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Title Page

Full Title: Interferon-α-mediated Activation of T Cells from Healthy and HIV-infected Individuals is Suppressed by Δ^9-Tetrahydrocannabinol[i]

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Running Title Page

Running Title: THC Suppresses T cell activation by IFNα and IL-7

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Abstract

HIV patients routinely use medicinal cannabinoids to treat neuropathic pain, anxiety, and HIV-associated wasting. However, Δ^9-Tetrahydrocannabinol (THC), the primary psychoactive cannabinoid in cannabis, suppresses T cell function and secretion of interferons, both critically important in the anti-viral immune response. Interferon-α (IFNα), a key cytokine in T cell activation and peripheral control of HIV infection, can potentiate responsiveness to IL-7, a crucial homeostatic cytokine for peripheral T cell maintenance. The objective of this investigation was to compare the response of T cells to stimulation by IFNα and IL-7 in T cells from healthy and HIV+ donors in the absence and presence of THC. To compare T cells responses between healthy and HIV+ donors signaling through IFNAR, IFNα-induced expression of IL-7Rα, cognate signaling through IL-7R, and on IL-7-mediated T cell proliferation were measured by by flow cytometry and RT-qPCR. $CD8^+$ T cells from HIV+ donors showed a diminished response to IFNα-induced pSTAT1 compared to $CD8^+$ T cells from healthy donors while $CD4^+$ T cells from HIV+ donors and healthy donors were comparable. Treatment with IFNα promoted IL-7R expression and potentiated IL-7-induced STAT5 phosphorylation to augment IL-7-mediated proliferation by T cells from healthy and HIV+ donors. Finally, HIV+ donors exhibited reduced sensitivity to THC-mediated suppression by IFNα and IL-7-mediated stimulation compared to healthy donors. These results further support THC as immune suppressive while identifying putatively beneficial aspects of cannabinoid-based therapies in HIV+ patients.

Introduction:

Approximately, 37 million people globally are infected with HIV(Karim, 2017) of which 1.1 million reside in the United States (Hess et al., 2017). $CD4^+$ T cell leukocytopenia is a hallmark of HIV infection, with $CD4^+$ T cells being a primary cellular target for HIV (Dragic et al., 1996; Sousa et al., 2002). The perturbation of $CD4^+$ T cells causes a loss of adaptive immune integrity including loss of cytotoxic T lymphocyte (CTL/$CD8^+$ T cell) function (Roederer et al., 1995) culminating in acquired immune disorder syndrome (AIDS) (Koot et al., 1993). Since the mid-1990's, the standard of care following HIV diagnosis is anti-retroviral therapy (ART) (Autran et al., 1997; Iacob et al., 2017). ART facilitates $CD4^+$ T cell restoration and, by extension, $CD8^+$ T cell restoration (Oxenius et al., 2000). However, 15-20% of HIV patients continue to have T cell deficiencies despite successful ART therapy (Kelley et al., 2009; Serrano-Villar et al., 2014).

One reported T cell deficiency in HIV patients exhibiting a low $CD4^+$ T cell nadir is reduced expression of interleukin-7 receptor (IL-7R) (MacPherson et al., 2001; Nguyen et al., 2016; Hartling et al., 2017). IL-7 is a crucial cytokine for T cell integrity as it drives both differentiation and peripheral maintenance of T cells(Tan et al., 2001). Likewise, IL-7 enhances expansion of T cells from HIV+ donors(Bazdar and Sieg, 2007; Levy et al., 2009; Sereti et al., 2009). Clinically, the use of IL-7 in HIV patients reversed T cell leukocytopenia and restored gut lumen integrity (*i.e.* Leaky-gut syndrome)(Sereti et al., 2014). The mechanisms underlying IL-7R expression deficiency in HIV-infected T cells is poorly understood, but is correlated with the phenomenon termed "T-cell exhaustion", which is associated with chronic and extensive antigenic stimulation (Lang et al., 2005).

IL-7R expression is tightly regulated with type 1 interferons playing a critical role in that regulation through a Type-1-interferon-inducible promoter region called an interferon sensitive

responsible element (ISRE)(Mazzucchelli and Durum, 2007). Type 1 interferons are composed of interferon (IFN)α and IFNβ and possess anti-viral activity (García-Sastre and Biron, 2006). Plasmacytoid dendritic cells (pDC) are the primary source of IFNα secreting (Colonna et al., 2004) and have a direct influence on maintaining T cell integrity during HIV infection. Typically the circulating numbers of pDC and $CD4^+$ T cell are correlated in a positive manner (Donaghy et al., 2001). During chronic HIV infection the is a reduction of pDC number and function resulting in a decrease capacity to produce IFNα (Chehimi et al., 2002). IFNα has also been shown to suppress HIV expansion(Poli et al., 1989) and provided protection for $CD4^+$ T cells from HIV-mediated depletion in a humanized mouse model(Lapenta et al., 1999). Furthermore, pDC promoted T cell activation and protection against certain viral infections when using a Fc-fused IL-7(Kang et al., 2017).

In addition to the complications arising from chronic HIV infection, HIV patients routinely utilize medicinal cannabinoids to treat: HIV-associated wasting, as an appetite stimulant; neuropathic pain, from use of some HIV reverse transcriptase inhibitors as part of ART regimens; and generally reduce anxiety (Abrams, 2000; Prentiss et al., 2004; Haney et al., 2007; Ellis et al., 2009). The primary psychoactive cannabinoid in cannabis, Δ^9-tetrahydrocannabinol (THC), and synthetic THC, dronabinol (*i.e.* marinol), exhibits potent anti-inflammatory activity and is also immunosuppression (Klein et al., 1998; Tanasescu and Constantinescu, 2010). It is well established that THC can suppress T cell responses to viral infections (Reiss, 2010; Eisenstein and Meissler, 2015), including HIV (Roth et al., 2005). Additionally, pDC secretion of IFNα is acutely sensitive to THC-mediated suppression and pDC from HIV+ donors show increased sensitivity to THC-mediated suppression than pDC from healthy donors (Henriquez et al., 2017).

The objective of this investigation was to compare the response of T cells to stimulation by IFNα and IL-7 in T cells from healthy and HIV+ donors in the absence and presence of THC. Specifically, studies were conducted to determine whether *in vitro* stimulation of T cells by IFNα would drive the expression of IL-7Rα, thereby potentiating IL-7-mediated signaling and proliferation. Furthermore, the effect of THC on the stimulation of T cells by IFNα and IL-7 was evaluated. Last, the responses to IFNα and IL-7, in the absence and presence of THC in T cells from healthy and HIV+ donors was compared.

Materials and Methods

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 with Hanks Balanced Salt Solution (HBSS) from Gibco™ (Grand Island, NY) and layered on Ficoll Paque Plus™ (GE Healthcare Life Sciences, Pittsburgh, PA) in SepMate™ tubes by StemCell Technologies™ (Vancouver, BC, Canada). Leukocytes were re-suspended in complete-Rosewell Park Memorial Institute (C-RPMI) Media from Gibco™ containing 5% Human AB Serum (Sigma-Aldrich, St. Louis, MO), 1% Penicillin-Streptomycin (Gibco), and 0.1% β-mercaptoethanol. T cells were identified using antibodies by BioLegend® (San Diego, CA) as $CD3^+$ cells and as either $CD4^+$ or $CD8^+$. Memory cells were identified as $CD45RO^+$ and non-memory cells were identified as $CD45RO^-$.

T cell purification by Magnetic Activated Cell Sorting (MACS): T cells were isolated using MACs CD3-T cell isolation kits from BioLegend. In short, following PBMC isolation, the cell concentration was determined using a Coulter Particle-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 magnet and T cells were collected in the flow through.

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 - 0.03% ethanol) prepared in C-RPMI. Cells were incubated at 37^{o}C and 5% CO_2 for 30 min before being stimulated (below).

Stimulation of T cells: Following treatment with THC or VC, PBMC or isolated T cells were stimulated as follows: a) to measure the phosphorylation of STAT1, cells were stimulated with 100U/ml of universal IFNα (PBL Assay Science, Piscataway, NJ) for 30 min before harvesting for phospho-protein detection (below); b) to measure IFNα-induced IL-7Rα mRNA and protein expression, cells were treated with 100U/ml of IFNα for 48 hr before harvesting and measurement of respective endpoints (below); c) IL-7-induced phosphorylation of STAT5 on day 0 or 48 hr post IFNα stimulation (100U/ml) was measured by stimulating cells with 10 ng/ml of IL-7 for 15 min before harvesting for phospho-protein detection (below); and d) for measuring IL-7-augmented proliferation of T cells (below), cells were stimulated with 100U/ml of IFNα, 2.5 μg/ml of mouse anti-human CD3 antibody (Biolegend), and 2.5 μg/ml mouse anti-human CD28 antibody for 48 hr followed by stimulation with 10 ng/ml of IL-7 or vehicle control (sterile endotoxin-free water from Invivogen, San Diego, CA) and cells were incubated for another 48 hr before harvesting.

Gene Expression Analysis: RNA was isolated using RNeasy® kits (Qiagen, Hilden, Germany) per the manufacturer's instructions. Briefly, T cells were lysed using lysing buffer containing β-mercaptoethanol and stored at -20^{o}C. Lysates were 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^{o}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.

Phospho-Protein and IL-7Ra Detection: 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 min at 37^{o}C, permeabilized with 1x of Phosflow™ perm buffer IV, stained for 1 hr 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 FACS buffer, and then immediately analyzed by flow cytometry. IL-7Rα surface expression was determined by surface staining with mouse anti-human antibodies (Biolegend).

Detection of T cell proliferation: Prior to activation (above) PBMC were treated with violet CellTrace™ dye by Thermo-Fisher. In brief, the dye was resuspended in DMSO and diluted in warm, sterile, PBS (0.02% DMSO). PBMC were incubated in the PBS/dye mixture at 37^{o}C for 20 min, washed with C-RPMI. Cells were then centrifuged, washed with RPMI, and resuspended in C-RPMI before stimulation (above). T cell proliferation for all T cell populations was determined by dye dilution and presented as the division index (DI), which was generated by the FlowJo v. 10 (FlowJo, LLC, Ashland, OR) proliferation tool. Specifically, the DI is the average number of cell divisions that a cell in the original population has undergone and includes all cells in the population.

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 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 subject, questionnaire, 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.

Data Analysis. Prism 5.0 by GraphPad Software, Inc (La Jolla, CA) 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 figure legends).

Results

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

As indicated in the materials and methods, HIV+ donors for this study were chosen based on the following criteria: 1) not using medicinal or recreational cannabinoids, 2) CD4+ T cell counts (>500cts/μl), 3) CD4:CD8 ratios (>1), 4) not co-infected with Hepatitis (A, B or C), and 5) currently on ART with non-detectable viral burdens. It is noteworthy that the number of $CD4^+$ and $CD8^+$ T cells provides only a partial view of the overall T cell repertoire. T cell were also evaluated for CD45RO expression, which identifies memory T cells (Fig.1A). 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 (Fig.1B).

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 (Hardy et al., 2009). Moreover, T cells from HIV+ donors without antiretroviral therapy have an altered response to IFNα compared to T cells from healthy donors (Hardy et al., 2009). 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 (Fig.2A). By contrast, no differences were observed in the expression of IFNAR2 comparing $CD8^+$ T cells from healthy and HIV+ donors.

Presently, it is unknown how T cells from HIV+ donors on ART respond to IFNα or THC. To address this, the phosphorylation of signal transducer and activator of transcription 1 (STAT1) in

response to treatment with IFNα was quantified (Fig.2B). $CD4^+$ T cells from HIV+ patients trended towards elevated background levels of pSTAT1 compared to $CD4^+$ T cells from healthy donors (Fig.2C-2E) and this difference was statistically significant in the $CD45RO^-$ (non-memory) $CD4^+$ T cells (Fig.2E). Upon addition of IFNα, similar induction of pSTAT1 was observed in $CD4^+$ T cells from HIV+ donors and healthy donors (Fig.2C-2E). Conversely, $CD8^+$ cells from HIV+ donors had diminished IFNα-induced pSTAT1 compared to healthy donors (Fig.2E). Furthermore, treatment with THC significantly suppressed IFNα-induced pSTAT1 in $CD4^+$ and $CD8^+$ T cells from both HIV+ and healthy donors (Fig.2C-2H), 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 (Fig.2E).

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(Mazzucchelli and Durum, 2007), 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 (Fig.3A). Interestingly, we found that the effects of IFNα on IFNAR2 expression were insensitive to THC treatment in T cells from healthy donors (Supplemental Figure 1).

Studies were also performed to determine the direct effects of THC on IFNα-induced IL-7Rα protein expression (Fig3B). 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 (Fig.3C-H). In addition, THC produced 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 compared to matched T cells from HIV+ donors and memory ($CD45RO^+$) cells (Fig.3E & 3H).

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 described by the "Spare Receptor Theory". By extension, the magnitude of IL-7Rα expression is not necessarily indicative of receptor function or delineates differences between T cells from healthy and HIV+ donors. Therefore, studies were performed to evaluate the effect of IFNα and THC on IL-7-induced signaling by quantifying the magnitude of STAT5 phosphorylation (Fig.4A). These studies showed that $CD4^+$ T cells from HIV+ donors had diminished IL-7-induced pSTAT5 before IFNα stimulation compared to $CD4^+$ T cells from healthy donors (Fig.4B). 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 (Fig.4C-4H). 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 both total and non-memory ($CD45RO^-$) $CD4^+$ (Fig.4C & 4H) and $CD8^+$ (Fig.4F & 4H) T cells.

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 of T cells is poorly characterized (Catalfamo et al., 2011). 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 a division index (Fig.5A). Stimulation with IFNα had minimal augmentation of CD3/CD28-induced proliferation in isolation (Fig 5B & 5C). However, stimulation with IFNα before the 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 (Fig 5B). This phenomenon was also observed in $CD8^+$ T cells from HIV+ donors (Fig. 5C). Stimulation with CD3/CD28/IFNα also increased the proportion of $CD45RO^+$ (memory) cells in $CD4^+$ and $CD8^+$ T cells and was more pronounced in HIV+ donors, but which was not significantly affected by treatment with IL-7 (Fig.5D & 5E). In the presence of THC, $CD4^+$ and $CD8^+$ T cell from healthy donors showed a diminished proliferative response to control treated cells (Fig.5F-5K). Interestingly, THC-mediated suppression of the proliferative response was diminished in $CD4^+$ T cells from HIV+ compared to healthy donors (Fig.5F-5H). By contrast, $CD8^+$ T cells from HIV+ donors showed comparable suppression in the presence of THC to $CD8^+$ T cells from healthy donors (Fig.5I-5K).

Discussion

Presented here is the first report of differential 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 (Donaghy et al., 2001; Chehimi et al., 2002; Gulzar and Copeland, 2004; Benlahrech and Patterson, 2011; Catalfamo et al., 2011; Février et al., 2011; Rizzo et al., 2017). Therefore, we investigated the responsiveness of T cells to IFNα, which is crucial to maintaining T cell homeostasis and is a critical mediator of antiviral responses. We found that 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 possessed 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 (Rodriguez et al., 2006). 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 (Brinkmann et al., 1993).

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 (Marrack et al., 1999; Kolumam et al., 2005; Huber and David Farrar, 2011) and potentially synergizes with IL-7 to stimulate HIV+ donor T cells (Catalfamo et al., 2011). We show here that IFNα drives IL-7R expression and potentiates IL-7 signaling, as evidenced by augmented IL-7-induced pSTAT5, in cells treated with IFNα. We also show that IL-7 drove robust proliferation of T cells treated with IFNα. These results partially agree with previous findings (Cha et al., 2014) and strengthen the link between IFNα, pDC number, and $CD4^+$ T cell number in HIV+ patients (Lapenta et al., 1999; Rissoan et al., 1999; Donaghy et al., 2001; Rodriguez et al., 2006). Specifically, circulating pDC secrete IFNα which may play a role in sensitizing T cells in their response to IL-7.

HIV+ patients routinely utilize medicinal cannabinoids (Abrams, 2000; Prentiss et al., 2004; Haney et al., 2007; Ellis et al., 2009). Cannabis use reduces the efficacy of IFNα as a therapeutic (Gross et al., 1991). 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 (Kozela et al., 2010). Likewise, THC also suppresses 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 suppresses the effects of IL-7 on proliferation, likely through suppression 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, endpoints temporally distal to STAT1 phosphorylation 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 (Abrams et al., 2003). Conversely, $CD8^+$ T cells from HIV+ patients showed marked suppression of proliferation by THC 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 (Ammirati et al., 2012). Furthermore, we did not distinguish the T regulatory (Treg) $CD4^+$ T cells from non Treg-$CD4^+$ T cells. It is noteworthy that IFNα can suppress Treg function (Becker et al., 2013). Lastly, 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 (Lo et al., 2013).

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 rage (>1); c) no co-infection with any strain of hepatitis; 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 a) the effects of HIV infection in: female HIV+ patients, which have different immunological responses to HIV infection compared to men (Berghöfer et al., 2006; Meier et al., 2009; Addo and Altfeld, 2014); b) HIV patients treated successfully with ART without restoration of $CD4^+$ T cell counts, which have elevated $CD8^+$ T cell activity and higher HIV-related mortality(Kelley et al., 2009; Serrano-Villar et al., 2014); c) patients co-infected with a virus, since infections with Hepatitis C Virus can alter interferon responses and T cell activation (Lincoln et al., 2003; Dolganiuc et al., 2006)); d) HIV patients suffering from early versus established T cell exhaustion, since the response to IL-7 likely changes as IL-7R expression is lost as T cell exhaustion progresses (Yi et al., 2010); and e) patients utilizing medicinal cannabinoids, since chronic THC exposure can lead to tolerance through various pharmacodynamic mechanisms (González et al., 2005), but it is unknown if chronic cannabis use can lead to THC tolerance in leukocytes. Finally, these studies were designed to address the effects of THC treatment on early signaling events by IFNα, CD3/CD28, and IL-7-mediated stimulation by single dose pretreatment with THC and do not address the effects of repeat treatment of THC or THC treatment on established effector cell functions. Further studies will be required to characterize the effects of THC in these various patient and cell populations to understand the consequences of cannabinoid use by HIV+ patients.

The findings presented in this article are the first to show a direct link between IFNα and IL-7-mediated augmentation of CD3/CD28-induced 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 (Henriquez et al., 2017) and elevated activation of pDC in women with HIV is linked to faster T cell depletion (Berghöfer et al., 2006), which is associated with more severe neurocognitive deficiency (Burlacu et al., 2017). Additionally, peripheral immune activation of $CD8^+$ T cells (Kessing et al., 2017) and monocytes is tied to the development of HIV-associated neural inflammation, which could explain why cannabis users have reduced inflammatory monocyte numbers (Rizzo et al., 2017). 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 largely unaffected.

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Author Contributions

Participated in research design: Henriquez, Rizzo, Crawford, Gulick and Kaminski

Conducted experiments: Henriquez, Rizzo

Performed data analysis: Henriquez

Wrote or contributed to the writing of the manuscript: Henriquez, Kaminski

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Footnotes

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Legends for Figures

Figure 1. 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 possessed $CD4^+$ and $CD8^+$ T cell numbers that where comparable to the healthy donors, and comparable memory ($CD45RO^+$)/non-memory ($CD45RO^-$) cell compositions.

Figure 2. 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+ donors were isolated by Ficoll Paque™ density gradient centrifugation and used for either determination of IFNAR2 surface expression or IFNα-induced STAT1 phosphorylation (pSTAT1). A) IFNAR2 expression was quantified by flow cytometry using the mean-fluorescence intensity (MFI). For pSTAT1, PBMC were treated with either vehicle (0.03% EtOH) or THC (1, 5, or 10 μM) in 0.03% EtOH for 30 min before stimulation with 100 U/ml of IFNα for 30 min. B) Representative experiment of IFNα-mediated pSTAT1 induction and THC (10 μM)-mediated suppression in T cells from a healthy donor. C-H) 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 T cells from healthy donors. For pSTAT1, Asterisks indicate statistically significant differences of the treatment with the HIV status-matched vehicle control (0 THC) (*$p < 0.5$; **p

< 0.01; ***p < 0.001). Daggers indicate statistically significant differences of treatment-matched groups between T cells from Healthy and HIV+ donors (†p < 0.5) (2-way analysis of variance with Bonferroni multiple comparison's posttest).

Figure 3. THC suppresses IFNα induced expression of IL-7Rα mRNA in T cells from healthy donors but differentially affects IFNα-induced surface expression of IL-7Rα in T cells from healthy vs HIV+ T donors. 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 (0.03% EtOH) or various concentrations of THC (1, 5, or 10 μM) for 30 min. After treatment, cells were stimulated with IFNα (100U/ml), incubated for 48 hr and harvested for quantification of IL-7Rα mRNA levels by RT-qPCR. For determination of IL-7Rα surface expression, PBMC from healthy and HIV+ 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 48 hr. B) Example of IFNα-mediated IL-7Rα expression and THC (10 μM)-mediated suppression in a healthy donor. C-H) The effects of THC on the expression level (MFI) of IL-7Rα in T cells from healthy and HIV+ donors in: 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 ≤ 0.5; **p ≤ 0.01; ***p ≤ 0.001). Daggers indicate statistically significant differences of treatment-matched groups between Healthy and HIV+ T cells (†p ≤ 0.5; ††p ≤ 0.01) (2-way analysis of variance with Bonferroni multiple comparison's posttest).

Figure 4. THC suppresses IFNα-mediated augmentation of IL-7-induced STAT5 phosphorylation. PBMC from healthy and HIV+ donors were isolated through Ficoll Paque™ density gradient centrifugation. Cells were either: 1) immediately used for detection of IL-7-induced pSTAT5 (D0) by treating with IL-7 (10ng/ml) for 15 min then rapidly fixed; or 2) treated with either vehicle (0.03% EtOH) or various concentrations of THC (1, 5, or 10 μM) for 30 min, stimulated with IFNα (100U/ml), incubated for 48, and then used for detection of IL-7-induced pSTAT5 as described above. A) Representative experiment of IL-7Rα-induced pSTAT5 and THC (10 μM)-mediated suppression in a healthy donor. B) Levels (MFI) of pSTAT5 in T cells was determined by flow cytometry on day 0. C-H) The effects of THC on the IL-7-induced pSTAT5 level following IFNα stimulation of T cells from healthy and HIV+ donors in: 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 between treatments with the HIV status-matched vehicle control (0 THC) (*$p \leq 0.5$; **$p \leq 0.01$; ***$p \leq 0.001$). Daggers indicate statistically significant differences of treatment-matched groups between Healthy and HIV+ T cells (†$p \leq 0.5$) (2-way analysis of variance with Bonferroni multiple comparison's posttest).

Figure 5. IL-7 augmented CD3/CD28/IFNα-induced T cell proliferation is suppressed by THC. PBMC from healthy and HIV+ donors were isolated through Ficoll Paque™ density gradient centrifugation. Cells were stained with violet CellTrace™ dye and then treated with either vehicle (0.03% EtOH) or various concentrations of THC (1, 5, or 10 μM) for 30 min. After treatment, cells were stimulated with IFNα (100U/ml) and anti-CD3 and anti-CD28 antibodies (2.5ug/ml each) for 48 hr, treated with IL-7 (10ng/ml) or vehicle (endotoxin-free

H_2O), and incubated for another 48 hr before harvesting. T cell proliferation is represented as the division index (DI) as determined by the FlowJo® proliferation tool. A) Representative experiment 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. B & C) The effects of treatment with IFNα and IL-7 on anti-CD3/CD28-mediated T cell proliferation in: B) $CD4^+$ and C) $CD8^+$ T cells from healthy and HIV+ donors. D & E) The effect of IL-7 stimulation total $CD3^+$ T cell composition and between memory ($CD45RO^+$) and non-memory ($CD45RO^-$) cells in: D) $CD4^+$ and E) $CD8^+$ T cells. F-K) The effects of THC on the IL-7-induced augmentation of CD3/CD28/IFNα-induced proliferation of T cells from healthy and HIV+ donors in: 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) (*$p \leq 0.5$; **$p \leq 0.01$; ***$p \leq 0.001$). Daggers indicate statistically significant differences of treatment matched groups between Healthy and HIV+ T cells (†$p \leq 0.5$; ††$p \leq 0.01$) (2-way analysis of variance with Bonferroni multiple comparison's posttest).

Figures

JPET Fast Forward. Published on July 19, 2018 as DOI: 10.1124/jpet.118.250308
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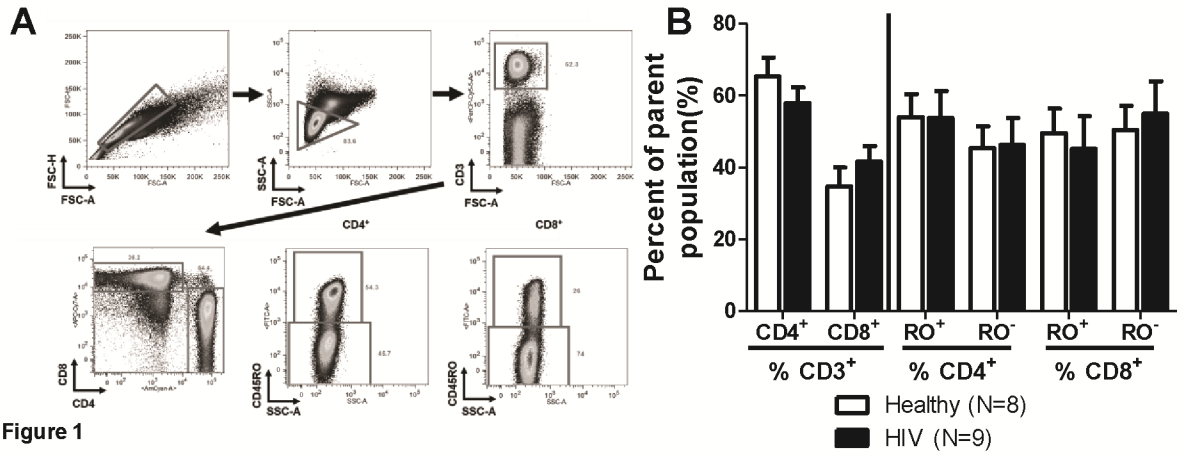

Figure 1

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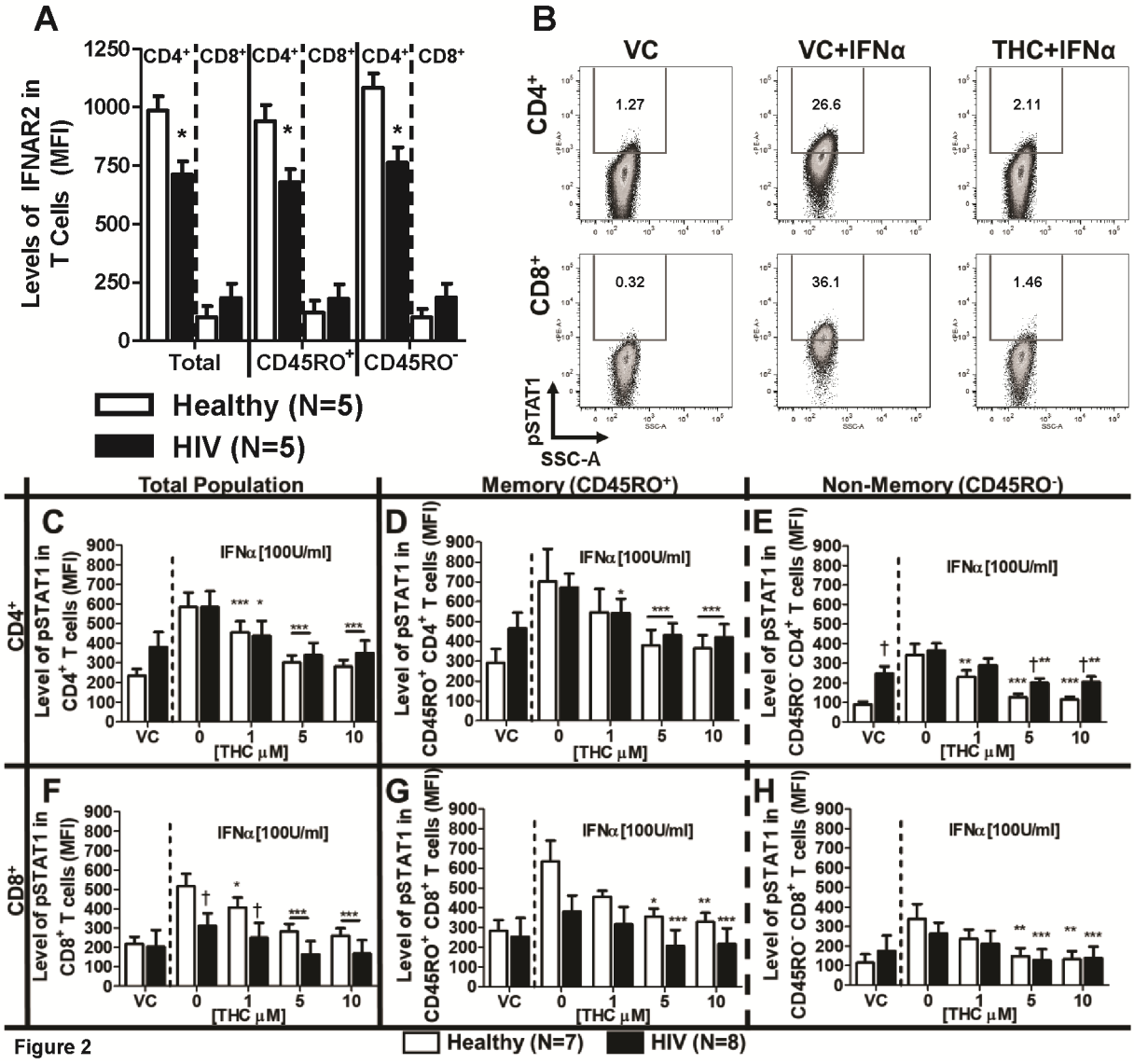

Figure 2

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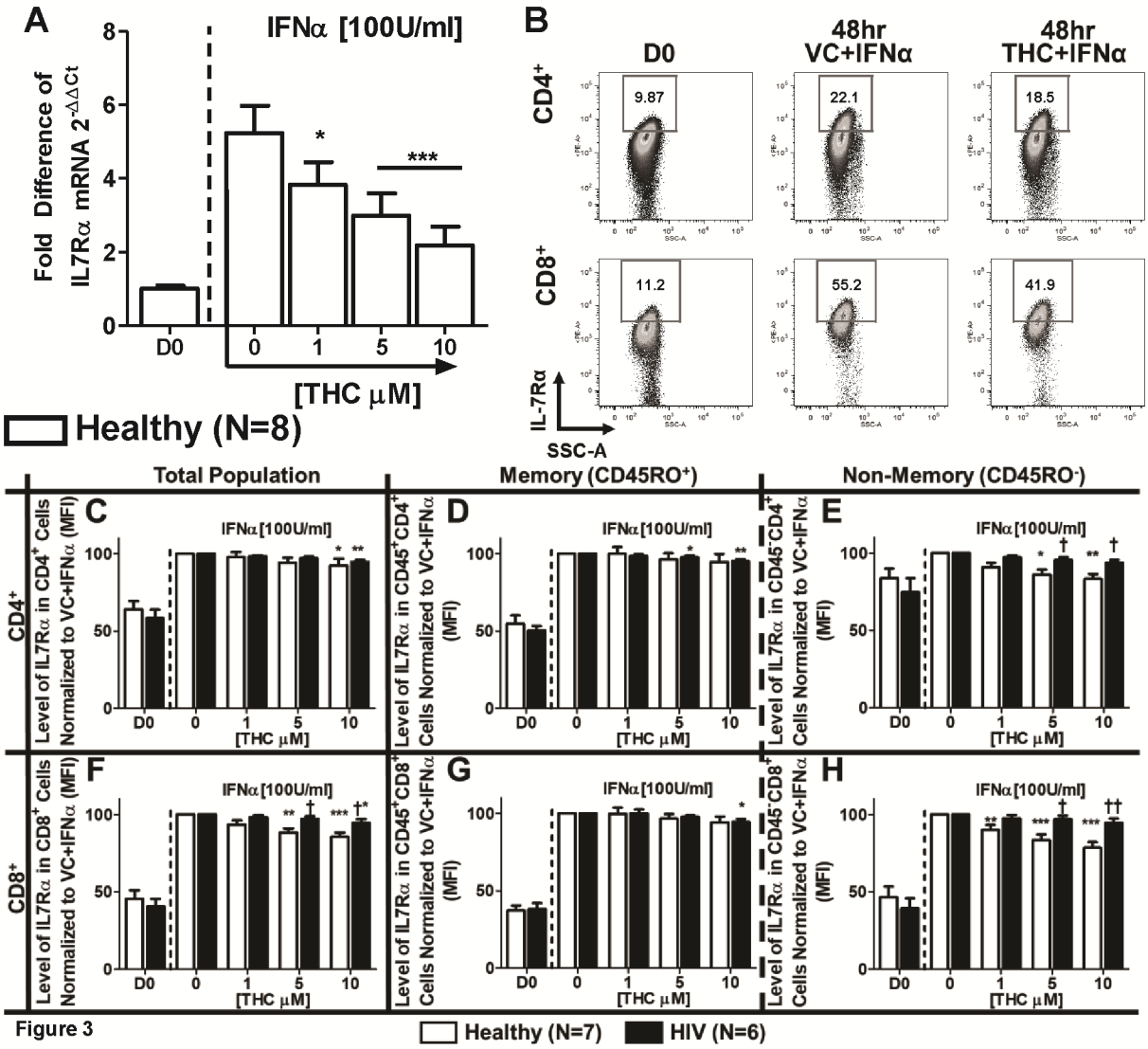

Figure 3

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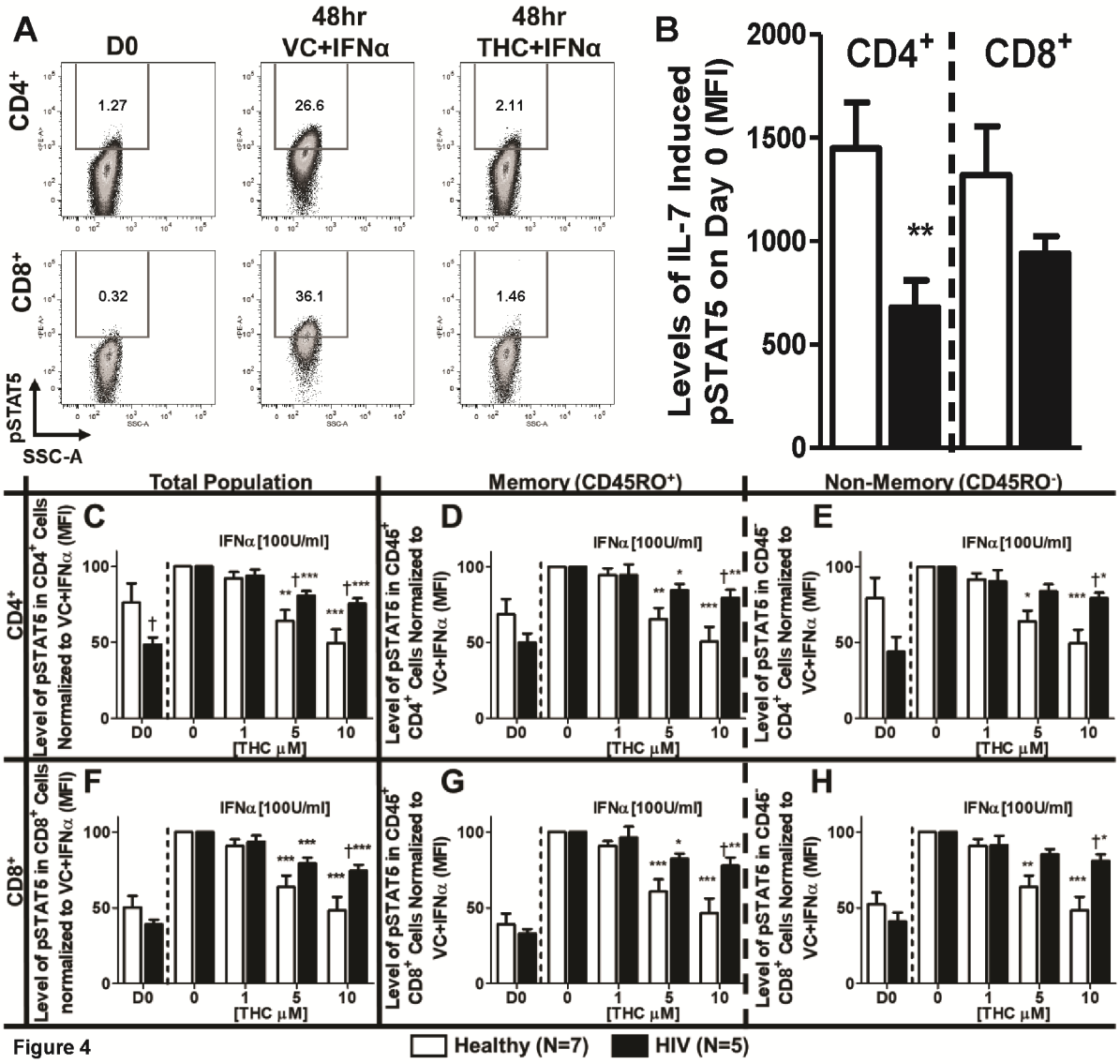

Figure 4

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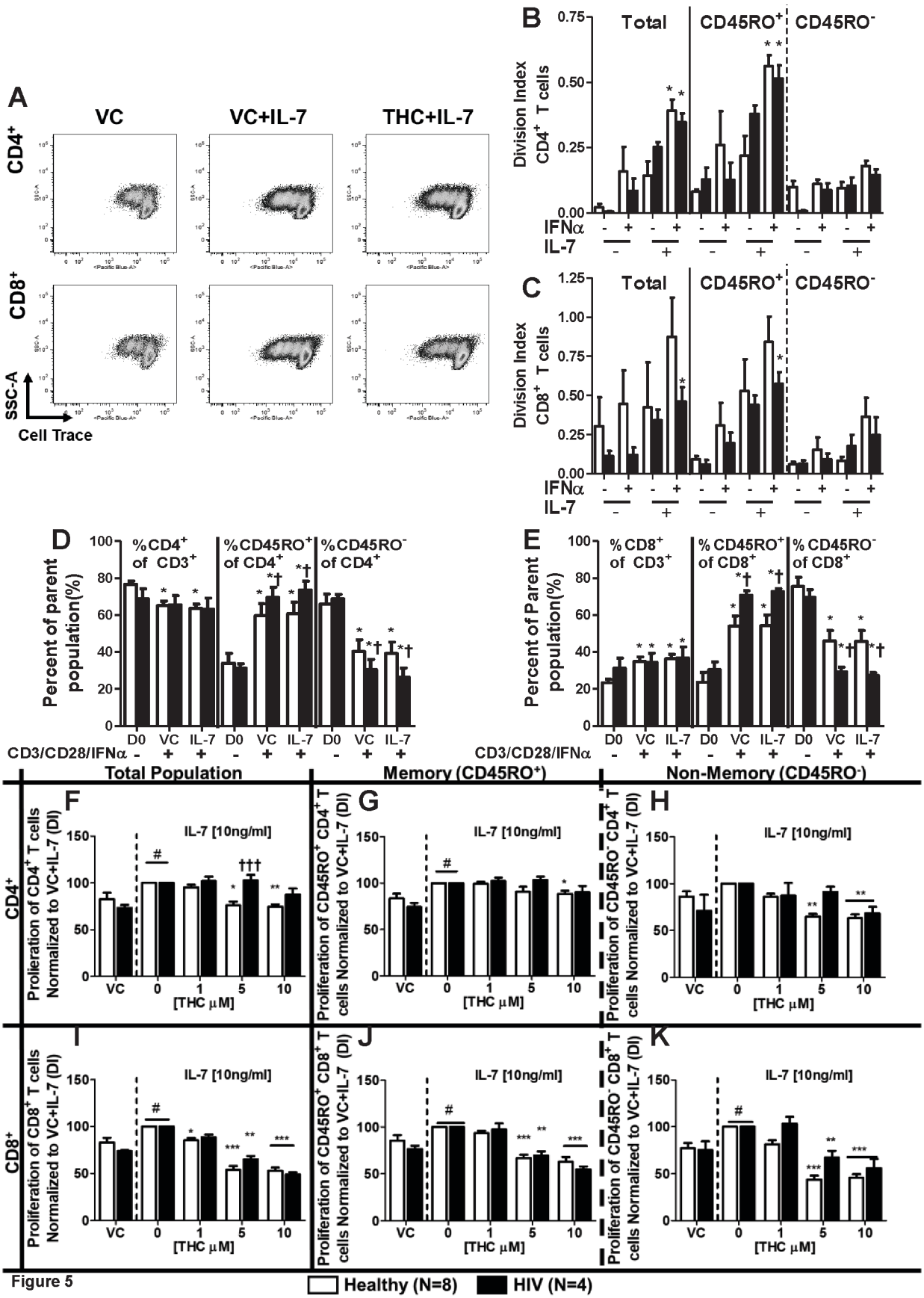
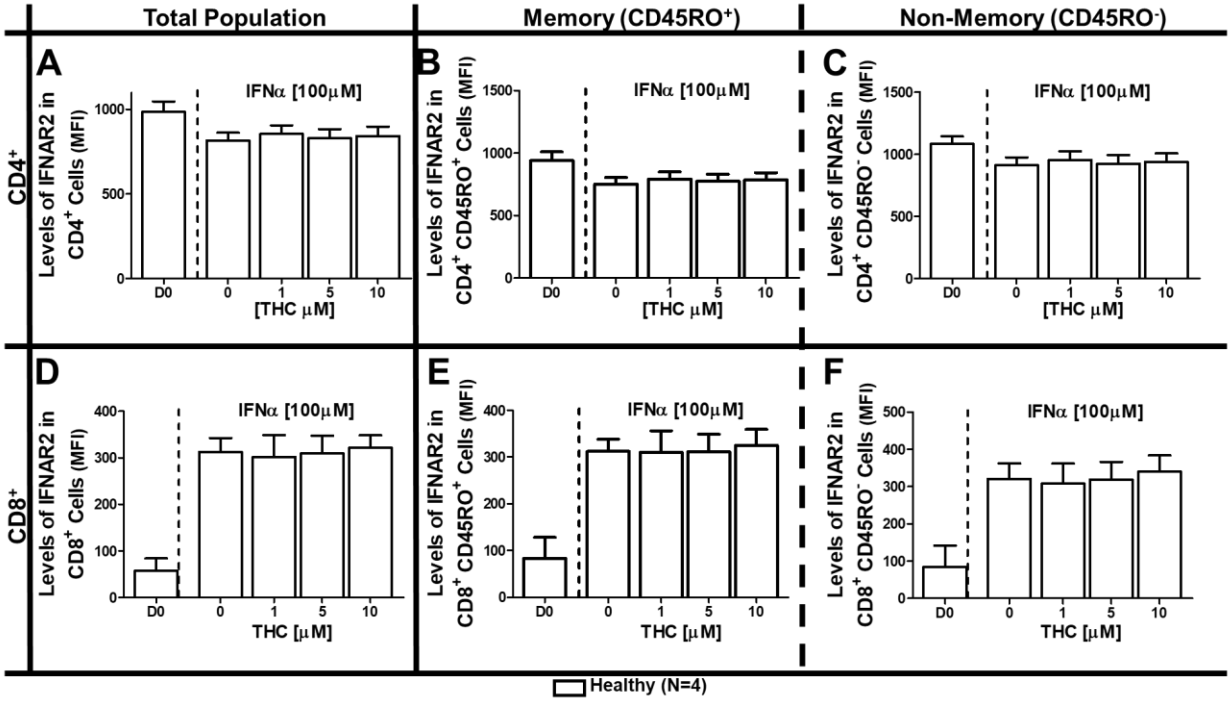


Figure 5

Article Title: Interferon-α-mediated Activation of T Cells from Healthy and HIV-infected Individuals is Suppressed by Δ^9-Tetrahydrocannabinol[i]

Authors: Joseph E. Henriquez, Michael D. Rizzo, Robert B. Crawford, Peter Gulick, & Norbert E. Kaminski,

Journal: *Journal of Pharmacology and Experimental Therapeutics*



Supplemental Digital Content 1. Stimulation of T cells with IFNα reduces IFNAR2 expression of CD4+ cells and enhances expression on CD8+ T cells while treatment with THC has no effect on IFNα-induced modulation of IFNAR2 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 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 hrs at which point cells were harvested and stained as described above. A-F) The surface expression of IFNAR2 was determined by flow cytometric analysis in healthy: A) total , B) memory and C) non-memory $CD4^+$ cells; and D) total, E)memory, and F) non-memory $CD8^+$ cells.