock of the PBS/Dye mixture was utilized for 1 million PBMC and the final

DMSO concentration was 0.02%. PBMC were incubated in the PBS/Dye mixture in the dark at 37ºC for 20 min and then diluted with complete RPMI containing 5% human Ab serum and incubated in the dark at 37ºC for another 5 minutes. Cells were then centrifuged, washed with incomplete RPMI, and resuspended in complete RPMI before stimulation (above). T cell proliferation was determined by dye dilution using the FlowJo v. 10 (FlowJo, LLC, Ashland, OR) proliferation tool.

H. T cell effector function: Isolated CD8$^+$ T cells, either treated with THC or not, were stimulated with anti-CD3 antibodies, anti-CD28 antibodies, IFNα (100U/ml), and IL-2 (1ng/ml) for 4 days in 96 well polystyrene tissue culture flasks. At 90hr of stimulation, T cells for “delayed” THC treatment were treated with THC or vehicle. CD8$^+$ T cells were then stimulated with phorbol 12-myristate 13-acetate (PMA) (50ng/ml) and ionomycin (1µg/ml). For cells being used for intracellular staining, they were treated with a protein transport blocker cocktail, which consisted of brefeldin (5µg/ml) and monensin (2nM) in 0.02% methanol when diluted to 1X, and incubated for 5 hr. Cells where then harvested and stained for CD8, CD45RO, and CD107a (LAMP-1) using the standard surface staining protocol.

I. Data analysis. GraphPad Prism 5.0 was used for all data analysis. Where appropriate, samples were normalized to VC + IFNα/IL-7 which was considered 100% maximum response for each individual donor and the appropriate statistical test was performed (see Figures).

IV. Techniques for astrocytes

A. Culturing: U251 cells, an astrocyte-derived glioblastoma cell line, were purchased from Kerafast® Inc (Boston, MA). 1 x 10^6 Cells were seeded in a T225 tissue culture flask from Gibco and allowed to grow to confluency (5 days) before being aliquoted into 1x10^6 cells/ aliquot, stored in freezing media consisting of complete DMEM (below) containing 10% Dimethyl sulfoxide (DMSO), then frozen at -196Co, in liquid nitrogen, for long term storage. Cells were rapidly thawed and seeded into T225 flasks and allowed to culture in Dulbecco's minimal essential media (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were harvested 1 day before use in cytokine induction assays. Cells were cultured until p10 at which they were discarded, and another aliquot thawed to maintain consistency.

B. Astrocyte activation by cytokines. U251 cells were seeded at 7x10^4 cells/well of a 24 well polystyrene tissue culture plate from Gibco 24 hr before the start of the assay. This allowed U251 cells to reach 1x10^5 cells/ well without potentially disrupting their response by stressing the cells via over-crowding (contact inhibition) or trypsinization. Cells were then treated with either TNFα (1, 5, 10, 50, 100 ng/ml) or IFNγ (1, 5, 10, 50, 100 U/ml) for individual stimulation or combinations of TNFα (1, 5, 10 ng/ml) and IFNγ (I, 5, 10 U/ml) when stimulating concurrently. After 18 hr of incubation, U251 astrocytes were treated with 1x brefeldin/monensin transport blocker and incubated for 6 hr then intracellularly stained for either IL-6, Interferon γ inducible protein-10 (IP-10), and monocyte chemotactic protein 1 (MCP-1) using the intracellular staining protocol indicated above.

C. T cell and astrocyte co-culture: U251 cells were seeded at 7x10^4 cells/well of a 24 well polystyrene tissue culture plate from Gibco 24 hr before the start of the assay. This allowed U251 cells to reach 1x10^5 cells/ well without potentially disrupting their response to incubation with T cells by stressing the cells with over seeding or trypsinization the day of coculture start. CD8$^+$ T cells were isolated and stimulated with anti-CD3 antibody, anti-CD28 antibody, IFNα, and IL-2 as indicated above. After 3 days of stimulation, CD8$^+$ T cells were removed from their well and 1x10^5 T cells were incubated with the astrocytes for a 1:1 ratio of CD8:U251 cells in 500µl of RPMI containing 5% human ab serum and 1% penicillin/streptomycin. T cells were then stimulated with PMA/Ionomycin (PMA/IO) (6.25 ng/ml/125 ng/ml) for 24 hr. After 18 hr, astrocytes were treated with 1x brefeldin/monensin transport blocker and incubated for 6 hr. Following incubation, U251 astrocytes were suspended using 200 µl of warm 0.2% trypsin DMEM for 2 minutes and then 100 µl of RPMI containing 5% human ab serum was added to each well. Cells were then utilized for intracellular staining as indicated above. For THC treatment, CD8$^+$ T cells were either treated with THC at the time of CD3/CD28/IFNα/IL-2 or 30 min before stimulation with PMA/IO.

D. Direct THC-mediated suppression of cytokine-mediated astrocyte activation. U251 cells were seeded at 7x10^4 cells/well of a 24 well polystyrene tissue culture plate from Gibco 24 hr before the start of the assay. This allowed U251 cells to reach 1x10^5 cells/ well without potentially disrupting their response by stressing the cells via over-crowding (contact inhibition) or trypsinization. Cells were treated with different concentrations of THC (1, 5, or 10 µM) for 30 minutes and then treated with TNFα (1 ng/ml), IFNγ (10 U/ml) or both. After 18 hr of incubation, U251 astrocytes were treated with 1x brefeldin/monensin transport blocker and incubated for 6 hr then intracellularly stained for either IL-6, Interferon γ inducible protein-10 (IP-10), and monocyte chemotactic protein 1 (MCP-1) using the intracellular staining protocol indicated above.

RESULTS

I. SA1: THC-mediated suppression of pDC activation in healthy and HIV+ donors

A. pDC have variable responses to different TLR agonists

pDC secrete large amounts of IFNα following stimulation through the endosomal TLRs (TLR3,7,8, and 9) [164]. Upon stimulation, human pDC will internalize CD303 and CD304, their hallmark surface markers, which is a regulatory mechanism and reduces the secretion of IFNα [367]. However, the optimal concentration for inducing IFNα secretion before losing the expression of CD303 is unknown. To determine these concentrations, PBMC were stimulated with: Polyinosinic-polycytidylic acid (poly-IC), a TLR3 agonist; Imiquimod (MedChem® Express, Monmouth Junction, NJ), a TLR7 agonist; Motolimod (MedChem®) a TLR8 agonist; and CpG-ODN type A 2216 (InvivoGen®), a powerful TLR9 agonist for 6 hr in PBMC or for 24 hr in a highly purified pDC preparation. Collectively, it was found that pDC respond to endosomal TLR activation by secretion of IFNα, after 6 hr of stimulation and did not lose CD303 expression as evidenced by consistent percentage of CD303$^+$ cells in the PBMC preparation. Furthermore, CpG-ODN-A-2216 induced the most significant IFNα response when comparing between the tested TLR agonists (Figure.11A and 11B).

IFNα secretion is the hallmark of pDC activation, but pDC can also express co-stimulatory proteins, like CD83 [368]. These studies revealed that while CpG (TLR9) induced CD83 expression, Motolimod (TLR8) induced the highest overall expression after 6 hr (Figure 12). However, treatment with both with H_2O and DMSO also induced a significant induction of CD83 compared to the unstimulated group. Despite the effects of

H_2O and DMSO, treatment with CpG and Motolimod induced higher expression of CD83 compared to their respective vehicle controls.

Following stimulation of endosomal TLRs (*e.g.* TLR3,7,8 & 9) by synthetic or pathogen-derived agonists, pDC will adopt a mature phenotype. The mature pDC phenotype includes the internalization of CD303 and the formation of dendritic spines [164]. Therefore, identifying pDC from PBMC can be a challenge when they adopt the mature phenotype. Furthermore, CD86 (B7.1), a canonical co-stimulatory molecule expressed on APC and which is critical for proper T cell activation [219], can be expressed by pDC 24 hr post stimulation. To identify pDC after prolonged, 24 hr stimulation by the aforementioned TLR agonists, pDC were isolated using MACS and stimulated for 24 hr with the highest concentration of each TLR agonist.

One of the most interesting observations from this set of studies was the presence of two distinct pDC populations, based upon size and granularity. Specifically, the larger and less granular pDC (population 1) secreted IFNα in response to stimulation via TLR7 and TLR9 (Figure 13A-B). Population 1 also expressed CD83 and CD86 in response to TLR 7, 8, and 9 (Figure 13C), but was less responsive to P.IC-mediated stimulation via TLR3 (Figure 13A-C). By contrast, population 2, the smaller and more granular pDC, showed strong poly-IC (TLR3)-induced IFNα secretion (Figure 13D). Furthermore, population 2 displayed mixed CD83 expression with all of the used TLR agonists (P.IC., IMI, MOT, and CpG) with especially strong induction of CD86 expression by IMI-mediated stimulation via TLR7 (Figure 13 D-F).

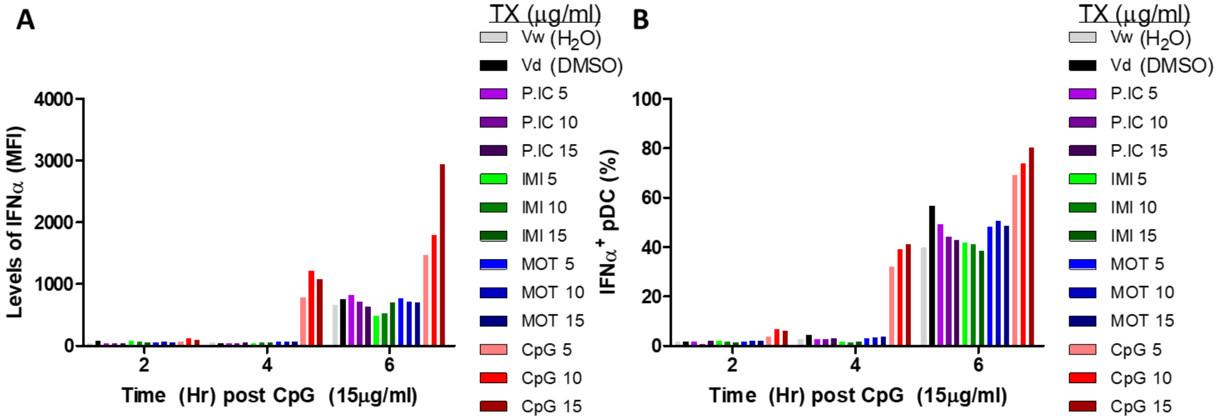

Figure 11. Activation of endosomal TLRs induce secretion of IFNα by pDC. PBMC were isolated via Ficoll-density gradient centrifugation and then treated with varying concentrations of either: Polyinosinic-polycytidylic acid (poly-IC), a TLR3 agonist; Imiquimod (MedChem® Express, Monmouth Junction, NJ), a TLR7 agonist; Motolimod (MedChem®) a TLR8 agonist; and CpG-ODN type A 2216 (InvivoGen®), a TLR9 agonist for 6 hr and then harvested for IFNα secretion (N=1). IFNα was determined using IFNα capture assay and measured via flow cytometric analysis. Controls were also included in the treatment group, specifically the water (H_2O) control served as the vehicle control for poly-IC (P.IC), imiquimod (IMI), and CpG-ODN 2216 (CpG), while 0.01% DMSO served as the control for Motolimod (MOT). A) Levels of IFNα following stimulation. B) %IFNα$^+$ pDC. No statistical analysis was possible for this experiment as these were preliminary experiments on a single donor (N=1).

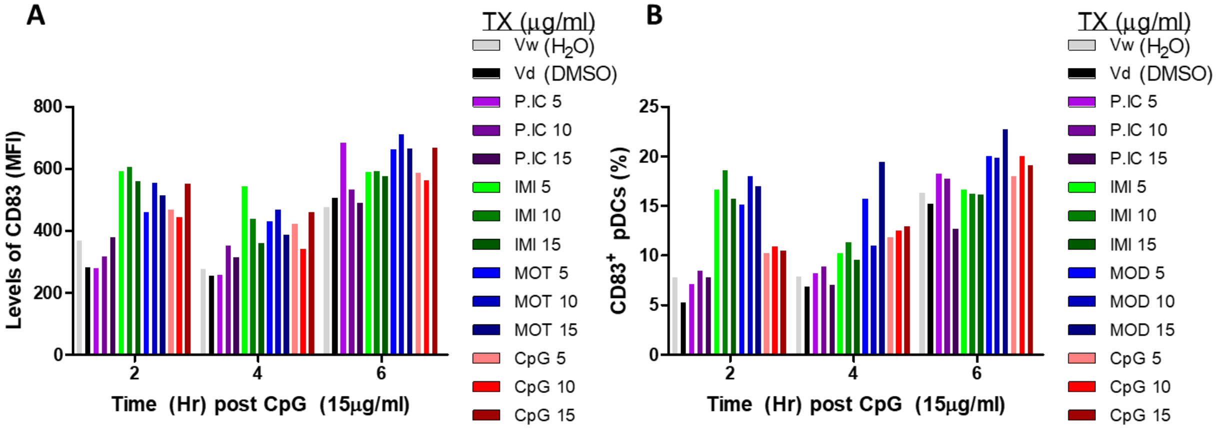

Figure 12. Activation of endosomal TLRs induce CD83 expression by pDC. PBMC were isolated via Ficoll-density gradient centrifugation and then treated with varying concentrations of either: Polyinosinic-polycytidylic acid (poly-IC), a TLR3 agonist; Imiquimod (MedChem® Express, Monmouth Junction, NJ), a TLR7 agonist; Motolimod (MedChem®) a TLR8 agonist; and CpG-ODN type A 2216 (InvivoGen®), a TLR9 agonist, for 6 hr (N=1). CD83 expression was measured via flow cytometric analysis. Controls were also included in the treatment group, specifically the water (H_2O) control served as the vehicle control for poly-IC (P.IC), imiquimod (IMI), and CpG-ODN 2216 (CpG), while 0.01% DMSO served as the control for Motolimod (MOT). A) Levels of CD83 following stimulation by TLR agonists. B) %$CD83^+$ pDC. No statistical analysis was possible for this experiment as these were preliminary experiments on a single donor (N=1).

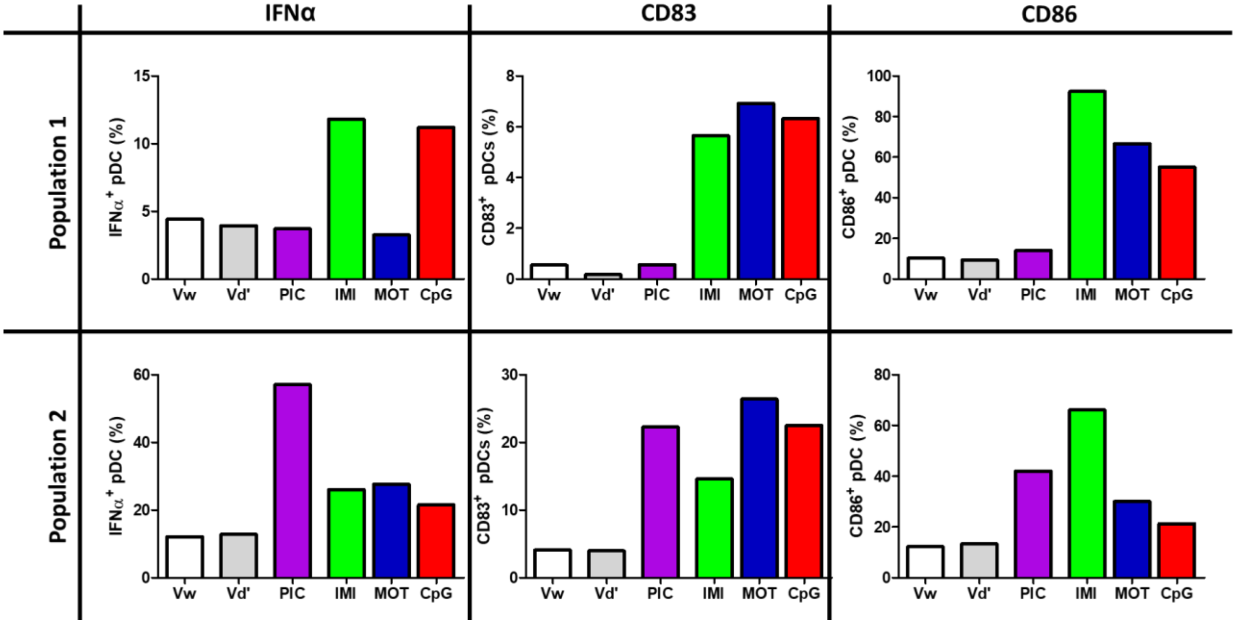

Figure 13. Stimulation of isolated pDC by endosomal TLR ligands induces differential IFNα, CD83, and CD86 expression. PBMC were isolated via Ficoll-density gradient centrifugation and then pDC were purified using MACs. Purified pDC were treated with varying concentrations of either: Polyinosinic-polycytidylic acid (poly-IC) at 5 µg/ml, Imiquimod (MedChem® Express, Monmouth Junction, NJ) at 10 µg/ml; Motolimod (MedChem®) at 10 µg/ml; and CpG-ODN type A 2216 (InvivoGen™) at 15 µg/ml (N=1). IFNα was determined using the IFNα capture assay and CD83/86 expression was measured via flow cytometric analysis. Controls were also included in the treatment group, specifically the water (H_2O) control served as the vehicle control for poly-IC (P.IC), imiquimod (IMI), and CpG-ODN 2216 (CpG), while 0.01% DMSO served as the control for Motolimod (MOT). A, B, C) % IFNα$^+$, CD83$^+$, and CD86$^+$ in population 1, respectively, D, E, F) % IFNα$^+$, CD83$^+$, and CD86$^+$ in population 2, respectively. No statistical analysis was possible for this experiment as these were preliminary experiments on a single donor (N=1).

B. The profile of *CNR1* and *CNR2* expression in pDC and PBMC from HIV+ donors versus healthy donors

The profile of cannabinoid receptor (CNR1 and CNR2) expression has not previously been characterized in human pDC and was therefore investigated using purified pDC and compared to PBMC from healthy donors (Figure 14A). Purified pDC were found to exhibit a very similar profile of CNR1 and CNR2 expression compared to other PBMC such that CNR2 mRNA levels were more highly expressed than CNR1 (Figure 14B). These studies were extended to also quantify CNR1 and CNR2 levels in HIV+ donors. PBMC from HIV+ donors showed significantly augmented CB1 mRNA levels compared to healthy donors (Figure 14C and 14D). By contrast, CB2 mRNA levels were similar in PBMC from healthy versus HIV+ donors (Figures 14C and 14D). These data suggest that the pDC from HIV+ donors may have elevated expression of CNR1 mRNA and protein. However, A sufficient amount of blood could not be collected from HIV+ donors to quantify CNR1 and CNR2 mRNA expression levels in purified pDC by RT/Qt-PCR.

C. THC inhibits CpG-ODN-induced IFNα secretion by pDC and pDC from HIV+ donors are more sensitive to THC-mediated suppression than pDC from healthy donors

HIV infection reduces both the number of circulating pDC and the ability for the remaining pDC to secrete IFNα [195, 201, 369]. To extend the prior observations, PBMCs from HIV+ patients were treated with CpG-ODN and the number of IFNα secreting pDC were quantified using the IFNα capture assay. THC is known to suppress interferon secretion in infection and inflammatory conditions [61]. Here, the effects of THC on IFNα secretion were determined in CpG-ODN-induced human primary pDC.

pDC were identified as CD303$^+$ CD123$^+$ cells (Figure 15A) and secretion of IFNα was then quantified by flow cytometry (Figure 15B). The induction of IFNα$^+$ pDC following CpG-ODN treatment from HIV+ donors was comparable to pDC from healthy donors (Figure 15C). Treatment of PBMCs with THC decreased the number of IFNα secreting pDC from both healthy and HIV+ donors (Figure 15D-15E). Conversely, the closely related cannabinoid congener cannabidiol (CBD), which possesses low affinity for both CB1 and CB2, produced no effect on the percentage of IFNα secreting cells in response to CpG-ODN activation (Figure 15D and 15E). Neither THC nor CBD exhibited cytotoxic effects on pDC at any of the concentrations used in these determinations.

HIV infection, and associated disease states, can cause prolonged stimulation of host immune cells and a chronic inflammatory state which can alter immune cell function. To determine possible differences in THC sensitivity of pDC between HIV+ and healthy donors, PBMCs from HIV+ donors were treated with THC and activated with CpG-ODN, as previously described. Treatment with THC significantly suppressed the number of IFNα secreting pDC from HIV+ donors (Figure 15E), and the degree of suppression was greater than the suppression in pDC from healthy donors (Figure 15F), indicating more pronounced sensitivity to cannabinoid-mediated suppression in pDC from HIV+ donors.

D. THC directly suppresses secretion of IFNα by pDC from healthy donors

Given that pDC are a minor population within the PBMC (Figure 15A), studies were conducted to determine whether THC acts directly on pDC to suppress IFNα production or indirectly through bystander cell effects. The aforementioned studies were repeated using highly purified pDC (Figure 16A) which showed that treatment with THC decreased the percent of IFNα secreting pDC in a manner comparable to that observed in the PBMC preparation (Figure 16B) indicating THC acts directly on pDC.

To determine if THC also suppressed the quantity of total secreted IFNα, LegendPlex™ cytometric bead array was used to quantify the amount of IFNα in the cell-culture supernatants from purified healthy pDC preparations. THC treatment significantly suppressed the amount of IFNα secreted by the highly purified pDC (Figure 16C).

E. THC directly reduces IFNα mRNA levels by impairment of interferon regulatory factor 7 (IRF-7) phosphorylation

To determine if the suppression of IFNα by THC was tied to decreased IFNα mRNA levels, PrimeFlow™, a flow cytometry-based method that allows quantification of gene specific mRNA levels on a per-cell basis, was employed (Figure 17A). THC suppressed the transcription of IFNA2, a member of the IFNα gene cassette, in healthy pDC in a manner that paralleled the decrease of secreted IFNα (Figure 17B).

Honda and coworkers demonstrated that phosphorylation of interferon regulatory factor 7 (IRF-7) is a master regulatory event of type I interferon responses [175]. In the present study, THC treatment suppressed the phosphorylation of IRF-7 in pDC from healthy and HIV+ donors in a concentration-dependent manner. Treatment with CBD had no effect on healthy pDC but did suppress pIRF7 in pDC from HIV+ donors at the highest concentration (Figure 17D and 17E). This provides further evidence that the pDC are sensitive to cannabinoid-mediated suppression likely though the elevated expression of the cannabinoid receptors. However, the possible influence of orphan receptors cannot be ruled out.

IFNα mRNA expression is dependent on nuclear translocation of pIRF-7, which is controlled, at least in part, through osteopontin (OPN) [370]. Specifically, osteopontin colocalizes with MyD88 following TLR9 activation and potentiates the activation, via phosphorylation, of IRF7 and subsequent nuclear translocation but does not translocate into the nucleus. Osteopontin also potentiates cross-presentation of antigen in pDC, thereby facilitating T cell activation. Furthermore, OPN knockout mice could not properly respond to Herpes simplex virus 1 (HSV1) by secreting type 1 interferon [370]. Treatment with both THC and CBD treatment had no significant effect on OPN levels in pDC from healthy donors (Figure 17C).

F. THC suppresses TLR-9-mediated induction of co-stimulatory molecule CD83 on pDC from healthy and HIV+ donors.

CD83 is a surface protein on myeloid lineage cells, including pDC, which serves as a co-stimulatory molecule to drive activation of other immune cells, including T cells [368, 371-374]. These studies revealed that CD83 is expressed early upon pDC activation by CpG-ODN (within 6 hr) and that THC suppressed the number of pDC expressing surface CD83 in both healthy and HIV+ donors (Figure 18A and B). Treatment with CBD did not alter CD83 expression by pDC from healthy donors (Figure 18A) but did suppress CD83 expression on pDC from HIV+ donors (Figure 18B).

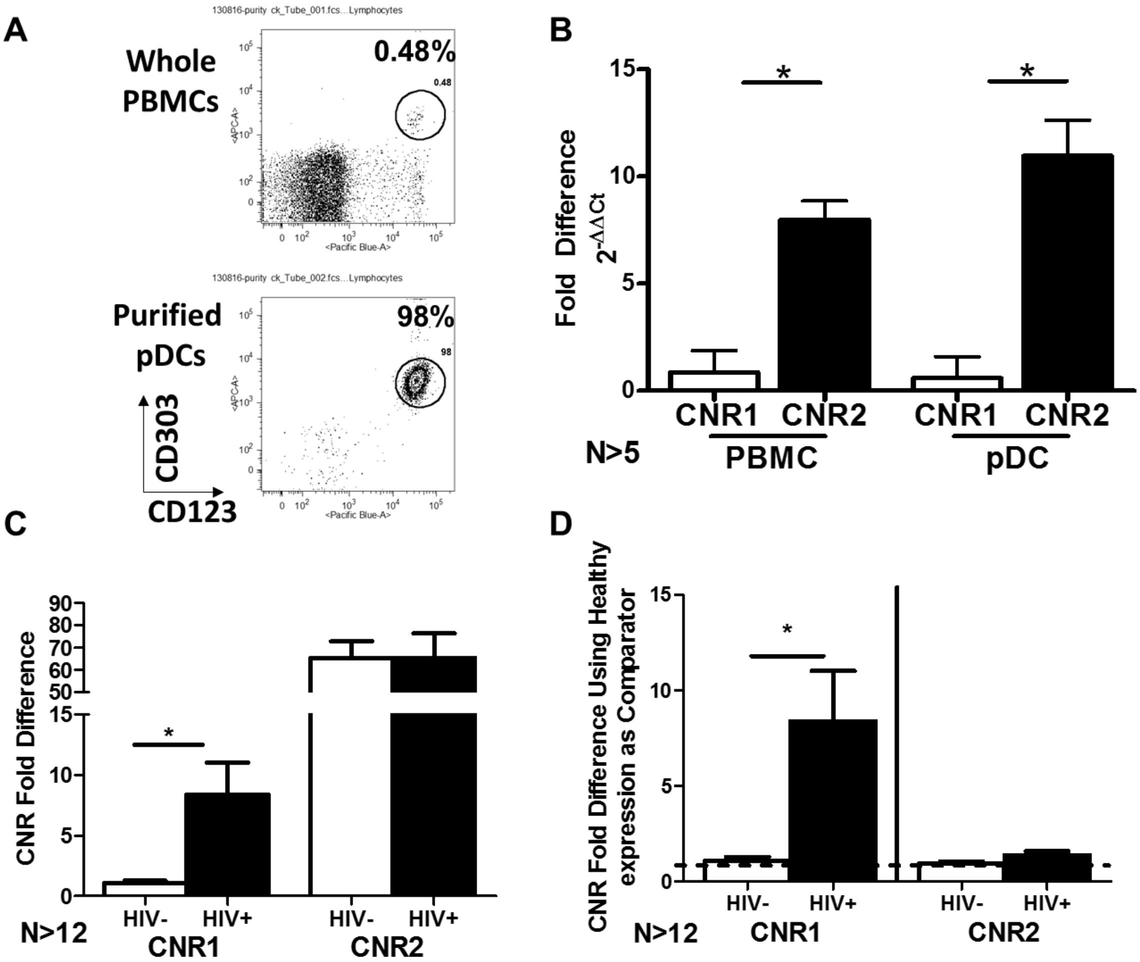

Figure 14. pDC exhibit the same expression pattern of cannabinoid receptors 1 and 2 as other PBMCs and the expression of CNR1, but not CNR2, is elevated in PBMC from HIV+ donors. CNR1 (N=5) and CNR2 (N=6) gene expression was determined by qPCR from human PBMCs and highly purified (>95%) pDC. A) Purification of pDC using MACS isolation by Miltenyi Biotec. B) Fold expression of CNR1 and CNR2 in whole PBMCs and pDC with CNR1 held as comparator. There was no statistically significant difference in CNR2 or CNR1 expression between isolated pDC and whole PBMC. C) Expression profiles of CNR1 and CNR2 in healthy (N=12) and HIV+ (N=15) PBMCs using CNR1 in healthy donors as comparator. D) Expression differences of CNR1 and CNR2 between healthy and HIV+ PBMC using expression of CNR1 and CNR2 in heathy donors as the respective gene comparator. Asterisks indicate statistically significant differences between healthy and HIV+ groups using Student's T test (*$p < 0.05$). (Image Source: Henriquez et al, 2017 [97])

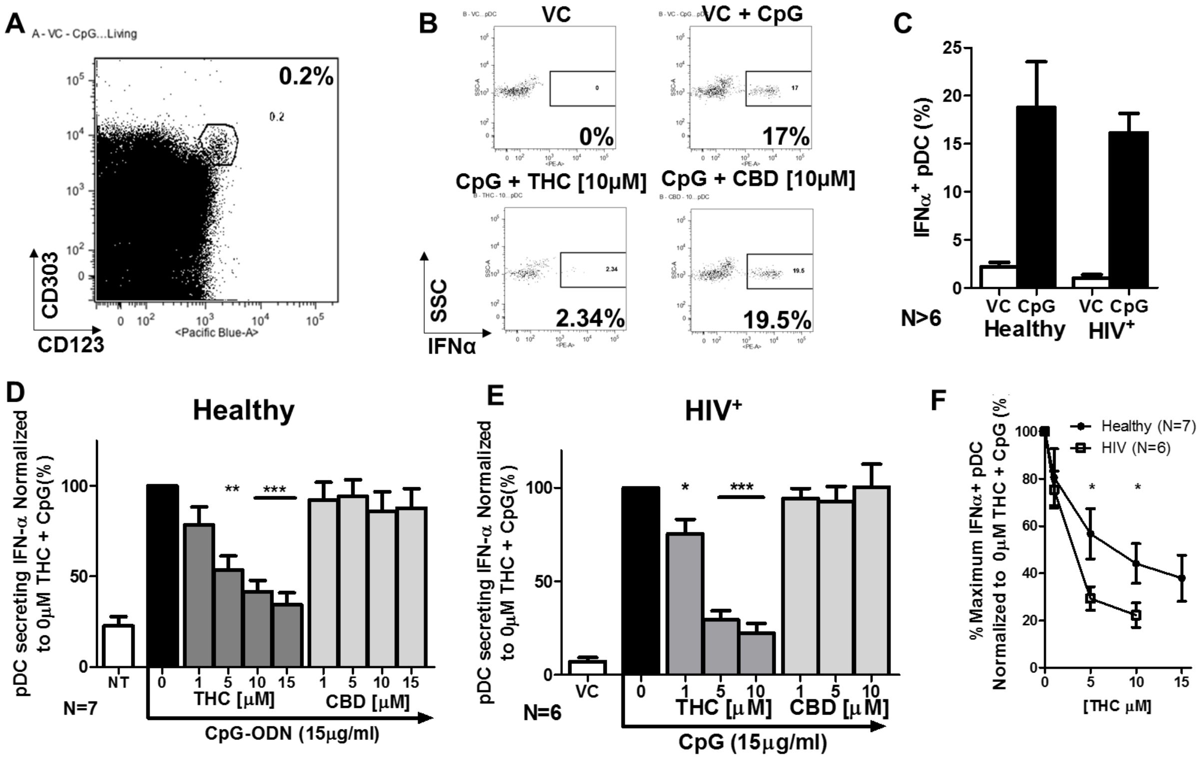

Figure 15. THC, but not CBD, suppresses IFNα secretion by pDC from healthy and HIV+ donors and pDC from HIV+ donors are more sensitive to THC-mediated suppression than pDC from healthy donors. Isolated human PBMCs were treated with either Vehicle control (VC; 0.026% Ethanol) or cannabinoid (THC or CBD) at 1, 5, 10, or 15 µM for 30 min, stimulated with CpG-ODN at 15µg/ml for 5 hr, and utilized for the IFNα capture assay by Miltenyi Biotec. A) pDC population identified as CD303$^+$/123$^+$ cells. B) Example of IFNα$^+$ pDC with 10µM of THC and CBD. C) General profile of CpG-ODN induced IFNα in healthy (N=7) and HIV+ (N=6) donors. There was no statistical difference in the number of IFNα$^+$ pDC in background (VC) or stimulated (CpG) when comparing between healthy and HIV+ donors. D) IFNα$^+$ pDC in healthy donors normalized to 0µM THC + CpG group. E) IFNα$^+$ pDC in HIV+ donors normalized to 0µM THC + CpG group. Asterisks indicate statistically significant differences in the number of IFNα$^+$ pDC compared to 0 THC with CpG group (1-way ANOVA with Dunnett's Posttest). F) Inhibition curves comparing percent of IFNα$^+$ pDC in healthy and HIV+ donors. Asterisks induce statistically significant using 2-Way ANOVA with Bonferroni's multiple comparison's posttest (*p < 0.05; **p < 0.01; ***p < 0.001). (Image source: Henriquez et al, 2017 [97])

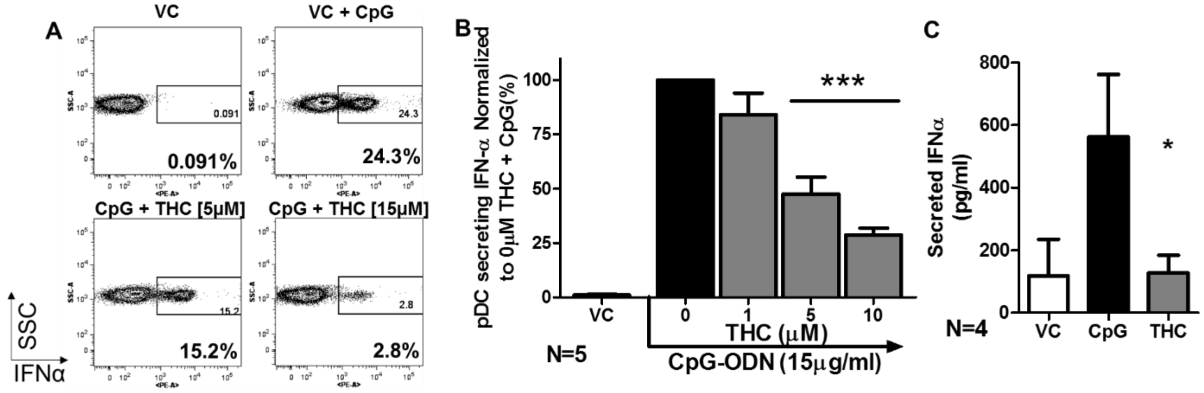

Figure 16. THC directly suppresses IFNα secretion in highly purified pDC. pDC were isolated from PBMC via MACS (Mitenyi Biotec©). Highly purified pDC (>95% purity) were then treated with 1, 5, 10, or 15μM THC for 30 min followed by stimulation with CpG-ODN for 5 hr. A) FACS scatter plot of CpG-ODN induced IFNα and concentration dependent suppression by THC. B) IFNα$^+$ pDC normalized to 0μM THC + CpG (N=5). Asterisks indicate significant differences compared to 0 μM THC + CpG (1-Way ANOVA with Dunnett's Posttest). C) Amount of Secreted IFNα as determined by LegendPlex™ secretion kit by BioLegend utilizing 1x10^5 isolated pDC (N=4) per treatment, treated with VC (0.026% EtOH), VC + CpG, or CpG+THC (15μM). Asterisks indicate statistically significant differences of treatment compared to 0 THC + CpG using 1-way ANOVA with Dunnett's posttest (*p < 0.05; **p < 0.01; ***p < 0.001). (Image source: Henriquez et al, 2017 [97])

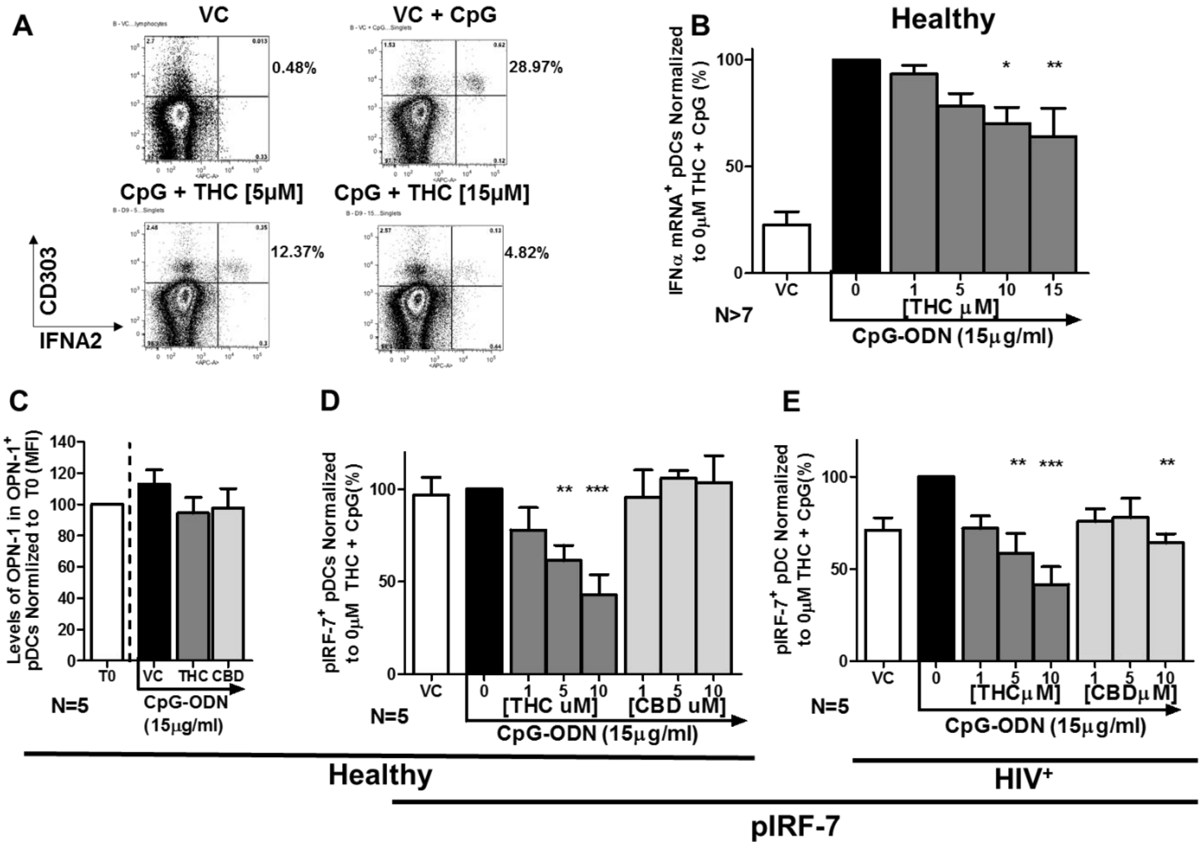

Figure 17. IFNA2 expression and phosphorylation of IRF-7 (pIRF-7) are suppressed by THC in pDC from both healthy and HIV+ donors. PBMCs were treated with THC at 1, 5, 10, 15 µM for 30 min and then stimulated with CpG-ODN for 5 hr. IFNA2 gene expression was determined using PrimeFlow RNA assay by Affymetrix. pDC were identified as CD303$^+$/123$^+$ cells. A) FACS scatter plot pDC undergoing CpG-ODN induced upregulation of IFNA2 expression in pDC and concentration dependent suppression by THC. B) pDC IFNA2 gene expression normalized to VC + CpG-ODN across multiple donors (VC & 0 µM: N=9; 1 & 5µM: N=8; 10 & 15µM: N=7). Asterisks indicate statistically significant differences (P<0.05) in IFNA2 expressing pDC compared to 0 THC with CpG group (1-Way ANOVA with Dunnett's posttest). Levels of Osteopontin (OPN) and pIRF-7$^+$ pDC were determined by flow cytometric analysis. pDC were identified as CD303$^+$/123$^+$ cells. C) Osteopontin (OPN) levels in pDC treated with THC and CBD at 10µM (N=5). D) Percent pIRF-7$^+$ pDC in from healthy donors (N=5). E) Percent pIRF-7$^+$ pDC from HIV+ donors (N=5). Asterisks indicate statistically significant differences in pIRF-7 expressing pDC compared to the 0 THC + CpG group using 1-Way ANOVA with Dunnett's posttest (*p < 0.05; **p < 0.01; ***p < 0.001). (Image source: Henriquez et al, 2017 [97])

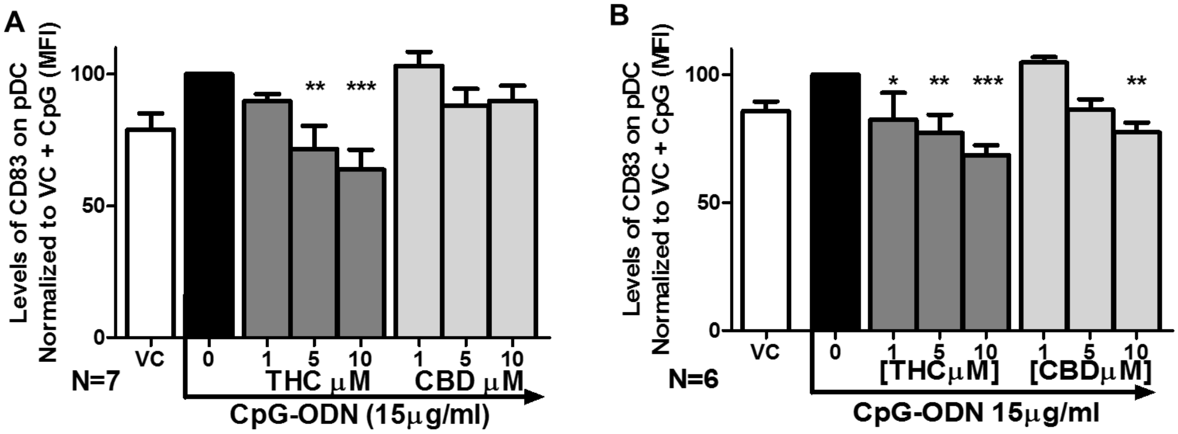

Figure 18. THC suppresses surface expression of CD83 in pDC from both healthy and HIV+ donors. Healthy and HIV+ PBMCs were treated with THC at 1, 5, 10, 15 µM for 30 min and then stimulated with CpG-ODN for 5 hr. pDC were identified as CD303$^+$/123$^+$ cells and CD83$^+$ pDC were determined by flow cytometric analysis. A) THC concentration dependent suppression of CD83 surface expression in pDC from healthy donors. B) THC concentration dependent suppression of CD83 surface expression in pDC from HIV+ donors. Asterisks indicate statistically significant differences in CD83 surface expression compared to 0 THC + CpG using 1-Way ANOVA with Dunnett's posttest (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). (Image source: Henriquez et al, 2017 [97])

G. THC inhibits resiquimod (R848)-mediated stimulation of pDC, a mimic for HIV-mediated activation

pDC can be stimulated by synthetic endosomal TLR agonists to secrete IFNα and express CD83. However, the previously used TLR-specific agonists (P.IC, IMI, MOT, and CpG) selectively stimulate single TLRs. While useful for determining the role of individual TLR in cell activation, isolated-TLR activation is not always physiologically relevant. Specifically, HIV encodes multiple sequences which are both TLR7 and TLR8 ligands [375]. Therefore, stimulation of pDC by HIV is not likely mediated through either TLR7 or TLR8 in isolation, but rather stimulated simultaneously through both TLR7 and TLR8. These studies were undertaken to determine the consequence of THC treatment during the initial stimulation of pDC during acute HIV infection and recurrent stimulation of pDC during chronic infection by HIV.

To address the consequences of THC utilization on pDC activation by co-stimulation through TLR7 and TLR8 ligation, resiquimod (R848), a TLR7/8 agonist, was used to mimic HIV-mediated stimulation of pDC. In these studies, R848 induced a more robust expression of CD83 in 6 hr post activation of pDC than did CpG (Figure 19A) while inducing a similar level of IFNα secretion (Figure 19B). Furthermore, treatment of pDC with THC, suppressed both the expression of CD83 (Figure 19A) and the secretion of IFNα (Figure 19B) while treatment with the CBD had no significant effect on either endpoint (Figure 19A and B).

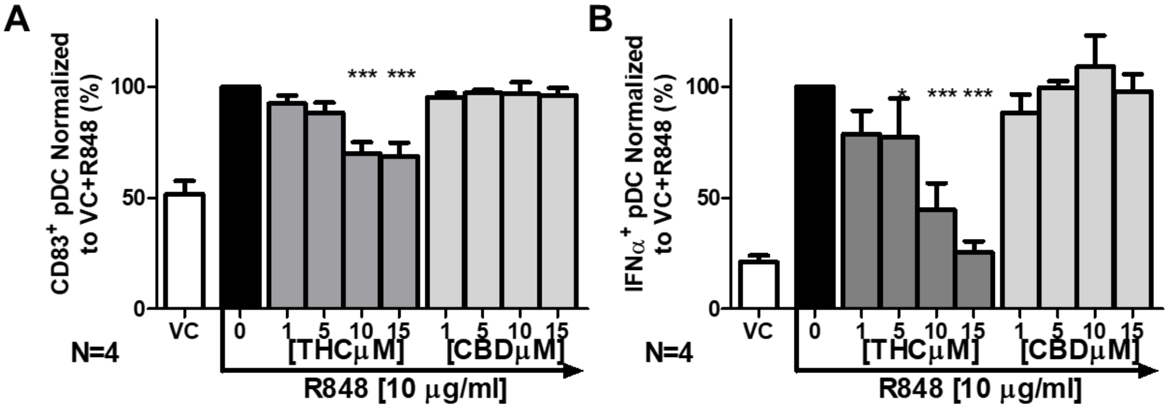

Figure 19.THC suppresses surface expression of CD83 and secretion of IFNα in pDC stimulated with resiquimod. Healthy PBMCs were treated with THC or CBD at 1, 5, 10, 15 µM for 30 min and then stimulated with 10µg/ml of resiquimod (R848) for 5 hr. pDC were identified as CD303$^+$/123$^+$ cells. CD83 expression and IFNα secretion by pDC were determined by flow cytometric analysis. A) THC concentration dependent suppression of CD83 surface expression in pDC from healthy donors. B) THC concentration dependent suppression of IFNα secretion in pDC from healthy donors. Asterisks indicate statistically significant differences in expression compared to 0 THC + CpG using 1-Way ANOVA with Dunnett's posttest (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

II. SA2: THC and CB2-selective agonists suppress TNFα and IFNα response in pDC from healthy donors

A. CpG mediated stimulation of pDC induces TNFα secretion which is suppressed by THC

As demonstrated previously, pDC can secrete detectable levels of IFNα by 6 hr post activation with CpG-ODN (Figure 16C). However, they can also secrete TNFα when stimulated with CpG, but not by the TLR7 agonist, imiquimod (IMI), within the same 6 hr timeframe (Figure 20A). Furthermore, CpG-induced secretion of TNFα can be suppressed by direct action of THC on highly purified pDC while CBD has no effect on TNFα secretion (Figure 20A and B).

B. CpG-mediated stimulation of pDC induced heterogeneous production of IFNα and TNFα

Previous experiments revealed the heterogeneity of the pDC population following activation with various TLR agonists (e.g. P.IC, IMI, MOT, and CpG). This observation agrees with previous studies which have found specialized functions in pDC and dendritic cells [376]. Therefore, to determine if TNFα-secreting pDC differed from IFNα-secreting pDC, pDC were stimulated with CpG for 6 hr and stained for both IFNα and TNFα. As previously observed, ~30% of pDC have detectable cytokine induction (IFNα or TNFα) following stimulation by CpG. Furthermore, of the cells which were secreting either cytokine, ~80% were IFNα$^+$ while ~ 20% were TNFα$^+$. In addition, those cells that were IFNα$^+$, ~60% were also TNFα$^+$ (Figure 21). Lastly, <16% of responding pDC were TNFα$^+$ only but they did express appreciable levels of TNFα (Figure 21).

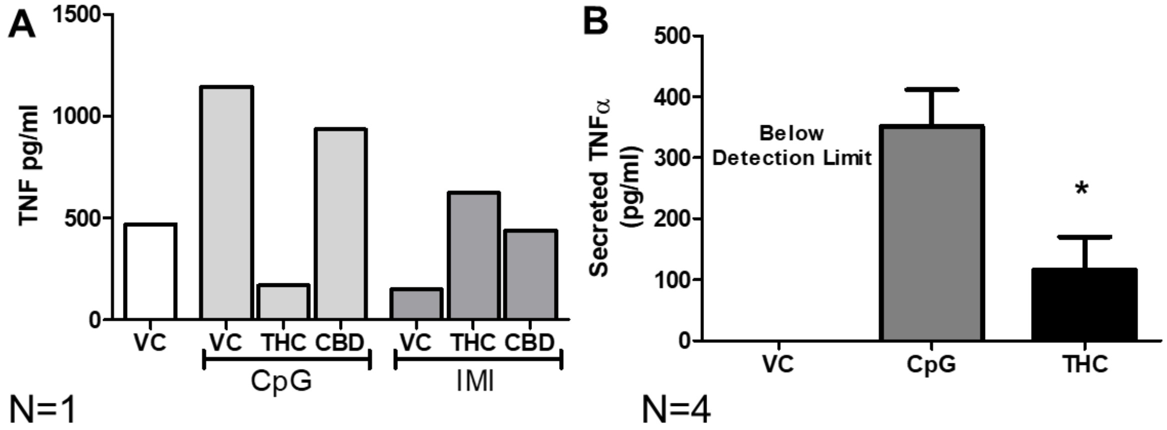

Figure 20. THC directly suppresses TNFα secretion in highly purified pDC. pDC were isolated from PBMC via MACS (Mitenyi Biotec©). A) Highly purified pDC (>95% purity) were then treated with 15µM THC or CBD for 30 min followed by stimulation with CpG-ODN (15µg/ml) or Imiquimod (IMI) (10µg/ml) for 5 hr. B) Highly purified pDC (>95% purity) were then treated with 15µM THC for 30 min followed by stimulation with CpG-ODN (15µg/ml) for 5 hr. Secreted TNFα was determined using LegendPlex by BioLegend and quantified via flow cytometric analysis. No statistical analysis was possible for figure 20A as this experiment was performed on a single donor (N=1). Asterisks on figure 20B indicate statistically significant differences in secreted TNFα CpG+VC using Students T test (*p < 0.05).

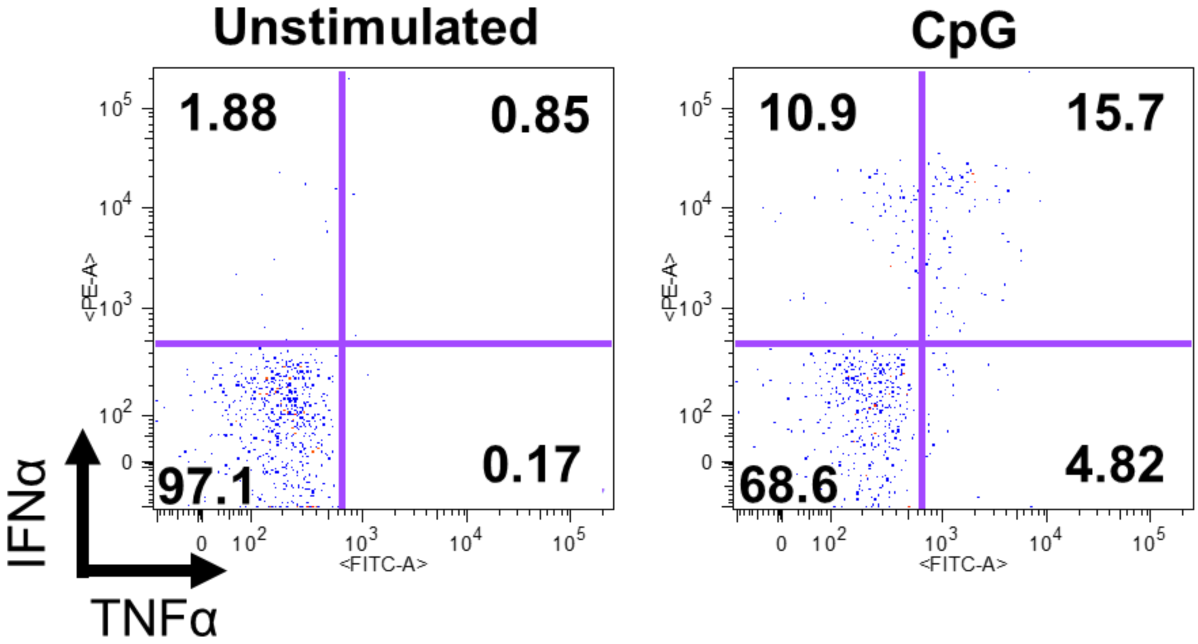

Figure 21. pDC secrete both IFNα and TNFα following stimulation by CpG. Healthy PBMC were either left without stimulation or stimulated with CpG-ODN for 6 hr. pDC were identified as CD303$^+$/123$^+$ cells. IFNα and TNFα production were determined by intracellular staining and visualized by flow cytometry using FlowJo™.

C. Treatment with THC, JWH-015 and JWH-133 suppressed CpG-ODN-induced production of IFNα and TNFα by pDC

The THC-mediated suppression IFNα secretion by pDC has been documented [97]. However, THC has roughly equal binding affinity for both CB1 and CB2. Despite the 10-40-fold higher expression of CB2 to CB1 in leukocytes, the possible influence of CB1 on THC-mediated suppression of pDC activity is unclear. Furthermore, CB1 agonists cause psychotropic effects and are regulated. Therefore, to address the potential for CB2 as a therapeutic target, the CB2 agonists JWH-015 and JWH-133 were used and compared to the known suppressive effects of THC.

As indicated previously, treatment with CpG-ODN induced the expression of IFNα and TNFα in pDC after 6 hr of incubation (Figure 22A). Treatment with THC, JWH-015 and JWH-133 suppressed the secretion of IFNα (Figure 22B). Furthermore, treatment with JWH-015 and JWH-133 caused significant suppression of the IFNα response at lower concentrations than THC (Figure 22B).

THC is known to suppress TNFα secretion in both human and animal models while JWH-133 has been shown to suppress TNFα in animal models of inflammatory disease[143]. In studies conducted here, treatment with THC, JWH-015 and JWH-133 suppressed CpG-induce TNFα response in a concentration dependent manner (Figure 22C). Interestingly, while THC suppressed IFNα and TNFα to a similar degree, the JWH-compounds elicited a greater degree of suppression on the IFNα response than the TNFα response at the same concentrations. (Figure 22B and C). Finally, CBD was used as a control for CB receptor involvement and, as previously described [97], had no effect on CpG-ODN induced IFNα and TNFα responses.

D. Treatment with THC, JWH-015 and JWH-133 suppressed the phosphorylation of IRF7 and TBK1, type I interferon-specific signaling events downstream of TLR-9 ligation by CpG-ODN

Treatment with THC suppressed the CpG-ODN-mediated phosphorylation of IRF7 [97] and IRF7 is the master regulator of IFNα response in pDC [175]. Therefore, to determine if either of the CB2-selective agonists, JWH-015 or JWH-133, reduced the phosphorylation of IRF7, pIRF7 was measured in pDC treated with either JWH-015 and JWH-133 and compared them to treatment with THC and CBD. These studies revealed that both JWH-015 and JWH-133 significantly diminished the phosphorylation of IRF7 (Figure 23A) in a concentration dependent manner (Figure 23B). Furthermore, JWH-015 and JWH-133 suppressed the phosphorylation of IRF7 at much lower concentrations than THC (Figure 23B).

TBK1 is most closely associated with TRAF3-mediated activation via TLR3 but can also be induced through TLR9 and is critical for IFNα response [174]. TBK1 can also phosphorylate both IRF3 and IRF7 and plays a role in IFNα response [178]. Treatment with THC, JWH-015, and JWH-133 significantly reduced the CpG-ODN-induced phosphorylation of TBK1 (Figure 24A). Furthermore, and as seen with IFNα and pIRF7, while THC and the CB2 selective agonists significantly suppressed TBK1 phosphorylation in a concentration dependent manner, the JWH compounds (133 and 015) suppressed TBK1 phosphorylation at lower concentrations than THC (Figure 24B).

E. Treatment with THC, JWH-015 and JWH-133 suppressed the phosphorylation of NFκB and IKKγ, signaling events downstream of TLR-9 ligation by CpG-ODN which can lead to TNFα production

TNFα secretion can be mediated through activation of NFκB and NFκB can be activated through TLR9 [377]. TBK-1 is a member of the NFκB family of signaling molecules and, as indicated above, TBK-1 phosphorylation is suppressed by THC and the CB2-selective agonists. Furthermore, while cannabinoid-modulation of NFκB activity is well documented in many immune cell types, no studies have been conducted to determine cannabinoid-mediated modulation of NFκB activation in pDC. To determine if CpG-ODN-mediated stimulation of pDC induces NFκB activation, the CpG-ODN-mediated phosphorylation of p65, a key event needed for optimum NFκB activation, was measured. These studies revealed that CpG induce p65 phosphorylation (Figure 25A), which was suppressed by THC, JWH-015, and JWH-133 in a concentration-dependent manner (Figure 25B). JWH-015 and JWH-133, significantly reduced p(65)NFκB at lower concentrations than THC (Figure 25B).

While the effects of cannabinoids on the activation of IKKγ, also known as NFκB essential modulator (NEMO), have been postulated [32, 33], few studies have directly shown cannabinoid modulation of IKKγ phosphorylation. Therefore, studies were conducted to determine if THC, JWH-015 or JWH-133 treatment would modulate phosphorylation IKKγ, a key event in the activation of NFκB. Results from these studies revealed that CpG-ODN induced phosphorylation of IKKγ (Figure 26A) was suppressed by THC, JWH-015 and JWH-133 (Figure 26B). As seen with the previous endpoints, while THC and the CB2-selective agonists, JWH-015 and JWH-133, significantly suppressed the phosphorylation of IKKγ in a concentration dependent manner, JWH-015 and JWH-133 suppressed pIKKγ at lower concentrations than THC (Figure 26B).

F. Treatment with THC, JWH-015 and JWH-133 differentially affected AKT phosphorylation at two key sites

Protein kinase B (PKB), also known as AKT, is a serine/threonine kinase which plays a critical role in both anti-apoptotic and activation processes [378]. Cannabinoid-mediated modulation of AKT related signaling has been suggested as a possible target for modulation in autoimmune disorders [49, 50, 209, 379]. In addition, AKT activation is principally controlled by phosphorylation of two residues, S473 and T308, and it is unknown if phosphorylation of these two sites are differentially controlled during TLR9-mediated activation of pDC and treatment with cannabinoids. These studies revealed that the S473 phosphorylation was not induced 6 hr post CpG-mediated activation of pDC (Figure 27). Further, treatment with THC significant reduced S473 phosphorylation while neither of the CB2-selective agonists had any effect (Figure 27). Conversely, phosphorylation of the T308 was induced by treatment with CpG and inhibited with THC and both CB2-selective agonists (Figure 28).

G. Treatment with THC, JWH-015 and JWH-133 suppressed the CpG-mediated induction of IFNA2 and TNFA mRNA expression

Treatment with THC suppressed the CpG-ODN-mediated induction of IFNα mRNA as evidenced by suppressed IFNA2 mRNA expression [97]. This finding is significant because it indicates that the suppression of CpG-mediated induction of gene transcription by THC likely results as a consequence of reduced IFNα gene transcription and not through a process like sequestration. However, while similar in results, the level of TNFα suppression, either transcription or translation, is unknown in this system. Furthermore, while the suppression of phosphoproteins indicates a shared mechanism, it is unknown if the CB2-selective agonists, JWH-015 and JWH-133, modulate gene induction and transcription. Therefore, to determine the effects of TNFα and IFNα mRNA induction by THC, JWH-015, and JWH-133 the induction of IFNA2 and TNFA mRNA was measured using PrimeFlow™ (Figure 29A). Interestingly, while treatment with THC, JWH-015, and JWH-133, trended towards suppression of IFNA2 mRNA induction (Figure 29B), the differences were not significant using the lower sensitivity reporter, Type 4 (FITC). However, treatment with THC and both CB2-selective agonists, JWH-015 and JWH-133, suppressed the induction of TNFA mRNA (Figure 29C), which utilizes the Type 1 (APC), high sensitivity reporter.

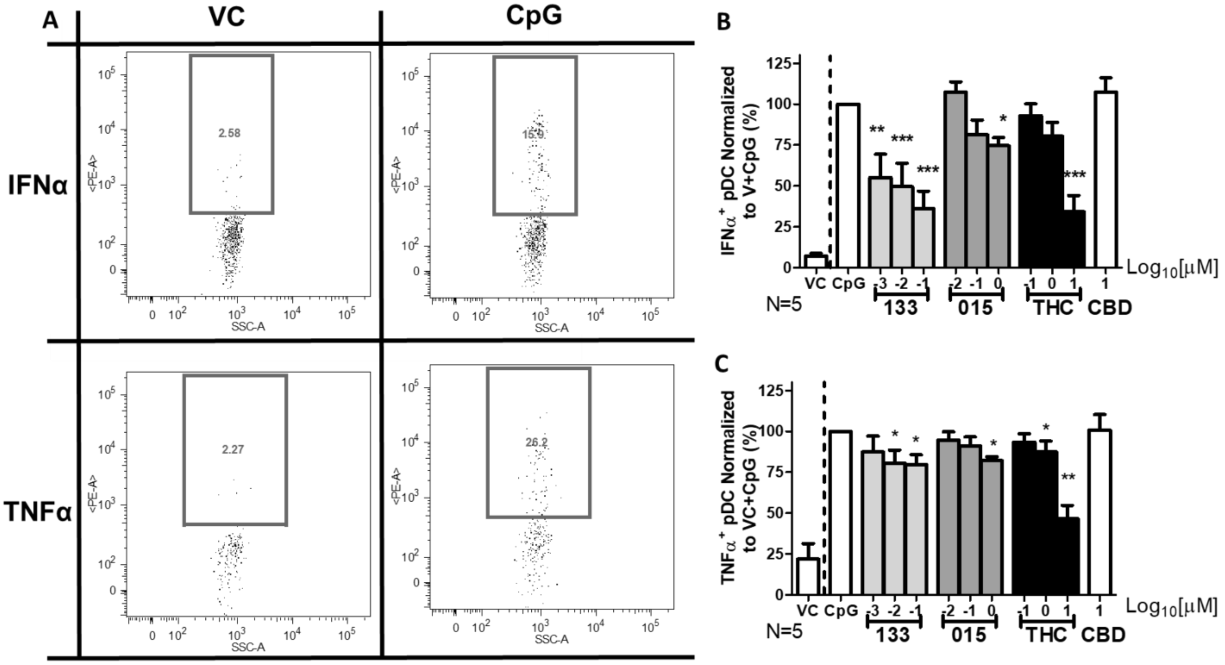

Figure 22. Treatment of pDC with THC or CB2-specific agonists, JWH-015 and JWH-133, suppressed CpG-ODN-induced production of IFNα and TNFα. Isolated human PBMCs were treated with either vehicle control (VC; 0.026% Ethanol), CBD (10 µM), THC (0.1, 1, or 10 µM), JWH-015 (10^{-2}, 10^{-1}, 10^{0} µM) or JWH-133 at (10^{-3}, 10^{-2}, 10^{-1} µM) for 30 min, stimulated with CpG-ODN at 15µg/ml for 6 hr, and intracellularly stained for either IFNα or TNFα. A) Example of gating for IFNα and TNFα with resting pDC, VC, and following CpG stimulation. B) CpG induced intracellular expression of IFNα which was suppressed by THC, JWH-015, and JWH-133 in a concentration dependent manner. C) CpG induced intracellular expression of TNFα which was suppressed by THC, JWH-015, and JWH-133 in a concentration dependent manner. Asterisks indicate statistically significant differences in IFNα or TNFα expression compared to 0 THC + CpG using 1-Way ANOVA with Dunnett's posttest (*p < 0.05; **p < 0.01; ***p < 0.001).

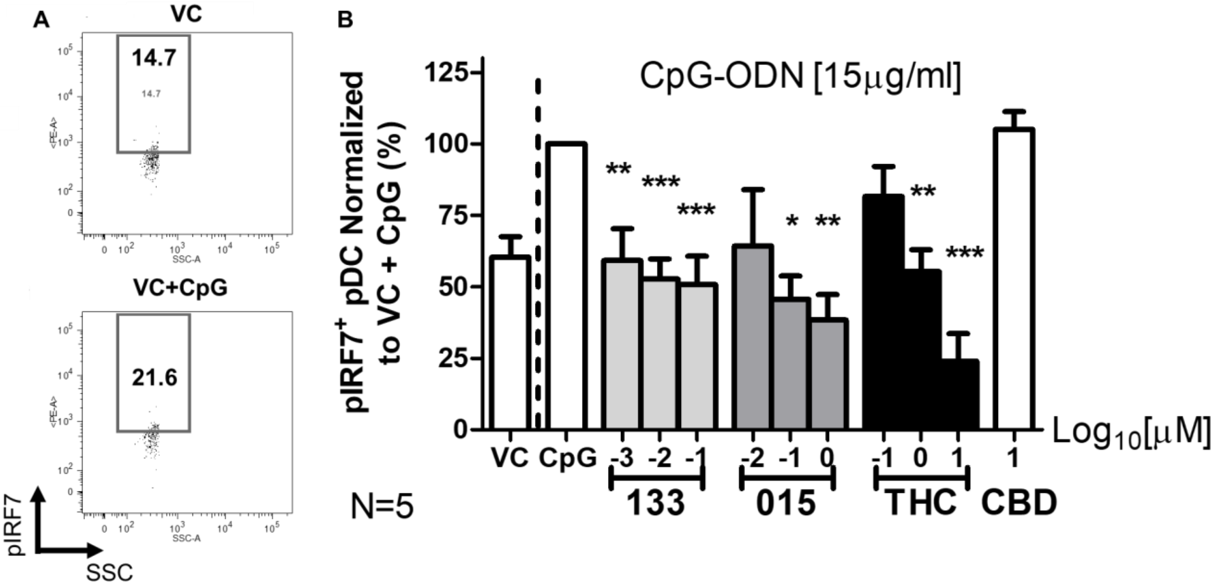

Figure 23. Treatment of pDC with THC or CB2-selective agonists, JWH-015 and JWH-133, suppressed the CpG-ODN-induced phosphorylation of IRF7. Isolated human PBMCs were treated with either vehicle control (VC; 0.026% Ethanol), CBD (10 µM), THC (0.1, 1, or 10 µM), JWH-015 (10^{-2}, 10^{-1}, 10^{0} µM) or JWH-133 at (10^{-3}, 10^{-2}, 10^{-1} µM) for 30 min, stimulated with CpG-ODN at 15µg/ml for 5 hr and intracellularly stained for pIRF7. A) Example of gating for pIRF7 with VC, CpG stimulation B) CpG induced intracellular expression of pIRF7 was suppressed by THC, JWH-015, and JWH-133 in a concentration dependent manner. Asterisks indicate statistically significant differences in pIRF7 expression compared to VC + CpG using 1-Way ANOVA with Dunnett's posttest (*p < 0.05; **p < 0.01; ***p < 0.001).

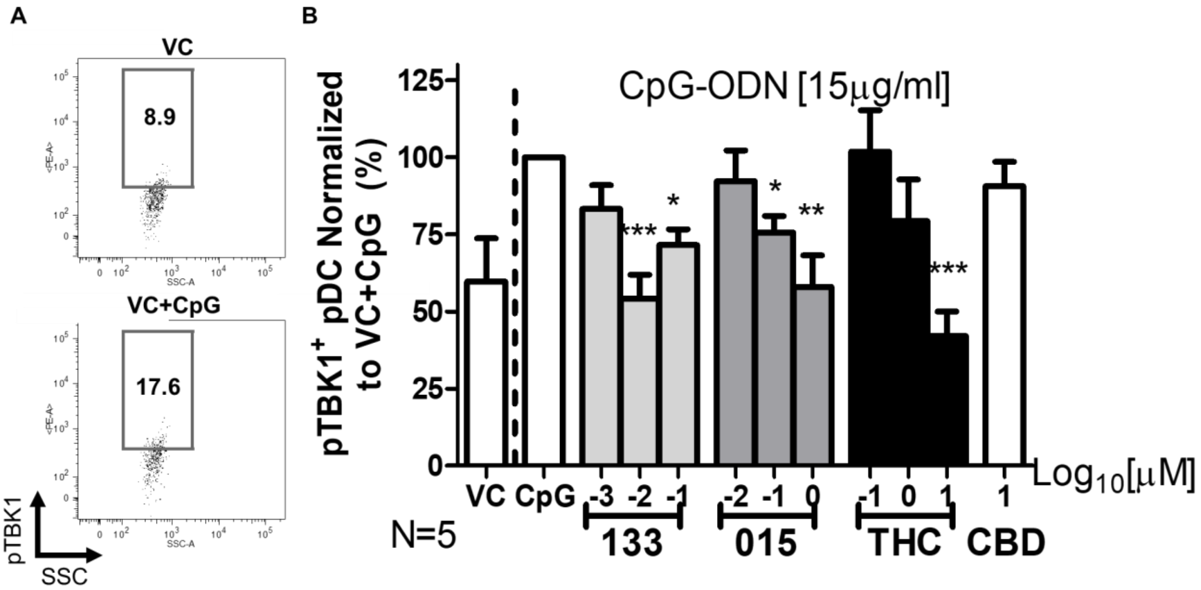

Figure 24. Treatment of pDC with THC or CB2-selective agonists, JWH-015 and JWH-133, suppressed the CpG-ODN-induced phosphorylation of TBK1. Isolated human PBMCs were treated with either vehicle control (VC; 0.026% Ethanol), CBD (10 μM), THC (0.1, 1, or 10 μM), JWH-015 (10^{-2}, 10^{-1}, 10^{0} μM) or JWH-133 at (10^{-3}, 10^{-2}, 10^{-1} μM) for 30 min, stimulated with CpG-ODN at 15μg/ml for 5 hr, and intracellularly stained for pTBK1. A) Example of gating for pTBK1 with VC and CpG stimulation B) CpG induced intracellular expression of pTBK1 was suppressed by THC, JWH-015, and JWH-133 in a mostly concentration dependent manner. Asterisks indicate statistically significant differences in pTBK1 expression compared to VC + CpG using 1-Way ANOVA with Dunnett's posttest (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

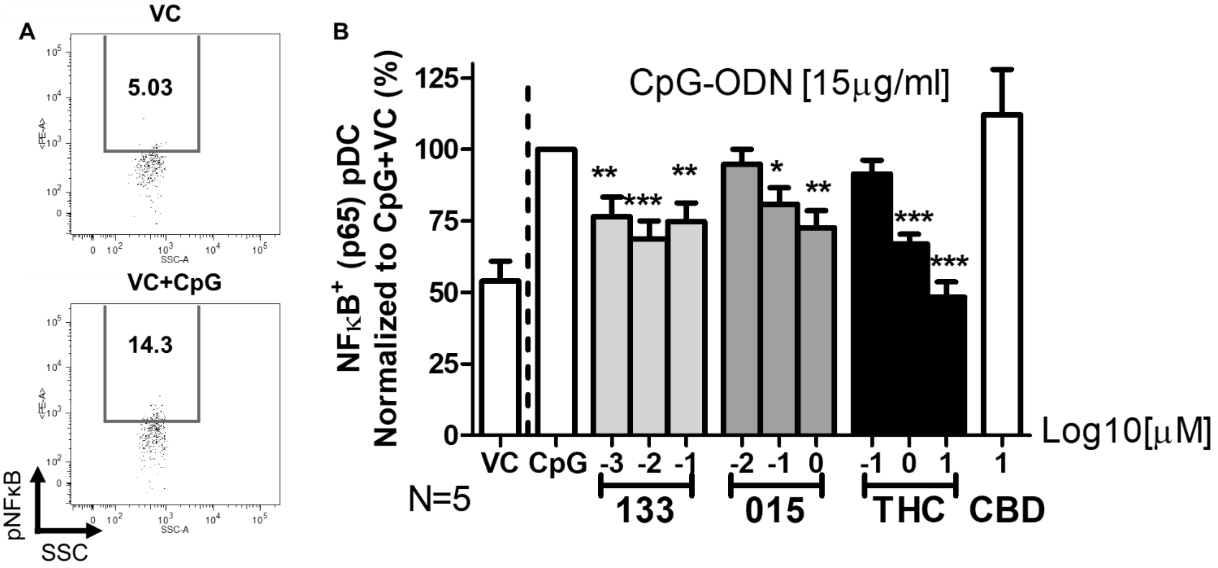

Figure 25. Treatment of pDC with THC or CB2-selective agonists, JWH-015 and JWH-133, suppressed the CpG-ODN-induced phosphorylation of the p65 subunit of NFκB. Isolated human PBMCs were treated with either vehicle control (VC; 0.026% Ethanol), CBD (10 μM), THC (0.1, 1, or 10 μM), JWH-015 (10^{-2}, 10^{-1}, 10^{0} μM) or JWH-133 at (10^{-3}, 10^{-2}, 10^{-1} μM) for 30 min, stimulated with CpG-ODN at 15μg/ml for 5 hr and intracellularly stained for p65 (pNFκB). A) Example of gating for p65 (pNFκB) with VC and CpG stimulation. B) CpG induced intracellular expression of pNFκB was suppressed by THC, JWH-015, and JWH-133 in a concentration dependent manner. Asterisks indicate statistically significant differences in p65 phosphorylation compared to VC + CpG using 1-Way ANOVA with Dunnett's posttest (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

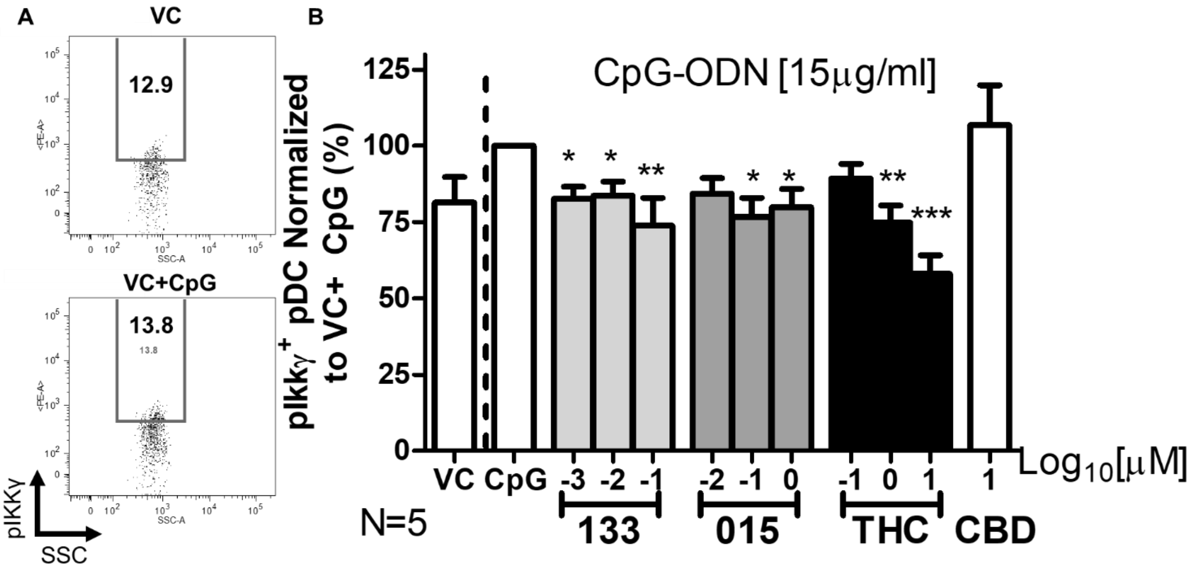

Figure 26. Treatment of pDC with THC or CB2-selective agonists, JWH-015 and JWH-133, suppressed the CpG-ODN-induced phosphorylation of IKKγ. Isolated human PBMCs were treated with either vehicle control (VC; 0.026% Ethanol), CBD (10 µM), THC (0.1, 1, or 10 µM), JWH-015 (10^{-2}, 10^{-1}, 10^{0} µM) or JWH-133 at (10^{-3}, 10^{-2}, 10^{-1} µM) for 30 min, stimulated with CpG-ODN at 15µg/ml for 5 hr, and intracellularly stained for pIKKγ. A) Example of gating for pIKKγ with VC and CpG stimulation B) CpG induced intracellular expression of IKKγ was suppressed by THC and both CB2-selective agonists in a concentration dependent manner. Asterisks indicate statistically significant differences in IKKγ expression compared to VC + CpG (1-Way ANOVA with Dunnett's posttest). Asterisks indicate statistically significant differences in p65 phosphorylation compared to VC + CpG using 1-Way ANOVA with Dunnett's posttest (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

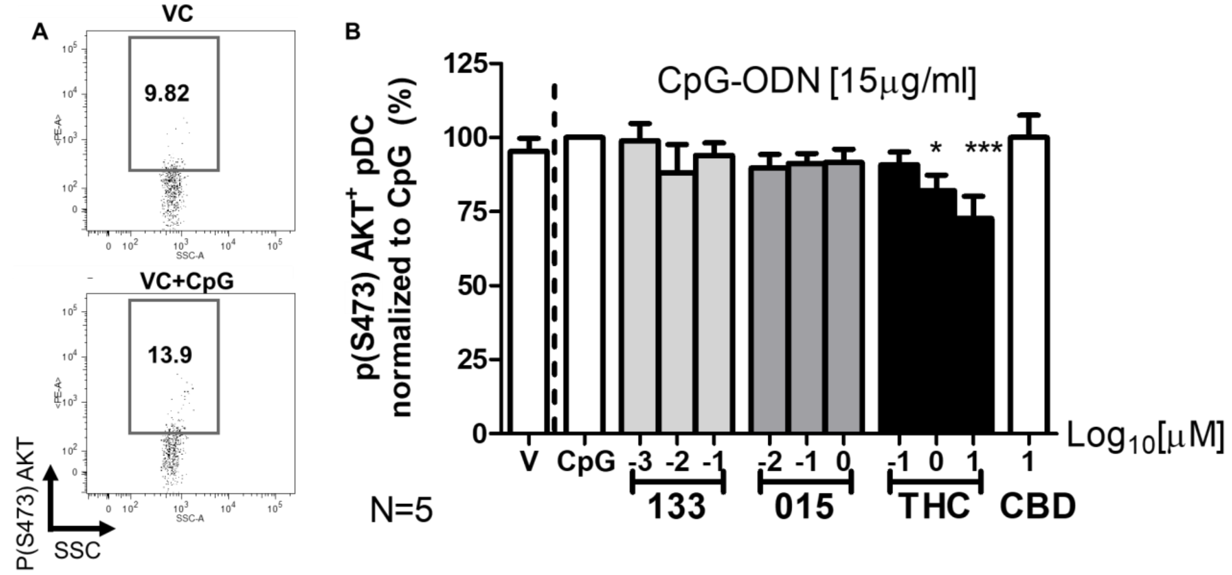

Figure 27. Treatment of pDC with THC but not the CB2-selective agonists, JWH-015 and JWH-133, suppressed the phosphorylation of AKT at the serine-473 (S473) residue. Isolated human PBMCs were treated with either vehicle control (VC; 0.026% Ethanol), CBD (10 µM), THC (0.1, 1, or 10 µM), JWH-015 (10^{-2}, 10^{-1}, 10^{0} µM) or JWH-133 at (10^{-3}, 10^{-2}, 10^{-1} µM) for 30 min, stimulated with CpG-ODN at 15µg/ml for 5 hr, and intracellularly stained for pAKT (S473). Treatment with THC suppressed pS473 in a concentration dependent manner while neither JWH-133 or JWH-015 had an effect. Asterisks indicate statistically significant differences in pS473-AKT expression compared to VC + CpG (1-Way ANOVA with Dunnett's posttest). Asterisks indicate statistically significant differences in pS473-AKT phosphorylation compared to VC + CpG using 1-Way ANOVA with Dunnett's posttest (*p < 0.05; ***p < 0.001).

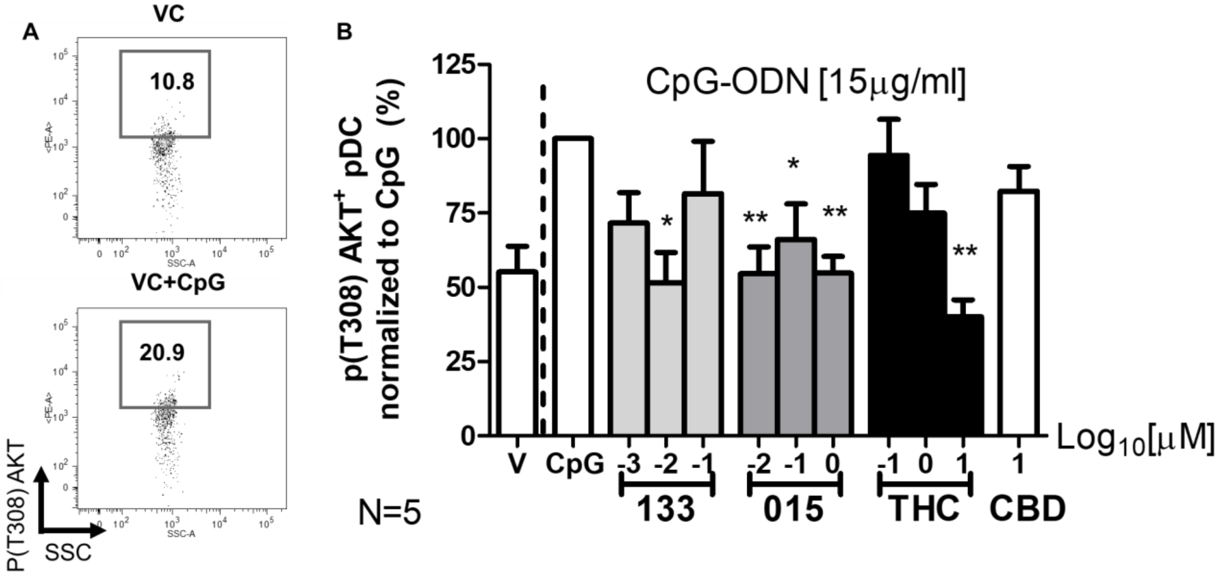

Figure 28. Treatment of pDC with THC or CB2-selective agonists, JWH-015 and JWH-133, suppressed the CpG-ODN-induced phosphorylation of AKT at the threonine-308 (T308) residue. Isolated human PBMCs were treated with either vehicle control (VC; 0.026% Ethanol), CBD (10 µM), THC (0.1, 1, or 10 µM), JWH-015 (10^{-2}, 10^{-1}, 10^{0} µM) or JWH-133 at (10^{-3}, 10^{-2}, 10^{-1} µM) for 30 min, stimulated with CpG-ODN at 15µg/ml for 5 hr, and intracellularly stained for pT308-AKT. CpG induced intracellular expression of pT308, which was suppressed by THC and both CB2-selective agonists. Asterisks indicate statistically significant differences in pT308-AKT expression compared to VC + CpG (1-Way ANOVA with Dunnett's posttest). Asterisks indicate statistically significant differences in pT308-AKT phosphorylation compared to VC + CpG using 1-Way ANOVA with Dunnett's posttest (*$p < 0.05$; **$p < 0.01$).

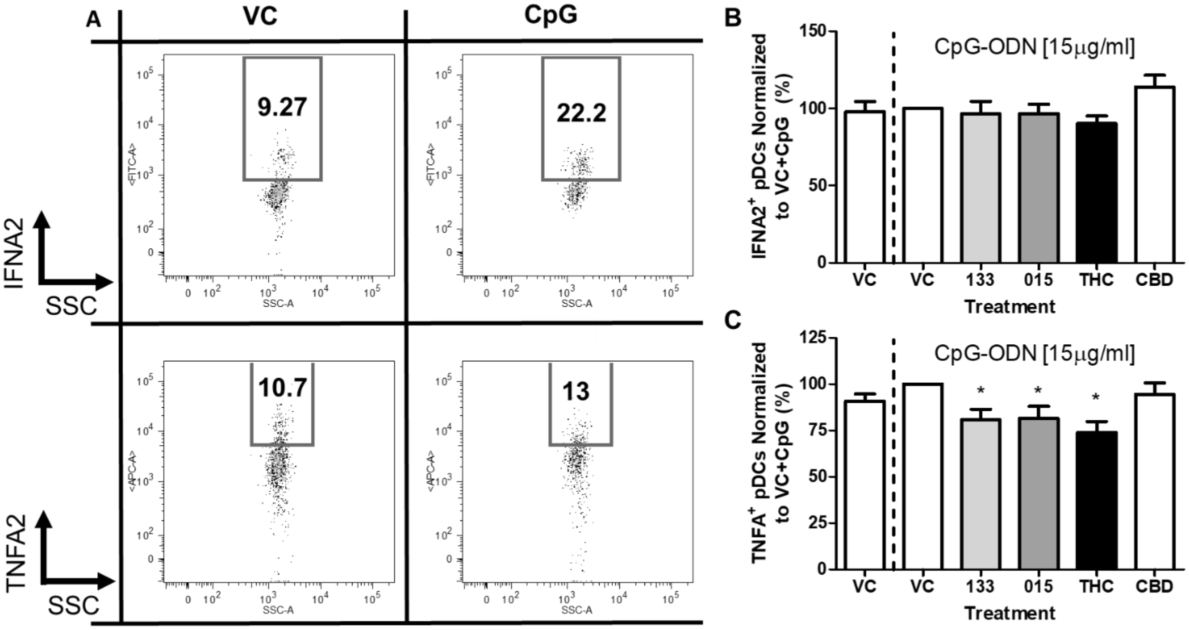

Figure 29. Treatment of pDC with THC or CB2-selective agonists, JWH-015 and JWH-133, suppressed the CpG-ODN-induced IFNA2 and TNFA mRNA expression. Isolated human PBMCs were treated with either vehicle control (VC; 0.026% Ethanol), CBD (10 µM), THC (10 µM), JWH-015 (10^0µM) or JWH-133 at (10^{-1} µM) for 30 min, stimulated with CpG-ODN at 15µg/ml for 5 hr, then used for PrimeFlow™. A) Example of CpG-induced IFNA2 and TNFA mRNA induction. B) CpG induced expression of IFNA2 was suppressed by THC and both CB2-selective agonists. C) CpG induced expression of TNFA was suppressed by THC and both CB2-selective agonists. Asterisks indicate statistically significant differences in p65 phosphorylation compared to VC + CpG using Student's T test (*p < 0.05).

III. SA3: Suppression of IFNα and IL-7-mediated T cell activation by THC

A. $CD4^+$ and $CD8^+$ T cells from healthy and HIV+ donors have comparable composition of memory and non-memory cells

The HIV+ donors in this study exhibited $CD4^+$ and $CD8^+$ T cell numbers in a similar range to healthy donors and possessed undetectable viral loads. However, the number of $CD4^+$ and $CD8^+$ T cells provides only a partial view of the overall T cell repertoire. To gain further insights in to potential differences in the T cell composition between healthy and HIV+ donors, cells were stained with anti-CD45RO antibody. CD45 is a protein tyrosine phosphatase, receptor C (PTPRC) which was originally known as leukocyte common antigen (LCA) and is found on all leukocytes. CD45 is a highly glycosylated protein which has multiple isoforms. These isoforms can be used to distinguish between memory ($CD45RO^+$) or non-memory ($CD45RO^-$) T cells. Upon activation, the CD45RA on naïve cells is replaced by CD45RO which is typically expressed by memory cells [380]. As HIV patients have had repeat exposure to HIV-related antigens, there was the possibility for more memory T cells ($CD45RO^+$) than naïve cells in circulation. This could skew the profile of cell activation as naïve and memory T cells have different gene expression and effector function in response to IFNα [381-383]. However, no significant differences were observed in the composition of memory and non-memory $CD4^+$ or $CD8^+$ T cells between healthy and HIV+ donors in this study (Figure 30B).

B. IFNα treatment has differential effects on the expression of IFNα receptor subunit 2 (IFNAR2) in CD4$^+$ and CD8$^+$ T cells from healthy donors.

Prolonged exposure to IFNα in culture may cause reduced receptor expression due to chronic stimulation and this reduced expression could affect the response to IFNα. Additionally, the effect of THC on IFNAR expression is unknown, especially in the context of chronic activation by IFNα.

Therefore, the surface expression of IFNAR2, the high affinity chain of the type1 interferon receptor complex, was determined by flow cytometric analysis. In these experiments, IFNα had differential effects on IFNAR expression in T cells. Specifically, CD4$^+$ T cells showed a marked suppression of IFNAR2 expression after IFNα stimulation. By contrast, CD8$^+$ T cells showed significant induction of IFNAR2 expression after IFNα treatment. Furthermore, the effects of IFNα on IFNAR2 expression were insensitive to treatment with THC in healthy donors (Figure 31). Conversely, expression of IFNα receptor (IFNAR) was reduced in patients with chronic HIV infection [384], which was specific to CD4$^+$ T cells but was not observed in CD8$^+$ T cells from HIV+ donors (Figure 32A).

C. IFNα-induced phosphorylation of STAT1 in CD4$^+$ and CD8$^+$ T cells from healthy donors was more sensitive to THC-mediated suppression than T cells from HIV+ donors.

The expression of IFNα receptor (IFNAR) is known to be diminished in patients with chronic HIV infection[384] and that T cells from HIV+ donors without antiretroviral therapy have an altered response to IFNα compared to T cells from healthy donors[384].

Our results confirm these findings such that CD4$^+$ T cells had diminished expression of interferon α receptor 2 (IFNAR2) in our cohort of HIV+ donors (Figure 32A). However, no differences in the expression of IFNAR2 in CD8$^+$ T cells between healthy and HIV+ donors were found.

Presently, it is not known if T cells from HIV+ donors on ART have an altered response to IFNα or THC. To address this, the phosphorylation of STAT1 in response to treatment with IFNα was quantified by flow cytometry (Figure 32B). CD4$^+$ T cells from HIV patients were found to have elevated background levels of pSTAT1, compared to CD4$^+$ T cells from healthy donors (Figure 32C-32E), and this difference was statistically significant in the CD45RO- (non-memory) CD4$^+$ T cells (Figure 32E). Upon addition of IFNα, CD4$^+$ T cells from HIV+ donors had a comparable response to CD4$^+$ T cells from healthy donors (Figure 32C-32E). Conversely, CD8$^+$ cells from HIV+ donors had diminished IFNα-induced pSTAT1 compared to CD8$^+$ cells from healthy donors (Figure 32F). Furthermore, treatment with THC significantly suppressed IFNα-induced pSTAT1 in CD4$^+$ and CD8$^+$ T cells from both HIV+ and healthy donors (Figure 32C-32H), but CD45RO$^-$ (non-memory) CD4$^+$ T cells from HIV+ donors were less sensitive to THC-mediated suppression compared to CD45RO$^-$ (non-memory) CD4$^+$ T cells from healthy donors (Figure 32E).

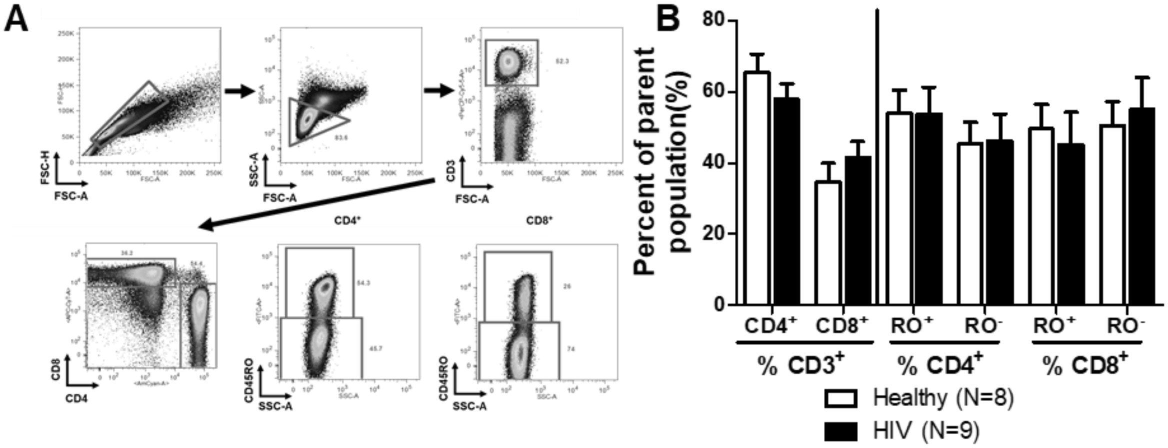

Figure 30. Healthy and HIV+ donors have comparable T cell compositions. A) T cells were identified as $CD3^+$ lymphocytes, and then classified as helper or Cytotoxic T lymphocytes (CTL) based upon the surface expression of CD4 and CD8 respectively. Memory T cells were identified as CD45RO+ and non-memory T cells were identified as $CD45RO^-$ for both $CD4^+$ and $CD8^+$ T cells. B) HIV+ donors used for this study had $CD4^+$ and $CD8^+$ T cell numbers that where comparable to the healthy donors, and comparable memory($CD45RO^+$)/non-memory($CD45RO^-$) cell compositions.

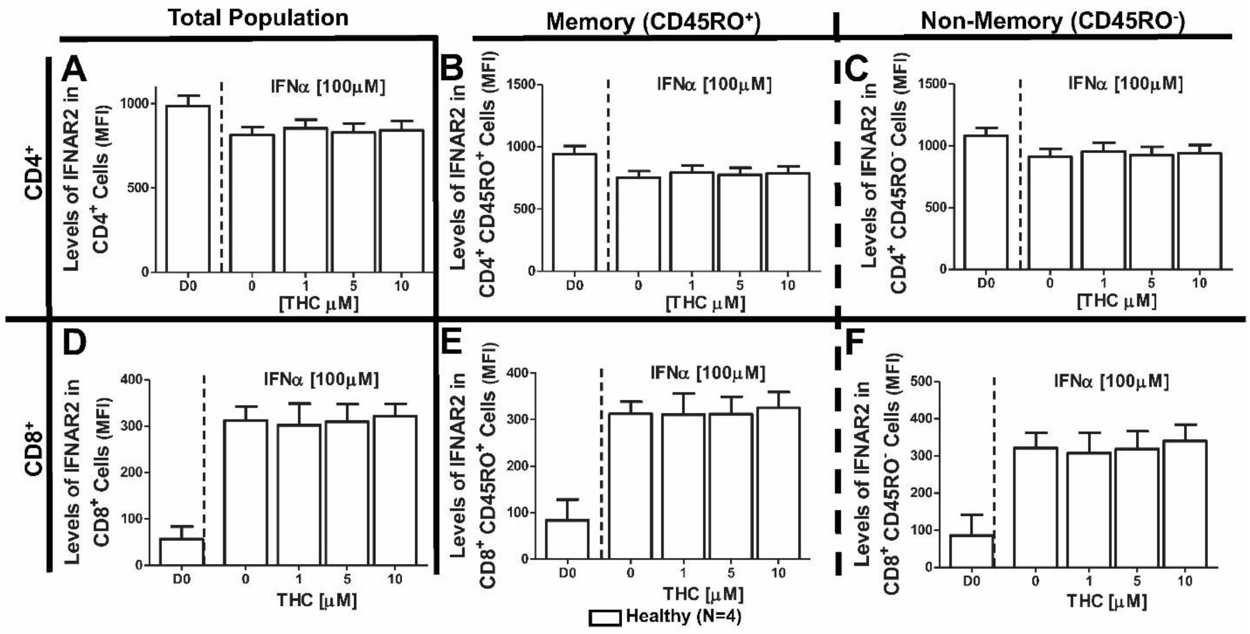

Figure 31. THC has no effect on IFNα-induced modulation of IFNAR2 in CD4$^+$ and CD8$^+$ T cells from Healthy donors. PBMC from healthy and HIV infected donors were isolated through Ficoll Paque™ density gradient centrifugation and either immediately stained for CD3, CD4, CD8, CD45RO, and IFNAR2 (D0) or treated with either vehicle (0.026% EtOH) or various concentrations of THC (1, 5, or 10 µM) for 30 min. Following treatment, cells were stimulated with 100U/ml of IFNα and incubated for 48 hr at which point cells were harvested and stained as described above. The surface expression of IFNAR2 was determined by flow cytometric analysis in healthy: total (A), memory (B) and non-memory (C) CD4$^+$ cells; and total (D), memory (E) and non-memory (F) CD8$^+$ cells.

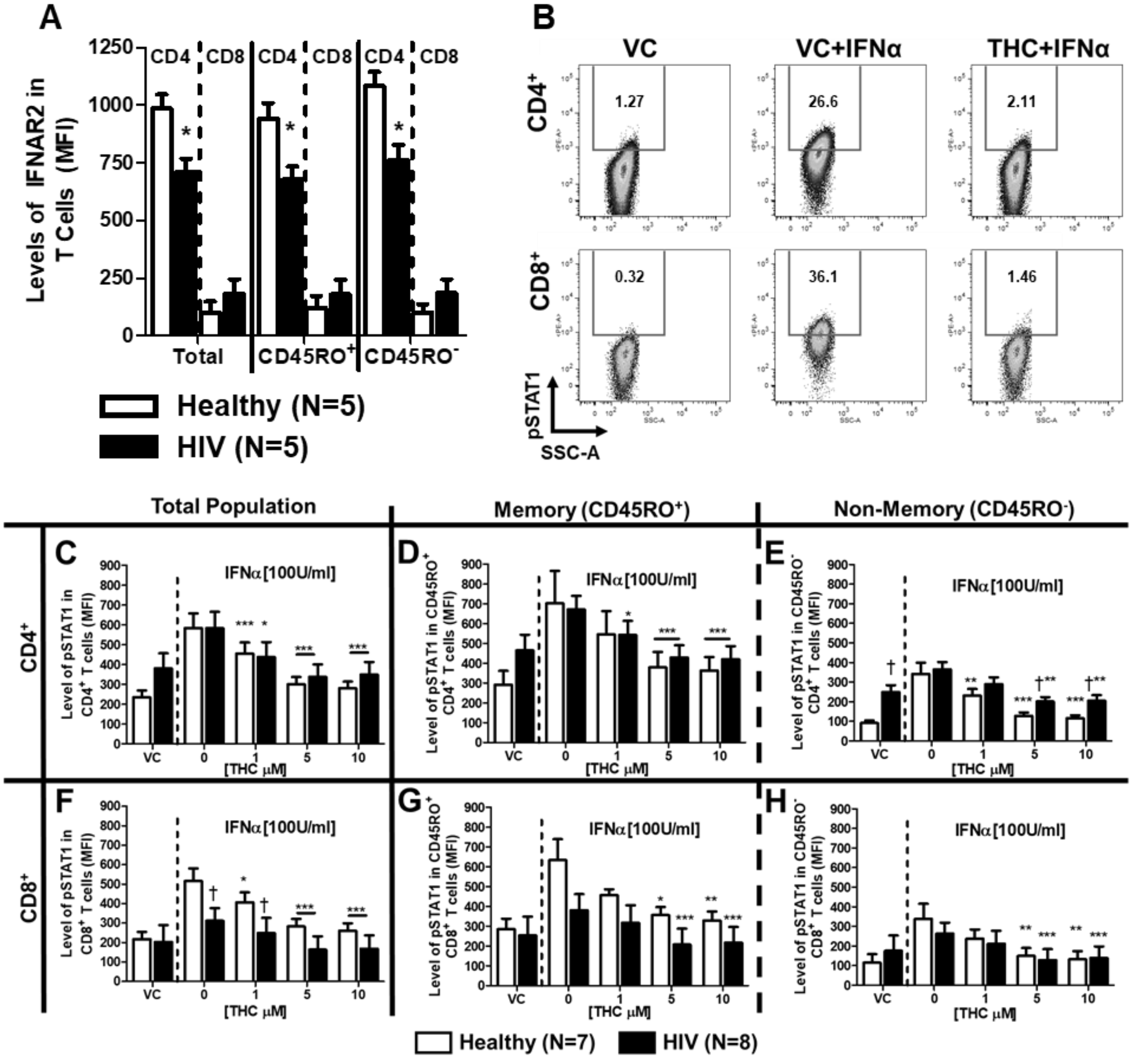

Figure 32. T cells from healthy and HIV+ donors exhibit different profiles of IFNAR2 expression and IFNα-induced STAT1 phosphorylation, which is suppressed by THC. PBMC from healthy and HIV infected donors were isolated through Ficoll Paque™ density gradient centrifugation and used for either determination of IFNAR2 surface expression or IFNα-induced STAT1 phosphorylation (pSTAT1). A) IFNAR2 expression was determined through flow cytometry using the mean-fluorescence intensity (MFI) as a metric expression level. For pSTAT1, PBMC were treated with either vehicle (0.026% EtOH) or various concentrations of THC (1, 5, or 10 μM) in 0.026% EtOH for 30 min before being stimulated with 100 U/ml of IFNα for 30 min. B) Example of IFNα-mediated

Figure 32 (cont'd)

pSTAT1 induction and THC (10 µM)-mediated suppression in a healthy donor. Cells were harvested and stained for CD3, CD4, CD8, CD45RO, and pSTAT1 to determine the effects of THC on IFNα-pSTAT1 induction in: C) Total; D) memory, and E) Non-memory $CD4^+$ T cells; and F) Total, G) memory, and H) non-memory $CD8^+$ T cells. For IFNAR2 expression, asterisks indicate statistically significant differences (*$p < 0.05$) of MFI in HIV compared to type matched healthy T cells. For pSTAT1, Asterisks indicate statistically significant differences of the treatment with the HIV status-matched vehicle control (0 THC) (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Daggers indicate statistically significant differences of treatment matched groups between Healthy and HIV+ T cells (†$p < 0.05$) (2-way analysis of variance with Bonferroni multiple comparison's posttest).

D. IFNα upregulates IL-7Rα expression in T cells from healthy and HIV+ donors, and T cells from healthy donors were more sensitive to THC-mediated suppression than T cells from HIV+ donors

As the IL-7Rα gene promoter region contains an ISRE [215], studies were conducted in purified T cells from healthy donors to determine the effects of THC on IFNα-induced IL-7Rα mRNA expression. IFNα treatment induced mRNA expression of IL-7Rα, which was significantly suppressed by THC (Figure 33A).

Due to sample size limitations, T cells could not be purified from HIV-infected donors. Moreover, mRNA does not always correlate with protein expression. Therefore, studies were performed to determine the direct effects of THC on IFNα-induced IL-7Rα protein expression using flow cytometry (Figure 33B). These studies showed that IFNα augmented the levels of cell surface IL-7Rα expression on memory and non-memory $CD4^+$ and $CD8^+$ T cells from healthy and HIV+ donors (Figure 33C - 33H). However, THC exerted differential effects between donor groups and T cell populations. Specifically, $CD45RO^-$ (non-memory) $CD4^+$ and $CD8^+$ T cells from healthy donors exhibited greater sensitivity to THC-mediated suppression when compared to matched T cells from HIV+ donors and memory ($CD45RO^+$) cells (Figure 33E & 33H).

E. IFNα augments IL-7-induced phosphorylation of STAT5 in $CD4^+$ and $CD8^+$ T cells from healthy and HIV+ donors, and T cells from healthy donors were more sensitive to THC-mediated suppression than T cells from HIV+ donors

Cell surface receptor expression does not necessarily correlate with biological activity according to the “Spare Receptor Theory”. By extension, the magnitude of IL-7Rα expression is not necessarily indicative of receptor function or delineate differences between T cells from healthy and HIV+ donors. Therefore, studies were performed to determine the effects of IFNα and THC on IL-7-induced signaling by measuring the phosphorylation of STAT5 by flow cytometry (Figure 34A). In these studies, CD4$^+$ T cells from HIV+ donors demonstrated diminished IL-7-induced pSTAT5 before IFNα stimulation when compared to CD4$^+$ T cells from healthy donors (Figure 34B). Treatment with IFNα augmented IL-7-induced pSTAT5 in both CD4$^+$ and CD8$^+$ T cells from healthy and HIV+ donors, which was suppressed by THC (Figure 34C-34H). Moreover, both CD4$^+$ and CD8$^+$ T cells from HIV+ donors were less sensitive to THC-mediated suppression than cells from healthy donors and the difference was significant when comparing between both total and non-memory (CD45RO$^-$) CD4$^+$ (Figure 34C & 34H) and CD8$^+$ (Figure 34F & 34H) T cells.

F. CD3/CD28/IFNα-induced proliferation was augmented by IL-7 and suppressed by THC in CD8$^+$ T cells regardless of HIV status while CD4$^+$ T cells from HIV+ donors were less sensitive to THC

The relationship between IFNα and IL-7 stimulation in T cells is known [302]. To better understand how IFNα may affect the homeostatic role of IL-7, studies were performed to address whether the IFNα-induced augmentation of IL-7R expression and cognate signaling resulted in an enhanced T cell proliferative response to IL-7. To mimic *in vivo* conditions using an *in vitro* system, T cells were stimulated using anti-CD3/CD28 antibodies and IFNα concurrently (*i.e.* Three Signal Hypothesis), then stimulated with IL-7 at the peak time of IL-7R expression (48 hr). T cell proliferation was quantified using an index of cell division, called the “Division Index” (DI), by flow cytometry (Figure 35A). Stimulation with IFNα had minimal augmentation of CD3/CD28-induced proliferation in isolation (Figure 35B & 35C). However, stimulation with IFNα before addition of IL-7 resulted in a significantly stronger proliferative response compared to anti-CD3/CD28 stimulation alone in $CD4^+$ T cells from both healthy and HIV+ donors (Figure 35B). This phenomenon was also observed in $CD8^+$ T cells from HIV+ donors (Figure 35C). Stimulation with CD3/CD28/IFNα also increased the proportion of $CD45RO^+$ cells in $CD4^+$ and $CD8^+$ T cells and this effect was more pronounced in HIV+ donors but was not significantly affected by treatment with IL-7 (Figure 35D & 35E). In the presence of THC, $CD4^+$ and $CD8^+$ T cell from healthy donors showed a diminished proliferative response to control treated cells (Figure 35F-35K). Interestingly, THC-mediated suppression of proliferation was diminished in $CD4^+$ T cells from HIV+ donors (Figure 35F-35H). By contrast, $CD8^+$ T cells from HIV+ donors showed comparable suppression in the presence of THC to $CD8^+$ T cells from healthy donors (Figure 35I-35K).

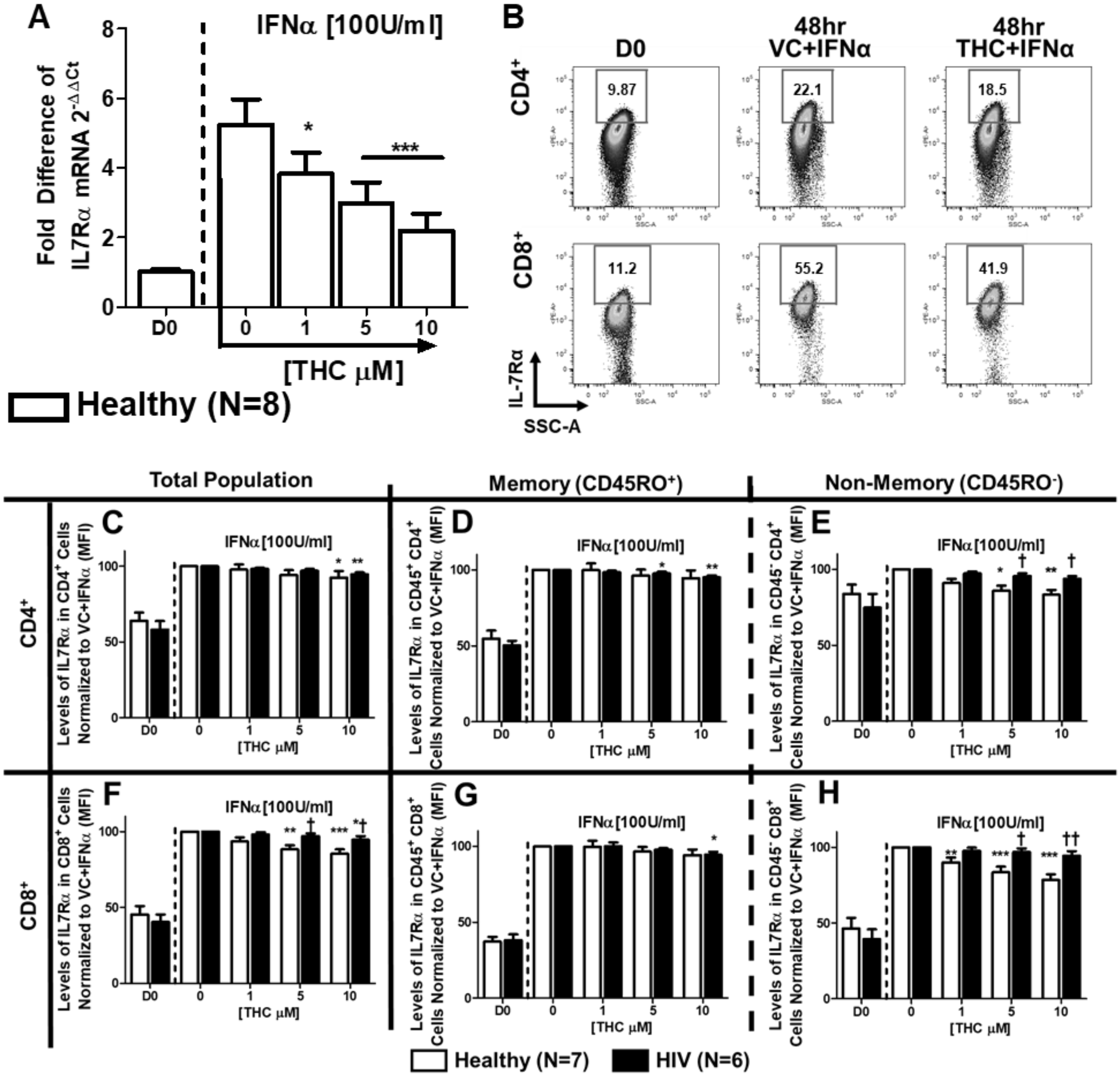

Figure 33. THC diminishes IFNα induced expression of IL-7Rα mRNA in T cells from healthy donors, but differentially affects IFNα-induced surface expression of IL-7Rα in healthy vs HIV+ T cells. A) To determine the effects of IFNα and THC on IL-7Rα mRNA expression, T cells were purified from healthy donors, treated with either vehicle (0.026% EtOH) or various concentrations of THC (1, 5, or 10 μM) for 30 min. Following treatment, cells were stimulated with 100U/ml of IFNα and incubated for 48 hr at which point cells were harvested and IL-7Rα mRNA expression was determined by RT-qPCR. For determination of IL-7Rα surface expression, PBMC from healthy and HIV infected donors were isolated through Ficoll Paque™ density gradient centrifugation and either immediately stained for CD3, CD4, CD8, CD45RO, and IL-7Rα (D0) or treated with THC and IFNα as described above and measured for IL-7Rα expression after 48Hr. B) Example of IFNα-mediated IL-7Rα expression and THC (10 μM)-mediated suppression in a healthy donor. The effects of THC on the expression level (MFI) of IL-7Rα in T cells

Figure 33 (cont'd)

from healthy and HIV+ donors were determined for: C) total, D) memory and E) non-memory CD4$^+$ cells; and F) total, G) memory, H) and non-memory CD8$^+$ cells. Asterisks indicate statistically significant differences of the treatment with the HIV status-matched vehicle control (0 THC) (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). Daggers indicate statistically significant differences of treatment matched groups between Healthy and HIV+ T cells (†p ≤ 0.05; ††p ≤ 0.01) (2-way analysis of variance with Bonferroni multiple comparison's posttest).

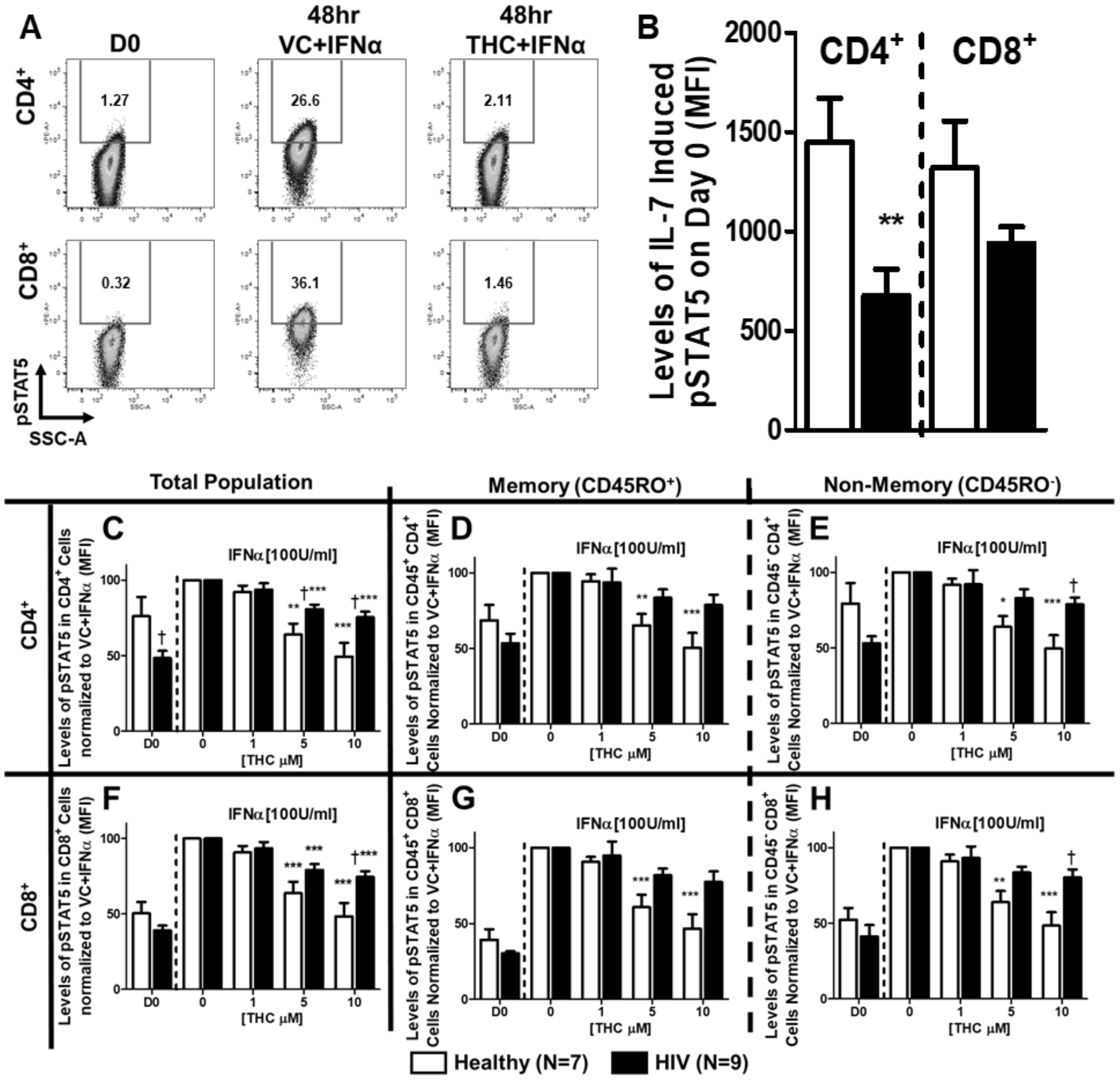

Figure 34. THC decreases IFNα-mediated augmentation of IL-7-induced STAT5 phosphorylation. PBMC from healthy and HIV infected donors were isolated through Ficoll Paque™ density gradient centrifugation. Cells were either immediately used for the detection IL-7 induced pSTAT5 (D0) or treated with vehicle (0.026% EtOH) or various concentrations of THC (1, 5, or 10 µM) for 30 min and then stimulated with 100U/ml of IFNα and incubated for 48 hr. For IL-7-induced pSTAT5, cells were treated with 10ng/ml of IL-7 for 15 min followed by rapid fixation. This process of IL-7-induced pSTAT5 was repeated following the 48 hr incubation with IFNα. A) Example of IL-7Rα-induced pSTAT5 and THC (10 µM)-mediated suppression in a healthy donor. B) Levels (MFI) of pSTAT5 in T was determined by flow cytometric analysis on day 0. The effects of THC on the IL-7-induced pSTAT5 level following IFNα stimulation of T cells from healthy and HIV+

Figure 34 (cont'd)

donors were determined for: C) total, D) memory and E) non-memory $CD4^+$ cells; and F) total, G) memory, H) and non-memory $CD8^+$ cells. Asterisks indicate statistically significant differences of the treatment with the HIV status-matched vehicle control (0 THC) (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). Daggers indicate statistically significant differences of treatment matched groups between Healthy and HIV+ T cells (†$p \leq 0.05$) (2-way analysis of variance with Bonferroni multiple comparison's posttest).

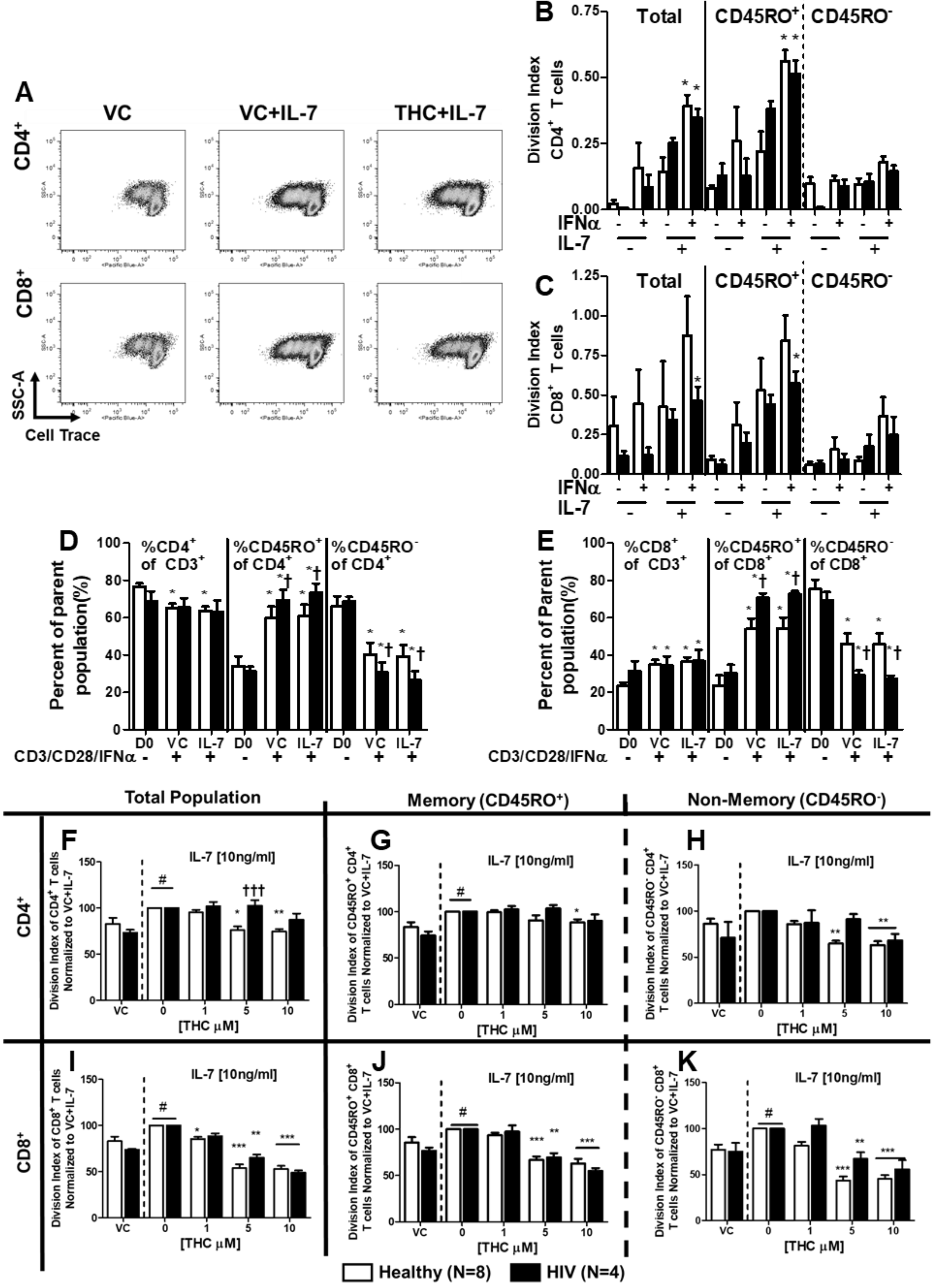

Figure 35. CD3/CD28/IFNα-induced T cell proliferation causes changes in the composition of CD4$^+$ and CD8$^+$ T cell populations and is augmented by IL-7 which is suppressed by THC. PBMC from healthy and HIV+ donors were isolated through Ficoll Paque™ density gradient centrifugation. Cells were stained with violet CellTrace™

Figure 35 (Cont'd)

dye and then treated with either vehicle (0.026% EtOH) or various concentrations of THC (1, 5, or 10 µM) for 30 min. Following THC and vehicle treatment, cells were stimulated with 100U/ml of IFNα and 2.5ug/ml of anti-CD3 and anti-CD28 antibodies then incubated for 48 hr. Cells were then treated with IL-7 (10ng/ml) or vehicle (endotoxin-free H_2O) and incubating for an additional 48 hr before harvesting. Proliferation was determined by CellTrace™ dye dilution and the proliferation tool of the FlowJo® 8.1 software by FlowJo, LLC. A) Example of IL-7-mediated augmentation of T cell proliferation in CD3/CD28/IFNα-stimulated T cells and THC (10 µM)-mediated suppression in a healthy donor. The effects of treatment with IFNα and IL-7 on anti-CD3/CD28 mediated T cell proliferation were determined B) $CD4^+$ and C) $CD8^+$ T cells from healthy and HIV+ donors. The effect of IL-7 stimulation on the composition of total $CD3^+$ T cells and within between memory ($CD45RO^+$) vs non-memory ($CD45RO^-$) cells was determined for both D) $CD4^+$ and E) $CD8^+$ T cells by flow cytometry after CD3/CD28/IFNα stimulation, with or without IL-7 treatment. The effects of THC on the IL-7-induced augmentation of CD3/CD28/IFNα-induced proliferation of T cells from healthy and HIV+ donors were determined for: F) total, G) memory and H) non-memory $CD4^+$ cells; and I) total, J) memory, K) and non-memory $CD8^+$ cells. Asterisks indicate statistically significant differences of the treatment with the HIV status-matched vehicle control (0 THC or D0) (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). Daggers indicate statistically significant differences of treatment matched groups between Healthy and HIV+ T cells (†$p \leq 0.05$; ††$p \leq 0.01$) (2-way analysis of variance with Bonferroni multiple comparison's posttest).

IV. SA4: THC-mediated suppression of CD8^{+} T cell effector function and CD8^{+} T cell-mediated stimulation of U251 astrocytes

A. IFNα/CD3/CD28/IL2-mediated stimulation of CD8^{+} T cells induces effector functions which are suppressed by THC

While important, proliferation is only one aspect of T cell function. According the three-signal hypothesis (1) antigen and (2) co-stimulatory molecules induce T cell proliferation while the effector function of T cells, CD8^{+} T cells especially, is largely influenced by (3) cytokines [383]. Specifically, stimulation by IFNα has been shown to influence T cell effector function such that both CD4^{+} and CD8^{+} T cells treated with IFNα will favor a more TH1 like response and secrete IFNγ [382]. However, THC has been known to modulate IFNγ secretion in CD8^{+} T cells [93, 385, 386].

To test the effect of THC on effector function, purified CD8^{+} T cells were stimulated with soluble anti-CD3 and anti-CD28 antibody (2.5ng/ml), IL-2 (1ng/ml), and IFNα (100U/ml) for 90 hr before being re-stimulated with 6.25ng/ml of Phorbol 12-myristate 13-acetate (PMA) and 125ng/ml of ionomycin (IO), which activate PKC and induces calcium flux promoting T cell cytokine secretion. Furthermore, THC was administered either concurrently with the CD3/28/IL-2/IFNα stimulation, to replicate naïve cell activation in the presence of THC, or 30 min before re-stimulation with PMA/IO, to simulate the effect of THC on differentiated effector cell function.

Results from these studies revealed that stimulation of CD3/28/IL-2/and IFNα drove CD8^{+} T cells to produce IFNγ (Figure 36) and that this response was primarily driven by CD45RO^{+} (memory) cells (Figure 36A). Moreover, regardless of when THC was added, the levels of IFNγ in the CD8$^+$ T cells were significantly suppressed in total, memory (CD45RO$^+$), and non-memory (CD45RO$^-$) cells (Figure 36B-D).

When THC was added at the time of initial stimulation with CD3/28/IL-2/IFNα, the percentage of PMA/IO-induced IFNγ$^+$ cells was suppressed in a concentration dependent manner (Figure 36E - 36G). When THC was added 30 min before PMA/IO re-stimulation, the number of IFNγ$^+$ CD45RO$^+$ cells was not suppressed (Figure 36F) but the number of IFNγ$^+$ CD45RO$^-$ cells was suppressed by THC (Figure 36G).

In addition to the IFNγ response, CD8$^+$ cells treated with IFNα will become more cytolytic[381]. Another way of detecting higher cytolytic function in CD8$^+$ cells is by the elevated presentation of lysosomal-associated membrane protein-1 (LAMP-1), which is present on cytolytic granules in CD8$^+$ T cells and is detectable on the cell membrane after degranulation. As before, purified CD8$^+$ T cells were stimulated with CD3/CD28/IL-2/IFNα and then re-stimulated with PMA/IO with THC added concurrently with initial stimulation or immediately before PMA/IO re-stimulation.

These studies demonstrated that stimulation by CD3/28/IL-2/and IFNα drove CD8$^+$ T cells to degranulate following PMA/IO re-stimulation as evidenced by increased LAMP-1 (CD107a) expression (Figure 37) and most of this response was driven by CD45RO$^+$ Cells (Figure 37A). Unlike the response with IFNγ, THC only suppressed the percent of LAMP-1$^+$ cells and levels when it was added concurrently with initial stimulation of CD3/CD28/IL-2/IFNα and not when added 30 min before PMA/IO re-stimulation (Figure 37).

Lastly CD8$^+$ T cells can also be induced to secrete TNFα following stimulation with IFNα [381, 383]. However, while important during the acute phase of an infection, elevated levels of TNFα can be cytotoxic and induce significant and severe inflammation. To determine the effects of THC on the TNFα response in CD8$^+$ T cells, as before, purified CD8$^+$ T cells were stimulated with CD3/CD28/IL-2/IFNα and then re-stimulated with PMA/IO with THC added either at the time of initial stimulation or immediately before PMA/IO re-stimulation.

These final studies demonstrated that stimulation by CD3/28/IL-2/and IFNα drove CD8$^+$ T cells to produce TNFα following PMA/IO re-stimulation (Figure 38) and, as with the previous effector responses, that this response was predominately driven by CD45RO$^+$ cells (Figure 38A). Unlike the response with IFNγ and LAMP-1, THC had no significant effect on the TNFα response (Figure 38B-38G).

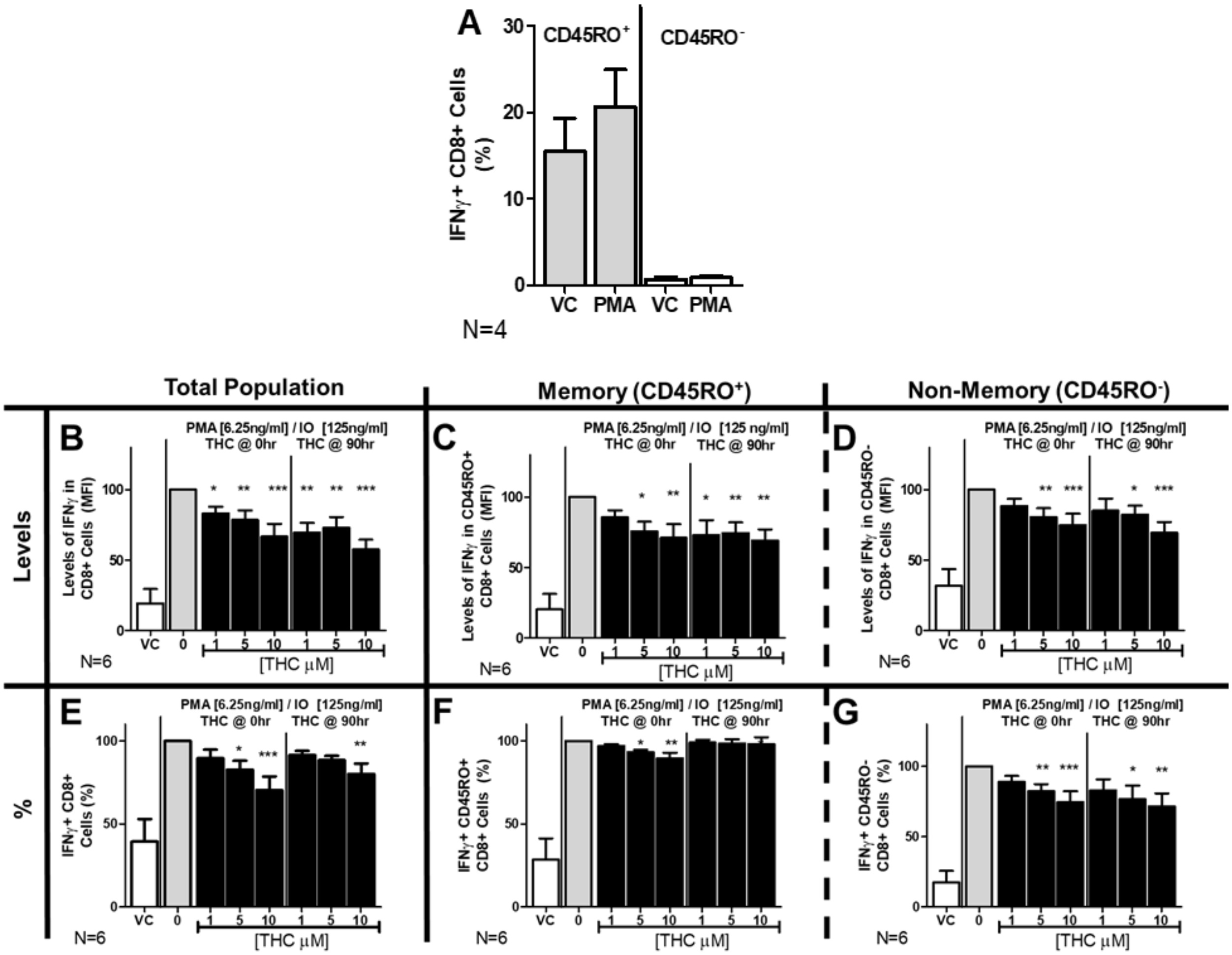

Figure 36. CD3/CD28/IL-2/IFNα-induced IFNγ response in CD8$^+$ T cells is suppressed by THC. PBMC from healthy donors were isolated through Ficoll Paque™ density gradient centrifugation. CD8$^+$ T cells were purified using MojoSort by BioLegend™. Cells were treated with THC (1, 5, or 10uM), vehicle (0.026% EtOH), or no treatment and concurrently stimulated with 100U/ml of IFNα, 2.5ug/ml of anti-CD3 and anti-CD28 antibodies, and 1ng/ml of IL-2. These cells are indicated as "THC @ 0Hr" on the panels. All cells were then incubated for 90 hr. A set of non-THC stimulated cells were then treated with THC as indicated above and indicated identified as "THC @ 90 hr" on the panels. Cells were then re-stimulated with PMA/IO (6.25ng/ml and 125ng/ml, respectively) and treated with brefeldin A/monensin transport blocking cocktail. The number IFNγ$^+$ cells and the levels of IFNγ were determined through flow cytometric analysis. A) Comparison of CD45RO$^+$ and CD45RO$^-$ which were IFNγ$^+$ following PMA/IO. The normalized levels of IFNγ (MFI) in each population of CD8$^+$ T cells are provided as follows: B) Total, C) Memory, and D) Non-memory. The normalized % of IFNγ$^+$ (%) in each population of CD8$^+$ T cells are provided as follows: E) Total, F) Memory, and G) Non-memory. Asterisks indicate statistically significant differences of the treatment (0 THC) (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001). (1-way analysis of variance with Dunnett's multiple comparison's posttest).

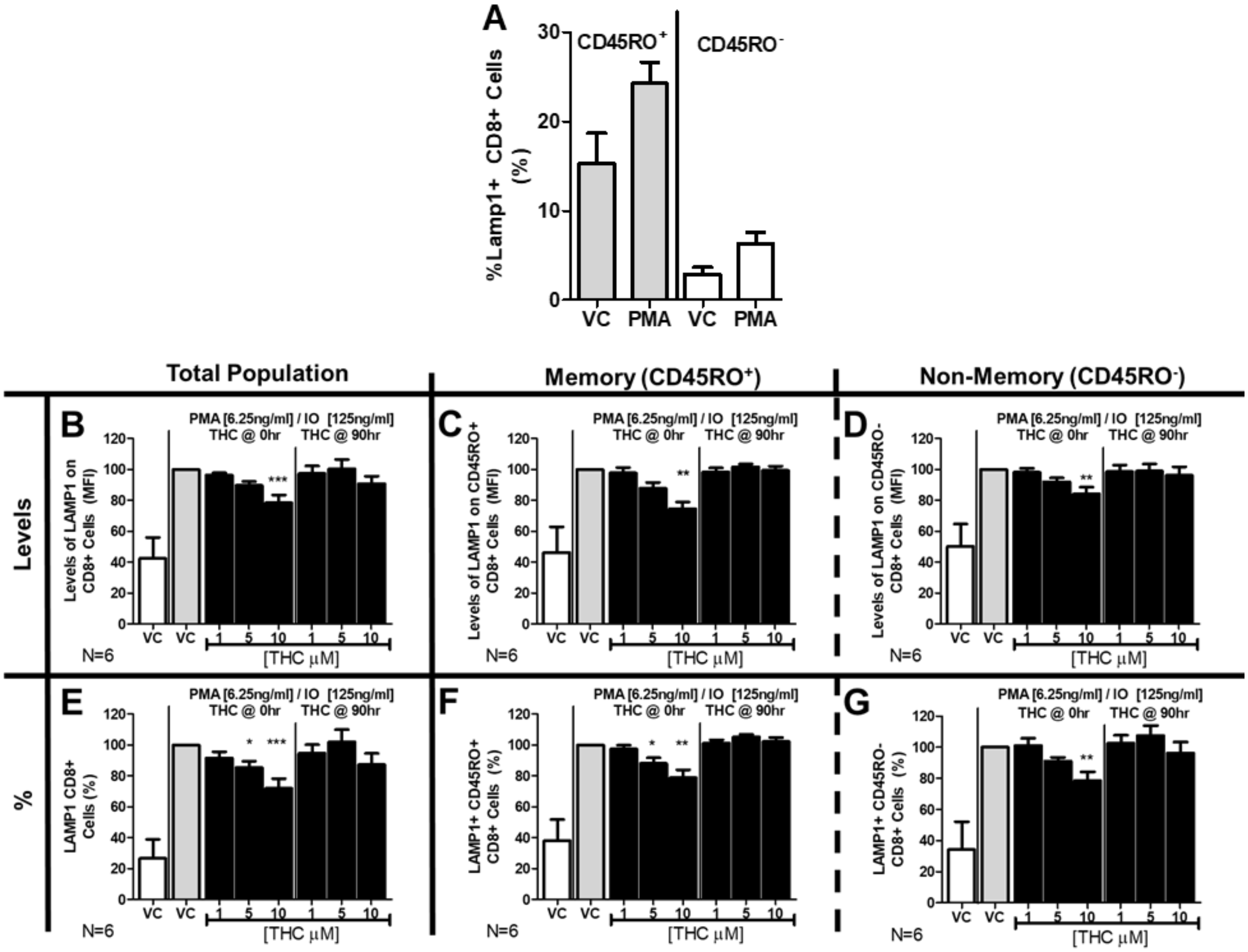

Figure 37. CD3/CD28/IL-2/IFNα-induced LAMP-1 response in $CD8^+$ T cells is suppressed by THC. PBMC from healthy donors were isolated through Ficoll Paque™ density gradient centrifugation. $CD8^+$ T cells were purified using MojoSort by BioLegend™. Cells were treated with THC (1, 5, or 10uM), vehicle (0.026% EtOH), or no treatment and concurrently stimulated with 100U/ml of IFNα, 2.5ug/ml of anti-CD3 and anti-CD28 antibodies, and 1ng/ml of IL-2. These cells are indicated as "THC @ 0Hr" on the panels. All cells were then incubated for 90 hr. A set of non-THC stimulated cells were then treated with THC as indicated above and indicated identified as "THC @ 90 hr" on the panels. Cells were then re-stimulated with PMA/IO (6.25ng/ml and 125ng/ml, respectively) and treated with brefeldin A/monensin transport blocking cocktail. The number LAMP1+ cells and the levels of LAMP-1 were determined through flow cytometric analysis. A) Comparison of $CD45RO^+$ and $CD45RO^-$ which were expressed LAMP-1 following PMA/IO. The normalized levels of LAMP-1 (MFI) in each population of $CD8^+$ T cells are provided as follows: B) Total, C) Memory, and D) Non-memory. The normalized % of LAMP-1 (%) in each population of $CD8^+$ T cells are provided as follows: E) Total, F) Memory, and G) Non-memory. Asterisks indicate statistically significant differences of the treatment (0 THC) (*$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$). (1-way analysis of variance with Dunnett's multiple comparison's posttest).

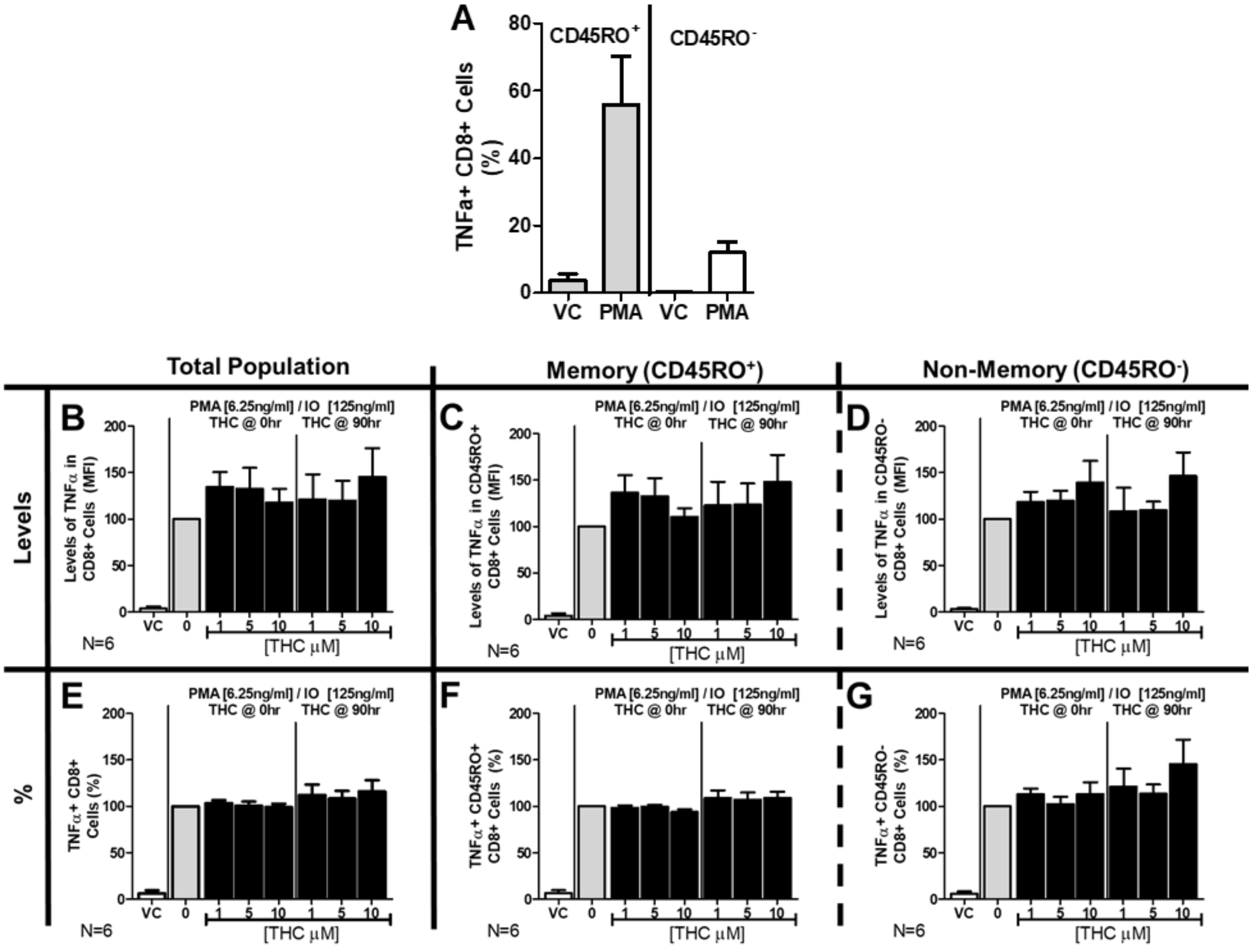

Figure 38. CD3/CD28/IL-2/IFNα-induced TNFα response in CD8$^+$ T cells is unaffected by treatment with THC. PBMC from healthy donors were isolated through Ficoll Paque™ density gradient centrifugation. CD8$^+$ T cells were purified using MojoSort by BioLegend™. Cells were treated with THC (1, 5, or 10uM), vehicle (0.026% EtOH), or no treatment and concurrently stimulated with 100U/ml of IFNα, 2.5ug/ml of anti-CD3 and anti-CD28 antibodies, and 1ng/ml of IL-2. These cells are indicated as “THC @ 0Hr” on the panels. All cells were then incubated for 90 hr. A set of non-THC stimulated cells were then treated with THC as indicated above and indicated identified as “THC @ 90 hr” on the panels. Cells were then re-stimulated with PMA/IO (6.25ng/ml and 125ng/ml, respectively) and treated with brefeldin A/monensin transport blocking cocktail. The number TNFα cells and the levels of TNFα were determined through flow cytometric analysis. A) Comparison of CD45RO$^+$ and CD45RO$^-$ which expressed TNFα following PMA/IO. The normalized levels of TNFα (MFI) in each population of CD8$^+$ T cells are provided as follows: B) Total, C) Memory, and D) Non-memory. The normalized % of TNFα (%) in each population of CD8$^+$ T cells are provided as follows: E) Total, F) Memory, and G) Non-memory.

B. IFNα/CD3/CD28/IL2-mediated stimulation of $CD8^+$ T cells induces secretion of inflammatory mediators which are differentially modulated by THC

T cell expression of cytokines like IFNγ and TNFα indicate effector function, but do not show the breadth of possible secreted factors. To determine the effects of stimulation and THC-mediated suppression a cytometric bead array was performed using LegendPlex™ by BioLegend for activated $CD8^+$ T cells and NK cells. Treatment with THC caused no significant change in the secretion of IL-2 (Figure 39A), IL-6 (Figure 37B), or IL-17A (Figure 39C). While there was a high degree of variability in the IFNγ response, THC significantly suppress IFNγ when added at time 0 (Figure 39D). Interestingly, there was a high degree of variability in the secretion of TNFα (Figure 39E) such that 1 and 5 µM THC added at 90 hr trended towards suppression, but 10 µM THC appeared to augment response. Additionally, soluble FAS (sFAS), a member of the TNF family, was also insensitive to modulation by THC (Figure 39F). Finally, while the granule components, Granzyme A (Figure 39G) and Granzyme B (Figure 39H) were not altered by treatment with THC, perforin (Figure 39I), was suppressed by THC when added at 0 Hr.

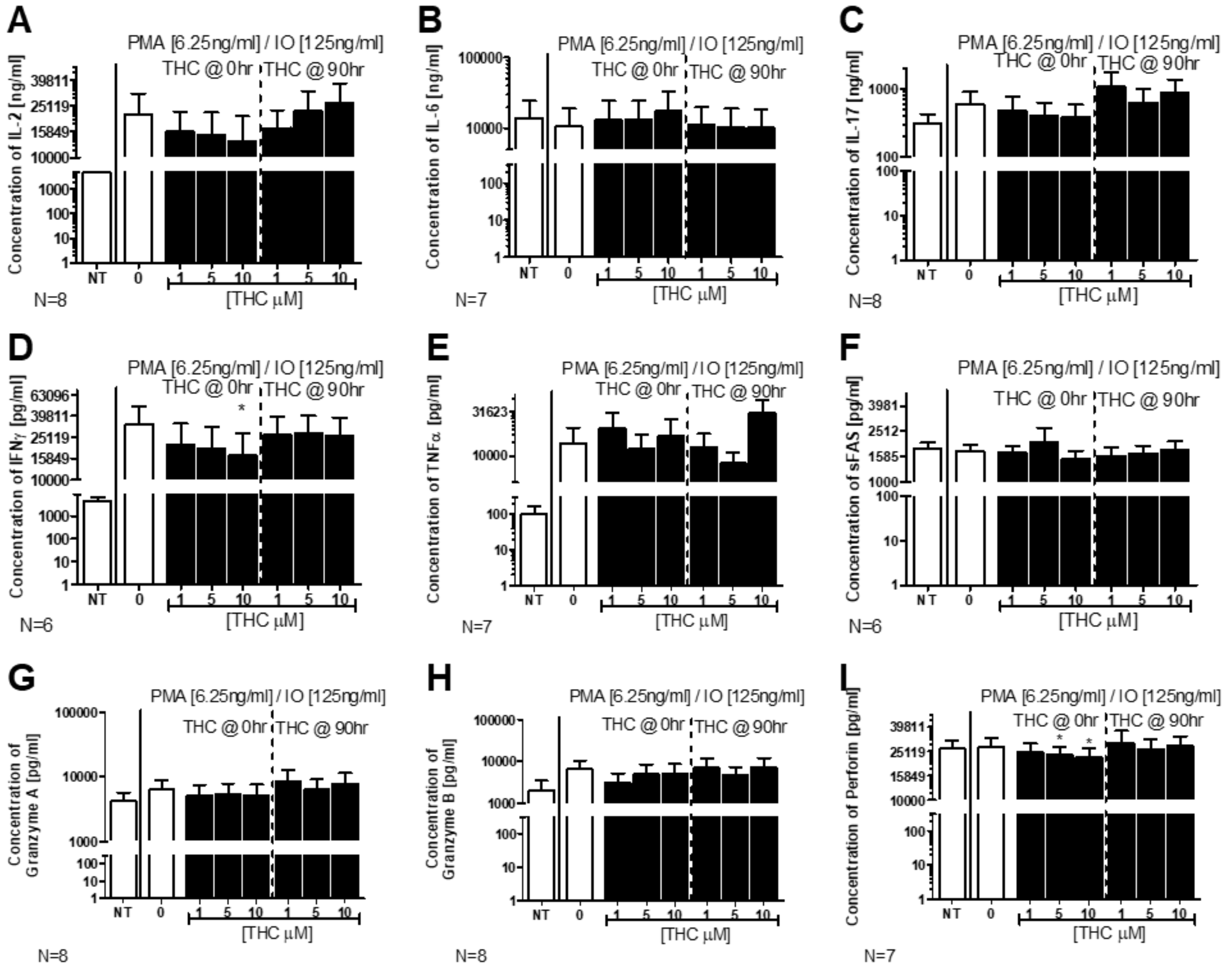

Figure 39. CD3/CD28/IL-2/IFNα-induced secretion of inflammatory and cytolytic factors by CD8$^+$ T cells are differentially modulated by treatment with THC. PBMC from healthy donors were isolated through Ficoll Paque™ density gradient centrifugation. CD8$^+$ T cells were purified using MojoSort by BioLegend™. Cells were treated with THC (1, 5, or 10uM), vehicle (0.026% EtOH), or no treatment and concurrently stimulated with 100U/ml of IFNα, 2.5ug/ml of anti-CD3 and anti-CD28 antibodies, and 1ng/ml of IL-2. These cells are indicated as "THC @ 0Hr" on the panels. All cells were then incubated for 90 hr. A set of non-THC stimulated cells were then treated with THC as indicated above and indicated identified as "THC @ 90 hr" on the panels. Cells were then re-stimulated with PMA/IO (6.25ng/ml and 125ng/ml, respectively). Supernatants were collected and utilized for LegendPlex™ assay to determine the levels of secreted: A) IL-2, B) IL-6, C) IL-17A, D) IFNγ, E) TNFα, F) sFAS, G) Granzyme A, H) Granzyme B, and I) Perforin. Asterisks indicate statistically significant differences of the treatment (0 THC) (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). (1-way analysis of variance with Dunnett's multiple comparison's posttest).

C. TNFα and IFNγ stimulate U251 astrocytes to secrete inflammatory cytokines

Astrocytes are a highly diverse group of glial cells which composes 20-40% of all glial cells in the central nervous system and have a number functions which include: reuptake of neurotransmitters, secretion of cytokines, and maintenance of the blood brain barrier [349]. Consequently, astrocytes are susceptible to dysregulation by inflammatory cytokines. Specifically, TNFα, either secreted by microglia or immune cells, causes the induction of inflammatory cytokine secretion by astrocytes and reduced reuptake of excitatory neurotransmitters, like glutamate, which can lead to excitotoxicity [334, 343, 347, 350].

To determine the sensitivity of the U251 astrocyte line to stimulation by TNFα and IFNγ, U251 cells were incubated with TNFα from HEK 293 cells (Sigma) or recombinant IFNγ (BioLegend™) at various concentrations for 24 hr. Following incubation, U251 astrocytes were stained for intracellular Interferon gamma inducible protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), and IL-6. These studies revealed that TNFα alone induced IL-6 and MCP-1 in a concentration dependent manner which plateaued at 10ng/ml of TNFα (Figure 40A) but TNFα did not induce IP-10. Conversely, IFNγ induced only IP-10 in the astrocytes while MCP-1 and IL-6 were not induced (Figure 40B).

D. TNFα and IFNγ act synergistically in driving the IP-10 and IL-6 responses but not MCP-1 in U251 astrocytes

The data from individual cytokine stimulation indicates that the U251 cells respond to the IFNγ and TNFα but does not reflect physiological relevant stimulation. Specifically, cytokines are often secreted either sequentially or concurrently. Furthermore, Tat-1 from HIV can synergize with IFNγ and TNFα to induce IP-10 [387].

To determine whether the IFNγ and TNFα-mediated stimulation synergizes to drive stronger induction of IP-10, IL-6 and MCP-1, U251 astrocytes were treated with varying concentrations of TNFα and IFNγ in combination and incubated for 24 hr. The MCP-1 response was only responsive to TNFα such that increasing the concentration of IFNγ did not result in an increase in the number of MCP-1$^+$ cells (Figure 41A) or an increase in MCP-1 levels (Figure 41B) above TNFα alone at the indicated concentrations. Interestingly, TNFα and IFNγ co-treatment was synergistic in driving the responses of both IP-10 (Figure 41C and 41D) and IL-6 (Figure 41E and 41F).

E. Stimulation with IFNγ and TNFα in combination alters the profile of STAT1 and NFκB phosphorylation compared to stimulation with IFNγ and TNFα in isolation

Maximum astrocyte induction of IP-10 requires stimulation of both TNFα and IFNγ, an observation other groups, and we, have made. The mechanism likely requires cooperation of STAT1, via IFNγ signaling, and NFκB, via TNFα, that synergize to drive IP-10 production [388].

To determine the effect of TNFα and IFNγ co-stimulation intracellular signaling events in U251 astrocytes, the induction of NFκB and STAT1 was investigated. These studies showed that STAT1 phosphorylation peaked 30 min post-stimulation with IFNγ in both the number of pSTAT1^{+} U251 astrocytes (Figure 42A) and the levels of pSTAT1 within the U251 astrocytes (Figure 42B). Furthermore, co-stimulation with TNFα induced a less robust pSTAT1 signal compared to IFNγ alone (Figure 42A and 42B).

To determine the profile of TNFα stimulation, U251 cells were treated with TNFα alone or in combination with IFNγ and measured for pNFκB. Similar to pSTAT1, NFκB levels (MFI) spiked at 30 min post stimulation and began to recede (Figure 43A) regardless of whether the U251 astrocytes were stimulated with TNFα in isolation or with TNFα and IFNγ concurrently. Interestingly, co-stimulation with TNFα induced a less robust pNFκB signal compared to TNFα alone (Figure 43A). Unlike pSTAT1 levels, which both increased and then decreased in the U251 astrocytes, the number of pNFκB^{+} U251 cells continued to accumulate over time regardless of single TNFα stimulation or simultaneous stimulation with TNFα and IFNγ (Figure 43B).

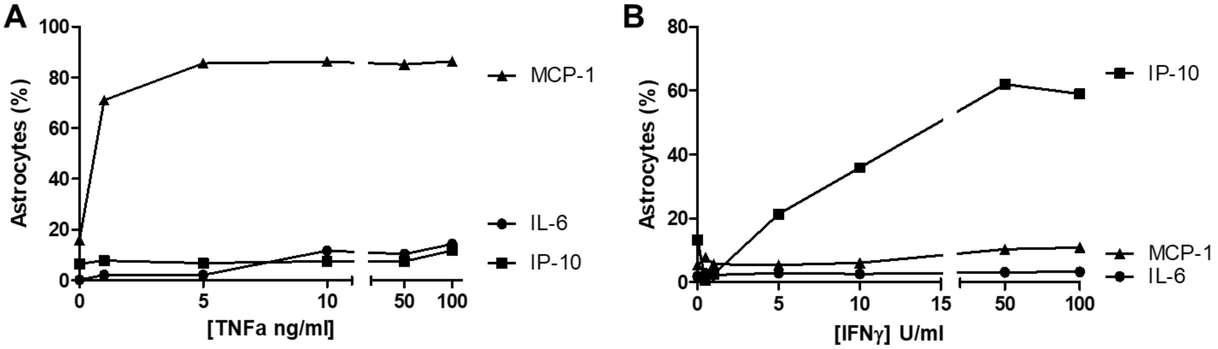

Figure 40. TNFα and IFNγ induce secretion of inflammatory cytokines from U251 astrocytes. U251 astrocytes were seeded in 24 well plates at a density of 7 x 10^4 cells/well and allow to grow to 1 x 10^5 cells/well over 24 hr. At which point the astrocytes were stimulated with different concentrations of either TNFα (1, 5, 10, 50, and 100ng/ml) or IFNγ (1, 5, 10, 50, and 100 U/ml) for 24 hr before harvesting. Cytokine expression was determined by flow cytometric analysis following intracellular staining. A) Cytokines induced by stimulation with TNFα. B) Cytokines induced by IP-10. No statistical analysis was possible for this experiment as these were preliminary experiments on a single donor (N=1).

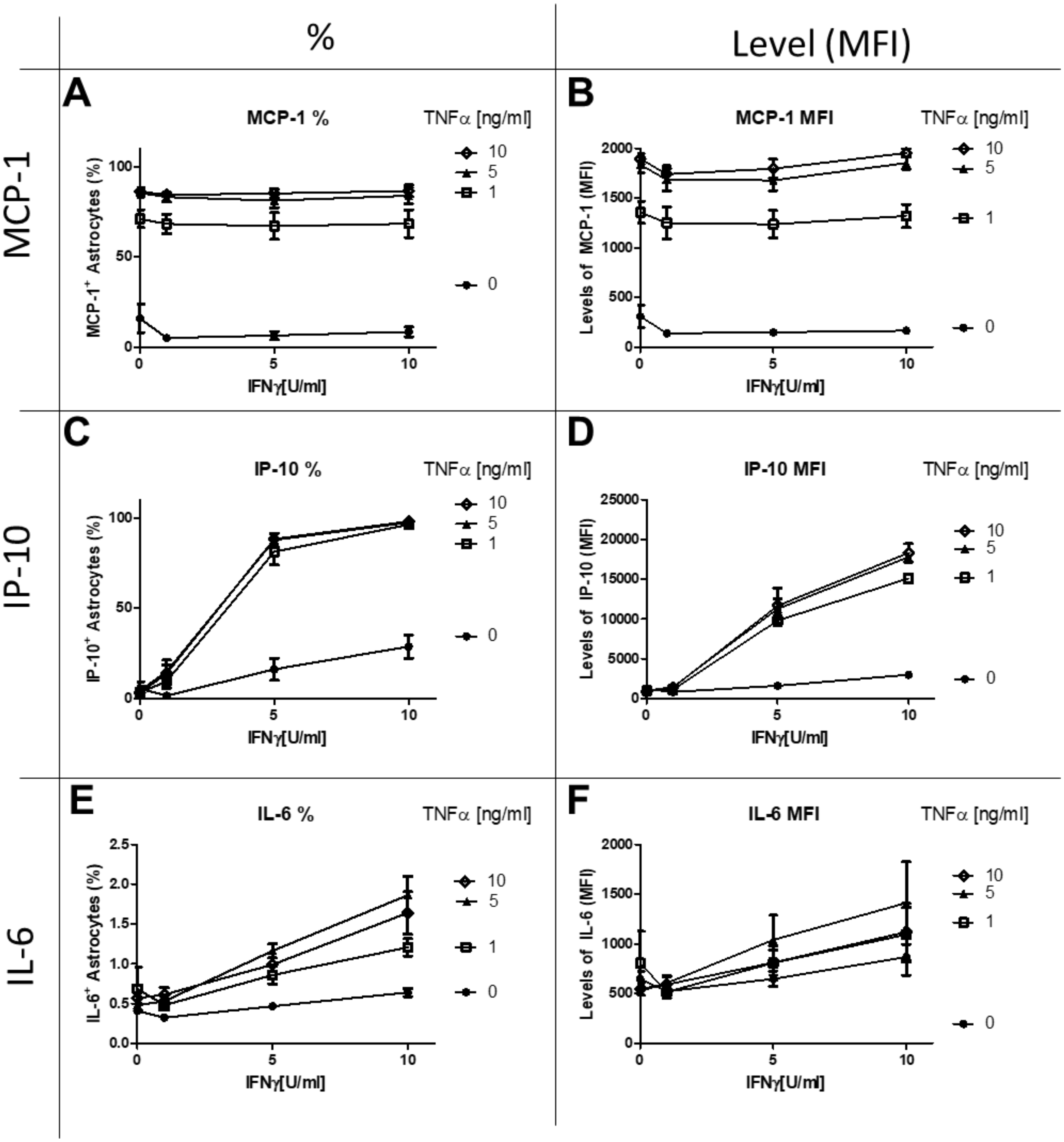

Figure 41. TNFα and IFNγ act synergistically in driving IP-10 and IL-6 responses but not MCP-1. U251 astrocytes were seeded in 24 well plates at a density of 7 x 10^4 cells/well and allow to grow to 1 x 10^5 cells/well over 24 hr (N=3). At which point the astrocytes were stimulated with different concentrations of either TNFα (1, 5, or 10 ng/ml) or IFNγ (1, 5, 10 U/ml) for 24 hr before harvesting. Cytokine expression was determined by flow cytometric analysis following intracellular staining. A) MCP-1^+ U251 astrocytes (%); B) Levels of MCP-1 in U251 astrocytes (MFI); C) IP-10^+ U251 astrocytes (%); D) Levels of IP-10 in U251 astrocytes (MFI); E) IL-6^+ U251 astrocytes (%); F) Levels of IL-6 in U251 astrocytes (MFI).

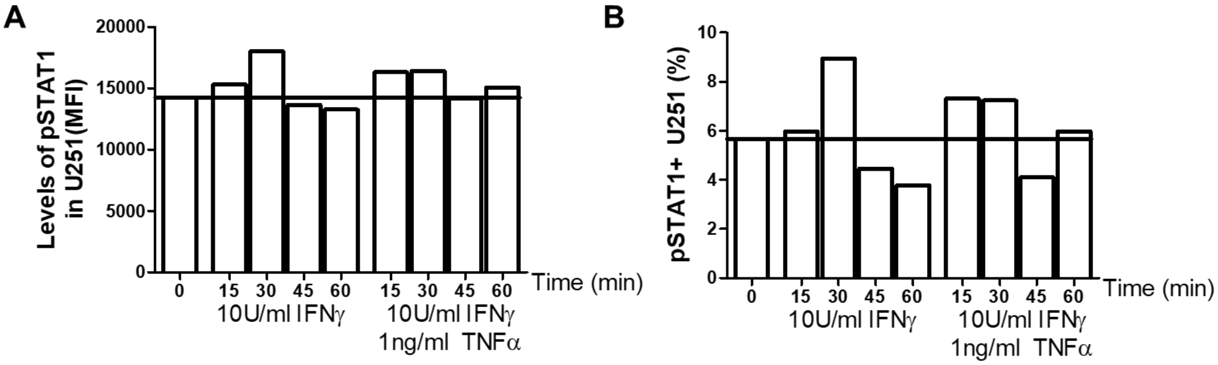

Figure 42. TNFα and IFNγ co-stimulation of U251 astrocytes alters the profile of STAT1 phosphorylation. U251 astrocytes were seeded in 24 well plates at a density of 7 x 10^4 cells/well and allow to grow to 1 x 10^5 cells/well over 24 hr. At which point the astrocytes were stimulated with different concentrations of either IFNγ (10 U/ml) or IFNγ + TNFα (1ng/ml) for 1 hr while harvesting in 15 min increments. pSTAT1 expression was determined by flow cytometric analysis following intracellular staining. A) Levels of pSTAT1 in U251 astrocytes (MFI). B) $pSTAT1^+$ U251 astrocytes (%). No statistical analysis was possible for this experiment as these were preliminary experiments on a single donor (N=1).

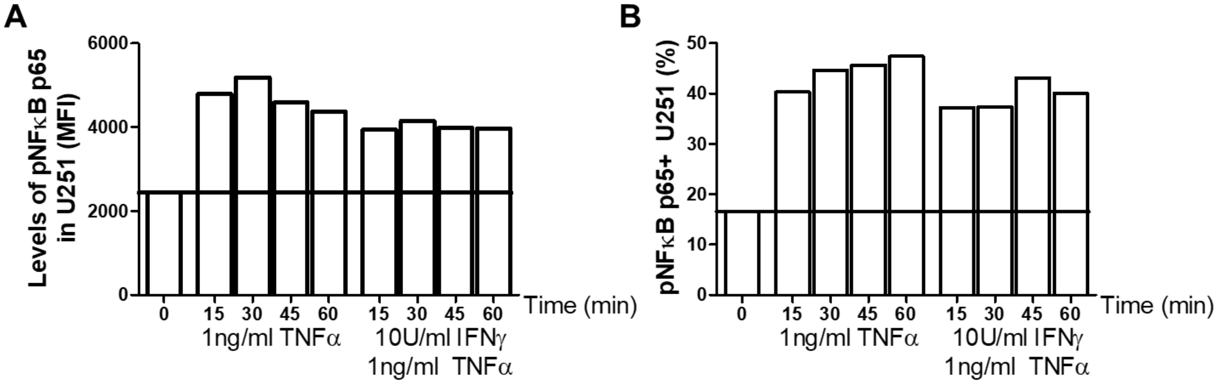

Figure 43. TNFα and IFNγ co-stimulation of U251 astrocytes alters the profile of NFκB phosphorylation. U251 astrocytes were seeded in 24 well plates at a density of 7 x 10^4 cells/well and allow to grow to 1 x 10^5 cells/well over 24 hr. At which point the astrocytes were stimulated with different concentrations of either TNFα (1ng/ml) or TNFα+IFNγ (10U/ml) for 1 hr while harvesting in 15 min increments. Phosphorylation of p65 subunit of NFκB was determined by flow cytometric analysis following intracellular staining. A) Levels of pNFκB in U251 astrocytes (MFI). B) pNFκB$^+$ U251 astrocytes. No statistical analysis was possible for this experiment as these were preliminary experiments on a single donor (N=1).

F. THC suppresses CD8$^+$ T cell-induced activation of U251 astrocytes

CD8$^+$ T cells can secrete both IFNγ and TNFα following stimulation with anti-CD3/CD28 antibodies and IFNα (Figure 34 and Figure 36). Further, THC suppressed CD8$^+$ T cells secretion of IFNγ but not TNFα. In addition, while adding THC directly before re-stimulation with PMA/IO had a negligible effect on the number of IFNγ secreting cells, it significantly reduced the levels of IFNγ being produced by effector CD8$^+$ T cells. Therefore, to address whether CD8$^+$ T cells could directly stimulate astrocytes and determine if THC had an effect of CD8$^+$ T cell stimulation, CD8$^+$ T cells were stimulated as mentioned above, treated with THC at different time points, added in a 1:1 ratio to U251 astrocytes, stimulated with PMA/I, and cocultured for 24 hr.

Astrocytes co-cultured with PMA/IO-treated CD8$^+$ T cells showed an elevated percent of MCP-1$^+$ (Figure 44A and 44B), IL-6$^+$ (Figure 44C and 44D), and IP-10$^+$ (Figure 44E and 44F) U251 astrocytes compared to astrocytes incubated with PMA/IO alone and no T cells (NT). MCP-1 was shown to be insensitive to THC-mediated suppression regardless of the time of addition (Figure 44B). THC did have an appreciable effect on the number of IL-6+ astrocytes such that THC generally reduced the number of IL-6$^+$ astrocytes (Figure 44D). However, due to the high variability between samples, this trend did not reach significance. Lastly, T-cell-induced IP-10$^+$ astrocytes were suppressed by THC in a concentration dependent manner (Figure 44F). Collectively, these results demonstrate that treatment with THC was generally suppressive towards CD8$^+$T cell-mediated IL-6 and IP-10 response in U251 cells regardless of the time of addition but had no effect on MCP-1.

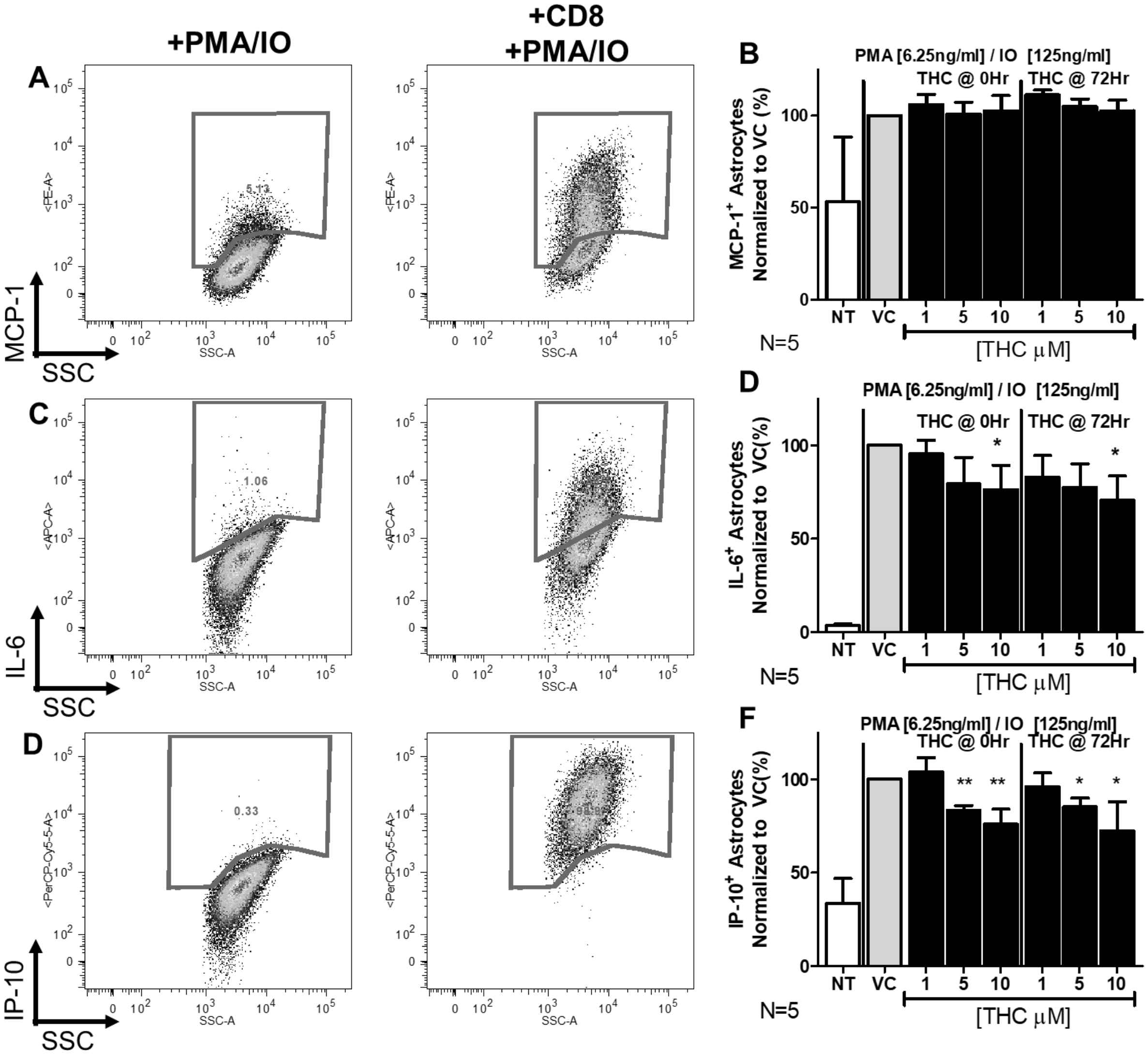

Figure 44. CD3/CD28/IL-2/IFNα-induced CD8$^+$ activation drives U251 astrocyte production of inflammatory cytokines which is suppressed by THC. PBMC from healthy donors were isolated through Ficoll Paque™ density gradient centrifugation. CD8$^+$ T cells were purified using MojoSort by BioLegend™. CD8$^+$ T Cells were treated with THC (1, 5, or 10uM), vehicle (0.026% EtOH), or no treatment and concurrently stimulated with 100U/ml of IFNα, 2.5ug/ml of anti-CD3 and anti-CD28 antibodies, and 1ng/ml of IL-2. These cells are indicated as “THC @ 0Hr” on the panels. All cells were then incubated for 72 hr. A set of non-THC stimulated cells were then treated with THC as indicated above and indicated identified as “THC @ 72 hr” on the panels. Astrocytes were seeded at 7 x 10^4 cells/well and grown to 1x10^5 cells/well for 24 hr to coincide with the beginning of T cell/astrocyte co-culture. After treatment with THC, 1 x 10^5 CD8$^+$ T cells were added to astrocytes and then re-stimulated with PMA/IO (6.25ng/ml and 125ng/ml, respectively) and treated with brefeldin A/monensin transport blocking cocktail. The number of MCP-1$^+$, IL-6$^+$, and IP-10$^+$ cells were determined through flow cytometric

Figure 44 (cont'd)

analysis. A and B) MCP-1$^+$ U251 astrocytes. C and D) IL-6$^+$ U251 astrocytes. E and F) IP-10$^+$ U251 astrocytes. Asterisks indicate statistically significant differences of the treatment (0 THC) (*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$). (1-way analysis of variance with Dunnett's multiple comparison's posttest).

G. THC directly inhibits individual, but not concurrent, stimulation of U251 astrocytes by IFNγ and TNFα

THC modulates inflammatory responses in a variety of cell types, including immune cells[389] and microglia[63]. Therefore, there are two possible routes by which THC may act upon CD8$^+$ T cell-induced IP-10 and IL-6 response in U251 astrocytes: 1) THC directly reduces CD8$^+$ T cell secretion of inflammatory cytokines and/or 2) THC directly diminishes astrocyte stimulation by inflammatory cytokines secreted from CD8$^+$ cells. Treatment with THC has already been shown to directly decrease CD8$^+$ T cell IFNγ and LAMP-1 responses while having no effect on TNFα. However, the direct effects of THC on astrocytes, which express canonical cannabinoid receptors [390] and are sensitive to cannabinoid mediated suppression [391], have not been evaluated.

To test the effects of THC on IFNγ and TNFα stimulation, U251 astrocytes were treated with THC, incubated with either IFNγ or TNFα for 24 hr, and quantified for MCP-1, IL-6, and IP-10 by flow cytometry. These studies revealed that MCP-1 was not induced by IFNγ (Figure 45A). Similarly, IL-6 showed minimal induction with IFNγ stimulation (Figure 45B). In contrast, IP-10 was induced by IFNγ and suppressed by THC in a concentration dependent manner (Figure 45C).

U251 astrocyte stimulation by TNFα alone was also sensitive to THC-mediated suppression, although the profiles differed considerably when compared to stimulation by IFNγ. Specifically, treatment with TNFα induced a robust MCP-1 response in U251 astrocytes (Figure 46A) which was insensitive to THC-mediated suppression. TNFα stimulation also induced IL-6 (Figure 46B) and, to a lesser extent, IP-10 (Figure 46C) which were both sensitive to THC-mediated suppression.

As previously demonstrated, co-stimulation with TNFα and IFNγ was synergistic in driving IP-10 and IL-6 responses in U251 astrocytes. However, the effects of THC on concurrent TNFα and IFNγ stimulation have not been determined in the U251 cells.

To test whether TNFα and IFNγ co-stimulation altered sensitivity to THC, U251 astrocytes were treated with THC and then stimulated with TNFα (1ng/ml) and IFNγ (10U/ml) in tandem for 24 hr, harvested and stained as previously described. TNFα-mediated induction of MCP-1 in U251 astrocytes was not enhanced by co-stimulation with IFNγ and, as with TNFα stimulation alone, the response was insensitive to THC (Figure 47A). IFNγ and TNFα co-stimulation augmented the induction of IL-6 and the response was sensitive to THC-mediated suppression (Figure 47B). Finally, the IP-10 response in U251 astrocytes was also synergistically augmented by co-stimulation with IFNγ and TNFα. Interestingly, co-stimulation by TNFα and IFNγ rendered the IP-10 response insensitive to THC-mediated suppression (Figure 47C).

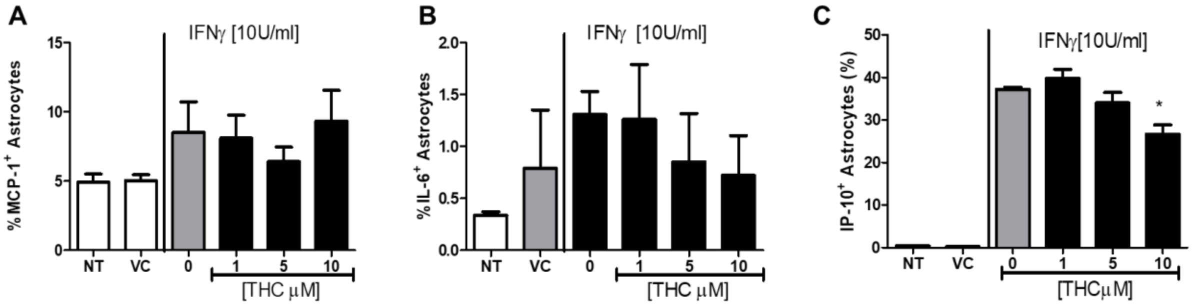

Figure 45. THC inhibits inflammatory cytokine response in IFNγ-stimulated U251 astrocytes. U251 astrocytes were seeded in a 24 well plate at a density of 7 x 10^4 cells/well and allowed to grow to 1 x 10^5 cells/well over 24 hr, at which point the astrocytes were stimulated with 10U/ml IFNγ for 24 hr before harvesting (N=3). Inflammatory cytokine production determined by flow cytometric analysis following intracellular staining. A) MCP-1^+ U251 Astrocytes (%). B) IL-6^+ U251 astrocytes (%). C) IP-10^+ U251 Astrocytes (%). Asterisks indicate statistically significant differences of the treatment (0 THC) ($*p \leq 0.05$). (1-way analysis of variance with Dunnett's multiple comparison's posttest).

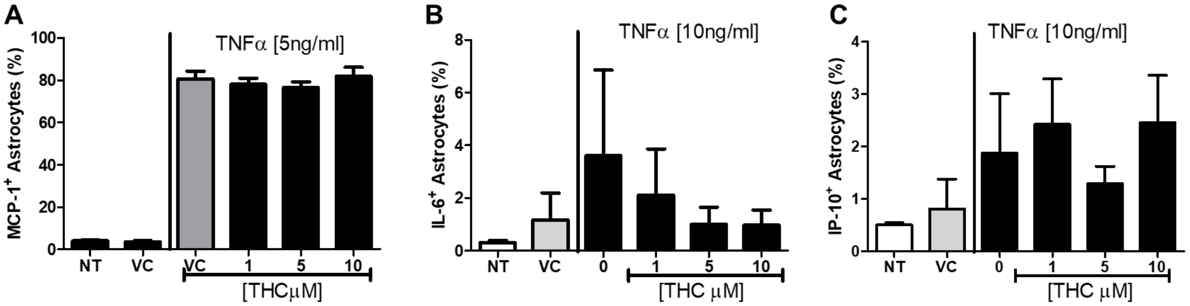

Figure 46. THC inhibits inflammatory cytokine response in TNFα-stimulated astrocytes. U251 astrocytes were seeded in a 24 well plate at a density of 7 x 10^4 cells/well and allowed to grow to 1 x 10^5 cells/well over 24 hr, at which point the astrocytes were stimulated with 10ng/ml TNFα for 24 hr before harvesting (N=3). Inflammatory cytokine production determined by flow cytometric analysis following intracellular staining. A) MCP-1$^+$ U251 Astrocytes (%). B) IL-6$^+$ U251 astrocytes (%). C) IP-10$^+$ U251 Astrocytes (%).

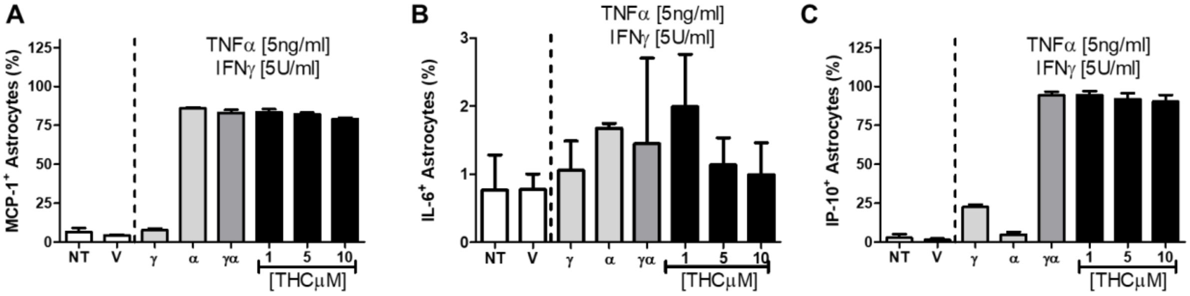

Figure 47. THC does not suppress the inflammatory cytokine response in U251 astrocytes concurrently stimulated with IFNγ and TNFα. U251 astrocytes were seeded in a 24 well plate at a density of 7 x 10^4 cells/well and allowed to grow to 1 x 10^5 cells/well over 24 hr, at which point the astrocytes were stimulated with 10ng/ml TNFα for 24 hr before harvesting (N=3). Inflammatory cytokine production determined by flow cytometric analysis following intracellular staining. A) MCP-1$^+$ U251 Astrocytes (%). B) IL-6$^+$ U251 astrocytes (%). C) IP-10$^+$ U251 Astrocytes (%).

DISCUSSION

I. The effects of THC on pDC from healthy and HIV+ Donors

Presented here is the first report of cannabinoid receptor expression and modulation by THC of pDC function. pDC expression of the canonical cannabinoid receptors (CNR1 and CNR2) was found to be comparable to other PBMC, with greater expression of CNR2 than CNR1. Treatment with THC, and not cannabidiol (CBD), caused a concentration-dependent suppression of IFNα secretion by pDC in healthy donors but only had an effect at higher concentrations in pDC from HIV+ donors. Because CBD has much lower affinity for both CB1 and CB2 than THC, suppression of pDC secretion of IFNα by THC suggests the involvement of cannabinoid receptors rather than non-specific mechanisms. Moreover, THC impaired IFNα secretion by purified pDC, ruling out the possibility for a bystander effect by other cell types. The direct suppression by THC of pDC-secreted IFNα is in agreement with previous findings showing pDC modulation by the endogenous cannabinoid, anandamide [392].

The mechanism underlying the modulation of immune cell function by cannabinoids has been partially elucidated by our and other labs [88, 90, 393]. Here, studies have demonstrated that THC diminishes the phosphorylation of IRF-7, the master regulator of IFNα secretion, in pDC and that this suppression results in the loss of IFNα gene transcription. IRF-7 can be phosphorylated by IRAK [394], phosphoinositide 3-kinase (PI3K) [395] and IκB kinase-α (IKK-α) [396]. PI3K signaling in particular has been identified in modulation of the innate immune-cell response and is a putative target for the development of therapeutics [378]. Activation of the cannabinoid receptors has been shown to directly modulate mTOR-AKT-PI3K signaling in neuronal cell differentiation and survival [50, 397] and disrupt T cell stimulation by keratinocytes through suppression of the same pathway [398]. Given the critical role of PI3K in IFNα secretion in pDC and the conservation of cannabinoid receptor-mediated suppression of mTOR-AKT-PI3K signaling across different cell types, the suppression of the mTOR-AKT-PI3K signaling axis is likely a means by which IFNα secretion is suppressed in pDC by THC. However, a comprehensive phosphoproteomic approach will be needed to elucidate the complexity surrounding the cannabinoid-mediated modulation of this signaling pathway.

pDC from HIV+ donors were found to be more sensitive to suppression by THC compared to pDC from healthy donors. This increased cannabinoid sensitivity may be linked to the significantly higher expression of CNR1 mRNA, and therefore higher CB1 receptor, in PBMC from HIV+ donors compared to healthy donors. The higher expression of CNR1 mRNA might be linked to the chronic inflammatory state experienced by many HIV+ patients as activation of T cells results in the upregulation of CNR1 and not CNR2 [399]. This elevated expression of CB1 may lead to elevated sensitivity to THC-mediated suppression as CB1 and CB2 share many of the same signaling cascades due to both receptors signaling through Gi/o proteins [46]. HIV patients, even those successfully treated by anti-retroviral therapy, experience a variety of inflammatory conditions (e.g. “Leaky Gut Syndrome”) that can lead to systemic inflammation and higher levels of circulating inflammatory cytokines [400, 401]. It is tempting to speculate that higher levels of inflammatory cytokines lead to increased expression of CNR1, but pro-inflammatory cytokines can induce expression of both CNR1 and CNR2 [64]. Furthermore, it is noteworthy that in the current studies CB1 and CB2 expression was quantified solely at the mRNA level (CNR1 and CNR2 respectively). Once reliable and validated CB1 and

CB2 antibodies become available for flow cytometry, additional studies will be needed to confirm these findings at the protein level.

pDC can stimulate other immune cells by secretion of IFNα and through the expression of costimulatory molecules (CD83, CD86, CD80, and HLA-DR) [402]. Expression of CD83 by pDC has been associated with stimulation of both T and B cells [166]. The conducted studies have revealed that THC can impair CD83 surface expression by pDC within 6 h post activation by CpG-ODN. Similarly, when CD83 signaling is ablated, dendritic cell induction of T cell expansion was significantly reduced [368, 372]. Therefore, our results indicate that cannabinoid-based therapies may diminish pDC activation of the adaptive immune response by suppressing both the secretion of IFNα and the expression of a key costimulatory molecule, CD83. Future studies will reveal whether the suppression of CD83 by THC contributes to a functional deficit in pDC-mediated T cell effector function.

The use of cannabis remains controversial in both healthy and HIV+ populations. The results presented here suggest that THC directly impairs pDC function, which may further compromise HIV patients in responding to opportunistic viral infections. However, the actual implications of these results are mixed. HIV-Associated Neurocognitive Disorders (HAND) affect HIV patients [403, 404] regardless of ART and these neurocognitive deficits have been linked with a chronic neuroinflammatory state [126, 400]. pDC have been implicated in neuroinflammatory disease [392, 405-407] and elevated levels of IFNα in neuronal tissue have been associated with neuroinflammation and neurodegeneration [408, 409]. Though the direct role of pDC on IFNα levels in the CNS is unclear, the suppression of pDC activation may be protective against neuroinflammation associated with prolonged HIV infection. Furthermore, and consistent with the premise of medicinal marijuana use as potentially neuroprotective, cannabinoids have been shown to help maintain the integrity of the blood brain barrier in HIV patients [410], potentially reducing the migration of inflammatory cells from the periphery to the brain.

The results from HIV+ donors presented here were obtained using PBMC provided by male donors exclusively, which comprise 80% of HIV patients in the US. However, over 240,000 women are infected with HIV in the US and modulation of pDC activity is of particular interest for these patients. Women progress more quickly from the establishment of HIV infection to the development of AIDS than men [112]. Interestingly, pDC from women have an augmented IFN response compared to men when stimulated through TLR-7 [124] and this difference may underlie the accelerated development of AIDS [112]. Collectively, the presented data imply that the use of cannabinoids may be beneficial for suppressing the activity of pDC, which play a role in the persistent activation of the immune system of HIV patients that have been successfully treated by ART.

II. The effects of CB2-selective agonists on pDC from healthy donors

Cannabinoid-based therapeutics are a growing area of interest for the treatment of inflammatory conditions. The studies presented in this dissertation are the first to report CB2-selective agonists being used to inhibit the production of inflammatory cytokines by pDC. Specifically, CpG-mediated induction of both IFNα and TNFα from pDC can be suppressed in a CB2 specific manner using JWH-133 and JWH-015. While critical during the acute phase of the immune response, elevated levels of both IFNα and TNFα have been found in association with autoimmune diseases [115, 118, 120-122, 165, 190, 246, 307, 405]. Though the studies were conducted in male donors, the therapeutic implications of these findings are of particular importance for women's health, as many autoimmune diseases disproportionately affect women. Specifically, lupus erythematosus, scleroderma and rheumatoid arthritis disproportionately affect women and elevated pDC has been identified in each of these conditions [121, 123].

The suppression of IFNα and TNFα by THC, JWH-015, and JWH-133 support the potential for cannabinoid-based therapies in treating inflammatory conditions. However, as mentioned previously, the utilization of cannabinoids for the treatment of inflammation has already been suggested and medicinal cannabinoids are already recommended for some inflammatory conditions [411] and our results offer further support. To better understand how these compounds suppress cytokine secretion and identify other putative targets for therapeutic intervention, phosphorylation of key regulators in the pathway from TLR-9 ligation to the secretion of each cytokine was measured. Previous studies have identified THC as a potent suppressor of IRF7 phosphorylation, the master regulatory event in IFNα response in pDC [97]. Here, evidence is provided that signaling through

CB2 can lead to the suppression of pIRF7, as evidenced by suppression of pIRF7 by JWH-133 and JWH-015. Further, IRF7 can be phosphorylated by TBK1 and pTBK1 induction by CpG-ODN is suppressed by THC, JWH-133, and JWH-015. These results are congruent with the literature [412, 413] and suggest that signaling through CB2 can suppress the phosphorylation of TBK1 and subsequent phosphorylation of IRF7. In addition, pIRF7 can also be phosphorylated by phosphoinositide-3-kinase (PI3K), a central kinase to many cell metabolic processes, which plays a key role in IFNα response [180]. Modulation of the AKT-PI3K-mTOR pathways by cannabinoids have already been found various cell models and suggested as a putative target of cannabinoid therapy in immune disorders [49, 50, 378, 414].

Similar to the results for the pathway leading to IFNα secretion, the phosphorylation of key intermediates for TNFα production, NFκB and IKKγ, were also suppressed by THC, JWH-133, and JWH-015. While the modulation of NFκB by cannabinoids is known [365], the suppression of IKKγ is a novel finding.. IKKγ is also a member of an activation complex which includes both TANK and TBK1 [177, 178]. Therefore, suppression of IKKγ may play a role in the suppression of other cytokines, including IFNα.

In this set of experiments, treatment with THC, a partial CBR agonist, resulted in greater suppression of NFκB and IKKγ phosphorylation than did treatment either JWH-133 and JWH-015, full CB2 agonists. While direct comparison between the compounds is not possible due to differences in the effective concentrations, the suppressive effect of the JWH compounds appeared to plateau at the concentrations used in studying the NFκB-associated signaling pathways but not the IRF7-associated pathways. This indicates that signaling through CB1, and other orphan cannabinoid receptors (e.g. GPR19, GPR18, GPR55), by THC may also play a role in suppressing immune cell activation via NFκB-modulation. The possibility of orphan-receptor involvement in immune modulation by cannabinoids has been previously suggested in the literature [412]. Overall, our results suggest that pDC function can be modulated by targeting key intermediates in the NFκB pathway and provides an additional target in IKKγ. These findings are also significant in that they provide support for CB2 as a therapeutic target since NFκB has a range of effects in cells [415] and the significant suppression by THC may cause unforeseen consequences on homeostatic processes in cells. This is further suggested by the studies conducted on AKT. Specifically, AKT plays a role in many cellular processes which are key to proper cell function and persistence[416]. Treatment with THC alone reduced the phosphorylation of the S473 residue, which was not induced by stimulation with CpG. The results from studying the effects of the tested cannabinoids on AKT phosphorylation indicate that THC suppresses the phosphorylation of a constitutively phosphorylated residue and suggest that prolonged exposure to a non-CB2-selecitve agonist, like THC, cause disruption and eventual death of immune cells instead of just a reduction in immune cell function.

From these studies, further evidence of the immunosuppressive effects of cannabinoids has been provided. In addition, the results from treatment with JWH-015 and JWH-133, CB2 specific agonists, have demonstrated the immunosuppressive potential of these specific synthetic-cannabinoids. While the anti-inflammatory potential of this class of compounds is known[136], these studies have demonstrated the suppression of two key inflammatory cytokines, IFNα and TNFα, by pDC. As mentioned, robust and chronic activation of pDC, and the secretion of IFNα and TNFα, have been found in several autoimmune diseases. Further, evidence is provided in supporting cannabinoid-dependent suppression of key signaling events for the induction of both IFNα and TNFα responses. These studies have elucidated key events within the CB2-related mechanism of cytokine suppression and revealed putative targets for future therapeutics. Collectively, our findings demonstrate the potential for CB2 targeted therapies for treatment of inflammatory conditions involving pDC as CB2 agonists can be potent anti-inflammatory compounds with minimal psychotropic activity.

III. The effects of THC on T cells from healthy and HIV+ donors

Presented here is the first report of THC-mediated suppression in response to IFNα by T cells from healthy and HIV-infected donors. Our goals were to investigate whether HIV infection affects the role of IFNα in maintaining peripheral T cell populations and to determine if cannabinoids can influence these processes. To address these goals, donors included in this study had no detectable HIV viral load, were not co-infected with any screened pathogen, did not utilize cannabinoids, and had comparable $CD4^+$ as well as $CD8^+$ T cell counts.

While the similarity of $CD4^+$ and $CD8^+$ T cell composition was critical for making comparisons between healthy and HIV+ donors, HIV infection is known to alter the number and function of certain immune cells [109, 110, 125, 201, 255, 302, 417]. Therefore, the responsiveness of resting T cells to IFNα, which is crucial to maintaining T cell homeostasis and is a critical mediator of antiviral responses was investigated. Specifically, IFNα-induced phosphorylation of STAT1, one of the most proximal biological events in response to ligation of the IFNAR2, differed between healthy and HIV+ $CD8^+$ T cells. Specifically, $CD8^+$ T cells from HIV+ donors were less responsive to IFNα as evidenced by reduced pSTAT1. Moreover, this difference was not observed in $CD4^+$ T cells even though HIV-derived $CD4^+$ T cells possess lower IFNAR2 expression than those from healthy donors. $CD8^+$ T cells from HIV+ donors also had lower pSTAT1 induction compared to $CD8^+$ cells from healthy donors despite having comparable IFNAR2 expression. These observations agree with previous findings which demonstrated that $CD4^+$ and $CD8^+$ T cells from HIV+ patients had differential responses to IFNα-mediated stimulation [418]. These data also indicate that $CD8^+$ T cells in HIV+ donors have a diminished response to IFNα-mediated activation while strengthening the link between the role of IFNα in directing CD4$^+$ T cells in viral infection [419].

The differential effects of IFNα in stimulating T cell subtypes is significant in HIV infection as IFNα plays a key role in maintaining activated T cell populations [381, 383, 420] and potentially synergizes with IL-7 stimulation in HIV+ donor derived T cells [302]. These data demonstrated that treatment with IFNα induces IL-7R expression and potentiates IL-7 signaling, as evidenced by augmented IL-7-induced pSTAT5, in cells treated with IFNα. Further, IL-7 drove robust proliferation of T cells treated with IFNα. These results partially agree with previous findings [421] and strengthen the link between IFNα, pDC number, and CD4$^+$ T cell number in HIV+ patients [108, 201, 418, 422]. Specifically, circulating pDC secrete IFNα which may play a role in sensitizing T cells to stimulation by IL-7.

HIV+ patients routinely utilize medicinal cannabinoids [104, 106, 423, 424]. Cannabis use reduces the efficacy of IFNα as a therapeutic [425]. Within healthy donors, the observed suppressive effect of THC on T cell activation by IFNα is mediated, at least in part, by decreased STAT1 phosphorylation. This observation agrees with previous work on IFNβ, which also binds IFNAR [412]. Likewise, THC also reduces the induction of IL-7Rα mRNA and protein expression, putatively mediated through the loss of both homo- and hetero-STAT-dimer formation and subsequent gene transcription. Additionally, THC significantly inhibits the effects of IL-7 on proliferation, likely through impairment of IL-7-induced STAT5 phosphorylation. Interestingly, THC had no effect on the IFNα-induced expression of IFNAR2, indicating that THC has a specific effect on the IFNα-IL-7 axis.

The most surprising finding of these studies was the reduced sensitivity of T cells from HIV patients to THC-mediated suppression. While initial suppression of IFNα-induced pSTAT1 showed similar trends in both healthy and HIV infected donors, later endpoints demonstrated reduced sensitivity to THC-mediated suppression in T cells from HIV-infected donors. This trend was most pronounced in CD4$^+$ T cells from HIV donors, especially with respect to proliferation. This finding, while unexpected, agrees with previous studies showing that CD4$^+$ T cell number was not affected in HIV+ patients using medicinal marijuana [105]. Conversely, CD8$^+$ T cells from HIV+ patients showed marked THC-mediated suppression of proliferation despite being less sensitive to THC-mediated impairment of other endpoints.

The limitations of these studies underlie possible reasons for the observed differences. First, the composition of the memory and non-memory cells could produce some of the differences in IFNα-mediated activation and sensitivity to THC. Memory T cells can be divided into central and effector memory and non-memory cells can be divided into naive and effector cells by using surface expression of CD62L [426]. Second, proliferation was induced by simulating a T cell receptor (TCR) like response using antibodies directed against CD3 and CD28, which differs from antigen-specific stimulation [427].

Most significantly, our studies were designed to limit the number of confounding factors by utilizing only male HIV+ patients with: a) CD4$^+$ T cell counts comparable with healthy donors; b) CD4:CD8 T cell ratios within the normal range (>1), c) no co-infection with any hepatitis strain; and d) no medicinal or current recreational cannabinoid use. While these parameters enabled a direct comparison with healthy donors, the profiles for

T cell activation presented in this article may vary significantly from other HIV+ patient populations. Specifically, our data does not address the effects of HIV infection in: a) female HIV+ patients, which have different immunological responses to HIV infection compared to men [112, 124, 280]; b) HIV patients treated successfully with ART without restoration of CD4$^+$ T cell counts, also called “immunologically discordant patients” [272, 428]; c) patients co-infected with a virus, since infections with Hepatitis C Virus can alter interferon responses and T cell activation [429, 430]; and d) in patients utilizing medicinal cannabinoids, since chronic THC exposure can lead to tolerance through various pharmacodynamic mechanisms [431], like receptor downregulation and decoupling. Presently, it is unclear if chronic cannabis use can make leukocytes tolerant to THC. Further studies will be required to characterize both acute and chronic effects of THC in these various patient populations including HIV+ patients.

These findings are the first to show a direct link between IFNα and IL-7-mediated augmentation of T cell proliferation. This work is also the first to show differences in the sensitivity to THC-mediated modulation of T cell stimulation from healthy and HIV-infected donors. The implications of this work are complex and multifaceted. Specifically, IFNα secretion by pDC from HIV+ donors is acutely sensitive to THC-mediated suppression [97] and elevated activation of pDC in women with HIV is linked to faster T cell depletion [124], which is associated with more severe neurocognitive deficiency [281]. Additionally, peripheral immune activation of CD8$^+$ T cells [432] and monocytes is tied to the development of HIV-associated neuroinflammation. Interestingly, HIV+ cannabis users have reduced inflammatory monocyte numbers [125] but the consequence of this reduced inflammatory population of monocytes on neurocognitive function is unknown.

Collectively, our findings imply that the use of cannabinoids by HIV+ patients undergoing ART treatment may be beneficial within the context of suppressing the activation of cells association with neural inflammation while maintaining $CD4^+$ T cells that are largely unaffected.

IV. The effects of THC on CD8$^+$ T cell-mediated activation of U251 astrocytes

HAND is a significant concern for many HIV patients in the era of ART. As HIV patients continue to live longer, healthier lives, a significant number have turned to using medicinal cannabinoids for the remediation of HIV-associated wasting [131] and neuropathic pain [424]. While recent publications have implicated a possible role in cannabinoid therapy for suppressing the activation of monocytes [125], which is associated with HAND development, it is not known if cannabinoid therapy has a direct effect on the neuroimmune interactions in chronically infected HIV patients. The studies presented in this dissertation have provided evidence for THC-mediated suppression of CD8$^+$ T cell-derived activation in a human astrocyte cell line (U251). These results are significant as they are the first to model the CD8$^+$ T cell-mediated activation of astrocytes using an *in vitro* system, which mimic the type of neuroimmune interactions seen in neuroinflammatory states like HAND [347, 353, 387].

In these studies, treatment with THC suppressed the effector function of CD8$^+$ T cells, which agrees with previous findings [386, 433]. Specifically, treatment with THC is known to suppress T cell effector function by inhibiting the secretion of IFNγ and IL-12, while upregulating TGFβ and IL-10 [95]. THC-mediated suppression of T cell effector function can proceed through either: a) inhibition of T cell activation, by pretreating with THC; or b) by direct suppression of differentiated effector cell function. To address at which point THC treatment can cause its impairment of CD8$^+$ T cell effector function, CD8$^+$ T cells were treated with THC at the time of CD3/28/IFNα stimulation, thereby acting on the initial activation, or after 72 hr after CD3/28/IFNα stimulation. Interestingly, THC reduced the levels of IFNγ secretion regardless of when cells are treated with THC, but the number of IFNγ$^+$ effector cells was not as affected when THC was added at 72 Hr. Collectively, THC treatment suppressed effector cell function. Furthermore, results from the aforementioned studies agree with T cell biology, such that differentiation of naïve into effector function causes global epigenetic changes which are not readily reversed [434]. Likewise, LAMP-1, an indicator of cytolytic degranulation by CD8$^+$ cells [248, 435], was decreased only when naïve CD8$^+$ T cells were treated with THC and prevented from developing into effector cells. Therefore, while LAMP-1 and IFNγ are both used as indicators of effector function [243, 435], they can be differentially modulated by drugs like THC. Surprisingly, THC had no effect on the secretion of TNFα by CD8$^+$ T cells in this system, further suggesting that THC does not cause pan suppression of all effector functions. This is an important finding as IFNγ and TNFα secreting cells are inflammatory in conditions like HAND. Specifically, IFNγ can induce astrocytes to secrete inflammatory factors like IP-10 and IL-6 [325, 333, 334, 343, 347, 350, 353, 383, 387, 432]. By contrast, cytolytic cells, those cells which release cytolytic granules and are likely LAMP-1$^+$, are considered protective in neuroinflammatory conditions [436, 437]. Thus, THC can act to suppress the activity of inflammatory CD8$^+$ T cells, which secrete IFNγ, while having minimal effect on the function of differentiated, cytolytic CD8$^+$ T cells.

The differential effect of THC on T cell effector function has implications for the consequences of CD8$^+$ T cell-mediated activation of astrocytes, as both TNFα and IFNγ can stimulate astrocytes [333, 334, 343, 387]. This study revealed that while MCP-1 was only induced by TNFα, IP-10 is primary induced by IFNγ, and both TNFα and IFNγ can induce IL-6. Furthermore, co-stimulation with TNFα and IFNγ synergistically augmented the IL-6 and IP-10 response while IFNγ had no effect on TNFα-induced MCP-1. This synergy of TNFα and IFNγ on the IP-10 response by astrocytes has been reported previously [388] and correlated with altered STAT1 and NFκB signaling [438].

The effect of THC on IFNγ secretion by $CD8^+$ T cells combined with the IFNγ-mediated stimulation of U251 astrocytes gave justification for testing a co-culture system. THC suppressed $CD8^+$ T cell-mediated induction of IL-6 and IP-10 responses in U251 astrocytes regardless of when it was added while having no effect on MCP-1. Results from the co-culture system agree with the data from studies using $CD8^+$ T cells in isolation and U251 cells in isolation. Specifically, MCP-1 is driven only by TNFα in astrocytes and TNFα secretion by $CD8^+$ T cells is not affected by treatment with THC. However, both $CD8^+$ T cell-induced IP-10 and IL-6 responses in U251 astrocytes were suppressed by treatment with THC.

The results from the coculture experiments demonstrated that THC can suppress $CD8^+$ T cell-mediated activation of U251 astrocytes, which has promising implications for the utilization of cannabinoids in treating $CD8^+$ T cell-mediated neuroinflammation. However, the system used in these studies allowed for two possible routes by which THC could affect U251 astrocyte stimulation by $CD8^+$ T cells: 1) by directly suppressing $CD8^+$ T cell effector function, thereby reducing U251 astrocyte activation; and 2) by suppressing U251 astrocyte stimulation by inflammatory cytokines, thereby having a direct effect on the astrocytes. The second possibility was plausible since astrocytes express cannabinoid and cannabinoid-like receptors [439, 440]. Furthermore, suppression by synthetic cannabinoids of inflammatory cytokines derived from astrocytes is known [441]. Therefore, studies were conducted to determine whether THC had a direct effect on TNFα and IFNγ stimulation of U251 astrocytes. Surprisingly, TNFα-induced MCP-1 production was insensitive to THC regardless of the mode of activation, which further confirmed the results from prior co-culture experiments.

The IP-10 response induced by IFNγ alone was sensitive to THC-mediated suppression while the IP-10 response induced via stimulation with TNFα and IFNγ in tandem was insensitive to THC. These data suggest that suppression of IP-10 in the CD8/U251 co-culture was likely mediated through suppression of $CD8^+$ T cells since the IP-10 response in U251 astrocytes is insensitive to THC-mediated abatement. Therefore, the significant reduction of the IFNγ response in $CD8^+$ T cells by THC treatment is likely the most substantial contributor to suppression of IP-10 response seen in the CD8/astrocyte co-culture experiments.

The IL-6 response in U251 astrocytes was sensitive to THC regardless of stimulation by TNFα alone, IFNγ alone, or with both concomitantly and likely accounts for the high degree of variability observed in the IL-6 response by astrocytes in the co-culture system. Interestingly, stimulation of the U251 astrocytes with IFNγ or TNFα alone did not completely mimic the IL-6 response induced in the co-culture system. This would suggest that there is another signal in the co-culture system that is lacking when the astrocytes are stimulated with recombinant TNFα and IFNγ. This unknown stimulus could be yet another cytokine or mediated through cell-cell contact. Further, we found that the $CD8^+$ T cells secrete a variety of cytokines in this system and it is possible that stimulation by those other cytokines may induce an IL-6 response by astrocytes. For example, IL-17 and IL-6 have been shown to act synergistically in driving IL-6 responses in astrocytes [442]. Therefore, the robust IL-6 response by astrocytes in the co-culture could have been the result of back stimulation of astrocyte-derived IL-6 synergizing with $CD8^+$ T cell-derived IL-17. By narrowing our focus to TNFα and IFNγ, other signals, which are present in the co-culture system, were likely excluded in these studies. Regardless of the underlying mechanism, the IL-6 response from the co-culture experiments were sensitive to THC-mediate suppression which supports the use of cannabinoids in suppressing the $CD8^+$ T cell-derived inflammatory response in astrocytes.

It is noteworthy that there are several limitations to the above studies. Specifically, the $CD8^+$ T cells were harvested from healthy donors. As seen in previous experiments, $CD8^+$ T cells from HIV+ donors differ in their composition following stimulation by CD3/CD28/IFNα. Therefore, the effector functions of $CD8^+$ T cells from HIV+ patients might vary between HIV+ patients and when compared to healthy donors. Furthermore, CD3/CD28/IFNα-mediated stimulation in absence of $CD4^+$ T cells and properly licensed antigen presenting cells is highly artificial. Specifically, CD3/CD28/IFNα-mediated stimulation of $CD8^+$ T cells does not fully recapitulate an antigen specific response [427] that would occur in HIV patients or others suffering from a neuroinflammatory condition [315, 435, 443]. Further, the astrocytes *in vivo* are an inherently heterogenous population of cells and U251 astrocytes are a glioma-derived cell line with known differences to primary astrocytes [444]. Interestingly, even fetal-derived primary astrocytes have significant limitations when comparing their responses to mature adult astrocyte populations[445]. Hence, the results generated by these experiments, or any astrocyte co-culture system, may not fully encapsulate the interactions between astrocytes and leukocytes *in vivo*. Finally, the process of $CD8^+$ T cell-astrocyte interactions has been modeled as unidirectional, such that $CD8^+$ T cells activate astrocytes. However, astrocytes can stimulate regulatory T cells to mitigate CNS inflammation [446] and may be stimulating the $CD8^+$ T cells *in vivo* to contribute to or abate inflammation.

Despite the limitations, these studies have revealed that THC can act on both U251 astrocytes and $CD8^+$ T cells to reduce both the secretion of cytokines by $CD8^+$ T cells and the response to those inflammatory cytokines by the U251 astrocytes. This observation agrees with the literature that both $CD8^+$ T cells and astrocytes are sensitive to cannabinoid-mediated suppression [386, 433, 441]. The studies presented here are the first step in understanding the role of $CD8^+$ T cells in neuroinflammation and offer support for cannabinoid-based therapeutics in mitigating neuroimmune inflammatory responses. Specifically, THC can suppress the inflammatory function of $CD8^+$ T cells without negatively impacting the role of cytolytic, protective, $CD8^+$ T cells. This suppression of IFNγ secretion leads to reduced stimulation of astrocytes and reduces the subsequent production of astrocyte-derived inflammatory factors. Lastly, THC can act directly on astrocytes to suppress the induction of inflammatory cytokine production. Collectively, these results suggest that cannabinoids, like THC, have potential in suppressing the peripheral stimulation of $CD8^+$ T cells and subsequent stimulation of astrocytes which is common in conditions like HAND.

V. Concluding remarks

The studies reported in this dissertation demonstrated the potent immunosuppressive effect of THC on the pDC-T cell-astrocyte axis which may play a role in conditions like HAND. Specifically, secretion of IFNα following endosomal TLR activation in pDC is acutely sensitive to suppression by THC and this phenomenon is likely mediated through inhibition of IRF7 phosphorylation. Further, THC reduces TNFα and CD83 expression by pDC, likely through reduced NFκB signaling, which both influence T cell activation [368]. Interestingly, pDC from HIV+ donors were more sensitive to THC-mediated suppression compared to those from healthy donors. This result is likely due to augmented CB1 expression in PBMC from HIV+ donors, although differences in CB1/CB2 expression between immune cell types could not be determined.

While the influence of blocking CD83 has already been demonstrated by Pinho and colleagues in 2014 [368], the literature surrounding the role of IFNα on T cell activation during HIV infection contains many conflicting studies. Specifically, IFNα has been shown to be both protective against HIV-mediated depletion of $CD4^+$ T cells [108] while also driving T cells towards exhaustion[302]. IFNα can also induce expression IL-7R [215]. IL-7 is a key homeostatic T cell cytokine [287], but T cells from HIV+ patients lose responsiveness to IL-7 not IFNα as HIV infection progresses [286]. Furthermore, IFNα has been shown to interfere with IL-7-induced T cell proliferation [421] while also augmenting T cell proliferation during viral infection[382]. Despite the retained sensitivity of $CD4^+$ T cells to IFNα during HIV infection and the ability for IFNα to drive IL-7R expression, loss of IL-7R is a hallmark of T cell exhaustion [285]. The loss of IL-7R in patients suffering from chronic HIV infection may be protective since IL-7 is a potent inducer of latent HIV infection [288]. However, treatment with IL-7 has been found to reduce T cell leukocytopenia in HIV+ patients and improve their prognosis [289, 291]. In summary, there is no consensus on the effects of IFNα on T cells in HIV+ patients or how stimulation via IFNα and IL-7 intersect in the regulation of T cell health.

To address the possible role of IFNα on the health of T cells from HIV+ patients, studies were performed to determine the effect of treatment with IFNα and IL-7 on T cell activation and CD3/CD28-induced T cell proliferation. Treatment with IFNα induced IL-7R expression and augmented the response of T cells to IL-7. Furthermore, concurrent stimulation with IFNα and anti-CD3/CD28 antibodies, followed by addition of IL-7, significantly augmented the proliferation of T cells compared to just CD3/CD28 and IL-7 alone. When considering the sequence of events during T cell activation, these results are not surprising. Specifically, IFNα is an acute-phase cytokine that would be present during the initiation of viral infection, as evidenced by the rapid (6 hr) production of IFNα by pDC following endosomal TLR stimulation. Furthermore, T cells need to be stimulated at the immunological synapse with three signals to be fully differentiated: 1) antigen, 2) costimulatory molecules, and 3) cytokines. The IFNα/CD3/CD28-mediated activation used in these studies simulated this type of stimulation. Following antigen exposure, T cells would then be exposed to IL-7 secreted by dendritic cells in lymphoid tissue [287]. Furthermore, there is precedent for this assertion as a recent publication demonstrated the synergy of IL-7 and pDC function on T cell activation [305]. Collectively, these studies have advanced our understanding IFNα and IL-7 on T cell activation. Further, these studies have revealed one way by which pDC may influence the restoration of T cell numbers during HIV infection and provide some explanation underlying the correlation of pDC number and function with CD4$^+$ T cell number in HIV and HIV/HCV coinfected patients [195]. Specifically, pDC secretion of IFNα is reduced during chronic viral infection due to chronic stimulation. This reduced IFNα response results in loss of IFNα mediated stimulation of T cells and subsequent loss of IFNα-induced IL-7R expression. The loss of IFNα-induced IL-7R expression results in diminished proliferation response by T cells.

With a greater understanding of IFNα and IL-7-mediated activation of T cells, the effect of THC on this system was investigated. These series of experiments revealed that treatment with THC suppressed the activation and proliferation of T cells by suppressing the phosphorylation of STAT1 downstream of IFNAR activation and STAT5 downstream of IL-7R activation. Interestingly, these studies also revealed that T cells from HIV+ donors were less sensitive to suppression by THC when compared to T cells from healthy donors. This finding was surprising as it was the opposite of what was found in pDC. Moreover, these experiments revealed that despite reduced sensitivity for most of the endpoints in both CD4$^+$ and CD8$^+$ T cells from HIV+ donors when compared to cells from healthy donors, CD8$^+$ T cells from healthy and HIV+ donors retained equivalent sensitivity to THC-mediated inhibition of proliferation. This difference between CD4$^+$ and CD8$^+$ T cells likely rests with the differences in cannabinoid receptor expression. Specifically, CD8$^+$ T cells have higher expression of CB2 than CD4$^+$ T cells [23, 57, 58] and could be more sensitive to the immunosuppressive effects of THC. Furthermore, CD8$^+$ T cells may also express more of the orphan cannabinoid receptors, but there are no studies to confirm this possibility. Further, inflammatory cytokines can induce the expression of CB1 and CB2 on immune cells [64]. As CD8$^+$ T cells are acutely sensitive to IFNα-mediated stimulation, it is possible that treatment with IFNα differentially induced the expression of

CB receptors on $CD4^+$ and $CD8^+$ T cells resulting in increased sensitivity to THC in $CD8^+$ T cells from HIV+ donors. However, these studies were conducted with very restricted access to HIV+ samples and this possibility could not be addressed.

Proliferation is one aspect of the T cell response, secretion of cytokines is another. Previous research has already demonstrated that THC can impair T cell effector function [386]. However, it was not clear if THC inhibited the differentiation of naïve T cells into effector cells or if THC directly suppressed the function of differentiated effector T cells. To delineate between these two possibilities, T cells were treated with THC either at the time of or after 72 hr post IFNα/CD3/CD28 stimulation. These studies revealed that while treatment with THC partially ablated $CD8^+$ T cells effector functions, it did not suppress all effector functions equally. Specifically, THC suppressed LAMP-1 surface expression, an indicator of cytolytic degranulation, only when THC was added concurrently with CD3/CD28/IFNα stimulation. Conversely, levels of IFNγ secretion could be suppressed with THC regardless of when THC was added, but the number of IFNγ secreting $CD8^+$ T cells could only be suppressed if THC was added during initial stimulation. Interestingly, TNFα secretion by these $CD8^+$ T cells was insensitive to THC-mediated suppression. These results demonstrated that the suppressive effect of THC is selective and does not cause “pan-suppression” of effector function. Collectively, these results indicated that some of the inflammatory effects of $CD8^+$ T cells may be preferentially suppressed by THC while not affecting all cytolytic function, as both degranulation and TNFα can lead to target-cell death[244, 436, 437].

HIV+ donors routinely utilize medicinal cannabinoids [10] and suffer from HAND [404]. HAND development is complex and involves both peripheral immune cells and glia

[387, 447, 448]. One of the glial cells which plays a significant role in neural immune activation is the astrocyte [334, 347, 387]. Astrocytes are sensitive to IFNγ and TNFα[334, 387], two of the major cytokines secreted by effector CD8^{+} T cells. To investigate the activation of astrocytes by CD8^{+} T cells and the effects of THC on astrocyte activation, CD8^{+} T cells and astrocytes were cocultured and astrocytes were stimulated with recombinant TNFα and IFNγ. These studies revealed that IFNγ and TNFα cooperatively induce IP-10 and IL-6 responses in U251 astrocytes while MCP-1 was driven by TNFα alone. Likewise, CD8^{+} T cell coculture with U251 astrocytes resulted in induction of IP-10, MCP-1, and IL-6. THC treatment suppressed both IL-6 and IP-10, both in coculture and when astrocytes were treated with exogenous TNFα and IFNγ but had no effect on MCP-1. Interestingly, the IL-6 response was diminished when astrocytes were stimulated with TNFα and IFNγ alone, indicating another factor may play a role eliciting the IL-6 response during co-culture. Regardless of the degree of activation, the results generated from these studies agreed with the CD8^{+} T cell effector function assay such that THC did not suppress the CD8^{+} T cell secretion of TNFα, the principal driver of MCP-1 in U251 astrocytes but did suppress secretion of IFNγ. Collectively, these results suggest that THC can suppress CD8^{+} T cell function and astrocyte activation directly.

CD8^{+} T cells secreting IFNy are considered inflammatory and have been implicated in HAND, while cytolytic CD8^{+} T cells are viewed as protective [436, 437]. Therefore, these results indicate that cannabinoids may suppress the type of effector function that causes inflammation while having minimal effect on the beneficial aspects of effector function. This is especially pertinent when considering cannabinoid treatment in HIV patients. Specifically, CD8^{+} T cells from HIV+ donors have already been stimulated to adopt an anti-viral response comprised of IFNγ secretion and cytolytic granule release. The present results show that once CD8$^+$ T cell effector function is set, the IFNγ response can be reduced by treatment with THC while the release of cytolytic granules, as evidenced by LAMP-1 expression, is insensitive to THC-mediated suppression. Therefore, treatment with THC, or other cannabinoids, may foster the protective, cytolytic, functions of CD8$^+$ T cells while reducing inflammation associated with IFNγ secretion. Beyond HAND, these results have implications for cannabinoid-based therapeutics in the treatment of neuroimmune activation in other neuroinflammatory conditions. For example, multiple sclerosis is a debilitating neuroinflammatory autoimmune disease [137] which presents with augmented pDC activity [405], elevated CD8$^+$ T cell activation [449], and neurocognitive deficits [450].

The studies in this dissertation provide evidence that THC can modulate the pDC-T cell-astrocyte axis (Figure 46). THC can suppress pDC-mediated stimulation of T cells by: 1) directly suppressing the TLR9-mediated induction of IFNα and CD83 expression in pDC; 2) directly suppressing T cell activation by IFNα; and 3) by suppressing IL-7-mediated stimulation through either suppressed receptor expression and/or suppressed STAT5 signaling. Next, THC can inhibit CD8$^+$ T cell activation of astrocytes by: 1) suppressing the secretion of IFNγ by differentiated effector T cells; and 2) directly suppressing astrocyte activation by IFNγ and TNFα (Figure 48).

While great strides have been made in characterizing an aspect of the pDC-T cell interaction, there are nuances to pDC and T cell activation and sensitivity to cannabinoids which have not fully addressed in the studies presented here. Specifically, the underlying reason for the differential sensitivity of pDC and T cells to THC when comparing between healthy and HIV+ donors and between T cell populations has not been explained by these studies. In addition, to remove confounding factors, only patients mono-infected with HIV+ were utilized and many HIV+ patients are coinfected with HCV, or other viruses, which can perturb pDC function [429, 430, 451]. Furthermore, HIV patients vary in their immunological response by sex [112, 124], restoration of $CD4^+$ T cells following ART [254, 261, 272, 428], and drug use (both ART [452] and illicit [453]) which are factors that were not included in these studies. The impact of these various factors on chronic HIV infection and immune function need to be addressed in the years to come.

The studies described in this dissertation have led to novel findings concerning cannabinoid receptor expression during HIV infection and cannabinoid-receptor mediated modulation of immune responses, but they have also led to new questions. Specifically, CpG-ODN mediated activation of pDC can be suppressed using CB2-selective agonists. These results suggest the involvement of CB1 and, possibly, orphan cannabinoid receptors in the suppression of pDC activation by THC. Further studies are still needed to elucidate the contribution of both CB1 and, at least, five proposed orphan cannabinoid receptors (GPR19, GPR18, GPR55, VR1, and TRPV1) in immune cell modulation. Likewise, while these studies have contributed to the field of cannabinoid-immunotoxicology, they have been focused on THC and only two CB2-selective agonists, JWH-015 and JWH-133. There are 67 known phytocannabinoid congeners in *Cannabis sativa* and hundreds of synthetic cannabinoids. Collectively, understanding how these receptors and cannabinoids interact to impact various immune cell populations is a concern which researchers will continue to face for years to come. Investigating various combinations of cannabinoid strategies could yield new therapeutic compounds, new therapeutic targets, and an ever-greater understanding of how cannabinoids affect immune cell function.

While there are limitations to the studies presented in this dissertation, they have shown that there may be a place for cannabinoid use by HIV patients and have elucidated mechanisms by which cannabinoids can suppress aberrant immune function. Specifically, in HIV patients with ART-controlled infection, cannabinoids may help suppress the peripheral activation of immune cells, a process which is related to inflammation, HAND, and T cell exhaustion. A recent publication indicated that cannabis use in HIV patients lead to reduced circulating inflammatory immune cells [452]. Furthermore, THC can suppress the IFNα-mediated transition of resting monocytes into inflammatory monocytes, a process found in HIV patients and associated with HAND. Lastly, cannabis using HIV patients have already been shown to have fewer inflammatory monocytes than non-cannabis using HIV+ patients [125].

While the activation of immune cells in HIV patients were the focus of these studies, elevated pDC activation and inflammatory leukocytes are also found in patients with autoimmune disorders like arthritis [454, 455] and lupus [116, 122, 307]. While cannabis has been suggested for use in autoimmune disorders, use of medical marijuana for the remediation of these conditions is not widely accepted. Clearly, the public opinion concerning therapeutic application of cannabinoids is slowly changing. On May 4th, 2018, a state board of Michigan finally recommended the use of cannabis for the treatment of rheumatoid arthritis, a full 2000 years after the Chinese used cannabis for the same purpose. Despite growing acceptance, the use of medical marijuana still poses legal troubles. In such cases, the non-DEA controlled CB2-selective agonists present an alternative immunosuppressive agent. As evidenced by the results shown, CB2-selective agonists have the potential to suppress IFNα and TNFα responses in pDC, two key cytokines in several autoimmune disorders [115, 190, 246].

Collectively, the studies presented here suggest cannabinoids have potential in treating chronic inflammation from infectious diseases and autoimmune disorders. If the data presented in this dissertation have shown anything, it is that the disease state can alter immune responses and sensitivity to cannabinoid-mediated suppression. Therefore, further research needs to be conducted in patients suffering from autoimmune disorders to determine the efficacy of cannabinoid-based therapies in remediating their inflammatory conditions.

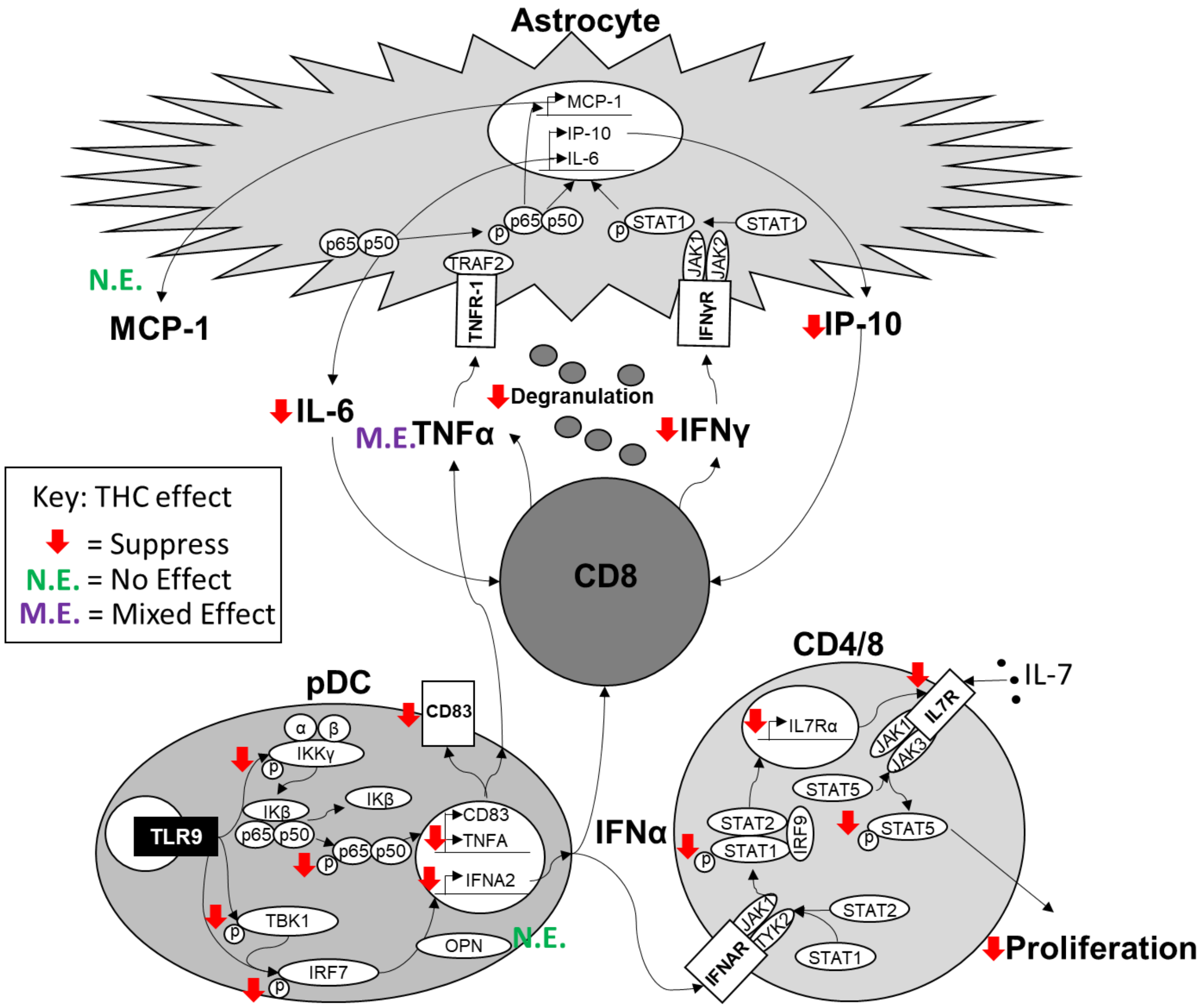

Figure 48. Schematic diagram summarizing the possible mechanisms by which THC inhibits pDC-mediated stimulation of T cells and subsequent activation of astrocytes. THC directly suppressed the activation of pDC by CpG-ODN via suppressing the phosphorylation of IKKγ, NFκB, TBK1, and IRF7, likely indicating inhibited initiation of TLR9 signaling. THC also suppression the IFNα-mediated activation of CD4$^+$ and CD8$^+$ T cells via suppression of IFNα-induced pSTAT1, IL-7-induced pSTAT5, and proliferation. Furthermore, THC suppressed IFNγ and degranulation of activated CD8$^+$ T cells. Lastly, THC suppressed astrocyte activation and secretion of inflammatory cytokines by suppressing both the secretion of IFNγ by CD8$^+$ T cells and the direct stimulation of astrocytes by TNFα and IFNγ.

APPENDICES

APPENDIX A. Antibodies

TABLE 1. List of antibodies used in this dissertation

Target	Fluorophore	Clone	Host	Reactivity	Supplier
CD3	N/A	HIT3a	Mouse	Human	BioLegend
CD3	PerCP/Cy5.5	OKT3	Mouse	Human	BioLegend
CD4	Brilliant Violet 510	SK3	Mouse	Human	BioLegend
CD8	APC/Fire™ 750	SK1	Mouse	Human	BioLegend
CD28	N/A	CD28.2	Mouse	Human	BioLegend
CD45RO	Alexa Fluor® 488	UCHL1	Mouse	Human	BioLegend
CD45RO	PE/Cy7	UCHL1	Mouse	Human	BioLegend
CD83	Alexa Fluor® 488	HB15e	Mouse	Human	BioLegend
LAMP-1	Brilliant Violet 510	H4A3	Mouse	Human	BioLegend
CD123	Brilliant Violet 421	6H6	Mouse	Human	BioLegend
CD123	VioBlue	AC145	Mouse	Human	Miltenyi Biotec
IL-7Rα	PE	A019D5	Mouse	Human	BioLegend
CD303	APC	REA693	Mouse	Human	Miltenyi Biotec
CD303	PE	REA693	Mouse	Human	Miltenyi Biotec
IFNα	PE	REA1013	Mouse	Human	Miltenyi Biotec
IFNγ	PE	B27	Mouse	Human	BioLegend
TNFα	PE	MAb11	Mouse	Human	BioLegend
TNFα	APC	MAb11	Mouse	Human	BioLegend
MCP-1	PE	5D3-F7	Mouse	Human	BioLegend

Table 1 Continued

IP-10	PerCP-eFluor 710	4NY8UN	Mouse	Human	ThermoFIsher
IL-6	APC	MQ2-13A5	Mouse	Human	BioLegend
pTBK1	PE	J133-587	Mouse	Human	BD Biosciences
pIRF7	PE	K40-321	Mouse	Human	BD Biosciences
pIKKy	PE	N19-39	Mouse	Human	BD Biosciences
pNFκB	PE	K10-895	Mouse	Human	BD Biosciences
pSTAT1	PE	4a	Mouse	Human	BD Biosciences
pSTAT5	PE	47/Stat5(pY694)	Mouse	Human	BD Biosciences

APPENDIX B. Kits

TABLE 2. List of kits used in this dissertation

Kit Name	Target	Supplier	Cat #
IFNα-secretion assay	Human	Miltenyi Biotec	130-049-161
Diamond pDC isolation kit II	Human	Miltenyi Biotec	130-097-240
Pan T cell Isolation Kit	Human	Miltenyi Biotec	130-096-535
MojoSort™ CD3 T cell isolation kit	Human	BioLegend	480022
MojoSort™ CD8 T cell isolation kit	Human	BioLegend	480065
LegendPlex™ Custom IFNα2, TNFα, IL-6 panel	Human	BioLegend	----
LegendPlex™ CD8/NK panel	Human	BioLegend	740267
PrimeFlow™ RNA assay kit	Human	ThermoFisher	88-18005-210
Cell-Trace™ Violet Proliferation Kit	----	Invitrogen™	C34571

APPENDIX C. Gene expression primers and probes

TABLE 3: List of primers and probes used to measure gene expression in this dissertation

Assay	Gene	Catalogue Number	RefSeq	Reporter
TaqMan	18s	4319413E	X03205.1	VIC-MGB
TaqMan	CNR1	Hs00275634_m1	NM_016083.4	FAM-MGB
TaqMan	CNR2	Hs00275635_m1	NM_001841.2	FAM-MGB
TaqMan	IL-7RA	Hs00902334_m1	NM_002185.3	FAM-MGB
PrimeFlow	IFNA2	VA1-12800-PF	NM_000605.3	Type 1 (AF647)
PrimeFlow	IFNA2	VA1-12800-PF	NM_000605.3	Type 4 (AF488)
PrimeFlow	TNFA2	VA1-10481-PF	NM_000594.3	Type 1 (AF647)

BIBLIOGRAPHY

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1. Lu X, Clarke RC. The cultivation and use of hemp (Cannabis sativa L.) in ancient China. *Journal of the International Hemp Association* 1995; 2(1):26-30.

2. Russo E. Cannabis in India: ancient lore and modern medicine. In: *Cannabinoids as therapeutics*: Springer; 2005. pp. 1-22.

3. Russo EB. History of cannabis and its preparations in saga, science, and sobriquet. *Chemistry & biodiversity* 2007; 4(8):1614-1648.

4. Bonnie RJ, Whitebread CH. The marihuana conviction: A history of marihuana prohibition in the United States. University Press of Virginia Charlottesville; 1974.

5. Stolberg V. Comprehensive Drug Abuse Prevention and Control Act. *Encyclopedia of substance abuse prevention, treatment, and recovery* 2009:224-225.

6. Kilmer B, Caulkins JP, Pacula RL, MacCoun RJ, Reuter P. Altered state?: assessing how marijuana legalization in California could influence marijuana consumption and public budgets. RAND Santa Monica, CA; 2010.

7. Model KE. The effect of marijuana decriminalization on hospital emergency room drug episodes: 1975–1978. *Journal of the American Statistical Association* 1993; 88(423):737-747.

8. Chadi N, Weitzman ER, Levy S. Understanding the Impact of National and State Medical Marijuana Policies on Adolescents. *Current Addiction Reports* 2018:1-9.

9. Doblin RE, Kleiman M. Marijuana as antiemetic medicine: a survey of oncologists' experiences and attitudes. *Journal of Clinical Oncology* 1991; 9(7):1314-1319.

10. Riggs PK, Vaida F, Rossi SS, Sorkin LS, Gouaux B, Grant I, et al. A pilot study of the effects of cannabis on appetite hormones in HIV-infected adult men. *Brain research* 2012; 1431:46-52.

11. Noyes R, Brunk S, Baram DA, Canter A. Analgesic Effect of Delta-9-Tetrahydrocannabinol. *The Journal of Clinical Pharmacology* 1975; 15(2-3):139-143.

12. Maa E, Figi P. The case for medical marijuana in epilepsy. *Epilepsia* 2014; 55(6):783-786.

13. Blake D, Robson P, Ho M, Jubb R, McCabe C. Preliminary assessment of the efficacy, tolerability and safety of a cannabis-based medicine (Sativex) in the treatment of pain caused by rheumatoid arthritis. *Rheumatology* 2006; 45(1):50-52.

14. Malfait A, Gallily R, Sumariwalla P, Malik A, Andreakos E, Mechoulam R, et al. The nonpsychoactive cannabis constituent cannabidiol is an oral anti-arthritic therapeutic in murine collagen-induced arthritis. *Proceedings of the National Academy of Sciences* 2000; 97(17):9561-9566.

15. Flach AJ. Delta-9-tetrahydrocannabinol (THC) in the treatment of end-stage open-angle glaucoma. *Transactions of the American Ophthalmological Society* 2002; 100:215.

16. Wei D, Dinh D, Lee D, Li D, Anguren A, Moreno-Sanz G, et al. Enhancement of anandamide-mediated endocannabinoid signaling corrects autism-related social impairment. *Cannabis and cannabinoid research* 2016; 1(1):81-89.

17. Kurz R, Blaas K. Use of dronabinol (delta-9-THC) in autism: a prospective single-case-study with an early infantile autistic child. *Cannabinoids* 2010; 5(4):4-6.

18. Englund A, Morrison PD, Nottage J, Hague D, Kane F, Bonaccorso S, et al. Cannabidiol inhibits THC-elicited paranoid symptoms and hippocampal-dependent memory impairment. *Journal of Psychopharmacology* 2013; 27(1):19-27.

19. Thacore VR, Shukla S. Cannabis psychosis and paranoid schizophrenia. *Archives of General Psychiatry* 1976; 33(3):383-386.

20. Bhargava HN. The effects of thyrotropin releasing hormone and histidyl-proline diketopiperazine on delta-9-tetrahydrocannabinol-induced hypothermia. *Life sciences* 1980; 26(11):845-850.

21. Hampson RE, Deadwyler SA. Cannabinoids, hippocampal function and memory. *Life sciences* 1999; 65(6-7):715-723.

22. Arseneault L, Cannon M, Poulton R, Murray R, Caspi A, Moffitt TE. Cannabis use in adolescence and risk for adult psychosis: longitudinal prospective study. *Bmj* 2002; 325(7374):1212-1213.

23. Pertwee RG. Cannabinoid pharmacology: the first 66 years. *British journal of pharmacology* 2006; 147(S1).

24. Pertwee RG. Pharmacology of cannabinoid receptor ligands. *Current medicinal chemistry* 1999; 6:635-664.

25. Dewey WL. Cannabinoid pharmacology. *Pharmacological reviews* 1986; 38(2):151-178.

26. Brenneisen R. Chemistry and analysis of phytocannabinoids and other Cannabis constituents. In: *Marijuana and the Cannabinoids*: Springer; 2007. pp. 17-49.

27. Seely KA, Lapoint J, Moran JH, Fattore L. Spice drugs are more than harmless herbal blends: a review of the pharmacology and toxicology of synthetic cannabinoids. *Progress in Neuro-psychopharmacology and biological psychiatry* 2012; 39(2):234-243.

28. Di Marzo V, Melck D, Bisogno T, De Petrocellis L. Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends in neurosciences* 1998; 21(12):521-528.

29. Howlett A, Barth F, Bonner T, Cabral G, Casellas P, Devane W, et al. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacological reviews* 2002; 54(2):161-202.

30. Raduner S, Majewska A, Chen J-Z, Xie X-Q, Hamon J, Faller B, et al. Alkylamides from Echinacea are a new class of cannabinomimetics Cannabinoid type 2 receptor-dependent and-independent immunomodulatory effects. *Journal of Biological Chemistry* 2006; 281(20):14192-14206.

31. Spelman K. The Pharmacodynamics, Pharmacokinetics and Clinical Use of Echinacea purpurea.

32. Gertsch J. Anti-inflammatory cannabinoids in diet. *Commun Integr Biol* 2008; 1(1):26-28.

33. Gertsch J, Schoop R, Kuenzle U, Suter A. Echinacea alkylamides modulate TNF-α gene expression via cannabinoid receptor CB2 and multiple signal transduction pathways. *FEBS letters* 2004; 577(3):563-569.

34. Jia W, Meng X, Qian Z, Hua Z, Li T, Liu C. Identification of three cannabimimetic indazole and pyrazole derivatives, APINACA 2H-indazole analogue, AMPPPCA, and 5F-AMPPPCA. *Drug testing and analysis* 2017; 9(2):248-255.

35. Castaneto MS, Gorelick DA, Desrosiers NA, Hartman RL, Pirard S, Huestis MA. Synthetic cannabinoids: epidemiology, pharmacodynamics, and clinical implications. *Drug & Alcohol Dependence* 2014; 144:12-41.

36. Blankman JL, Simon GM, Cravatt BF. A comprehensive profile of brain enzymes that hydrolyze the endocannabinoid 2-arachidonoylglycerol. *Chemistry & biology* 2007; 14(12):1347-1356.

37. Okamoto Y, Wang J, Morishita J, Ueda N. Biosynthetic pathways of the endocannabinoid anandamide. *Chemistry & biodiversity* 2007; 4(8):1842-1857.

38. Johansson E, AGURELL S, HOLLISTER LE, HALLDIN MM. Prolonged apparent half-life of Δ1-tetrahydrocannabinol in plasma of chronic marijuana users. *Journal of Pharmacy and Pharmacology* 1988; 40(5):374-375.

39. Cravatt BF, Demarest K, Patricelli MP, Bracey MH, Giang DK, Martin BR, et al. Supersensitivity to anandamide and enhanced endogenous cannabinoid signaling in mice lacking fatty acid amide hydrolase. *Proceedings of the National Academy of Sciences* 2001; 98(16):9371-9376.

40. Park B, Gibbons H, Mitchell M, Glass M. Identification of the CB1 cannabinoid receptor and fatty acid amide hydrolase (FAAH) in the human placenta. *Placenta* 2003; 24(10):990-995.

41. Kita Y, Yoshida K, Tokuoka SM, Hamano F, Yamazaki M, Sakimura K, et al. Fever is mediated by conversion of endocannabinoid 2-arachidonoylglycerol to prostaglandin E2. *PloS one* 2015; 10(7):e0133663.

42. Tanimura A, Yamazaki M, Hashimotodani Y, Uchigashima M, Kawata S, Abe M, et al. The endocannabinoid 2-arachidonoylglycerol produced by diacylglycerol lipase α mediates retrograde suppression of synaptic transmission. *Neuron* 2010; 65(3):320-327.

43. Kim J, Alger BE. Inhibition of cyclooxygenase-2 potentiates retrograde endocannabinoid effects in hippocampus. *Nature neuroscience* 2004; 7(7):697.

44. Melis M, Pistis M, Perra S, Muntoni AL, Pillolla G, Gessa GL. Endocannabinoids mediate presynaptic inhibition of glutamatergic transmission in rat ventral tegmental area dopamine neurons through activation of CB1 receptors. *Journal of Neuroscience* 2004; 24(1):53-62.

45. Mo FM, Offertáler L, Kunos G. Atypical cannabinoid stimulates endothelial cell migration via a Gi/Go-coupled receptor distinct from CB1, CB2 or EDG-1. *European journal of pharmacology* 2004; 489(1-2):21-27.

46. Bayewitch M, Avidor-Reiss T, Levy R, Barg J, Mechoulam R, Vogel Z. The peripheral cannabinoid receptor: adenylate cyclase inhibition and G protein coupling. *FEBS letters* 1995; 375(1-2):143-147.

47. Moranta D, Esteban S, García-Sevilla JA. Acute, chronic and withdrawal effects of the cannabinoid receptor agonist WIN55212-2 on the sequential activation of MAPK/Raf-MEK-ERK signaling in the rat cerebral frontal cortex: Short-term regulation by intrinsic and extrinsic pathways. *Journal of neuroscience research* 2007; 85(3):656-667.

48. Howlett A. Cannabinoid receptor signaling. In: *Cannabinoids*: Springer; 2005. pp. 53-79.

49. Ellert-Miklaszewska A, Kaminska B, Konarska L. Cannabinoids down-regulate PI3K/Akt and Erk signalling pathways and activate proapoptotic function of Bad protein. *Cellular signalling* 2005; 17(1):25-37.

50. Gomez O, Sanchez-Rodriguez A, Le M, Sanchez-Caro C, Molina-Holgado F, Molina-Holgado E. Cannabinoid receptor agonists modulate oligodendrocyte differentiation by activating PI3K/Akt and the mammalian target of rapamycin (mTOR) pathways. *British journal of pharmacology* 2011; 163(7):1520-1532.

51. Twitchell W, Brown S, Mackie K. Cannabinoids inhibit N-and P/Q-type calcium channels in cultured rat hippocampal neurons. *Journal of neurophysiology* 1997; 78(1):43-50.

52. Lauckner JE, Hille B, Mackie K. The cannabinoid agonist WIN55, 212-2 increases intracellular calcium via CB1 receptor coupling to Gq/11 G proteins. *Proceedings of the National Academy of Sciences of the United States of America* 2005; 102(52):19144-19149.

53. Li R-J, Xu J, Fu C, Zhang J, Zheng YG, Jia H, et al. Regulation of mTORC1 by lysosomal calcium and calmodulin. *Elife* 2016; 5.

54. Domenici MR, Azad SC, Marsicano G, Schierloh A, Wotjak CT, Dodt H-U, et al. Cannabinoid receptor type 1 located on presynaptic terminals of principal neurons in the forebrain controls glutamatergic synaptic transmission. *Journal of Neuroscience* 2006; 26(21):5794-5799.

55. Seagar M, Lévêque C, Charvin N, Marquèze B, Martin–Moutot N, Boudier JA, et al. Interactions between proteins implicated in exocytosis and voltage–gated calcium channels. *Philosophical Transactions of the Royal Society B: Biological Sciences* 1999; 354(1381):289-297.

56. Naderi N, Ahmad-Molaei L, Mazar-Atabaki A, Ronaghi A, Shirazi-zand Z, Motiei-Langroudi SM, et al. L-type calcium channel mediates anticonvulsant effect of cannabinoids in acute and chronic murine models of seizure. *Neurochemical research* 2012; 37(2):279-287.

57. Pertwee R. The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Δ9-tetrahydrocannabinol, cannabidiol and Δ9-tetrahydrocannabivarin. *British journal of pharmacology* 2008; 153(2):199-215.

58. Galiegue S, Mary S, Marchand J, Dussossoy D, Carriere D, Carayon P, et al. Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *The FEBS Journal* 1995; 232(1):54-61.

59. Buchweitz JP, Karmaus PW, Williams KJ, Harkema JR, Kaminski NE. Targeted deletion of cannabinoid receptors CB1 and CB2 produced enhanced inflammatory responses to influenza A/PR/8/34 in the absence and presence of Δ9-tetrahydrocannabinol. *Journal of leukocyte biology* 2008; 83(3):785-796.

60. Cabral GA, Pettit DAD. Drugs and immunity: cannabinoids and their role in decreased resistance to infectious disease. *Journal of neuroimmunology* 1998; 83(1):116-123.

61. Croxford JL, Yamamura T. Cannabinoids and the immune system: potential for the treatment of inflammatory diseases? *Journal of neuroimmunology* 2005; 166(1):3-18.

62. Maestroni GJ. The endogenous cannabinoid 2-arachidonoyl glycerol as in vivo chemoattractant for dendritic cells and adjuvant for Th1 response to a soluble protein. *The FASEB journal* 2004; 18(15):1914-1916.

63. Ehrhart J, Obregon D, Mori T, Hou H, Sun N, Bai Y, et al. Stimulation of cannabinoid receptor 2 (CB 2) suppresses microglial activation. *Journal of neuroinflammation* 2005; 2(1):29.

64. Jean-Gilles L, Braitch M, Latif ML, Aram J, Fahey AJ, Edwards LJ, et al. Effects of pro-inflammatory cytokines on cannabinoid CB1 and CB2 receptors in immune cells. *Acta Physiologica* 2015; 214(1):63-74.

65. Chioccarelli T, Cacciola G, Altucci L, Lewis SE, Simon L, Ricci G, et al. Cannabinoid receptor 1 influences chromatin remodeling in mouse spermatids by affecting content of transition protein 2 mRNA and histone displacement. *Endocrinology* 2010; 151(10):5017-5029.

66. Idris AI, van't Hof RJ, Greig IR, Ridge SA, Baker D, Ross RA, et al. Regulation of bone mass, bone loss and osteoclast activity by cannabinoid receptors. *Nature medicine* 2005; 11(7):774.

67. Morgese MG, Cassano T, Cuomo V, Giuffrida A. Anti-dyskinetic effects of cannabinoids in a rat model of Parkinson's disease: role of CB1 and TRPV1 receptors. *Experimental neurology* 2007; 208(1):110-119.

68. Zygmunt PM, Petersson J, Andersson DA, Chuang H-h, Sørgård M, Di Marzo V, et al. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature* 1999; 400(6743):452.

69. Ryberg E, Larsson N, Sjögren S, Hjorth S, Hermansson NO, Leonova J, et al. The orphan receptor GPR55 is a novel cannabinoid receptor. *British journal of pharmacology* 2007; 152(7):1092-1101.

70. Console-Bram L, Brailoiu E, Brailoiu GC, Sharir H, Abood ME. Activation of GPR18 by cannabinoid compounds: a tale of biased agonism. *British journal of pharmacology* 2014; 171(16):3908-3917.

71. Dobovišek L, Hojnik M, Ferk P. Overlapping molecular pathways between cannabinoid receptors type 1 and 2 and estrogens/androgens on the periphery and their involvement in the pathogenesis of common diseases. *International journal of molecular medicine* 2016; 38(6):1642-1651.

72. Grotenhermen F. Pharmacokinetics and pharmacodynamics of cannabinoids. *Clinical pharmacokinetics* 2003; 42(4):327-360.

73. Huestis MA, Henningfield JE, Cone EJ. Blood cannabinoids. I. Absorption of THC and formation of 11-OH-THC and THCCOOH during and after smoking marijuana. *Journal of analytical Toxicology* 1992; 16(5):276-282.

74. Huestis MA. Cannabis(Marijuana)- Effects on Human Behavior and Performance. *Forensic science review* 2002; 14(1):15-60.

75. Kelly P, Jones RT. Metabolism of tetrahydrocannabinol in frequent and infrequent marijuana users. *Journal of analytical toxicology* 1992; 16(4):228-235.

76. Matsunaga T, Iwawaki Y, Watanabe K, Yamamoto I, Kageyama T, Yoshimura H. Metabolism of Δ9-tetrahydrocannabinol by cytochrome P450 isozymes purified from hepatic microsomes of monkeys. *Life sciences* 1995; 56(23-24):2089-2095.

77. Narimatsu S, Watanabe K, Matsunaga T, Yamamoto I, Imaoka S, Funae Y, et al. Cytochrome P-450 isozymes involved in the oxidative metabolism of delta 9-tetrahydrocannabinol by liver microsomes of adult female rats. *Drug metabolism and disposition* 1992; 20(1):79-83.

78. WATANABE K, MATSUNAGA T, YAMAMOTO I, FUNAE Y, YOSHIMURA H. Involvement of CYP2C in the metabolism of cannabinoids by human hepatic microsomes from an old woman. *Biological and Pharmaceutical Bulletin* 1995; 18(8):1138-1141.

79. Alburges M, Peat M. Profiles of Δ 9-Tetrahydrocannabinol Metabolites in Urine of Marijuana Users: Preliminary Observations by High Performance Liquid Chromatography-Radioimmunoassay. *Journal of Forensic Science* 1986; 31(2):695-706.

80. Williams P, Moffat A. Identification in human urine of Δ9-tetrahydrocannabinol-11-oic acid glucuronide: a tetrahydrocannabinol metabolite. *Journal of Pharmacy and Pharmacology* 1980; 32(1):445-448.

81. Pistis M, Ferraro L, Pira L, Flore G, Tanganelli S, Gessa GL, et al. Δ9-Tetrahydrocannabinol decreases extracellular GABA and increases extracellular glutamate and dopamine levels in the rat prefrontal cortex: an in vivo microdialysis study. *Brain research* 2002; 948(1-2):155-158.

82. Nutt DJ, Lingford-Hughes A, Erritzoe D, Stokes PR. The dopamine theory of addiction: 40 years of highs and lows. *Nature Reviews Neuroscience* 2015; 16(5):305.

83. Little P, Compton D, Johnson M, Melvin L, Martin B. Pharmacology and stereoselectivity of structurally novel cannabinoids in mice. *Journal of Pharmacology and Experimental Therapeutics* 1988; 247(3):1046-1051.

84. McCallum R, Soykan I, Sridhar K, Ricci D, Lange R, Plankey M. Delta-9-tetrahydrocannabinol delays the gastric emptying of solid food in humans: a double-blind, randomized study. *Alimentary Pharmacology and Therapeutics* 1999; 13(1):77-80.

85. Clark S. Marihuana and the cardiovascular system. *Pharmacology Biochemistry and Behavior* 1975; 3(2):299-306.

86. Pope HG, Gruber AJ, Hudson JI, Huestis MA, Yurgelun-Todd D. Cognitive Measures in long-term cannabis users. *The Journal of Clinical Pharmacology* 2002; 42(S1).

87. Renault PF, Schuster CR, Heinrich R, Freeman DX. Marihuana: standardized smoke administration and dose effect curves on heart rate in humans. *Science* 1971; 174(4009):589-591.

88. Klein TW, Newton C, Friedman H. Cannabinoid receptors and immunity. *Immunology today* 1998; 19(8):373-381.

89. Massi P, Vaccani A, Parolaro D. Cannabinoids, immune system and cytokine network. *Current pharmaceutical design* 2006; 12(24):3135-3146.

90. Tanasescu R, Constantinescu CS. Cannabinoids and the immune system: an overview. *Immunobiology* 2010; 215(8):588-597.

91. Mishkin E, Cabral G. Delta-9-tetrahydrocannabinol decreases host resistance to herpes simplex virus type 2 vaginal infection in the B6C3F1 mouse. *Journal of general virology* 1985; 66(12):2539-2549.

92. Morahan P, Klykken P, Smith S, Harris L, Munson A. Effects of cannabinoids on host resistance to Listeria monocytogenes and herpes simplex virus. *Infection and Immunity* 1979; 23(3):670-674.

93. Buchweitz JP, Karmaus PW, Harkema JR, Williams KJ, Kaminski NE. Modulation of airway responses to influenza A/PR/8/34 by Δ9-tetrahydrocannabinol in C57BL/6 mice. *Journal of Pharmacology and Experimental Therapeutics* 2007; 323(2):675-683.

94. Reiss CS. Cannabinoids and viral infections. *Pharmaceuticals* 2010; 3(6):1873-1886.

95. Eisenstein TK, Meissler JJ. Effects of cannabinoids on T-cell function and resistance to infection. *Journal of Neuroimmune Pharmacology* 2015; 10(2):204-216.

96. Roth MD, Tashkin DP, Whittaker KM, Choi R, Baldwin GC. Tetrahydrocannabinol suppresses immune function and enhances HIV replication in the huPBL-SCID mouse. *Life sciences* 2005; 77(14):1711-1722.

97. Henriquez JE, Rizzo MD, Schulz MA, Crawford RB, Gulick P, Kaminski NE. Δ9-Tetrahydrocannabinol Suppresses Secretion of IFNα by Plasmacytoid Dendritic Cells From Healthy and HIV-Infected Individuals. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 2017; 75(5):588-596.

98. Palella Jr FJ, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, et al. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. *New England Journal of Medicine* 1998; 338(13):853-860.

99. Ammassari A, Murri R, Pezzotti P, Trotta MP, Ravasio L, De Longis P, et al. Self-reported symptoms and medication side effects influence adherence to highly active antiretroviral therapy in persons with HIV infection. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 2001; 28(5):445-449.

100. Tang AM, Forrester J, Spiegelman D, Knox TA, Tchetgen E, Gorbach SL. Weight loss and survival in HIV-positive patients in the era of highly active antiretroviral therapy. *Journal of acquired immune deficiency syndromes (1999)* 2002; 31(2):230-236.

101. Wanke C, Silva M, Knox T, Forrester J, Speigelman D, Gorbach S. Weight loss and wasting remain common complications in individuals infected with human immunodeficiency virus in the era of highly active antiretroviral therapy. *Clinical Infectious Diseases* 2000; 31(3):803-805.

102. Ciesla JA, Roberts JE. Meta-analysis of the relationship between HIV infection and risk for depressive disorders. *American Journal of Psychiatry* 2001; 158(5):725-730.

103. Calcagni E, Elenkov I. Stress system activity, innate and T helper cytokines, and susceptibility to immune-related diseases. *Annals of the New York Academy of Sciences* 2006; 1069(1):62-76.

104. Haney M, Gunderson EW, Rabkin J, Hart CL, Vosburg SK, Comer SD, et al. Dronabinol and marijuana in HIV-positive marijuana smokers: caloric intake, mood, and sleep. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 2007; 45(5):545-554.

105. Abrams DI, Hilton JF, Leiser RJ, Shade SB, Elbeik TA, Aweeka FT, et al. Short-Term Effects of Cannabinoids in Patients with HIV-1 InfectionA Randomized, Placebo-Controlled Clinical Trial. *Annals of internal medicine* 2003; 139(4):258-266.

106. Abrams DI. Potential interventions for HIV/AIDS wasting: an overview. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 2000; 25:S74-S80.

107. Kosel BW, Aweeka FT, Benowitz NL, Shade SB, Hilton JF, Lizak PS, et al. The effects of cannabinoids on the pharmacokinetics of indinavir and nelfinavir. *Aids* 2002; 16(4):543-550.

108. Lapenta C, Santini SM, Proietti E, Rizza P, Logozzi M, Spada M, et al. Type I interferon is a powerful inhibitor of in vivo HIV-1 infection and preserves human CD4+ T cells from virus-induced depletion in SCID mice transplanted with human cells. *Virology* 1999; 263(1):78-88.

109. Benlahrech A, Patterson S. HIV-1 infection and induction of interferon alpha in plasmacytoid dendritic cells. *Current Opinion in HIV and AIDS* 2011; 6(5):373-378.

110. Chehimi J, Campbell DE, Azzoni L, Bacheller D, Papasavvas E, Jerandi G, et al. Persistent decreases in blood plasmacytoid dendritic cell number and function despite effective highly active antiretroviral therapy and increased blood myeloid dendritic cells in HIV-infected individuals. *The Journal of Immunology* 2002; 168(9):4796-4801.

111. Donaghy H, Gazzard B, Gotch F, Patterson S. Dysfunction and infection of freshly isolated blood myeloid and plasmacytoid dendritic cells in patients infected with HIV-1. *Blood* 2003; 101(11):4505-4511.

112. Meier A, Chang JJ, Chan ES, Pollard RB, Sidhu HK, Kulkarni S, et al. Sex differences in the Toll-like receptor–mediated response of plasmacytoid dendritic cells to HIV-1. *Nature medicine* 2009; 15(8):955-959.

113. Orange JS, Biron CA. Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection. *The Journal of Immunology* 1996; 156(12):4746-4756.

114. Kimura M, Haisa M, Uetsuka H, Takaoka M, Ohkawa T, Kawashima R, et al. TNF combined with IFN-α accelerates NF-κB-mediated apoptosis through enhancement of Fas expression in colon cancer cells. *Cell Death & Differentiation* 2003; 10(6):718-728.

115. Gilliet M, Cao W, Liu Y-J. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nature Reviews Immunology* 2008; 8(8):594-606.

116. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA–peptide complexes in systemic lupus erythematosus. *Science translational medicine* 2011; 3(73):73ra19-73ra19.

117. Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang Y-H, Homey B, et al. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* 2007; 449(7162):564-569.

118. Meyer O. Interferons and autoimmune disorders. *Joint Bone Spine* 2009; 76(5):464-473.

119. Kollias G, Kontoyiannis D. Role of TNF/TNFR in autoimmunity: specific TNF receptor blockade may be advantageous to anti-TNF treatments. *Cytokine & growth factor reviews* 2002; 13(4):315-321.

120. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. Induction of dendritic cell differentiation by IFN-α in systemic lupus erythematosus. *Science* 2001; 294(5546):1540-1543.

121. Crispín JC, Liossis S-NC, Kis-Toth K, Lieberman LA, Kyttaris VC, Juang Y-T, et al. Pathogenesis of human systemic lupus erythematosus: recent advances. *Trends in molecular medicine* 2010; 16(2):47-57.

122. Farkas L, Beiske K, Lund-Johansen F, Brandtzaeg P, Jahnsen FL. Plasmacytoid dendritic cells (natural interferon-α/β-producing cells) accumulate in cutaneous lupus erythematosus lesions. *The American journal of pathology* 2001; 159(1):237-243.

123. Koffler D, Agnello V, Thoburn R, Kunkel H. Systemic lupus erythematosus: prototype of immune complex nephritis in man. *Journal of Experimental Medicine* 1971; 134(3):169-179.

124. Berghöfer B, Frommer T, Haley G, Fink L, Bein G, Hackstein H. TLR7 ligands induce higher IFN-α production in females. *The Journal of Immunology* 2006; 177(4):2088-2096.

125. Rizzo MD, Crawford RB, Henriquez JE, Aldhamen Y, Gulick P, Amalfitano A, et al. HIV-infected cannabis users have lower circulating CD16+ monocytes and IP-10 levels compared to non-using HIV patients. *AIDS* 2017.

126. Gannon P, Khan MZ, Kolson DL. Current understanding of HIV-associated neurocognitive disorders pathogenesis. *Current opinion in neurology* 2011; 24(3):275.

127. Ancuta P, Kamat A, Kunstman KJ, Kim E-Y, Autissier P, Wurcel A, et al. Microbial translocation is associated with increased monocyte activation and dementia in AIDS patients. *PloS one* 2008; 3(6):e2516.

128. Ashton JC. Cannabinoids for the treatment of inflammation. *Current opinion in investigational drugs (London, England: 2000)* 2007; 8(5):373-384.

129. Kalant H. Medicinal use of cannabis: history and current status. *Pain Research and Management* 2001; 6(2):80-91.

130. Kaufmann R, Kraft B, Frey R, Winkler D, Weiszenbichler S, Bäcker C, et al. Acute psychotropic effects of oral cannabis extract with a defined content of Δ9-tetrahydrocannabinol (THC) in healthy volunteers. *Pharmacopsychiatry* 2010; 43(01):24-32.

131. Timpone JG, Wright DJ, Li N, Egorin MJ, Enama ME, Mayers J, et al. The safety and pharmacokinetics of single-agent and combination therapy with megestrol acetate and dronabinol for the treatment of HIV wasting syndrome. *AIDS research and human retroviruses* 1997; 13(4):305-315.

132. Izzo AA, Borrelli F, Capasso R, Di Marzo V, Mechoulam R. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. *Trends in pharmacological sciences* 2009; 30(10):515-527.

133. EVERY-PALMER S. Warning: legal synthetic cannabinoid-receptor agonists such as JWH-018 may precipitate psychosis in vulnerable individuals. *Addiction* 2010; 105(10):1859-1860.

134. Hanuš L, Breuer A, Tchilibon S, Shiloah S, Goldenberg D, Horowitz M, et al. HU-308: a specific agonist for CB2, a peripheral cannabinoid receptor. *Proceedings of the National Academy of Sciences* 1999; 96(25):14228-14233.

135. Valenzano KJ, Tafesse L, Lee G, Harrison JE, Boulet JM, Gottshall SL, et al. Pharmacological and pharmacokinetic characterization of the cannabinoid receptor 2 agonist, GW405833, utilizing rodent models of acute and chronic pain, anxiety, ataxia and catalepsy. *Neuropharmacology* 2005; 48(5):658-672.

136. Lombard C, Nagarkatti M, Nagarkatti P. CB2 cannabinoid receptor agonist, JWH-015, triggers apoptosis in immune cells: potential role for CB2-selective ligands as immunosuppressive agents. *Clinical immunology* 2007; 122(3):259-270.

137. Arévalo-Martín Á, Vela JM, Molina-Holgado E, Borrell J, Guaza C. Therapeutic action of cannabinoids in a murine model of multiple sclerosis. *Journal of Neuroscience* 2003; 23(7):2511-2516.

138. Maresz K, Pryce G, Ponomarev ED, Marsicano G, Croxford JL, Shriver LP, et al. Direct suppression of CNS autoimmune inflammation via the cannabinoid receptor CB 1 on neurons and CB 2 on autoreactive T cells. *Nature medicine* 2007; 13(4):492.

139. Aso E, Juvés S, Maldonado R, Ferrer I. CB 2 cannabinoid receptor agonist ameliorates Alzheimer-like phenotype in AβPP/PS1 mice. *Journal of Alzheimer's Disease* 2013; 35(4):847-858.

140. Murikinati S, Jüttler E, Keinert T, Ridder DA, Muhammad S, Waibler Z, et al. Activation of cannabinoid 2 receptors protects against cerebral ischemia by inhibiting neutrophil recruitment. *The FASEB journal* 2010; 24(3):788-798.

141. Ramírez BG, Blázquez C, del Pulgar TG, Guzmán M, de Ceballos ML. Prevention of Alzheimer's disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. *Journal of Neuroscience* 2005; 25(8):1904-1913.

142. Croxford JL, Pryce G, Jackson SJ, Ledent C, Giovannoni G, Pertwee RG, et al. Cannabinoid-mediated neuroprotection, not immunosuppression, may be more relevant to multiple sclerosis. *Journal of neuroimmunology* 2008; 193(1):120-129.

143. Xu H, Cheng CL, Chen M, Manivannan A, Cabay L, Pertwee RG, et al. Anti-inflammatory property of the cannabinoid receptor-2-selective agonist JWH-133 in a rodent model of autoimmune uveoretinitis. *Journal of leukocyte biology* 2007; 82(3):532-541.

144. Guillot A, Hamdaoui N, Bizy A, Zoltani K, Souktani R, Zafrani ES, et al. Cannabinoid receptor 2 counteracts interleukin-17-induced immune and fibrogenic responses in mouse liver. *Hepatology* 2014; 59(1):296-306.

145. Nagarkatti P, Pandey R, Rieder SA, Hegde VL, Nagarkatti M. Cannabinoids as novel anti-inflammatory drugs. *Future medicinal chemistry* 2009; 1(7):1333-1349.

146. Dhabhar FS, Miller AH, McEwen BS, Spencer RL. Effects of stress on immune cell distribution. Dynamics and hormonal mechanisms. *The Journal of Immunology* 1995; 154(10):5511-5527.

147. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature* 1998; 392(6673):245.

148. Schultz KT, Grieder F. Structure and function of the immune system. *Toxicologic pathology* 1987; 15(3):262-264.

149. Delves PJ, Roitt IM. The immune system. *New England journal of medicine* 2000; 343(1):37-49.

150. Tran C, Buchanan D, Cullen R, Searl A, Jones A, Donaldson K. Inhalation of poorly soluble particles. II. Influence of particle surface area on inflammation and clearance. *Inhalation toxicology* 2000; 12(12):1113-1126.

151. Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proceedings of the National Academy of Sciences* 1997; 94(10):5320-5325.

152. Kondo M. Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. *Immunological reviews* 2010; 238(1):37-46.

153. Shortman K, Sathe P, Vremec D, Naik S, O'Keeffe M. Plasmacytoid dendritic cell development. In: *Advances in immunology*: Elsevier; 2013. pp. 105-126.

154. Gasque P. Complement: a unique innate immune sensor for danger signals. *Molecular immunology* 2004; 41(11):1089-1098.

155. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annual review of immunology* 2003; 21(1):335-376.

156. Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. *Nature Reviews Immunology* 2005; 5(8):617.

157. Pandey JP. Major histocompatibility complex. *Medical Immunology* 2007; 6:23-34.

158. Howell W, Carter V, Clark B. The HLA system: immunobiology, HLA typing, antibody screening and crossmatching techniques. *Journal of clinical pathology* 2010; 63(5):387-390.

159. Carrington M, Nelson GW, Martin MP, Kissner T, Vlahov D, Goedert JJ, et al. HLA and HIV-1: heterozygote advantage and B* 35-Cw* 04 disadvantage. *Science* 1999; 283(5408):1748-1752.

160. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nature Reviews Immunology* 2013; 13(4):227.

161. Shih T-AY, Meffre E, Roederer M, Nussenzweig MC. Role of BCR affinity in T cell–dependent antibody responses in vivo. *Nature immunology* 2002; 3(6):570.

162. Smith CM, Wilson NS, Waithman J, Villadangos JA, Carbone FR, Heath WR, et al. Cognate CD4+ T cell licensing of dendritic cells in CD8+ T cell immunity. *Nature immunology* 2004; 5(11):1143.

163. Barchet W, Blasius A, Cella M, Colonna M. Plasmacytoid dendritic cells. *Immunologic research* 2005; 32(1-3):75-83.

164. Colonna M, Trinchieri G, Liu Y-J. Plasmacytoid dendritic cells in immunity. *Nature immunology* 2004; 5(12):1219-1226.

165. Kioon MDA, Tripodo C, Fernandez D, Kirou KA, Spiera RF, Crow MK, et al. Plasmacytoid dendritic cells promote systemic sclerosis with a key role for TLR8. *Science translational medicine* 2018; 10(423):eaam8458.

166. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nature Reviews Immunology* 2015; 15(8):471-485.

167. Krug A, Rothenfusser S, Hornung V, Jahrsdörfer B, Blackwell S, Ballas ZK, et al. Identification of CpG oligonucleotide sequences with high induction of IFN-α/β in plasmacytoid dendritic cells. *European journal of immunology* 2001; 31(7):2154-2163.

168. Bauer S, Kirschning CJ, Häcker H, Redecke V, Hausmann S, Akira S, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proceedings of the National Academy of Sciences* 2001; 98(16):9237-9242.

169. Kerkmann M, Rothenfusser S, Hornung V, Towarowski A, Wagner M, Sarris A, et al. Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells. *The Journal of Immunology* 2003; 170(9):4465-4474.

170. Vollmer J, Weeratna R, Payette P, Jurk M, Schetter C, Laucht M, et al. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. *European journal of immunology* 2004; 34(1):251-262.

171. Hartmann G, Battiany J, Poeck H, Wagner M, Kerkmann M, Lubenow N, et al. Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-α induction in plasmacytoid dendritic cells. *European journal of immunology* 2003; 33(6):1633-1641.

172. Häcker H, Redecke V, Blagoev B, Kratchmarova I, Hsu L-C, Wang GG, et al. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* 2006; 439(7073):204-207.

173. Kim SS-Y, Lee K-G, Chin C-S, Ng S-K, Pereira NA, Xu S, et al. DOK3 is required for IFN-β production by enabling TRAF3/TBK1 complex formation and IRF3 activation. *The Journal of Immunology* 2014; 193(2):840-848.

174. Oganesyan G, Saha SK, Guo B, He JQ, Shahangian A, Zarnegar B, et al. Critical role of TRAF3 in the Toll-like receptor-dependent and-independent antiviral response. *Nature* 2006; 439(7073):208-211.

175. Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, et al. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 2005; 434(7034):772-777.

176. Hoebe K, Beutler B. TRAF3: a new component of the TLR-signaling apparatus. *Trends in molecular medicine* 2006; 12(5):187-189.

177. Huang J, Liu T, Xu LG, Chen D, Zhai Z, Shu HB. SIKE is an IKKε/TBK1-associated suppressor of TLR3-and virus-triggered IRF-3 activation pathways. *The EMBO journal* 2005; 24(23):4018-4028.

178. Guo B, Cheng G. Modulation of the interferon antiviral response by the TBK1/IKKi adaptor protein TANK. *Journal of Biological Chemistry* 2007; 282(16):11817-11826.

179. Unterholzner L, Sumner RP, Baran M, Ren H, Mansur DS, Bourke NM, et al. Vaccinia virus protein C6 is a virulence factor that binds TBK-1 adaptor proteins and inhibits activation of IRF3 and IRF7. *PLoS pathogens* 2011; 7(9):e1002247.

180. Guiducci C, Ghirelli C, Marloie-Provost M-A, Matray T, Coffman RL, Liu Y-J, et al. PI3K is critical for the nuclear translocation of IRF-7 and type I IFN production by human plasmacytoid predendritic cells in response to TLR activation. *Journal of Experimental Medicine* 2008; 205(2):315-322.

181. Fitzgerald-Bocarsly P, Dai J, Singh S. Plasmacytoid dendritic cells and type I IFN: 50 years of convergent history. *Cytokine & growth factor reviews* 2008; 19(1):3-19.

182. Liu Y-J. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 2005; 23:275-306.

183. Gerosa F, Gobbi A, Zorzi P, Burg S, Briere F, Carra G, et al. The reciprocal interaction of NK cells with plasmacytoid or myeloid dendritic cells profoundly affects innate resistance functions. *The Journal of Immunology* 2005; 174(2):727-734.

184. Megjugorac NJ, Young HA, Amrute SB, Olshalsky SL, Fitzgerald-Bocarsly P. Virally stimulated plasmacytoid dendritic cells produce chemokines and induce migration of T and NK cells. *Journal of leukocyte biology* 2004; 75(3):504-514.

185. Havenar-Daughton C, Kolumam GA, Murali-Krishna K. Cutting Edge: The direct action of type I IFN on CD4 T cells is critical for sustaining clonal expansion in response to a viral but not a bacterial infection. *The Journal of Immunology* 2006; 176(6):3315-3319.

186. Yrlid U, Milling SW, Miller JL, Cartland S, Jenkins CD, MacPherson GG. Regulation of intestinal dendritic cell migration and activation by plasmacytoid dendritic cells, TNF-α and type 1 IFNs after feeding a TLR7/8 ligand. *The Journal of Immunology* 2006; 176(9):5205-5212.

187. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, et al. Tumor necrosis factor-α is required in the protective immune response against Mycobacterium tuberculosis in mice. *Immunity* 1995; 2(6):561-572.

188. Wong GH, Goeddel DV. Tumour necrosis factors α and β inhibit virus replication and synergize with interferons. *Nature* 1986; 323(6091):819-822.

189. Pasparakis M, Alexopoulou L, Episkopou V, Kollias G. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *Journal of Experimental Medicine* 1996; 184(4):1397-1411.

190. Chen X, Oppenheim JJ. Contrasting effects of TNF and anti-TNF on the activation of effector T cells and regulatory T cells in autoimmunity. *FEBS letters* 2011; 585(23):3611-3618.

191. Wang C-Y, Mayo MW, Baldwin Jr AS. TNF-and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kB. *Science* 1996; 274(5288):784.

192. Liu H, Sidiropoulos P, Song G, Pagliari LJ, Birrer MJ, Stein B, et al. TNF-α gene expression in macrophages: regulation by NF-κB is independent of c-Jun or C/EBPβ. *The Journal of Immunology* 2000; 164(8):4277-4285.

193. Choi YH, Kim G-Y, Lee HH. Anti-inflammatory effects of cordycepin in lipopolysaccharide-stimulated RAW 264.7 macrophages through Toll-like receptor 4-mediated suppression of mitogen-activated protein kinases and NF-κB signaling pathways. *Drug design, development and therapy* 2014; 8:1941.

194. Norris PJ, Pappalardo BL, Custer B, Spotts G, Hecht FM, Busch MP. Elevations in IL-10, TNF-α, and IFN-γ from the earliest point of HIV type 1 infection. *AIDS Research & Human Retroviruses* 2006; 22(8):757-762.

195. Anthony DD, Yonkers NL, Post AB, Asaad R, Heinzel FP, Lederman MM, et al. Selective impairments in dendritic cell-associated function distinguish hepatitis C virus and HIV infection. *The Journal of Immunology* 2004; 172(8):4907-4916.

196. Cha L, Berry CM, Nolan D, Castley A, Fernandez S, French MA. Interferon-alpha, immune activation and immune dysfunction in treated HIV infection. *Clinical & Translational Immunology* 2014; 3(2):e10.

197. Kwa S, Kannanganat S, Nigam P, Siddiqui M, Shetty RD, Armstrong W, et al. Plasmacytoid dendritic cells are recruited to the colorectum and contribute to immune activation during pathogenic SIV infection in rhesus macaques. *Blood* 2011; 118(10):2763-2773.

198. Fong L, Mengozzi M, Abbey NW, Herndier BG, Engleman EG. Productive infection of plasmacytoid dendritic cells with human immunodeficiency virus type 1 is triggered by CD40 ligation. *Journal of virology* 2002; 76(21):11033-11041.

199. Feldman S, Stein D, Amrute S, Denny T, Garcia Z, Kloser P, et al. Decreased interferon-α production in HIV-infected patients correlates with numerical and functional deficiencies in circulating type 2 dendritic cell precursors. *Clinical immunology* 2001; 101(2):201-210.

200. Loré K, Smed-Sörensen A, Vasudevan J, Mascola JR, Koup RA. Myeloid and plasmacytoid dendritic cells transfer HIV-1 preferentially to antigen-specific CD4+ T cells. *The Journal of experimental medicine* 2005; 201(12):2023-2033.

201. Donaghy H, Pozniak A, Gazzard B, Qazi N, Gilmour J, Gotch F, et al. Loss of blood CD11c+ myeloid and CD11c− plasmacytoid dendritic cells in patients with HIV-1 infection correlates with HIV-1 RNA virus load. *Blood* 2001; 98(8):2574-2576.

202. Constantinescu SN, Croze E, Wang C, Murti A, Basu L, Mullersman JE, et al. Role of interferon alpha/beta receptor chain 1 in the structure and transmembrane signaling of the interferon alpha/beta receptor complex. *Proceedings of the National Academy of Sciences* 1994; 91(20):9602-9606.

203. Colamonici OR, Uyttendaele H, Domanski P, Yan H, Krolewski JJ. p135tyk2, an interferon-alpha-activated tyrosine kinase, is physically associated with an interferon-alpha receptor. *Journal of Biological Chemistry* 1994; 269(5):3518-3522.

204. Darnell JE, Kerr IM, Stark GR. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 1994; 264(5164):1415-1421.

205. Jaks E, Gavutis M, Uzé G, Martal J, Piehler J. Differential receptor subunit affinities of type I interferons govern differential signal activation. *Journal of molecular biology* 2007; 366(2):525-539.

206. De Weerd NA, Vivian JP, Nguyen TK, Mangan NE, Gould JA, Braniff S-J, et al. Structural basis of a unique interferon-β signaling axis mediated via the receptor IFNAR1. *Nature immunology* 2013; 14(9):901.

207. Lavoie TB, Kalie E, Crisafulli-Cabatu S, Abramovich R, DiGioia G, Moolchan K, et al. Binding and activity of all human alpha interferon subtypes. *Cytokine* 2011; 56(2):282-289.

208. Rani MS, Leaman DW, Han Y, Leung S, Croze E, Fish EN, et al. Catalytically active TYK2 is essential for interferon-β-mediated phosphorylation of STAT3 and interferon-α receptor-1 (IFNAR-1) but not for activation of phosphoinositol 3-kinase. *Journal of Biological Chemistry* 1999; 274(45):32507-32511.

209. Badr G, Saad H, Waly H, Hassan K, Abdel-Tawab H, Alhazza IM, et al. Type I interferon (IFN-α/β) rescues B-lymphocytes from apoptosis via PI3Kδ/Akt, Rho-A, NFκB and Bcl-2/BclXL. *Cellular immunology* 2010; 263(1):31-40.

210. Huynh L, Wang L, Shi C, Park-Min K-H, Ivashkiv LB. ITAM-coupled receptors inhibit IFNAR signaling and alter macrophage responses to TLR4 and Listeria monocytogenes. *The Journal of Immunology* 2012; 188(7):3447-3457.

211. Yan H, Krishnan K, Greenlund A, Gupta S, Lim J, Schreiber R, et al. Phosphorylated interferon-alpha receptor 1 subunit (IFNaR1) acts as a docking site for the latent form of the 113 kDa STAT2 protein. *The EMBO journal* 1996; 15(5):1064-1074.

212. Moro H, Otero DC, Tanabe Y, David M. T cell-intrinsic and-extrinsic contributions of the IFNAR/STAT1-axis to thymocyte survival. *PloS one* 2011; 6(9):e24972.

213. Fu X-Y, Kessler DS, Veals SA, Levy DE, Darnell J. ISGF3, the transcriptional activator induced by interferon alpha, consists of multiple interacting polypeptide chains. *Proceedings of the National Academy of Sciences* 1990; 87(21):8555-8559.

214. Levy DE, Kessler D, Pine R, Darnell J. Cytoplasmic activation of ISGF3, the positive regulator of interferon-alpha-stimulated transcription, reconstituted in vitro. *Genes & development* 1989; 3(9):1362-1371.

215. Mazzucchelli R, Durum SK. Interleukin-7 receptor expression: intelligent design. *Nature Reviews Immunology* 2007; 7(2):144-154.

216. Germain RN. T-cell development and the CD4–CD8 lineage decision. *Nature reviews immunology* 2002; 2(5):309.

217. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. *Annual review of immunology* 2003; 21(1):139-176.

218. Suda T, Zlotnik A. IL-7 maintains the T cell precursor potential of CD3-CD4-CD8- thymocytes. *The Journal of Immunology* 1991; 146(9):3068-3073.

219. Itano AA, Jenkins MK. Antigen presentation to naive CD4 T cells in the lymph node. *Nature immunology* 2003; 4(8):733.

220. Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. *Blood* 2008; 112(5):1557-1569.

221. Sioud M, Fløisand Y. TLR agonists induce the differentiation of human bone marrow CD34+ progenitors into CD11c+ CD80/86+ DC capable of inducing a Th1-type response. *European journal of immunology* 2007; 37(10):2834-2846.

222. Kubo T, Hatton RD, Oliver J, Liu X, Elson CO, Weaver CT. Regulatory T cell suppression and anergy are differentially regulated by proinflammatory cytokines

produced by TLR-activated dendritic cells. *The Journal of immunology* 2004; 173(12):7249-7258.

223. Lanzavecchia A, Sallusto F. Regulation of T cell immunity by dendritic cells. *Cell* 2001; 106(3):263-266.

224. Clark EA, Ledbetter JA. How B and T cells talk to each other. *Nature* 1994; 367(6462):425.

225. Mosmann TR, Coffman R. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual review of immunology* 1989; 7(1):145-173.

226. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunology today* 1996; 17(3):138-146.

227. Licona-Limón P, Henao-Mejia J, Temann AU, Gagliani N, Licona-Limón I, Ishigame H, et al. Th9 cells drive host immunity against gastrointestinal worm infection. *Immunity* 2013; 39(4):744-757.

228. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annual review of immunology* 2009; 27:485-517.

229. Basu R, O'Quinn DB, Silberger DJ, Schoeb TR, Fouser L, Ouyang W, et al. Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. *Immunity* 2012; 37(6):1061-1075.

230. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. *Nature Reviews Immunology* 2010; 10(7):490.

231. Gondek DC, Lu L-F, Quezada SA, Sakaguchi S, Noelle RJ. Cutting edge: contact-mediated suppression by CD4+ CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *The Journal of Immunology* 2005; 174(4):1783-1786.

232. Fife BT, Bluestone JA. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunological reviews* 2008; 224(1):166-182.

233. Horwitz DA, Zheng SG, Wang J, Gray JD. Critical role of IL-2 and TGF-β in generation, function and stabilization of Foxp3+ CD4+ Treg. *European journal of immunology* 2008; 38(4):912-915.

234. Wong P, Pamer EG. CD8 T cell responses to infectious pathogens. *Annual review of immunology* 2003; 21(1):29-70.

235. Fu J, Xu D, Liu Z, Shi M, Zhao P, Fu B, et al. Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology* 2007; 132(7):2328-2339.

236. Norment AM, Salter RD, Parham P, Engelhard VH, Littman DR. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature* 1988; 336(6194):79.

237. Cresswell P, Ackerman AL, Giodini A, Peaper DR, Wearsch PA. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunological reviews* 2005; 207(1):145-157.

238. Heath WR, Carbone FR. Cross-presentation, dendritic cells, tolerance and immunity. *Annual review of immunology* 2001; 19(1):47-64.

239. Jusforgues-Saklani H, Uhl M, Blachère N, Lemaître F, Lantz O, Bousso P, et al. Antigen persistence is required for dendritic cell licensing and CD8+ T cell cross-priming. *The Journal of Immunology* 2008; 181(5):3067-3076.

240. Bourgeois C, Veiga-Fernandes H, Joret AM, Rocha B, Tanchot C. CD8 lethargy in the absence of CD4 help. *European journal of immunology* 2002; 32(8):2199-2207.

241. Shedlock DJ, Shen H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* 2003; 300(5617):337-339.

242. Kalams SA, Walker BD. The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses. *Journal of Experimental Medicine* 1998; 188(12):2199-2204.

243. Kambayashi T, Assarsson E, Lukacher AE, Ljunggren H-G, Jensen PE. Memory CD8+ T cells provide an early source of IFN-γ. *The Journal of Immunology* 2003; 170(5):2399-2408.

244. Ratner A, Clark WR. Role of TNF-alpha in CD8+ cytotoxic T lymphocyte-mediated lysis. *The Journal of Immunology* 1993; 150(10):4303-4314.

245. Sartor RB. Cytokines in intestinal inflammation: pathophysiological and clinical considerations. *Gastroenterology* 1994; 106(2):533-539.

246. Bradley J. TNF-mediated inflammatory disease. *The Journal of pathology* 2008; 214(2):149-160.

247. Zaidi MR, Merlino G. The two faces of interferon-γ in cancer. *Clinical cancer research* 2011; 17(19):6118-6124.

248. Hersperger AR, Makedonas G, Betts MR. Flow cytometric detection of perforin upregulation in human CD8 T cells. *Cytometry Part A* 2008; 73(11):1050-1057.

249. Nowacki TM, Kuerten S, Zhang W, Shive CL, Kreher CR, Boehm BO, et al. Granzyme B production distinguishes recently activated CD8+ memory cells from resting memory cells. *Cellular immunology* 2007; 247(1):36-48.

250. Hadida F, Vieillard V, Mollet L, Clark-Lewis I, Baggiolini M, Debré P. Cutting edge: RANTES regulates Fas ligand expression and killing by HIV-specific CD8 cytotoxic T cells. *The Journal of immunology* 1999; 163(3):1105-1109.

251. Topham D, Tripp R, Doherty P. CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. *The Journal of Immunology* 1997; 159(11):5197-5200.

252. Peter ME, Krammer P. The CD95 (APO-1/Fas) DISC and beyond. *Cell death and differentiation* 2003; 10(1):26.

253. Dragic T, Litwin V, Allaway GP, Martin SR, Huang Y, Nagashima KA, et al. HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 1996; 381(6584):667-673.

254. Sousa AE, Carneiro J, Meier-Schellersheim M, Grossman Z, Victorino RM. CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. *The Journal of Immunology* 2002; 169(6):3400-3406.

255. Gulzar N, Copeland KF. CD8+ T-cells: function and response to HIV infection. *Current HIV research* 2004; 2(1):23-37.

256. Cohen GB, Gandhi RT, Davis DM, Mandelboim O, Chen BK, Strominger JL, et al. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity* 1999; 10(6):661-671.

257. Herbeuval J-P, Hardy AW, Boasso A, Anderson SA, Dolan MJ, Dy M, et al. Regulation of TNF-related apoptosis-inducing ligand on primary CD4+ T cells by HIV-1: role of type I IFN-producing plasmacytoid dendritic cells. *Proceedings of the National Academy of Sciences of the United States of America* 2005; 102(39):13974-13979.

258. Herbeuval J-P, Shearer GM. HIV-1 immunopathogenesis: how good interferon turns bad. *Clinical immunology* 2007; 123(2):121-128.

259. Hardy AW, Graham DR, Shearer GM, Herbeuval J-P. HIV turns plasmacytoid dendritic cells (pDC) into TRAIL-expressing killer pDC and down-regulates HIV coreceptors by Toll-like receptor 7-induced IFN-α. *Proceedings of the National Academy of Sciences* 2007; 104(44):17453-17458.

260. Stary G, Klein I, Kohlhofer S, Koszik F, Scherzer T, Müllauer L, et al. Plasmacytoid dendritic cells express TRAIL and induce CD4+ T-cell apoptosis in HIV-1 viremic patients. *Blood* 2009; 114(18):3854-3863.

261. Brenchley JM, Schacker TW, Ruff LE, Price DA, Taylor JH, Beilman GJ, et al. CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *Journal of Experimental Medicine* 2004; 200(6):749-759.

262. Fauci AS, Mavilio D, Kottilil S. NK cells in HIV infection: paradigm for protection or targets for ambush. *Nature Reviews Immunology* 2005; 5(11):835.

263. Chung AW, Navis M, Isitman G, Wren L, Silvers J, Amin J, et al. Activation of NK cells by ADCC antibodies and HIV disease progression. *Journal of acquired immune deficiency syndromes (1999)* 2011; 58(2):127.

264. Roederer M, Dubs JG, Anderson MT, Raju PA, Herzenberg LA, Herzenberg LA. CD8 naive T cell counts decrease progressively in HIV-infected adults. *Journal of Clinical Investigation* 1995; 95(5):2061.

265. Moir S, Fauci AS. B cells in HIV infection and disease. *Nature Reviews Immunology* 2009; 9(4):235.

266. Koot M, Keet IP, Vos AH, de Goede RE, Roos MTL, Coutinho RA, et al. Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. *Annals of internal medicine* 1993; 118(9):681-688.

267. Autran B, Carcelain G, Li TS, Blanc C, Mathez D, Tubiana R, et al. Positive effects of combined antiretroviral therapy on CD4+ T cell homeostasis and function in advanced HIV disease. *Science* 1997; 277(5322):112-116.

268. Iacob SA, Iacob DG, Jugulete G. Improving the adherence to antiretroviral therapy, a difficult but essential task for HIV treatment success. *Frontiers in Pharmacology* 2017; 8:831.

269. Fischl MA, Richman DD, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, et al. The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *New England Journal of Medicine* 1987; 317(4):185-191.

270. Oxenius A, Price DA, Easterbrook PJ, O'Callaghan CA, Kelleher AD, Whelan JA, et al. Early highly active antiretroviral therapy for acute HIV-1 infection preserves immune function of CD8+ and CD4+ T lymphocytes. *Proceedings of the National Academy of Sciences* 2000; 97(7):3382-3387.

271. Collaboration ATC. Life expectancy of individuals on combination antiretroviral therapy in high-income countries: a collaborative analysis of 14 cohort studies. *The Lancet* 2008; 372(9635):293-299.

272. Serrano-Villar S, Sainz T, Lee SA, Hunt PW, Sinclair E, Shacklett BL, et al. HIV-infected individuals with low CD4/CD8 ratio despite effective antiretroviral therapy exhibit altered T cell subsets, heightened CD8+ T cell activation, and increased risk of non-AIDS morbidity and mortality. *PLoS pathogens* 2014; 10(5):e1004078.

273. Wherry EJ. T cell exhaustion. *Nature immunology* 2011; 12(6):492.

274. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. *Nature Reviews Immunology* 2015; 15(8):486.

275. Yi JS, Cox MA, Zajac AJ. T-cell exhaustion: characteristics, causes and conversion. *Immunology* 2010; 129(4):474-481.

276. Day CL, Kaufmann DE, Kiepiela P, Brown JA, Moodley ES, Reddy S, et al. PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. *Nature* 2006; 443(7109):350.

277. Nakamoto N, Cho H, Shaked A, Olthoff K, Valiga ME, Kaminski M, et al. Synergistic reversal of intrahepatic HCV-specific CD8 T cell exhaustion by combined PD-1/CTLA-4 blockade. *PLoS pathogens* 2009; 5(2):e1000313.

278. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nature immunology* 2009; 10(1):29.

279. Lang KS, Recher M, Navarini AA, Harris NL, Löhning M, Junt T, et al. Inverse correlation between IL-7 receptor expression and CD8 T cell exhaustion during persistent antigen stimulation. *European journal of immunology* 2005; 35(3):738-745.

280. Addo MM, Altfeld M. Sex-based differences in HIV type 1 pathogenesis. *The Journal of infectious diseases* 2014; 209(suppl_3):S86-S92.

281. Burlacu R, Umlauf A, Luca A, Gianella S, Radoi R, Ruta SM, et al. Sex based differences in neurocognitive functioning in HIV infected young adults. *AIDS (London, England)* 2017.

282. Macal M, Sankaran S, Chun T, Reay E, Flamm J, Prindiville T, et al. Effective CD4+ T-cell restoration in gut-associated lymphoid tissue of HIV-infected patients is associated with enhanced Th17 cells and polyfunctional HIV-specific T-cell responses. *Mucosal immunology* 2008; 1(6):475.

283. Hunt PW, Sinclair E, Rodriguez B, Shive C, Clagett B, Funderburg N, et al. Gut epithelial barrier dysfunction and innate immune activation predict mortality in treated HIV infection. *The Journal of infectious diseases* 2014; 210(8):1228-1238.

284. MacPherson PA, Fex C, Sanchez-Dardon J, Hawley-Foss N, Angel JB. Interleukin-7 receptor expression on CD8+ T cells is reduced in HIV infection and partially restored with effective antiretroviral therapy. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 2001; 28(5):454-457.

285. Hartling HJ, Jespersen S, Gaardbo JC, Sambleben C, Thorsteinsson K, Gerstoft J, et al. Reduced IL-7R T cell expression and increased plasma sCD127 in late presenting HIV-infected individuals. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 2017; 74(1):81-90.

286. Nguyen TP, Shukla S, Asaad R, Freeman ML, Lederman MM, Harding CV, et al. Responsiveness to IL-7 but not to IFN-α is diminished in CD4+ T cells from treated HIV infected patients who experience poor CD4+ T-cell recovery. *AIDS* 2016; 30(13):2033-2042.

287. Tan JT, Dudl E, LeRoy E, Murray R, Sprent J, Weinberg KI, et al. IL-7 is critical for homeostatic proliferation and survival of naive T cells. *Proceedings of the National Academy of Sciences* 2001; 98(15):8732-8737.

288. Sereti I, Dunham RM, Spritzler J, Aga E, Proschan MA, Medvik K, et al. IL-7 administration drives T cell–cycle entry and expansion in HIV-1 infection. *Blood* 2009; 113(25):6304-6314.

289. Levy Y, Lacabaratz C, Weiss L, Viard J-P, Goujard C, Lelièvre J-D, et al. Enhanced T cell recovery in HIV-1–infected adults through IL-7 treatment. *The Journal of clinical investigation* 2009; 119(4):997.

290. Bazdar DA, Sieg SF. Interleukin-7 enhances proliferation responses to T-cell receptor stimulation in naive CD4+ T cells from human immunodeficiency virus-infected persons. *Journal of virology* 2007; 81(22):12670-12674.

291. Sereti I, Estes JD, Thompson WL, Morcock DR, Fischl MA, Croughs T, et al. Decreases in colonic and systemic inflammation in chronic HIV infection after IL-7 administration. *PLoS pathogens* 2014; 10(1):e1003890.

292. Lin J-X, Migone T-S, Tseng M, Friedmann M, Weatherbee JA, Zhou L, et al. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. *Immunity* 1995; 2(4):331-339.

293. Palmer MJ, Mahajan VS, Trajman LC, Irvine DJ, Lauffenburger DA, Chen J. Interleukin-7 receptor signaling network: an integrated systems perspective. *Cellular & molecular immunology* 2008; 5(2):79.

294. Barata JT, Silva A, Brandao JG, Nadler LM, Cardoso AA, Boussiotis VA. Activation of PI3K is indispensable for interleukin 7–mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. *Journal of Experimental Medicine* 2004; 200(5):659-669.

295. Li WQ, Jiang Q, Khaled AR, Keller JR, Durum SK. Interleukin-7 inactivates the pro-apoptotic protein Bad promoting T cell survival. *Journal of Biological Chemistry* 2004; 279(28):29160-29166.

296. Sharfe N, Roifman CM. Differential association of phosphatidylinositol 3-kinase with insulin receptor substrate (IRS)-1 and IRS-2 in human thymocytes in response to IL-7. *The Journal of Immunology* 1997; 159(3):1107-1114.

297. O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. *Cell* 2002; 109(2):S121-S131.

298. Grange M, Giordano M, Mas A, Roncagalli R, Firaguay G, Nunes JA, et al. Control of CD8 T cell proliferation and terminal differentiation by active STAT5 and CDKN2A/CDKN2B. *Immunology* 2015; 145(4):543-557.

299. Verdeil G, Puthier D, Nguyen C, Schmitt-Verhulst A-M, Auphan-Anezin N. STAT5-mediated signals sustain a TCR-initiated gene expression program toward differentiation of CD8 T cell effectors. *The Journal of Immunology* 2006; 176(8):4834-4842.

300. Park S-H, Liu X, Hennighausen L, Davey HW, Waxman DJ. Distinctive roles of STAT5a and STAT5b in sexual dimorphism of hepatic P450 gene expression impact of Stat5a gene disruption. *Journal of Biological Chemistry* 1999; 274(11):7421-7430.

301. Teglund S, McKay C, Schuetz E, Van Deursen JM, Stravopodis D, Wang D, et al. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell* 1998; 93(5):841-850.

302. Catalfamo M, Wilhelm C, Tcheung L, Proschan M, Friesen T, Park J-H, et al. CD4 and CD8 T cell immune activation during chronic HIV infection: roles of homeostasis, HIV, type I IFN, and IL-7. *The Journal of Immunology* 2011; 186(4):2106-2116.

303. García-Sastre A, Biron CA. Type 1 interferons and the virus-host relationship: a lesson in detente. *Science* 2006; 312(5775):879-882.

304. Poli G, Orenstein JM, Kinter A, Folks TM, Fauci AS. Interferon-alpha but not AZT suppresses HIV expression in chronically infected cell lines. *Science* 1989; 244(4904):575-577.

305. Kang MC, Park HW, Choi D-H, Choi YW, Park Y, Sung YC, et al. Plasmacytoid Dendritic Cells Contribute to the Protective Immunity Induced by Intranasal Treatment with Fc-fused Interleukin-7 against Lethal Influenza Virus Infection. *Immune network* 2017; 17(5):343-351.

306. Woelk CH, Ottones F, Plotkin CR, Du P, Royer CD, Rought SE, et al. Interferon gene expression following HIV type 1 infection of monocyte-derived macrophages. *AIDS Research & Human Retroviruses* 2004; 20(11):1210-1222.

307. Cairns AP, Crockard AD, Bell AL. The CD14+ CD16+ monocyte subset in rheumatoid arthritis and systemic lupus erythematosus. *Rheumatology international* 2002; 21(5):189-192.

308. Clifford DB, Ances BM. HIV-associated neurocognitive disorder. *The Lancet infectious diseases* 2013; 13(11):976-986.

309. Heaton R, Clifford D, Franklin D, Woods S, Ake C, Vaida F, et al. HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy CHARTER Study. *Neurology* 2010; 75(23):2087-2096.

310. Antinori A, Arendt G, Becker J, Brew B, Byrd D, Cherner M, et al. Updated research nosology for HIV-associated neurocognitive disorders. *Neurology* 2007; 69(18):1789-1799.

311. Simioni S, Cavassini M, Annoni J-M, Abraham AR, Bourquin I, Schiffer V, et al. Cognitive dysfunction in HIV patients despite long-standing suppression of viremia. *Aids* 2010; 24(9):1243-1250.

312. Ances BM, Ellis RJ. Dementia and neurocognitive disorders due to HIV-1 infection. In: *Seminars in neurology*: Copyright© 2007 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA.; 2007. pp. 086-092.

313. Gisslén M, Price RW, Nilsson S. The definition of HIV-associated neurocognitive disorders: are we overestimating the real prevalence? *BMC infectious diseases* 2011; 11(1):356.

314. Kaul M, Garden GA, Lipton SA. Pathways to neuronal injury and apoptosis in HIV-associated dementia. *Nature* 2001; 410(6831):988.

315. Goudsmit J, Paul D, Lange JA, Speelman H, Van Der Noordaa J, Van Der Helm H, et al. Expression of human immunodeficiency virus antigen (HIV-Ag) in serum and cerebrospinal fluid during acute and chronic infection. *The lancet* 1986; 328(8500):177-180.

316. He J, Chen Y, Farzan M, Choe H, Ohagen A, Gartner S, et al. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* 1997; 385(6617):645.

317. Streit WJ, Mrak RE, Griffin WST. Microglia and neuroinflammation: a pathological perspective. *Journal of neuroinflammation* 2004; 1(1):14.

318. Herbein G, Coaquette A, Perez-Bercoff D, Pancino G. Macrophage activation and HIV infection: can the Trojan horse turn into a fortress? *Current molecular medicine* 2002; 2(8):723-738.

319. Yadav A, Collman RG. CNS inflammation and macrophage/microglial biology associated with HIV-1 infection. *Journal of neuroimmune pharmacology* 2009; 4(4):430-447.

320. McArthur JC, Brew BJ, Nath A. Neurological complications of HIV infection. *The Lancet Neurology* 2005; 4(9):543-555.

321. Schifitto G, McDermott M, McArthur J, Marder K, Sacktor N, McClernon D, et al. Markers of immune activation and viral load in HIV-associated sensory neuropathy. *Neurology* 2005; 64(5):842-848.

322. Roux-Lombard P, Modoux C, Cruchaud A, Dayer J-M. Purified blood monocytes from HIV 1-infected patients induce high levels of TNFα and IL-1. *Clinical immunology and immunopathology* 1989; 50(3):374-384.

323. Gardella S, Andrei C, Ferrera D, Lotti LV, Torrisi MR, Bianchi ME, et al. The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO reports* 2002; 3(10):995-1001.

324. Pedrazzi M, Melloni E, Sparatore B. Selective Pro-Inflammatory Activation of Astrocytes by High Mobility Group Box 1 Protein Signaling. In: *New Insights to Neuroimmune Biology*: Elsevier; 2010. pp. 53-72.

325. Ransohoff RM, Hamilton TA, Tani M, Stoler MH, Shick HE, Major JA, et al. Astrocyte expression of mRNA encoding cytokines IP-10 and JE/MCP-1 in experimental autoimmune encephalomyelitis. *The FASEB Journal* 1993; 7(6):592-600.

326. Taub DD, Lloyd AR, Conlon K, Wang JM, Ortaldo J, Harada A, et al. Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *Journal of Experimental Medicine* 1993; 177(6):1809-1814.

327. González H, Pacheco R. T-cell-mediated regulation of neuroinflammation involved in neurodegenerative diseases. *Journal of neuroinflammation* 2014; 11(1):201.

328. Balashov KE, Rottman JB, Weiner HL, Hancock WW. CCR5+ and CXCR3+ T cells are increased in multiple sclerosis and their ligands MIP-1α and IP-10 are expressed in demyelinating brain lesions. *Proceedings of the National Academy of Sciences* 1999; 96(12):6873-6878.

329. Meda L, Cassatella MA, Szendrei GI, Otvos Jr L, Baron P, Villalba M, et al. Activation of microglial cells by β-amyloid protein and interferon-γ. *Nature* 1995; 374(6523):647.

330. Barcia C, Ros C, Annese V, Gómez A, Ros-Bernal F, Aguado-Llera D, et al. IFN-γ signaling, with the synergistic contribution of TNF-α, mediates cell specific microglial and astroglial activation in experimental models of Parkinson's disease. *Cell death & disease* 2011; 2(4):e142.

331. Banati R, Schubert P, Rothe G, Gehrmann J, Rudolphi K, Valet G, et al. Modulation of intracellular formation of reactive oxygen intermediates in peritoneal macrophages and microglia/brain macrophages by propentofylline. *Journal of Cerebral Blood Flow & Metabolism* 1994; 14(1):145-149.

332. Qin L, Liu Y, Cooper C, Liu B, Wilson B, Hong JS. Microglia enhance β-amyloid peptide-induced toxicity in cortical and mesencephalic neurons by producing reactive oxygen species. *Journal of neurochemistry* 2002; 83(4):973-983.

333. Bezzi P, Domercq M, Brambilla L, Galli R, Schols D, De Clercq E, et al. CXCR4-activated astrocyte glutamate release via TNFα: amplification by microglia triggers neurotoxicity. *Nature neuroscience* 2001; 4(7):702.

334. Fine SM, Angel RA, Perry SW, Epstein LG, Rothstein JD, Dewhurst S, et al. Tumor Necrosis Factor α Inhibits Glutamate Uptake by Primary Human Astrocytes IMPLICATIONS FOR PATHOGENESIS OF HIV-1 DEMENTIA. *Journal of Biological Chemistry* 1996; 271(26):15303-15306.

335. Yuzefpolskiy Y, Baumann FM, Kalia V, Sarkar S. Early CD8 T-cell memory precursors and terminal effectors exhibit equipotent in vivo degranulation. *Cellular & molecular immunology* 2015; 12(4):400.

336. Freeman BE, Hammarlund E, Raué H-P, Slifka MK. Regulation of innate CD8+ T-cell activation mediated by cytokines. *Proceedings of the National Academy of Sciences* 2012; 109(25):9971-9976.

337. Kohlmeier JE, Cookenham T, Roberts AD, Miller SC, Woodland DL. Type I interferons regulate cytolytic activity of memory CD8+ T cells in the lung airways during respiratory virus challenge. *Immunity* 2010; 33(1):96-105.

338. Farrar MA, Schreiber RD. The molecular cell biology of interferon-gamma and its receptor. *Annual review of immunology* 1993; 11(1):571-611.

339. Silva CM, Lu H, Weber MJ, Thorner MO. Differential tyrosine phosphorylation of JAK1, JAK2, and STAT1 by growth hormone and interferon-gamma in IM-9 cells. *Journal of Biological Chemistry* 1994; 269(44):27532-27539.

340. Qing Y, Stark GR. Alternative activation of STAT1 and STAT3 in response to interferon-γ. *Journal of Biological Chemistry* 2004; 279(40):41679-41685.

341. Samardzic T, Jankovic V, Stosic-Grujicic S, Trajkovic V. STAT1 is required for iNOS activation, but not IL-6 production in murine fibroblasts. *Cytokine* 2001; 13(3):179-182.

342. Mander P, Brown GC. Activation of microglial NADPH oxidase is synergistic with glial iNOS expression in inducing neuronal death: a dual-key mechanism of inflammatory neurodegeneration. *Journal of neuroinflammation* 2005; 2(1):20.

343. Hsiao HY, Mak OT, Yang CS, Liu YP, Fang KM, Tzeng SF. TNF-α/IFN-γ-induced iNOS expression increased by prostaglandin E2 in rat primary astrocytes via EP2-evoked cAMP/PKA and intracellular calcium signaling. *Glia* 2007; 55(2):214-223.

344. Jackson SJ, Baker D, Cuzner ML, Diemel LT. Cannabinoid-mediated neuroprotection following interferon-gamma treatment in a three-dimensional mouse brain aggregate cell culture. *European Journal of Neuroscience* 2004; 20(9):2267-2275.

345. Walter L, Stella N. Cannabinoids and neuroinflammation. *British journal of pharmacology* 2004; 141(5):775-785.

346. Zarruk JG, Fernández-López D, García-Yébenes I, García-Gutiérrez MS, Vivancos J, Nombela F, et al. Cannabinoid type 2 receptor activation downregulates stroke-induced classic and alternative brain macrophage/microglial activation concomitant to neuroprotection. *Stroke* 2012; 43(1):211-219.

347. Conant K, Garzino-Demo A, Nath A, McArthur JC, Halliday W, Power C, et al. Induction of monocyte chemoattractant protein-1 in HIV-1 Tat-stimulated astrocytes and

elevation in AIDS dementia. *Proceedings of the National Academy of Sciences* 1998; 95(6):3117-3121.

348. Abbott NJ, Rönnbäck L, Hansson E. Astrocyte–endothelial interactions at the blood–brain barrier. *Nature Reviews Neuroscience* 2006; 7(1):41.

349. Janzer RC, Raff MC. Astrocytes induce blood–brain barrier properties in endothelial cells. *Nature* 1987; 325(6101):253.

350. Pellerin L, Magistretti PJ. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. *Proceedings of the National Academy of Sciences* 1994; 91(22):10625-10629.

351. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. *Journal of interferon & cytokine research* 2009; 29(6):313-326.

352. Penkowa M, Giralt M, Lago N, Camats J, Carrasco J, Hernández J, et al. Astrocyte-targeted expression of IL-6 protects the CNSagainst a focal brain injury. *Experimental neurology* 2003; 181(2):130-148.

353. Choi C, Park JY, Lee J, Lim J-H, Shin E-C, Ahn YS, et al. Fas ligand and Fas are expressed constitutively in human astrocytes and the expression increases with IL-1, IL-6, TNF-α, or IFN-γ. *The Journal of Immunology* 1999; 162(4):1889-1895.

354. Erdmann NB, Whitney NP, Zheng J. Potentiation of excitotoxicity in HIV-1-associated dementia and the significance of glutaminase. *Clinical neuroscience research* 2006; 6(5):315-328.

355. Haughey N, Nath A, Mattson M, Slevin J, Geiger J. HIV-1 Tat through phosphorylation of NMDA receptors potentiates glutamate excitotoxicity. *Journal of neurochemistry* 2001; 78(3):457-467.

356. Pitt D, Werner P, Raine CS. Glutamate excitotoxicity in a model of multiple sclerosis. *Nature medicine* 2000; 6(1):67.

357. Shaw P, Ince P. Glutamate, excitotoxicity and amyotrophic lateral sclerosis. *Journal of neurology* 1997; 244(2):S3-S14.

358. Bonventre JV. Roles of phospholipases A2 in brain cell and tissue injury associated with ischemia and excitotoxicity. *Journal of lipid mediators and cell signalling* 1996; 14(1-3):15-23.

359. Lok J, Martin LJ. Rapid subcellular redistribution of Bax precedes caspase-3 and endonuclease activation during excitotoxic neuronal apoptosis in rat brain. *Journal of neurotrauma* 2002; 19(7):815-828.

360. Palmer AM, Marion DW, Botscheller ML, Swedlow PE, Styren SD, DeKosky ST. Traumatic brain injury-induced excitotoxicity assessed in a controlled cortical impact model. *Journal of neurochemistry* 1993; 61(6):2015-2024.

361. Yi J-H, Hazell AS. Excitotoxic mechanisms and the role of astrocytic glutamate transporters in traumatic brain injury. *Neurochemistry international* 2006; 48(5):394-403.

362. Shaked I, Tchoresh D, Gersner R, Meiri G, Mordechai S, Xiao X, et al. Protective autoimmunity: interferon-γ enables microglia to remove glutamate without evoking inflammatory mediators. *Journal of neurochemistry* 2005; 92(5):997-1009.

363. Ngaotepprutaram T, Kaplan BL, Carney S, Crawford R, Kaminski NE. Suppression by Δ9-tetrahydrocannabinol of the primary immunoglobulin M response by human peripheral blood B cells is associated with impaired STAT3 activation. *Toxicology* 2013; 310:84-91.

364. Ngaotepprutaram T, Kaplan BL, Crawford RB, Kaminski NE. Differential Modulation by Delta 9-Tetrahydrocannabinol (Δ 9-THC) of CD40 Ligand (CD40L) Expression in Activated Mouse Splenic CD4+ T cells. *Journal of Neuroimmune Pharmacology* 2012; 7(4):969-980.

365. Ngaotepprutaram T, Kaplan BL, Kaminski NE. Impaired NFAT and NFκB activation are involved in suppression of CD40 ligand expression by Δ9-tetrahydrocannabinol in human CD4+ T cells. *Toxicology and applied pharmacology* 2013; 273(1):209-218.

366. Henriquez J, Zhou J, Li J, Crawford R, Kaminski N. Application of gene specific mRNA level determinations in individual cells using flow cytometry-based PrimeFlow™ in immunotoxicology. *Toxicology and Applied Pharmacology* 2017; 337:39-44.

367. Röck J, Schneider E, Grün JR, Grützkau A, Küppers R, Schmitz J, et al. CD303 (BDCA-2) signals in plasmacytoid dendritic cells via a BCR-like signalosome involving Syk, Slp65 and PLCγ2. *European journal of immunology* 2007; 37(12):3564-3575.

368. Pinho MP, Migliori IK, Flatow EA, Barbuto JAM. Dendritic cell membrane CD83 enhances immune responses by boosting intracellular calcium release in T lymphocytes. *Journal of leukocyte biology* 2014; 95(5):755-762.

369. Brown KN, Trichel A, Barratt-Boyes SM. Parallel loss of myeloid and plasmacytoid dendritic cells from blood and lymphoid tissue in simian AIDS. *The Journal of Immunology* 2007; 178(11):6958-6967.

370. Shinohara ML, Lu L, Bu J, Werneck MB, Kobayashi KS, Glimcher LH, et al. Osteopontin expression is essential for interferon-α production by plasmacytoid dendritic cells. *Nature immunology* 2006; 7(5):498-506.

371. Fujimoto Y, Tedder TF. CD83: a regulatory molecule of the immune system with great potential for therapeutic application. *Journal of medical and dental sciences* 2006; 53(2):85.

372. Hirano N, Butler MO, Xia Z, Ansén S, von Bergwelt-Baildon MS, Neuberg D, et al. Engagement of CD83 ligand induces prolonged expansion of CD8+ T cells and preferential enrichment for antigen specificity. *Blood* 2006; 107(4):1528-1536.

373. Zhou L-J, Tedder TF. Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *The Journal of Immunology* 1995; 154(8):3821-3835.

374. Lechmann M, Berchtold S, Steinkasserer A, Hauber J. CD83 on dendritic cells: more than just a marker for maturation. *Trends in immunology* 2002; 23(6):273-275.

375. Chang JJ, Altfeld M. TLR-mediated immune activation in HIV. *Blood* 2009; 113(2):269-270.

376. Bird L. Plasmacytoid dendritic cells: Division of labour. *Nature Reviews Immunology* 2017; 18(1):2.

377. Tsujimura H, Tamura T, Kong HJ, Nishiyama A, Ishii KJ, Klinman DM, et al. Toll-like receptor 9 signaling activates NF-κB through IFN regulatory factor-8/IFN consensus sequence binding protein in dendritic cells. *The Journal of Immunology* 2004; 172(11):6820-6827.

378. Weichhart T, Säemann M. The PI3K/Akt/mTOR pathway in innate immune cells: emerging therapeutic applications. *Annals of the rheumatic diseases* 2008; 67(Suppl 3):iii70-iii74.

379. Molina-Holgado E, Vela JM, Arévalo-Martín A, Almazán G, Molina-Holgado F, Borrell J, et al. Cannabinoids promote oligodendrocyte progenitor survival: involvement of cannabinoid receptors and phosphatidylinositol-3 kinase/Akt signaling. *Journal of Neuroscience* 2002; 22(22):9742-9753.

380. Santana MA, Esquivel-Guadarrama F. Cell biology of T cell activation and differentiation. *International review of cytology* 2006; 250:217-274.

381. Huber JP, David Farrar J. Regulation of effector and memory T-cell functions by type I interferon. *Immunology* 2011; 132(4):466-474.

382. Gallagher KM, Lauder S, Rees IW, Gallimore AM, Godkin AJ. Type I interferon (IFNα) acts directly on human memory CD4+ T cells altering their response to antigen. *The Journal of Immunology* 2009; 183(5):2915-2920.

383. Kolumam GA, Thomas S, Thompson LJ, Sprent J, Murali-Krishna K. Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection. *Journal of Experimental Medicine* 2005; 202(5):637-650.

384. Hardy GA, Sieg SF, Rodriguez B, Jiang W, Asaad R, Lederman MM, et al. Desensitization to type I interferon in HIV-1 infection correlates with markers of immune activation and disease progression. *Blood* 2009; 113(22):5497-5505.

385. Chen W, Kaplan BL, Pike ST, Topper LA, Lichorobiec NR, Simmons SO, et al. Magnitude of stimulation dictates the cannabinoid-mediated differential T cell response to HIVgp120. *Journal of leukocyte biology* 2012; 92(5):1093-1102.

386. Karmaus PW, Chen W, Kaplan BL, Kaminski NE. Δ 9-Tetrahydrocannabinol Suppresses Cytotoxic T Lymphocyte Function Independent of CB 1 and CB 2, Disrupting Early Activation Events. *Journal of Neuroimmune Pharmacology* 2012; 7(4):843-855.

387. Williams R, Yao H, Dhillon NK, Buch SJ. HIV-1 Tat co-operates with IFN-γ and TNF-α to increase CXCL10 in human astrocytes. *PLoS One* 2009; 4(5):e5709.

388. Ohmori Y, Hamilton TA. The interferon-stimulated response element and a kappa B site mediate synergistic induction of murine IP-10 gene transcription by IFN-gamma and TNF-alpha. *The Journal of Immunology* 1995; 154(10):5235-5244.

389. Klein TW. Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nature Reviews Immunology* 2005; 5(5):400-411.

390. Bouaboula M, Bourrié B, Rinaldi-Carmona M, Shire D, Le Fur G, Casellas P. Stimulation of cannabinoid receptor CB1 induces krox-24 expression in human astrocytoma cells. *Journal of Biological Chemistry* 1995; 270(23):13973-13980.

391. Curran NM, Griffin BD, O'Toole D, Brady KJ, Fitzgerald SN, Moynagh PN. The synthetic cannabinoid R (+) WIN 55,212-2 inhibits the interleukin-1 signaling pathway in human astrocytes in a cannabinoid receptor-independent manner. *Journal of Biological Chemistry* 2005; 280(43):35797-35806.

392. Chiurchiu V, Cencioni MT, Bisicchia E, Bardi M, Gasperini C, Borsellino G, et al. Distinct modulation of human myeloid and plasmacytoid dendritic cells by anandamide in multiple sclerosis. *Annals of neurology* 2013; 73(5):626-636.

393. Kaplan BLF, Rockwell CE, Kaminski NE. Evidence for cannabinoid receptor-dependent and-independent mechanisms of action in leukocytes. *Journal of Pharmacology and Experimental Therapeutics* 2003; 306(3):1077-1085.

394. Honda K, Yanai H, Mizutani T, Negishi H, Shimada N, Suzuki N, et al. Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling. *Proceedings of the National Academy of Sciences of the United States of America* 2004; 101(43):15416-15421.

395. Guiducci C, Ghirelli C, Marloie-Provost M-A, Matray T, Coffman RL, Liu Y-J, et al. PI3K is critical for the nuclear translocation of IRF-7 and type I IFN production by human plasmacytoid predendritic cells in response to TLR activation. *The Journal of experimental medicine* 2008; 205(2):315-322.

396. Hoshino K, Sugiyama T, Matsumoto M, Tanaka T, Saito M, Hemmi H, et al. IκB kinase-α is critical for interferon-α production induced by Toll-like receptors 7 and 9. *Nature* 2006; 440(7086):949-953.

397. Molina-Holgado E, Vela JM, Arévalo-Martín A, Almazán G, Molina-Holgado F, Borrell J, et al. Cannabinoids promote oligodendrocyte progenitor survival: involvement of cannabinoid receptors and phosphatidylinositol-3 kinase/Akt signaling. *The Journal of Neuroscience* 2002; 22(22):9742-9753.

398. Chiurchiù V, Rapino C, Talamonti E, Leuti A, Lanuti M, Gueniche A, et al. Anandamide Suppresses Proinflammatory T Cell Responses In Vitro through Type-1 Cannabinoid Receptor–Mediated mTOR Inhibition in Human Keratinocytes. *The Journal of Immunology* 2016; 197(9):3545-3553.

399. Börner C, Bedini A, Höllt V, Kraus J. Analysis of promoter regions regulating basal and interleukin-4-inducible expression of the human CB1 receptor gene in T lymphocytes. *Molecular pharmacology* 2008; 73(3):1013-1019.

400. Ipp H, Zemlin A. The paradox of the immune response in HIV infection: when inflammation becomes harmful. *Clinica chimica acta* 2013; 416:96-99.

401. Kaul M. HIV-1 associated dementia: update on pathological mechanisms and therapeutic approaches. *Current opinion in neurology* 2009; 22(3):315.

402. Jarrossay D, Napolitani G, Colonna M, Sallusto F, Lanzavecchia A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *European journal of immunology* 2001; 31(11):3388-3393.

403. Heaton RK, Franklin DR, Ellis RJ, McCutchan JA, Letendre SL, LeBlanc S, et al. HIV-associated neurocognitive disorders before and during the era of combination antiretroviral therapy: differences in rates, nature, and predictors. *Journal of neurovirology* 2011; 17(1):3-16.

404. Rumbaugh JA, Tyor W. HIV-associated neurocognitive disorders Five new things. *Neurology: Clinical Practice* 2015; 5(3):224-231.

405. Longhini AL, von Glehn F, Brandão CO, de Paula RF, Pradella F, Moraes AS, et al. Plasmacytoid dendritic cells are increased in cerebrospinal fluid of untreated patients during multiple sclerosis relapse. *Journal of neuroinflammation* 2011; 8(1):2.

406. McMahon EJ, Bailey SL, Miller SD. CNS dendritic cells: critical participants in CNS inflammation? *Neurochemistry international* 2006; 49(2):195-203.

407. Pashenkov M, Huang Y-M, Kostulas V, Haglund M, Söderström M, Link H. Two subsets of dendritic cells are present in human cerebrospinal fluid. *Brain* 2001; 124(3):480-492.

408. Sas AR, Bimonte-Nelson H, Smothers CT, Woodward J, Tyor WR. Interferon-α causes neuronal dysfunction in encephalitis. *The Journal of Neuroscience* 2009; 29(12):3948-3955.

409. Sas AR, Bimonte-Nelson HA, Tyor WR. Cognitive dysfunction in HIV encephalitic SCID mice correlates with levels of Interferon-α in the brain. *Aids* 2007; 21(16):2151-2159.

410. Lu T-S, Avraham HK, Seng S, Tachado SD, Koziel H, Makriyannis A, et al. Cannabinoids inhibit HIV-1 Gp120-mediated insults in brain microvascular endothelial cells. *The Journal of Immunology* 2008; 181(9):6406-6416.

411. Aggarwal SK, Carter GT, Sullivan MD, ZumBrunnen C, Morrill R, Mayer JD. Medicinal use of cannabis in the United States: historical perspectives, current trends, and future directions. *Journal of opioid management* 2009; 5(3):153-168.

412. Kozela E, Pietr M, Juknat A, Rimmerman N, Levy R, Vogel Z. Cannabinoids Δ9-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-κB and interferon-β/STAT proinflammatory pathways in BV-2 microglial cells. *Journal of biological chemistry* 2010; 285(3):1616-1626.

413. Downer EJ, Clifford E, Gran B, Nel HJ, Fallon PG, Moynagh PN. Identification of the Synthetic Cannabinoid R (+) WIN55, 212-2 as a Novel Regulator of IFN Regulatory Factor 3 Activation and IFN-β Expression RELEVANCE TO THERAPEUTIC EFFECTS IN MODELS OF MULTIPLE SCLEROSIS. *Journal of Biological Chemistry* 2011; 286(12):10316-10328.

414. Chappell WH, Steelman LS, Long JM, Kempf RC, Abrams SL, Franklin RA, et al. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. *Oncotarget* 2011; 2(3):135.

415. Zhang Q, Lenardo MJ, Baltimore D. 30 years of NF-κB: A blossoming of relevance to human pathobiology. *Cell* 2017; 168(1-2):37-57.

416. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007; 129(7):1261-1274.

417. Février M, Dorgham K, Rebollo A. CD4+ T cell depletion in human immunodeficiency virus (HIV) infection: role of apoptosis. *Viruses* 2011; 3(5):586-612.

418. Rodriguez B, Lederman MM, Jiang W, Bazdar DA, Garate K, Harding CV, et al. Interferon-α differentially rescues CD4 and CD8 T cells from apoptosis in HIV infection. *Aids* 2006; 20(10):1379-1389.

419. Brinkmann V, Geiger T, Alkan S, Heusser CH. Interferon alpha increases the frequency of interferon gamma-producing human CD4+ T cells. *Journal of Experimental Medicine* 1993; 178(5):1655-1663.

420. Marrack P, Kappler J, Mitchell T. Type I interferons keep activated T cells alive. *Journal of Experimental Medicine* 1999; 189(3):521-530.

421. Cha L, de Jong E, French MA, Fernandez S. IFN-α Exerts Opposing Effects on Activation-Induced and IL-7–Induced Proliferation of T Cells That May Impair Homeostatic Maintenance of CD4+ T Cell Numbers in Treated HIV Infection. *The Journal of Immunology* 2014; 193(5):2178-2186.

422. Rissoan M-C, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R, et al. Reciprocal control of T helper cell and dendritic cell differentiation. *Science* 1999; 283(5405):1183-1186.

423. Prentiss D, Power R, Balmas G, Tzuang G, Israelski DM. Patterns of marijuana use among patients with HIV/AIDS followed in a public health care setting. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 2004; 35(1):38-45.

424. Ellis RJ, Toperoff W, Vaida F, Van Den Brande G, Gonzales J, Gouaux B, et al. Smoked medicinal cannabis for neuropathic pain in HIV: a randomized, crossover clinical trial. *Neuropsychopharmacology* 2009; 34(3):672.

425. Gross G, Roussaki A, Ikenberg H, Drees N. Genital warts do not respond to systemic recombinant interferon alfa-2a treatment during cannabis consumption. *Dermatology* 1991; 183(3):203-207.

426. Ammirati E, Cianflone D, Vecchio V, Banfi M, Vermi AC, De Metrio M, et al. Effector memory T cells are associated with atherosclerosis in humans and animal models. *Journal of the American Heart Association* 2012; 1(1):27-41.

427. Lo Y-C, Edidin MA, Powell JD. Selective activation of antigen-experienced T cells by anti-CD3 constrained on nanoparticles. *The Journal of Immunology* 2013; 191(10):5107-5114.

428. Kelley CF, Kitchen CM, Hunt PW, Rodriguez B, Hecht FM, Kitahata M, et al. Incomplete peripheral CD4+ cell count restoration in HIV-infected patients receiving long-term antiretroviral treatment. *Clinical Infectious Diseases* 2009; 48(6):787-794.

429. Dolganiuc A, Chang S, Kodys K, Mandrekar P, Bakis G, Cormier M, et al. Hepatitis C virus (HCV) core protein-induced, monocyte-mediated mechanisms of reduced IFN-α and plasmacytoid dendritic cell loss in chronic HCV infection. *The Journal of Immunology* 2006; 177(10):6758-6768.

430. Lincoln D, Petoumenos K, Dore G. HIV/HBV and HIV/HCV coinfection, and outcomes following highly active antiretroviral therapy. *HIV medicine* 2003; 4(3):241-249.

431. González S, Cebeira M, Fernández-Ruiz J. Cannabinoid tolerance and dependence: a review of studies in laboratory animals. *Pharmacology Biochemistry and Behavior* 2005; 81(2):300-318.

432. Kessing CF, Spudich S, Valcour V, Cartwright P, Chalermchai T, Fletcher JL, et al. High Number of Activated CD8+ T Cells Targeting HIV Antigens Are Present in

Cerebrospinal Fluid in Acute HIV Infection. *JAIDS Journal of Acquired Immune Deficiency Syndromes* 2017; 75(1):108-117.

433. Buchweitz JP, Karmaus PW, Williams KJ, Harkema JR, Kaminski NE. Targeted deletion of cannabinoid receptors CB1 and CB2 produced enhanced inflammatory responses to influenza A/PR/8/34 in the absence and presence of Δ9-tetrahydrocannabinol. *Journal of leukocyte biology* 2008; 83(3):785-796.

434. Scharer CD, Barwick BG, Youngblood BA, Ahmed R, Boss JM. Global DNA methylation remodeling accompanies CD8 T cell effector function. *The Journal of Immunology* 2013; 191(6):3419-3429.

435. Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *Journal of immunological methods* 2003; 281(1-2):65-78.

436. Giorgi JV, Hultin LE, McKeating JA, Johnson TD, Owens B, Jacobson LP, et al. Shorter survival in advanced human immunodeficiency virus type 1 infection is more closely associated with T lymphocyte activation than with plasma virus burden or virus chemokine coreceptor usage. *The Journal of infectious diseases* 1999; 179(4):859-870.

437. Giorgi JV, Liu Z, Hultin LE, Cumberland WG, Hennessey K, Detels R. Elevated levels of CD38+ CD8+ T cells in HIV infection add to the prognostic value of low CD4+ T cell levels: results of 6 years of follow-up. The Los Angeles Center, Multicenter AIDS Cohort Study. *Journal of acquired immune deficiency syndromes* 1993; 6(8):904-912.

438. Cheshire JL, Baldwin AS. Synergistic activation of NF-kappaB by tumor necrosis factor alpha and gamma interferon via enhanced I kappaB alpha degradation and de novo I kappaBbeta degradation. *Molecular and cellular biology* 1997; 17(11):6746-6754.

439. Sánchez C, Rueda D, Ségui B, Galve-Roperh I, Levade T, Guzmán M. The CB1 cannabinoid receptor of astrocytes is coupled to sphingomyelin hydrolysis through the adaptor protein fan. *Molecular pharmacology* 2001; 59(5):955-959.

440. Stella N. Cannabinoid and cannabinoid-like receptors in microglia, astrocytes, and astrocytomas. *Glia* 2010; 58(9):1017-1030.

441. Sheng WS, Hu S, Min X, Cabral GA, Lokensgard JR, Peterson PK. Synthetic cannabinoid WIN55, 212-2 inhibits generation of inflammatory mediators by IL-1β-stimulated human astrocytes. *Glia* 2005; 49(2):211-219.

442. Ma X, Reynolds SL, Baker BJ, Li X, Benveniste EN, Qin H. IL-17 enhancement of the IL-6 signaling cascade in astrocytes. *The Journal of Immunology* 2010:ji_1000142.

443. Chen W, Crawford RB, Kaplan BL, Kaminski NE. Modulation of HIV GP120 Antigen-Specific Immune Responses In Vivo by Δ 9-Tetrahydrocannabinol. *Journal of Neuroimmune Pharmacology* 2015; 10(2):344-355.

444. Zhang S, Xie R, Wan F, Ye F, Guo D, Lei T. Identification of U251 glioma stem cells and their heterogeneous stem-like phenotypes. *Oncology letters* 2013; 6(6):1649-1655.

445. Sun W, McConnell E, Pare J-F, Xu Q, Chen M, Peng W, et al. Glutamate-dependent neuroglial calcium signaling differs between young and adult brain. *Science* 2013; 339(6116):197-200.

446. Trajkovic V, Vuckovic O, Stosic-Grujicic S, Miljkovic D, Popadic D, Markovic M, et al. Astrocyte-induced regulatory T cells mitigate CNS autoimmunity. *Glia* 2004; 47(2):168-179.

447. Karim QA. Current status of the HIV epidemic & challenges in prevention. *Indian Journal of Medical Research* 2017; 146(6):673.

448. Hess KL, Hu X, Lansky A, Mermin J, Hall HI. Lifetime risk of a diagnosis of HIV infection in the United States. *Annals of epidemiology* 2017; 27(4):238-243.

449. Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, et al. Clonal expansions of CD8+ T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. *Journal of Experimental Medicine* 2000; 192(3):393-404.

450. Zakzanis KK. Distinct neurocognitive profiles in multiple sclerosis subtypes. *Archives of Clinical Neuropsychology* 2000; 15(2):115-136.

451. Sethi A, Sterling RK. HIV-HCV Coinfection. *Gastroenterology & hepatology* 2006; 2(5):357.

452. Manuzak JA, Gott TM, Kirkwood JS, Coronado E, Hensley-McBain T, Miller C, et al. Heavy Cannabis Use Associated With Reduction in Activated and Inflammatory Immune Cell Frequencies in Antiretroviral Therapy–Treated Human Immunodeficiency Virus–Infected Individuals. *Clinical Infectious Diseases* 2018.

453. Edlin BR, Irwin KL, Faruque S, McCoy CB, Word C, Serrano Y, et al. Intersecting epidemics--crack cocaine use and HIV infection among inner-city young adults. *New England Journal of Medicine* 1994; 331(21):1422-1427.

454. Kawanaka N, Yamamura M, Aita T, Morita Y, Okamoto A, Kawashima M, et al. CD14+, CD16+ blood monocytes and joint inflammation in rheumatoid arthritis. *Arthritis & Rheumatology* 2002; 46(10):2578-2586.

455. Cavanagh LL, Boyce A, Smith L, Padmanabha J, Filgueira L, Pietschmann P, et al. Rheumatoid arthritis synovium contains plasmacytoid dendritic cells. *Arthritis Res Ther* 2005; 7(2):R230.