Δ^9-TETRAHYDROCANNABINOL SUPPRESSES HUMAN MONOCYTE ACTIVATION AND MONOCYTE-MEDIATED ASTROCYTE INFLAMMATION: IMPLICATIONS FOR HIV-ASSOCIATED NEUROINFLAMMATION

By

Michael Denton Rizzo

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ABSTRACT

Δ^9-TETRAHYDROCANNABINOL SUPPRESSES HUMAN MONOCYTE ACTIVATION AND MONOCYTE-MEDIATED ASTROCYTE INFLAMMATION: IMPLICATIONS FOR HIV-ASSOCIATED NEUROINFLAMMATION

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A hallmark of human immunodeficiency virus (HIV) infection is chronic immune activation and is believed to be one of the major contributors to neuroinflammation and HIV-associated neurocognitive disorder (HAND). Circulating activated monocytes, including those that are $CD16^+$, have been implicated in HIV-associated neuroinflammation. These activated monocytes become infected with HIV in the periphery, cross the blood-brain barrier (BBB) and release inflammatory factors, HIV virions and viral proteins. These factors lead to HIV infection and activation of brain-resident cells, including microglia and astrocytes, driving a pro-inflammatory environment in the brain. Ultimately, these processes contribute to neuronal dysfunction and death, ultimately resulting in cognitive decline in up to 50% of the HIV-infected population. Cannabis is widely used by the HIV-infected population at an estimated prevalence of 23-56% in the United States. Δ^9-Tetrahydrocannabinol (THC) and cannabidiol (CBD), two major constituents of cannabis, are known to have immune suppressive and anti-inflammatory properties. The overall objective of this project was to determine whether the cannabinoids, THC and CBD, could suppress monocyte activation and monocyte-mediated astrocyte inflammation, which are key processes implicated in chronic neuroinflammation and HAND. Herein, it is shown that HIV-infected donors using cannabis displayed a lower level of circulating activated ($CD16^+$) monocytes and plasma IP-10 compared to non-using HIV-infected donors. Furthermore, *in vitro* studies revealed that THC but not CBD suppressed monocyte expression

of CD16 and secretion of IP-10, suggesting that THC is the major cannabinoid in cannabis promoting the anti-inflammatory effects. To determine whether activated monocytes could promote inflammatory functions of brain-resident glial cells, we developed a human co-culture system utilizing primary monocytes and cell-line/primary fetal astrocytes with viral-related stimulators (IFNα and a TLR7 agonist – R837). Monocytes, together with IFNα and/or R837, promoted astrocyte secretion of MCP-1, IL-6 and IP-10. Furthermore, monocyte-derived IL-1β was critical for astrocyte secretion of pro-inflammatory factors, as neutralization of IL-1β strongly hampered the astrocyte response, while direct addition of recombinant IL-1β to astrocyte monocultures mimicked the actions of monocytes. *In vitro* THC treatment of the R837-stimulated co-culture resulted in decreased astrocyte production of MCP-1 and IL-6, while CBD increased IL-6 production and had no effect on MCP-1 production. With the use of separate monocyte and astrocyte monocultures, THC and CBD were shown to directly target both cell types. Interestingly, THC and CBD were both shown to decrease the percent of astrocytes producing IL-6 and MCP-1, which for THC, is concordant with the co-culture observation. However, the CBD-mediated decrease in IL-6 and MCP-1 production in the astrocyte monoculture differed from the observations in the CBD-treated co-culture. Our findings were explained when THC and CBD were shown to suppress and augment monocyte production of IL-1β, respectively. Furthermore, the CBD-mediated augmentation of monocyte-derived IL-1β was able to override the direct CBD suppression on the astrocytes. Collectively, THC but not CBD, impairs monocyte activation and monocyte-driven astrocyte inflammatory responses. In the context of HAND, cannabis use, in particular THC, may decelerate monocyte processes that are implicated in neuroinflammation and cognitive dysfunction.

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KEY TO ABBREVIATIONS

11-OH-THC: 11-hydroxy-THC

2-AG: 2-arachidonoylglycerol

7-AAD: 7-amino-actinomycin D

ABM: astrocyte basal media

AC: adenylate cyclase

ACD: acid citrate dextrose

ACK: ammonium-chloride-potassium

AEA: anandamide

ALCAM: activated leukocyte cell adhesion molecule

ANI: asymptomatic neurocognitive impairment

ANOVA: analysis of variance

AP-1: activator protein 1

APC: allophycocyanin

ART: antiretroviral therapy

ASC: apoptosis-associated speck-like protein

ATP: adenosine triphosphate

BBB: blood-brain barrier

BMEC: brain microvascular endothelial cells

BMI: body mass index

BSA: bovine serum albumin

cAMP: Cyclic adenosine 3,5-monophosphate

CB: cannabinoid receptor

CBD: cannabidiol

CBN: cannabinol

CCL3: C-C motif chemokine ligand 3

CCL5: C-C motif chemokine ligand 5

CCR1: C-C chemokine receptor type 1

CCR2: C-C chemokine receptor type 2

CCR5: C-C chemokine receptor type 5

CD: cluster of differentiation

CFR: code of federal regulations

CNS: central nervous system

COOH: carboxyl

CREB: cAMP response element binding protein

CSF: cerebrospinal fluid

CX3C1: CX3C chemokine receptor 1

CXCR4: C-X-C motif chemokine receptor 4

Cy: cyanine dye

CYP: cytochrome P450

DMEM: dulbecco's modified eagle medium

DNA: deoxyribonucleic acid

EAE: experimental autoimmune encephalomyelitis

ELISA: enzyme-linked immunosorbent assay

ERK: extracellular signal-regulated kinase

EtOH: ethanol

FAAH: fatty acid amide hydrolase

FACS: fluorescence-activated cell sorting

FITC: fluorescein isothiocyanate

FSC-A: forward scatter area

FSC-H: forward scatter height

GA-1000: gentamicin sulfate-amphotericin

GFAP: glial fibrillary acidic protein

$G_{i/o}$-protein: inhibitory G-protein

gp120: glycoprotein 120

GPR: G-protein coupled receptor

HAD: HIV-associated dementia

HAND: HIV-associated neurocognitive disorder

HIPAA: health insurance portability and accountability act of 1996

HIV: human immunodeficiency virus

HSC: hematopoietic stem cell

ICAM-1: intercellular adhesion molecule 1

IFNAR: IFNα receptor

IFNα: interferon alpha

IFNγ: interferon gamma

Ig: immunoglobulin

IκB: inhibitor of NF-κB

IKK: IκB kinase

IL-1β: interleukin 1 beta

IL-6: interleukin 6

IL-10: interleukin 10

IP-10/CXCL10: IFN-γ-inducible protein 10

IRAK: interleukin-1 receptor-associated kinase

IRB: institutional review board

ISG: interferon-stimulated gene

JAK1: januse kinase 1

JAM-A: junctional adhesion molecule-A

JNK: c-Jun N-terminal kinase

Ki: binding inhibitory constant

LEAF: low endotoxin, azide-free

LPS: lipopolysaccharide

MAGL: monoacylglycerol lipase

MAPK: mitogen-activated protein kinases

MCP-1/CCL-2: monocyte chemoattractant protein 1

MDM: monocyte-derived macrophage

MFI: mean fluorescence intensity

MIG: monokine induced by interferon gamma

MJ: cannabis

MMHC: Mid-Michigan HIV consortium

MND: mild neurocognitive disorder

MyD88: myeloid differentiation primary response 88

NEF: negative regulatory factor

NFκB: nuclear factor kappa B

NHA: normal human astrocytes

NK: natural killer

NLRP3: nucleotide-binding domain-like receptor protein 3

NO: nitric oxide

NS: non-stimulated

OH: hydroxyl

P2X7: P2X purinoceptor 7

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

PCR: polymerase chain reaction

pDC: plasmacytoid dendritic cell

PE: r-phycoerythrin

PerCP: peridinin chlorophyll protein complex

PI3K: phosphoinositide 3-kinase

PKA: protein kinase A

R837: imiquimod

REDcap: research data capture

rhEGF: recombinant human epidermal growth factor

RIG-I: retinoic acid-inducible gene I

RM: repeated measures

RNA: ribonucleic acid

ROS: reactive oxygen species

RPMI: roswell park memorial institute

RTU: ready-to-use

SD: standard deviation

SEM: standard error of the mean

SIV: simian immunodeficiency virus

SSC: side-scatter

ssRNA: single-stranded RNA

STAT: signal transducer and activator of transcription

TAB: TAK1-binding protein

TAK: transforming growth factor β-activated kinase

Tat: transactivator of transcription

THC: Δ^9-tetrahydrocannabinol

THC-COOH: 11-Nor-9-carboxy-THC

TIR: Toll/IL-1 receptor

TLR: toll-like receptor

TNFα: tumor necrosis factor alpha

TRAF: TNF receptor associated factor

TYK2: tyrosine kinase 2

U251: U-251 MG cell line

UGT: glucuronosyltransferase

LITERATURE REVIEW

I. Overview of monocytes and astrocytes

A. Function and subsets of human monocytes

Monocytes are a circulating leukocyte population derived from hematopoietic stems cells (HSCs) in the bone marrow. HSCs differentiate into a common myeloid progenitor and then to several intermediate stages including a granulocyte and macrophage precursor, macrophage and dendritic cell precursor, and a common monocyte progenitor, before exiting the bone marrow as a monocyte (1, 2). Monocytes, which make up 5-30% of peripheral blood mononuclear cells (PBMCs), are identified primarily by surface expression of CD14, which is a lipopolysaccharide (LPS) co-receptor that helps to initiate toll-like receptor 4 (TLR4) signal transduction and cellular activation in response to LPS (3). Monocytes are further divided into subsets based on surface expression of the low affinity Fc receptor, CD16. The three subsets include: classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{+}$) (4). The majority (85-95%) of circulating monocytes of healthy individuals are of the classical phenotype ($CD14^{+}CD16^{-}$), whereas $CD14^{+}CD16^{+}$ monocytes comprise the remaining 5-15% (4).

In humans, monocytes exit the bone marrow in the classical phenotype and when in circulation a small percentage of classical monocytes transition to the intermediate phenotype before final differentiation into a non-classical monocyte (5). However, the mechanism(s) or factors governing monocyte differentiation to $CD16^{+}$ aren't well established. Classical monocytes have a life span of 1 day in circulation before entering tissues, differentiating or undergoing cell death, while intermediate and non-classical monocytes have longer circulating life spans of 4.3 and 7.4 days, respectively (5).

Monocytes possess an extensive repertoire of pattern-recognition, chemokine, and cytokine receptors that facilitate activation, trafficking and effector functions for pathogen clearance. Effector functions include: phagocytosis, reactive oxygen species (ROS) production, cytokine secretion, and antigen presentation (4). All three subsets have the capacity to infiltrate tissues during an immune response; however, differences in monocyte subset recruitment and function are dependent on the specific pathogen and associated immune factors produced. Monocyte subsets have been shown to display differing pattern-recognition receptor and cytokine/chemokine receptor expression profiles which leads to differences in monocyte subset recruitment, activation and functional profile (4, 6-10). Specifically, classical monocytes display high expression of C-C chemokine receptor type 2 (CCR2) and low expression of CX3C chemokine receptor 1 (CX3CR1) whereas non-classical monocytes display the opposite expression profile (8). Intermediate monocytes express both CCR2 and CX3CR1, and express the chemokine receptor/HIV-co-receptor, C-C chemokine receptor type 5 (CCR5) (6, 8). Therefore, the ligand for CCR2, monocyte-chemoattractant protein 1 (MCP-1/CCL2), is a major chemokine promoting recruitment of classical monocytes while CX3CL1 recruits non-classical monocytes (11).

Classical monocytes are more potent at phagocytosis compared to the intermediate and non-classical subsets and also secrete specific cytokines (e.g., IL-10, CCL2, IL-6) upon LPS stimulation (4). $CD16^+$ monocytes (intermediate and non-classical) are often termed "inflammatory" monocytes due to their increased ability to secrete pro-inflammatory cytokines in response to LPS (e.g., IL-1β, IL-6 and TNFα) and promote T cell activation (4, 12). An increased frequency of $CD16^+$ monocytes in peripheral blood has been observed in several chronic inflammatory conditions, including: viremic human immunodeficiency virus (HIV),

atherosclerosis, multiple sclerosis and systemic lupus erythematosus (13-16). As these cells have increased pro-inflammatory potential, they are considered as a major contributor to chronic immune activation (17, 18).

B. Astrocyte function during neuroinflammation

Astrocytes are one of the most abundant cell populations in the human brain and originate from neural stem cells during development (19, 20). They contain numerous branch-like processes extending from the cell body allowing them to cover the brain in a non-overlapping manner and communicate with each other (21, 22). Despite being highly heterogeneous, astrocytes have been categorized into two major subtypes, protoplasmic and fibrous. Protoplasmic astrocytes are found in the gray matter and surround neuronal synapses while fibrous astrocytes are found in the white matter surrounding axons (21). Astrocytes are commonly identified in the brain by their expression of the intermediate filament protein, glial fibrillary acidic protein (GFAP) (21, 23). Astrocytes have numerous functions including neurotropic support, blood flow and blood-brain barrier (BBB) regulation, neurotransmitter uptake/release, energy storage (e.g., glycogen) and mediating inflammation (21, 24, 25).

During central nervous system (CNS) insults (e.g., viral infection), astrocytes undergo a process termed astrogliosis, which is characterized by increased expression of GFAP, cellular hypertrophy and proliferation. During severe insults, astrocytes form a scar around the site of injury, which serves as a protective barrier between normal CNS tissue and the injured/inflamed tissue (21). Furthermore, astrocytes play an important role in the inflammatory response to pathogen exposure (26). Astrocytes express a range of pattern recognition receptors, including several TLRs, along with cytokine receptors, which allows them to respond to pathogens and inflammatory factors. Their major pro-inflammatory functions include peripheral leukocyte

recruitment and cytokine secretion, but also have anti-inflammatory roles during tissue repair (24, 26-28). Specifically, astrocytes have been shown to be sensitive to the pro-inflammatory cytokines, IL-1β and TNFα, which promotes their secretion of several cytokines (e.g., IL-6) and chemokines (e.g., MCP-1 and IFN-γ-inducible protein 10 (IP-10)) that facilitate leukocyte infiltration into the brain (29, 30).

II. The role of monocytes in HIV-associated neurocognitive disorder (HAND)

A. Classification and epidemiology of HAND

Antiretroviral therapy (ART) has shifted HIV prognosis to a controllable disease; however, health complications remain and include cognitive decline, cardiovascular disease and malignancies (31). A characteristic of HIV pathogenesis is neurocognitive dysfunction and is identifiable in up to 50% of HIV patients, regardless of ART (32, 33). The range of neurocognitive abnormalities due to HIV infection is known as HIV-associated neurocognitive disorders (HAND). HAND is sub-classified into three categories and is diagnosed using neuropsychological testing and self-reported (or third party) symptoms (34, 35). HAND is classified into three categories and includes: (1) asymptomatic neurocognitive impairment (ANI); (2) mild neurocognitive disorder (MND); and (3) HIV-associated dementia (HAD) (34). The neuropsychological testing examines seven different areas of cognition, which includes attention-information processing, language, executive function, memory, sensory ability, and simple and complex motor skills. ANI is defined as one standard deviation (SD) below the mean of the normalized scores in the general population in at least two of the domains from above. For an individual to be defined as having MND, two cognitive domains are abnormal as in ANI but the cognitive impairment also mildly impacts daily functions. Finally, HAD is defined as two SDs below the mean of the normalized scores in the general population in at least two of the

domains from above and the cognitive impairment markedly affects daily activities (34). The major cognitive domains impacted in ART-treated individuals are executive function and memory (32, 36, 37) compared to impaired motor skills, cognitive speed and fluency in the pre-ART era (32).

Before ART, HIV-associated dementia (HAD) was most common, with an estimated prevalence of 20%. With ART, the percentage of HIV patients with HAND remains similar (50%), but the degree of impairment is less severe (50% ANI/MND and 5% HAD) suggesting that viral suppression slows the progression of HAND (32). Since HIV patients are living longer, how patients progress from mild to more severe forms (MND and HAD) of HAND as they age has become an important question. However, limited reports have addressed the progression of HAND in the ART era. One 5-year follow-up study demonstrated that 63% of patients had persistent neurocognitive impairment (36) and another revealed that patients already diagnosed with ANI had a 2-fold to 6-fold higher risk of developing MND compared to patients without neurocognitive impairment (38).

Prominent risk factors for the progression of HAND include CD4 nadir, age, substance abuse and depression (35). CD4 nadir, which is defined as the patient's lowest $CD4^+$ T cell count recorded, is arguably the most important risk factor for HAND as several studies have associated a low CD4 nadir with cognitive impairment (39, 40). Tan and colleagues found that older HIV-infected patients (>50 years old) have a higher risk of memory deficits (5-fold) compared to younger individuals (41), highlighting age as a probable risk factor. In addition, a large segment of the HIV-infected population suffers from depression (24-42%), which has been linked to cognitive decline (42).

Substance abuse (e.g., cocaine, cannabis and methamphetamine) amongst HIV-infected individuals is thought to be a significant contributor to neurocognitive impairment (43-49). Numerous reports have found methamphetamine and cocaine use to be associated with impaired cognition in HIV-infected individuals (50-55). However, there are mixed reports on cannabis use and cognitive decline in HIV-infected individuals, as cannabis use seems to affect memory when examining current or acute use while having no significant long-term effects (56). Specifically, a study found that current cannabis use amongst HIV-infected individuals was linked to impaired cognition in only one of many neurocognitive tests evaluated; however, lifetime cannabis use was not associated with impairment in any of the cognitive tests measured (57). This report would suggest that the acute psychotropic effect of cannabis promotes a transient impairment in cognition but not over the long term. Conversely, another study found daily cannabis use was associated with a decline in memory but only in HIV-infected individuals at advanced stages of disease progression (58). Furthermore, a recent 2016 report found that moderate-to-heavy HIV+ cannabis users had a decline in memory and learning compared to non-users (59).

B. The role of neuroinflammation in HAND

The etiology of HAND is not fully understood but is due, in part, to dysfunction, damage and ultimately death of neurons, in the absence of productive HIV infection of neurons (60, 61). A growing body of evidence supports chronic immune activation and CNS inflammation as major mechanisms underlying neuronal damage (62, 63). HIV-induced neuroinflammation is characterized by BBB dysregulation, persistent HIV entry, leukocyte infiltration into the CNS, and activation/HIV-infection of CNS-resident astrocytes, microglia and monocyte-derived

macrophages (61, 62, 64). In addition, there is some evidence that specific ART drugs may contribute to neuroinflammation and/or neurotoxicity (65-67).

The BBB is composed of brain microvascular endothelial cells (BMECs) connected via tight junctions. BMECs are surrounded by astrocytes, which are critical in BBB regulation as their end processes interact with BMECs to modulate blood flow and permeability (64, 68). The BBB becomes comprised during HIV infection; however, the underlying mechanisms are not well established. A proposed mechanism of BBB disruption is through viral protein and pro-inflammatory cytokine (e.g., TNFα and IL-1β)-mediated effects on BMECs, which can occur in the brain and blood (64). The above factors are thought to contribute to increased BBB permeability through the down regulation of tight junction proteins along with increased BMEC expression of cell adhesion molecules. Increased BBB permeability facilitates entry of infected/un-infected leukocytes and cell-free virus into the CNS, which are major contributors to HIV-associated neuroinflammation (61, 64, 69). In fact, HIV entry into CNS has been detected as early as 8 days post-infection and remains detectable despite ART (70-72). The mechanisms of HIV entry into the CNS are: (a) migration of peripherally infected immune cells across BBB; and (b) cell-free mechanisms (paracellular and transcellular) across the dysfunctional BBB (61, 62, 69, 73). Monocytes and T cells are the primary immune cells implicated in trafficking HIV into the CNS (74, 75).

Entry of activated immune cells and HIV into the CNS contributes to HIV-associated neuroinflammation. Once in the brain, activated immune cells release inflammatory factors including virions, viral proteins, reactive oxygen species and cytokines (TNFα, IL-1β and IL-6), which activates brain-resident cells including microglia and astrocytes (61, 62, 74, 76-78). The milieu of host and viral factors can directly promote neuronal injury or indirectly promote injury

due to the dysfunction of adjacent cells including astrocytes (61, 77, 79). HIV viral proteins, gp120 and transactivator of transcription (Tat), have been shown to promote cell death of primary human neurons *in vitro* (80). Furthermore, elevated IP-10 levels *in vitro* also promote neuronal apoptosis through a direct mechanism (81). By contrast, TNFα and IL-1β suppress glutamate uptake by astrocytes, which leads to increased extracellular glutamate and neuronal excitotoxicity (82). IL-1β and TNFα also promote astrocyte secretion of a battery of cytokines and chemokines including IL-6, MCP-1 and IP-10 (30, 83). The chronic release of these factors promotes further inflammation via the recruitment of additional activated/infected immune cells from circulation (84). Overall, prolonged production of neuroinflammatory factors, leukocyte recruitment and impaired glutamate control are major mechanisms driving neuronal dysfunction, damage, cell death and eventually HAND (61, 62, 77, 79).

C. The role of monocytes in HIV-associated neuroinflammation

Monocytes, especially those expressing CD16, are thought to contribute to HIV-associated neuroinflammation (62, 74, 76). Specifically, increased levels of activated $CD16^+$ monocytes in circulation have been observed in patients with chronic HIV infection and HIV-associated dementia (13, 85, 86). Circulating $CD16^+$ monocytes are highly susceptible to HIV infection when compared to $CD16^-$ monocytes, which seems in part, due to elevated expression of the HIV co-receptor, CCR5 (6, 87). Furthermore, the $CD16^+$ subset has been shown to be the primary monocyte population to harbor HIV *in vivo* despite patients receiving ART (87). Studies involving animal models and post-mortem HAND patients have identified an increased level of $CD16^+$ monocytes in the CNS, which may be due to surface expression of CCR2 (88-94). Specifically, CCR2 expression levels on $CD16^+$ monocytes has been shown to correlate with HAND (93) and to be an important contributor to HIV-infected $CD16^+$ monocytes when crossing

an *in vitro* BBB (92). Moreover, CCL2, the ligand for CCR2, is elevated in the plasma and CSF of HIV patients and correlates with neuronal injury and cognitive impairment (92, 95-98).

$CD16^+$ monocytes are also thought to be a major transport mechanism for HIV into the brain (74, 75) as these cells have been fluorescently tracked from the blood to the brain in a simian immunodeficiency virus (SIV) model, which paralleled HIV entry into the brain (91). In addition, $CD16^+$ monocytes detected in post-mortem human brain stain positive for HIV viral proteins and were associated with higher CSF viral loads (88, 94). Furthermore, blocking leukocyte trafficking into tissues with an anti-α4 antibody in a SIV model resulted in a decline in virally infected monocytes in the brain and was paralleled with less neuronal injury compared to untreated control animals (63).

Once in the CNS, monocytes differentiate into long-lived macrophages and serve as a viral reservoir (76, 88, 99, 100). Monocyte-derived macrophages release intact HIV virions, viral proteins, neurotoxic factors (ROS and NO) and secrete cytokines (TNFα, IL-1β, IL-6 and MCP-1), leading to activation and HIV-infection of microglia, astrocytes and macrophages (61, 74, 78). As noted in the previous section, the inflammatory factors can directly promote neuronal injury. In addition, monocyte-derived factors (e.g., IL-1β) in the brain may also have indirect effects on neuronal functions through promoting astrocyte activation and dysfunction (61, 74, 78).

In addition to CD16, another surface protein expressed by monocytes is CD163 and may be an important marker on CNS-bound $CD16^+$ monocytes during HIV infection (101, 102). CD163 is a scavenging receptor for hemoglobin-haptoglobin complexes, and is almost exclusively expressed on monocyte/macrophages (102, 103). In addition, CD163 has been shown to have an important role for monocyte adherence to endothelial cells while also serving as an

immune receptor to detect bacteria (104, 105). Co-expression of CD16 and CD163 on monocytes has been observed in post-mortem brain tissue of HIV+ individuals with cognitive impairment (89, 90). $CD16^{+}CD163^{+}$ monocytes are also elevated in circulation of HIV+ individuals with detectable viral loads (106), suggesting that CD163 is expressed on $CD16^{+}$ monocytes before entry into the brain. Other surface markers that may be indicative of monocyte trafficking into the CNS and/or neurocognitive impairment include CCR2 and the cell adhesion molecules, junctional adhesion molecule-A (JAM-A) and activated leukocyte cell adhesion molecule (ALCAM) (92, 93).

D. Chronic peripheral monocyte activation during HIV infection

Peripheral monocyte activation is readily identifiable in HIV patients, even in the ART era, and correlates with disease progression and neurocognitive impairment (93, 106-110). Notable monocyte activation markers that are elevated during HIV infection include CD16 and CD163, both of which are expressed on monocytes found in the blood and brain of HIV patients with cognitive impairment (13, 89, 90, 106). Furthermore, soluble proteins shed or secreted from activated monocytes are increased in circulation and/or CSF, including soluble CD14 (sCD14), sCD163 and IP-10, all of which are predictive of HAND and/or disease progression (107, 110-113).

Enhanced monocyte activation in circulation, including the process of transitioning to $CD16^{+}$, is crucial in maintaining monocyte infiltration into the CNS during chronic HIV infection, and is mediated through HIV-induced chronic immune activation (88, 114). Two major mechanisms promoting monocyte activation during HIV infection are microbial translocation at the damaged gut mucosal barrier and residual HIV (114). Microbial translocation and residual HIV can directly promote monocyte activation, and indirectly through stimulating

pro-inflammatory cytokine production (e.g., IFNα) from other leukocytes, which in turn, leads to monocyte activation (114, 115).

During HIV infection, especially in the acute phase, the principle mechanism of increased microbial translocation is a depletion of HIV-infected $CD4^+$ T cells in the gut, which isn't fully restored during ART (114, 116, 117). As intestinal $CD4^+$ T cells have an important role in gut homeostasis, the result of their depletion leads to increased gut permeability as well as impaired mucosal immunity (118). Consequently, microbial products (e.g., LPS) are released into circulation resulting in activation of circulating leukocytes via activation of pattern-recognition receptors (e.g., TLRs), with monocytes being substantially impacted (114, 119-121). Specifically, several reports have found a positive association between plasma levels of LPS and soluble CD14 (sCD14), which is shed during monocyte activation (122-125).

Despite effective treatment, residual HIV can be detected in locations such as the blood and CSF, and in cell reservoirs, including $CD4^+$ T cells and monocytes/macrophages (126-130). The location of these cell reservoirs includes the blood, gut and brain (127, 131). Monocyte activation by HIV occurs via different mechanisms including HIV ssRNA-mediated activation of TLRs and through cell recognition of viral proteins (132). Specifically, HIV ssRNA has been identified to stimulate monocyte activation via TLR 7 and 8 (133-138), which promotes cytokine secretion (TNFα, IL-6 and IL-1β) (139). HIV viral proteins known to stimulate monocytes are the envelope glycoprotein, gp120, as well as Tat and Negative Regulatory Factor (Nef) (140-144).

E. The role of interferon alpha on monocyte activation in circulation and the brain

IFNα is a potent cytokine produced during viral infection and when secreted, protects the host by inducing anti-viral defense mechanisms in neighboring cells (145). During HIV

infection, plasmacytoid dendritic cells (pDC) are the primary producers of IFNα in circulation, as they secrete a 1000-fold more IFNα on a per cell basis compared to other cells (115). IFNα secretion by pDC can be promoted by HIV itself and through microbial products derived from the compromised intestinal barrier (115). IFNα is a central component of the acute immune response against HIV infection, but sustained levels during chronic stages of infection contributes to immune activation and dysfunction (115). Specifically, interferon-stimulated genes (ISGs) have been shown to be upregulated in several different immune populations during chronic HIV infection including T cells, dendritic cells and monocytes (115). Furthermore, IFNα has also been shown to be elevated in the cerebrospinal fluid (CSF) of HIV patients and is thought to be an important contributor to HAND as it correlates with neuronal damage and neurocognitive impairment (146-149). Studies involving mouse models of HIV encephalitis treated with IFNα neutralizing antibodies have found a significant role of IFNα on neuronal damage and cognitive decline (150-152).

$CD16^-$ monocytes transition into the $CD16^+$ phenotype in circulation and this process is of interest due to the pathogenic nature of the $CD16^+$ monocyte subset during HIV infection (12, 74). However, the specific mechanism(s) of enhanced $CD16^-$ monocyte transition to $CD16^+$ during HIV infection remains unclear. Previous reports have identified a type I interferon gene signature in monocytes isolated from HIV-infected individuals, suggesting exposure to IFNα *in vivo* (109, 153). Additionally, the use of IFNα as a vaccine adjuvant in humans increased the percentage of $CD16^+$ monocytes (154). Collectively, the body of literature suggests a key role for IFNα on monocyte activation in the periphery and brain during HIV infection, including increasing the frequency of $CD16^+$ monocytes.

F. Astrocyte activation, dysfunction and chronic release of pro-inflammatory cytokines and chemokines during HAND

Several astrocyte functions are dysregulated during HIV pathogenesis including glutamate uptake, ionic (K^+ and Ca^{2+}) homeostasis, BBB regulation and chronic release of proinflammatory factors (77). In addition, astrogliosis (astrocyte activation) occurs as evidenced by increased markers of hypertrophy and proliferation in brain tissue of post-mortem HIV patients (89). The specific mediators implicated in promoting astrocyte activation and dysfunction are; (a) HIV virions; (b) viral proteins; and (c) cytokines derived from adjacent activated cells (e.g., macrophages) (77). In response to the above factors, astrocytes produce an array of pro-inflammatory cytokines and chemokines (24). As noted above, IL-1β and TNFα are key factors inducing astrocyte secretion of cytokines and chemokines including IL-6, MCP-1 and IP-10 (30, 83). When chronically elevated, these factors contribute to ongoing leukocyte infiltration, cytokine secretion and direct neuronal injury (24, 61, 78, 79, 81). Specifically during HIV infection, IL-6, MCP-1 and IP-10 are increased in the plasma and/or cerebrospinal fluid (CSF) and are markers associated with neuronal injury and/or cognitive impairment (96-98, 109, 112, 155-157).

G. Monocyte-mediated astrocyte inflammation

The inflammatory factors produced by monocytes in the brain may have a profound impact on neuronal injury by promoting astrocyte dysfunction, including chronic astrocyte production of pro-inflammatory cytokines/chemokines and impaired glutamate control (24, 61, 74, 77-79). However, there are limited human co-culture studies investigating monocyte-astrocyte interactions and whether monocytes, especially when activated, promote astrocyte production of specific pro-inflammatory mediators. Two studies have reported that human

monocytes or monocyte-derived macrophages (MDMs) promoted MCP-1 secretion by astrocytes when co-cultured (158, 159), while another demonstrated TNFα and IL-1β production in co-cultures containing HIV-infected MDMs and astrocytes (160).

H. The role of toll-like receptor 7 (TLR7) in chronic immune activation and neuroinflammation

Toll-like receptor 7 (TLR7) is a pathogen-recognition receptor expressed in the endosomes of cells, including leukocytes, and recognizes purine-rich single-stranded RNA, particularly that of viruses (161, 162). TLR7 is expressed in a range of human immune cells including pDC, monocytes, B cells, T cells and natural killer (NK) cells (139, 163). However, TLR7 expression in astrocytes has been shown to be minimal or non-detectable (28, 164).

Activation of TLR7 by genomic ssRNA has been implicated as a major pattern-recognition receptor activated during viral-induced neuroinflammation (27, 165, 166). A growing body of evidence has demonstrated a strong neuroinflammatory response with *in vivo* treatment of TLR7 agonists. Specifically, Butchi and colleagues demonstrated a CNS inflammatory response in mice with intracerebroventricular inoculation of the TLR7 agonist, imiquimod. This response was characterized, in part, by astrocyte activation, as well as temporal induction of several pro-inflammatory factors including IL-1β, MCP-1, IL-6 and IP-10 (167, 168). Furthermore, topical administration of imiquimod (Aldara^{TM}) to mice, which enters the brain hours post-treatment, also promoted a similar neuroinflammatory response profile (MCP-1, IL-6 and IP-10), which could be detected as early as 4 hours post treatment and up to 3-5 days thereafter (169). In addition, this study illustrated activation of both microglia and astrocytes (169). Topical administration of imiquimod also was shown to promote immune cell infiltration into the brain, including monocytes (170), which would be speculated to exacerbate CNS

inflammation. Interestingly, the TLR7-mediated pro-inflammatory CNS profile observed in these studies is similar to what is observed in HIV-infected patients that display neuroinflammation and cognitive dysfunction (88, 89, 96).

III. Monocyte signaling pathways and IL-1β production

A. Toll-like receptor 7 and 8 signaling pathway

TLR7 and 8 signaling is mediated through the myeloid differentiation primary response 88 (MyD88)-dependent pathway (121). Figure 1 illustrates key aspects of TLR7 and 8 signaling. Specifically, ligand (e.g., ssRNA viruses) or agonist (e.g., imiquimod/R837) binding to the TLR promotes the recruitment of the adapter protein, MyD88, which associates with the cytoplasmic portion of the TLR via Toll/IL-1 receptor (TIR) domain interactions (171, 172). Subsequently, interleukin-1 receptor-associated kinase 1 (IRAK-1), IRAK-4 and TNF receptor associated factor 6 (TRAF6) are recruited and IRAK-1 associates with MyD88 through interaction of their death domains. IRAK-4 then phosphorylates IRAK-1 promoting the dissociation of p-IRAK-1 and TNF receptor associated factor 6 (TRAF6) from the complex (171). TRAF6 forms a complex with multiple proteins including transforming growth factor β-activated kinase 1 (TAK1), TAK1-binding protein 1 (TAB1) and TAB2. The formation of this complex leads to the activation of TAK1, which then phosphorylates the IκB kinase (IKK) complex (IKKα/β/γ). In addition, TAK1 phosphorylates MAPKs, such as c-Jun N-terminal kinase (JNK), eventually leading to the phosphorylation of AP-1 (121, 171). The IKK complex phosphorylates IκB, which promotes the dissociation of IκB (inhibitor of nuclear factor kappa B (NF-κB)) and NFκB, leading to NFκB activation and translocation into the nucleus. AP-1 and NFκB act as transcription factors to modulate gene expression, including *IL1B* (121, 171).

B. TLR 7 and 8 expression and activation in human monocytes

Cros and colleagues confirmed that human monocytes express TLRs 7 and 8, and become activated in response to their ligands/agonists. Furthermore, monocytes were shown to produce IL-1β in response to ssRNA viruses as well as TLR7 and 8 agonists, 3M13 and 3M2, respectively (139). TLRs 7 and 8 have been shown to be involved in monocyte activation in response to HIV ssRNA (134, 137, 138), with TLR8 being the predominant receptor involved (138).

The $CD16^+$ monocyte subsets (intermediate and non-classical monocytes) were identified to produce substantially more IL-1β on a per cell basis compared to classical monocytes when stimulated with TLR7 and 8 agonists (139), suggesting that $CD16^+$ monocytes are the major producers of IL-1β when stimulated by HIV ssRNA. The differences in the amount of IL-1β produced between monocyte subsets is due to different signaling proteins becoming activated in response to TLR7 and 8 agonists, specifically, the MAP kinases activated by TAK1 (139). p38MAPK was identified to be preferentially activated in classical monocytes whereas extracellular signal-regulated kinase (ERK) and JNK were activated in $CD16^+$ monocytes (139). The functional outcome is that classical monocytes primarily produce IL-6 in response to TLR7/8 activation while $CD16^+$ monocytes produce IL-1β and TNFα (139).

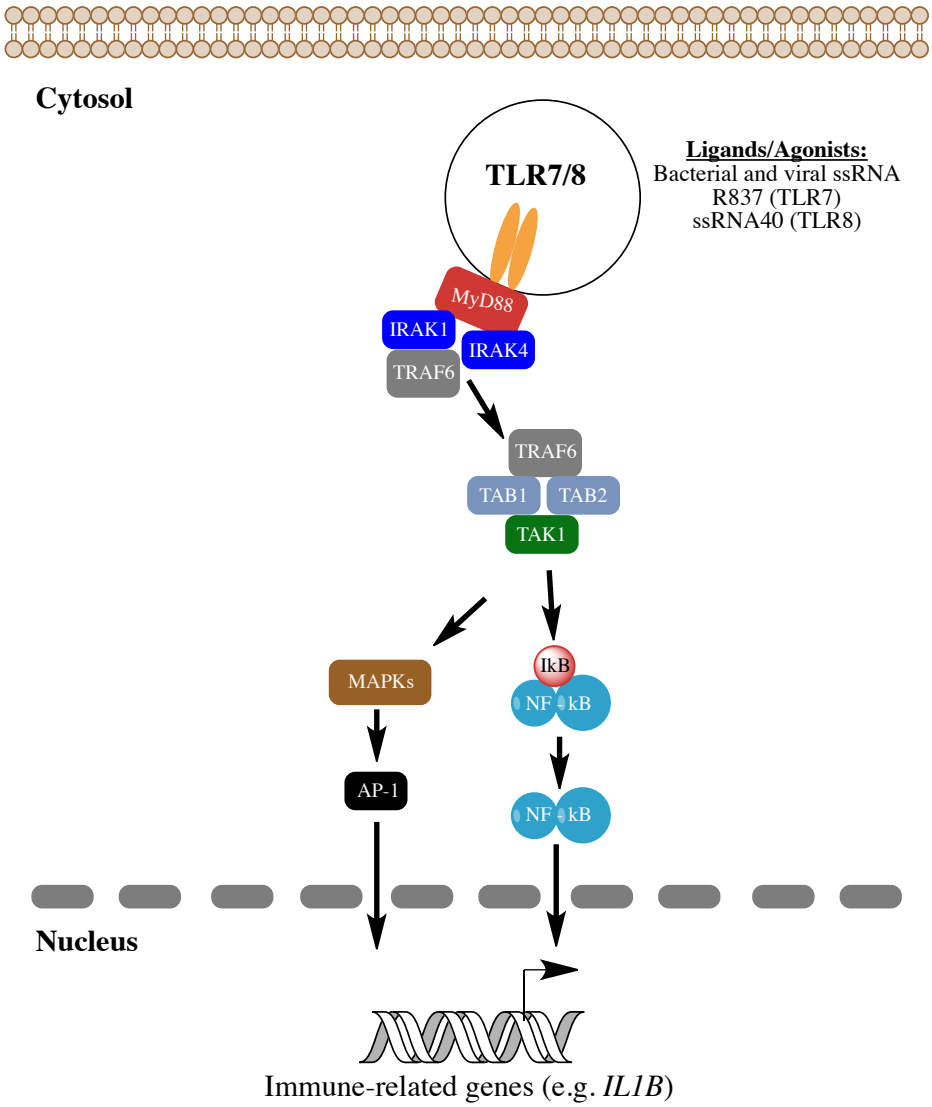

Figure 1. Overview of TLR 7 and 8 signaling pathway. TLR 7 and 8 ligands and agonists promote MyD88-mediated signal transduction ultimately leading to the activation of NFκB and AP-1 to induce (regulate) gene transcription.

C. IFNα receptor (IFNAR) expression and signaling

IFNα stimulates cells through binding to the IFNα receptor (IFNAR) (145) and human monocytes express IFNAR (156). IFNα stimulation of monocytes promotes the production of several inflammatory factors, including chemokines (IP-10 and monokine induced by interferon gamma (MIG)) (156, 173), and nitric oxide (NO) (174). Furthermore, IFNα has been shown to prime monocytes to become more responsive to microbial products (e.g., LPS) (175). IFNα binding to IFNAR promotes cross phosphorylation by tyrosine kinase 2 (TYK2) and janus kinase 1 (JAK1). Subsequently, TYK2 and JAK1 phosphorylate the cytoplasmic domain of IFNAR creating binding sites for signal transducer and activator of transcription 1 (STAT1) and STAT2 (145). STAT 1 and 2 become phosphorylated and p-STAT1/p-STAT2 heterodimers or p-STAT1 homodimers form. STAT dimers act as transcription factors and translocate to the nucleus to stimulate gene transcription. STAT1/2 dimers form a complex with IRF9 before entering the nucleus and stimulate transcription of antiviral genes while STAT1 dimers drive proinflammatory gene expression (145).

D. IL-1β production by monocytes

The major producer of IL-1β in circulation is the monocyte (176). IL-1β production is unique in comparison to other common cytokines, as it requires transcription and translation as well as inflammasome-mediated maturation (177). The two major steps to produce mature IL-1β are: (1) transcription and translation of pro-IL-1β; and (2) inflammasome-mediated caspase-dependent cleavage of pro-IL-1β (177). IL-1β production is commonly thought to occur via the two-signal model, in which two distinct activation stimuli are needed (Fig. 2) (178). The first activation signal is through PRRs (e.g., TLRs), which promotes NFκB activation leading to the production of pro-IL-1β and nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3),

a key part of the inflammasome complex (178, 179). In addition to NFκB, cAMP response element binding protein (CREB) and activator protein 1 (AP-1) are transcription factors involved in the modulation of *IL1B* expression levels (180-182). The second signal (e.g., extracellular ATP) promotes the activation of the inflammasome, which is mediated, in part, through a K^+ efflux mechanism (178). Specifically, the release of extracellular ATP and its binding to the purinergic receptor, $P2X_7$, promotes K^+ efflux and the low level of intracellular K^+ triggers the formation and activation of the inflammasome (183). Other postulated mechanisms of inflammasome activation include microbial products inducing ROS and increased intracellular Ca^{2+} (184). The most common inflammasome consists of NLRP3, associated speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase-1 (177, 178). NLRP3 interacts with ASC via homotypic interactions of pyrin domains, while ASC and pro-caspase-1 interact via caspase recruitment domains (184). Complex formation promotes the activation of caspases via self-cleavage, which then converts pro-IL-1β to mature IL-1β via proteolysis (184). Caspase 1 is the primary caspase involved in IL-1β production, however, caspase 8 has also been identified as a novel caspase to cleave pro-IL-1β (185). In addition, inflammasome activation normally results in a form of programmed cell death termed pyroptosis (186); however, a recent report has indicated that pyroptosis doesn't occur in TLR4-stimulated human monocytes (187). It remains unclear whether pyroptosis occurs in TLR7-stimulated monocytes.

In contrast to murine monocytes, human monocytes have been shown to produce mature IL-1β with TLR stimulation alone (187-189). The mechanism is similar to above; however, TLR4 ligand treatment (e.g., LPS) alone stimulates pro-IL-1β production (signal 1), while also promoting monocyte release of ATP (signal 2).

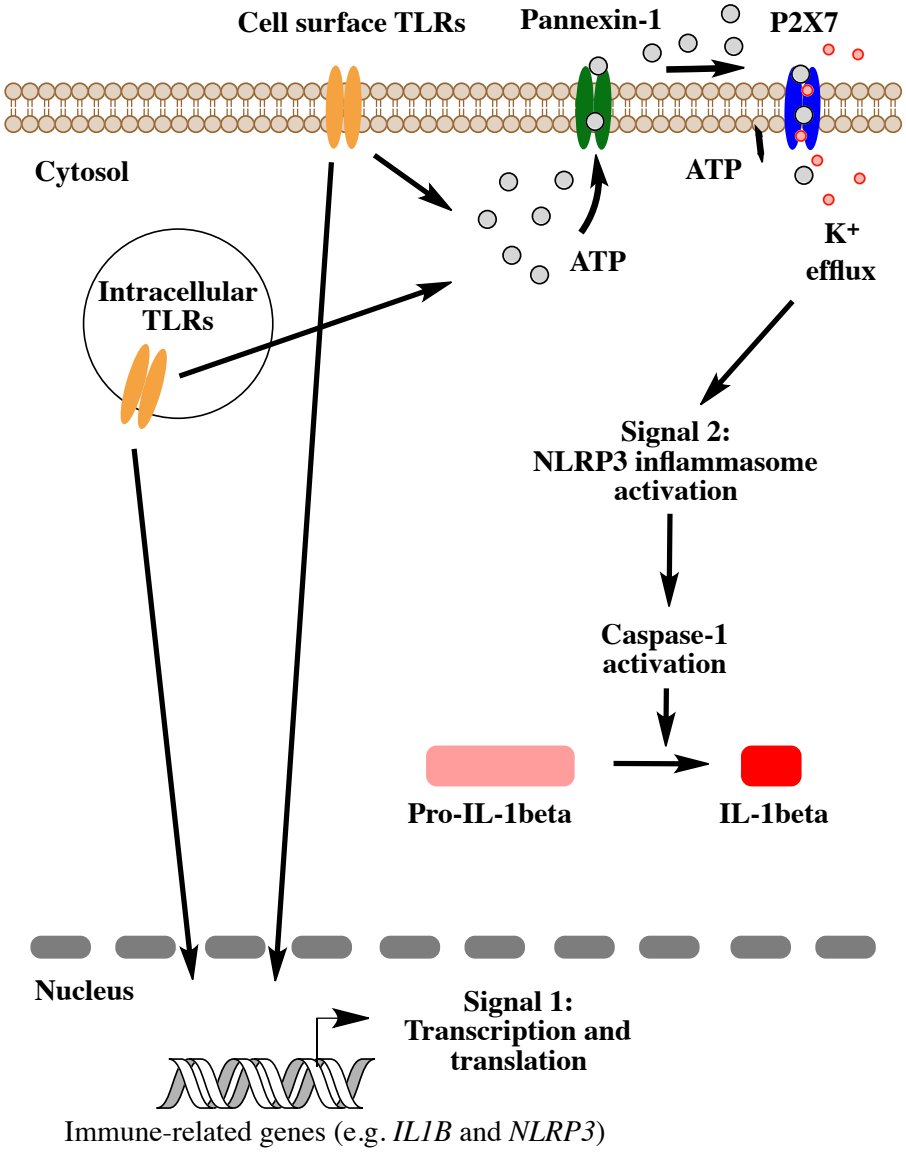

Figure 2. Two-signal model for production and maturation of IL-1β. The two major steps to produce mature IL-1β are: (1) transcription and translation of pro-IL-1β; and (2) inflammasome-mediated caspase-1-dependent cleavage of pro-IL-1β

Extracellular ATP has been identified to be responsible for inflammasome activation, as blocking its receptor (purinergic receptor $P2X_7$) was able to prevent IL-1β maturation (188). The mechanism by which TLRs 7 and 8 induces IL-1β maturation in human monocytes may be similar.

III. Cannabis use by HIV-infected individuals

A. Cannabis use by the HIV-infected population

Cannabis use is common amongst HIV-infected individuals in the United States, with an estimated prevalence of 23-56% (47, 190, 191). A 2018 report found that 22.9% of HIV-infected individuals who reported cannabis use were using cannabis daily while the remainder were non-daily (47). Furthermore, 26% of HIV+ cannabis users were using for medicinal purposes (47). HIV-infected individuals reportedly use cannabis to relieve a variety of symptoms including loss of appetite, anxiety, stress, depression, pain and nausea (192-194). However, there is a growing concern of the impact of cannabis use on medication adherence, cognition and immune function in HIV-infected individuals.

The current body of literature on the impact of cannabis use on medication adherence, is mixed, as several reports indicate an association between cannabis use and poor medication adherence (195-197), while others show no association (198-200). Furthermore, a recent systematic review determined that the effect of cannabis use on medication adherence was inconclusive based on the current evidence (201). There are also mixed reports on cannabis use and cognitive decline in HIV-infected individuals, which may be due to acute cognitive impairment immediately after use but not over the long-term (56). Specifically, one report found an association with memory deficits in current cannabis users while no correlations were found with lifetime use (57). In addition, a 2016 report found that HIV+ cannabis users were not at an

increased risk for brain or cognitive abnormalities when compared to HIV-negative cannabis users (202), suggesting that there is no synergistic or additive effect between HIV status and cannabis use. In terms of immune function, there have been two major reports examining immune cell phenotypes and function with short-term cannabis use in HIV-infected persons. Bredt and colleagues performed a 21-day clinical trial of cannabis use in HIV-infected individuals and concluded that no meaningful changes in immune cell numbers or function with cannabis use. This included no major changes in $CD4^+$ and $CD8^+$ T cell counts, leukocyte proliferation and NK cell function with cannabis use (203). Furthermore, another 21-day clinical trial of cannabis use in HIV+ patients found no major alterations in $CD4^+$ or $CD8^+$ T cells counts or HIV RNA levels (204). It remains to be elucidated whether long-term cannabis use results in immunological effects.

B. The major constituents of cannabis

Cannabis, also known as *Cannabis Sativa*, is made up of over 500 chemicals with 104 of them being defined as phytocannabinoids (205). In addition to cannabinoids, there are other compounds present in cannabis including terpenes and flavonoids (206). Two of the major cannabinoids within cannabis are Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), and as of 2014, make up 12% and 0.2%, respectively, of the plant material (207). THC but not CBD, is the main psychotropic component of cannabis, as it impairs aspects of cognition including short-term memory (208). However, THC and CBD do display similar effects elsewhere in the body, with both shown to have immune modulating/anti-inflammatory, antiemetic and antiepileptic activity (208).

C. Pharmacokinetics of THC and CBD after inhalation of cannabis

When cannabis is inhaled via smoking, THC and CBD, both of which are highly lipophilic, are rapidly absorbed through the lungs into the bloodstream. Bioavailability of THC after smoking is highly variable, ranging from 2-56%, which is dependent on several inter- and intra-individual variables (209, 210). Peak plasma concentration of THC has been shown to occur at about 10 minutes after first inhalation of a 3.55% THC cannabis cigarette and ranges from 76-267ng/ml (239-868nM) (209, 211). However, these values may not accurately reflect peak concentrations with present day cannabis use, as the amount of THC in cannabis as of 2014 was 12% (207). There is limited examination of CBD peak plasma concentrations via inhalation of cannabis cigarettes, which may be due to its minimal levels within the plant material. However, one study found peak plasma CBD levels ranged from 42-191ng/mL (132-601nM), after smoking a cigarette containing about 20mg of deuterium-labelled CBD (212). Blood levels of THC drop rapidly, decreasing to less than half of the peak concentration within 15 minutes after the last inhalation of cannabis, which is due to metabolism in the liver as well as rapid distribution into tissues (e.g., brain, adipose tissue, liver and heart) (209).

The half-life of cannabinoids after inhalation is dependent on the type of user, as infrequent cannabis users display a half-life of THC of approximately 1 day versus a 5-13 day half-life in frequent users (210). The half-life of CBD after smoking a cigarette containing roughly 20mg of deuterium-labelled CBD was 31 hours (212). Metabolism of cannabinoids primarily occurs in the liver where it is oxidized by various metabolic enzymes. THC is metabolized by cytochrome P450, 2C9 (CYP2C9), to 11-OH-THC and then COOH-THC (Phase I). Other CYPs involved in THC oxidation include CYP2C19 and CYP3A4 (209). Glucoronide addition to 11-OH-THC and THC-COOH occurs via glucuronosyltransferases (UGT) (Phase 2)

(209). Metabolism of CBD is similar to THC, as both hydroxylation (OH) and carboxylation (COOH) occur via CYPs, including CYP2C9, CYP2C19 and CYP3A4 (213). THC gets excreted from the body via both the feces and urine, with 65% being removed via feces (11-OH-THC) and 20% in the urine (glucuronide conjugate) (209). Similar routes of excretion occur with CBD, as one study determined that 16% and 33% of CBD was excreted via urine and feces, respectively (212).

D. Immune modulation by THC and CBD: Role of cannabinoid receptors 1 and 2

Both THC and CBD have been shown, primarily in laboratory animal studies, to modulate immune cell activity *in vivo* and *in vitro*, with most of the literature demonstrating immune suppressive and anti-inflammatory activity (214-218). THC and CBD modulate the activity of almost every immune cell type including T cells, B cells, monocyte/macrophages, NK cells and dendritic cells. Both cannabinoids suppress a wide range of immune functions including proliferation, chemotaxis, antibody responses, phagocytosis and cytokine production (216-221). Specific immune cell derived-cytokines modulated by cannabinoids include IL-1, TNF, IFNγ, IL-12 and IFNα (216, 219, 222). While most of the effects observed with cannabinoids are immune suppressive, under certain experimental conditions and/or drug concentrations, cannabinoids can exacerbate inflammation and immune function, including increasing cytokine production (216, 217, 223).

THC modulates immune cell activity primarily through the binding of two G-protein coupled receptors termed cannabinoid receptor 1 and 2 (CB1 and CB2) (224, 225); however, CB-independent cellular effects have been observed (226, 227). THC is a partial agonist to both cannabinoid receptors and has a Ki (binding inhibitory constant) of 25nM and 35nM to CB1 and CB2, respectively (224, 228, 229). CB1, which is highly expressed in the CNS, is responsible for

the psychotropic effects seen with cannabis use (230). Conversely, CB2 is expressed at lower levels in the CNS, with the exception of microglia, and is the major cannabinoid receptor expressed in the immune system (224). In immune competent cells, B cells display the highest level of cannabinoid receptor expression (CB1 and CB2) followed by NK cells, macrophage/monocytes, neutrophils and T cells (216, 224, 225).

After ligand binding (e.g., THC), CB1 and CB2 couple with inhibitory $G_{i/o}$-proteins, leading to Gi/o-protein activation (Fig. 3). A major downstream result of $G_{i/o}$-protein activation is the inhibition of adenylate cyclase (AC), which causes a reduction in intracellular cAMP. Decreased intracellular cAMP limits the activation of protein kinase A (PKA) (224, 231). Two transcription factors negatively impacted by decreased PKA activation are CREB and NFκB (224, 232). The decreased activity of these NFκB and CREB by cannabinoid receptor signaling highlights a mechanistic basis for the anti-inflammatory activity of cannabinoids, as these transcription factors are critically involved in the regulation of immune function and cell survival genes (233-235). Other features of cannabinoid receptor signaling include regulation of mitogen-activated protein kinase (MAPK) signaling, such as extracellular signal–regulated kinases (ERK), phosphoinositide 3-kinase (PI3K) and intracellular calcium levels (Fig. 3) (224, 231, 232).

Activation of cannabinoid receptors *in vivo* and *in vitro* with agonists, including CP55940, WIN55-212, JWH-015/133 and THC have been shown to impair several immune functions in a range of immune cell types, including suppressing cell migration, proliferation cytokine production and antibody responses (219, 221, 222, 236). In addition, cannabinoid receptors, especially CB2, have been under consideration as a therapeutic target for neuroinflammatory conditions, as activation of cannabinoid receptors has been shown to promote

anti-inflammatory effects in the brain (219, 224, 237-241). Cannabinoids have also been shown to directly suppress both astrocyte and microglia immune functions, including production of inflammatory factors (242, 243).

The mechanism by which CBD modulates immune cell function isn't well established, as there is limited evidence of receptors and signaling cascades involved. CBD has poor binding affinity to both CB1 and CB2 as compared to THC, with a Ki in the micromolar range (3-28μM) for both receptors (228, 229). Despite this, some evidence indicates that CBD can act as either an antagonist or inverse agonist of CB1 and CB2 (229). Other receptors potentially targeted by CBD include G-protein coupled receptor 55 (GPR55), serotonin 5-HT1A receptors and transient receptor potential vanilloid type 1 (TRPV1) receptors (227, 244-246).

In addition to altering immune cell-related gene expression, both THC and CBD have the potential to promote immune suppression through cannabinoid-induced apoptosis, which has been identified to occur at higher *in vitro* concentrations (e.g., 10μM) (247). In addition, the apoptotic effects of THC have also been observed *in vivo*, in which THC (10mg/kg) significantly increased the percent of apoptotic cells in the spleen and thymus (248). THC has been identified to induce apoptosis by acting on the CB2 receptor, as a CB2 antagonist (SR144528) was able to reverse the THC-induced apoptosis in murine splenocytes and human Jurkat T cells (248, 249). The CB2 agonist, JWH-015, also promotes immune cell apoptosis (250), further supporting CB2 as the receptor involved. CBD induces immune cell apoptosis (251); however, the mechanism of action is unknown (247). The specific cellular signaling pathways promoting apoptosis by cannabinoids is not well established; however, a commonality between THC, JWH-015 and CBD is the activation of specific caspases (247, 252, 253). Specifically, activation of caspases 1, 3 and 8 has been identified to be involved in THC-induced apoptosis of murine splenocytes or

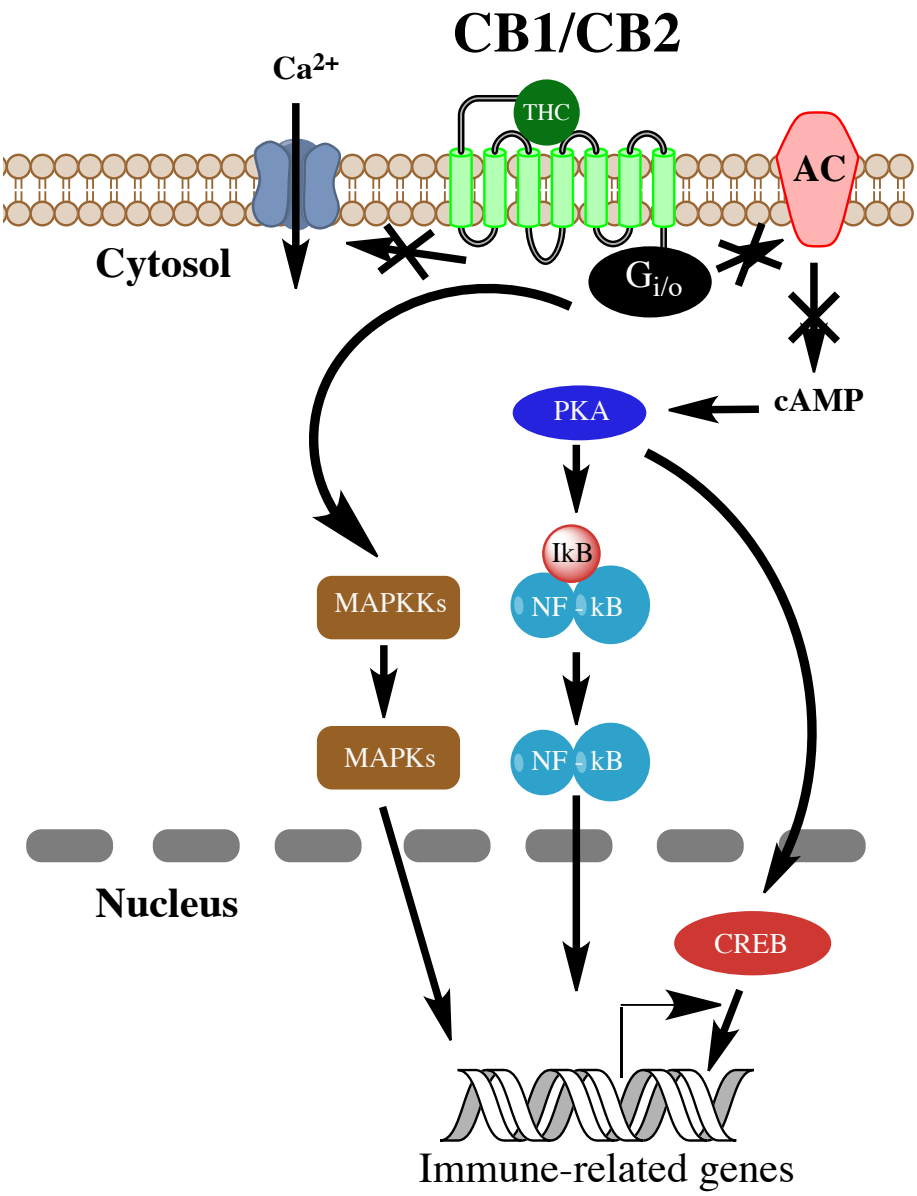

Figure 3. Overview of cannabinoid receptor (CB1/CB2) signaling. CB1 and CB2 are G-protein coupled receptors with seven transmembrane domains. Upon ligand (e.g., THC) binding, CB1 and CB2 promote the activation of Gi/o-proteins. A major downstream consequence of CB-signaling is the inhibition of adenylate cyclase (AC) leading to decreased cAMP production and protein kinase A (PKA) activation. Ultimately, decreased PKA activation leads to impaired activation of the transcription factors, NFκB and CREB, and thus suppresses the transcriptional induction of immune-related gene.

Jurkat T cells (249, 254) while caspases 3, 7 and 8 were important for JWH-015-induced apoptosis (250). In addition, activation of caspases 3 and 8 were shown to be crucial for CBD-induced apoptosis (251).

E. Endogenous ligands for CB1 and CB2

The two major endocannabinoids that interact with CB1 and/or CB2 are anandamide (AEA) and 2-arachidonoylglycerol (2-AG). Both AEA and 2-AG are lipid mediators that are produced in response to elevated intracellular calcium (255). Metabolism of AEA occurs primarily via fatty acid amide hydrolase (FAAH) while monoacylglycerol lipase (MAGL) hydrolyses 2-AG (219, 255). Both AEA and 2-AG are thought to play a role in immune cell function, as reports have identified that leukocytes have the ability to synthesize and respond to these lipid mediators (219, 256-258). The overall body of literature, including both *in vivo* and *in vitro* studies, has demonstrated primarily anti-inflammatory activity of AEA and 2-AG including suppression of leukocyte chemotaxis, cytokine production and ROS production (219). AEA and 2-AG have been identified to modulate immune cellular activity through CB1 and CB2 (255, 258); although CB-independent effects have been identified (259, 260). A meta-analysis in 2007 demonstrated that K_i values of AEA for human CB1 and CB2 were 239nM and 440nM, respectively, while Ki values for 2-AG were reported as 3400nM and 1194nM (228).

The use of CB1 and CB2 knockout mice have suggested an important role for these receptors and endocannabinoids in modulating immune function, as deletion of these receptors exacerbates immune responses towards pathogens (219). Notably, $CB1^{-/-}CB2^{-/-}$ mice challenged with influenza virus, PR8, displayed hyperactive immune responses compared to wild-type, consisting of increased bronchiolitis, pro-inflammatory gene expression, T cell activation and cytokine production (214, 261). Furthermore, FAAH and MAGL knockout mouse models and *in*

vivo treatment of FAAH/MAGL inhibitors, both of which elevate endocannabinoids due to impaired metabolism, have been shown to decrease pro-inflammatory immune cell responses, including cytokine production as well as neuroinflammatory responses (219). These findings suggest that endogenous ligands, such as AEA and/or 2-AG, and cannabinoid receptors play a critical regulatory role in normal immune responses against pathogens.

F. Human monocytes as a target of immune modulation by cannabinoids

Purified human monocytes have been shown to have higher CB2 mRNA expression in comparison to CB1 (262). Protein expression analysis of cannabinoid receptors on immune cells remains a challenge due to the absence of effective anti-CB1/CB2 monoclonal antibodies. Despite this, a few reports have detected protein expression of CB1 and/or CB2 on human monocytes (262-264). Several reports have shown human primary monocytes to be sensitive to THC treatment, with the evidence indicating a CB2-mediated mode of action. Specifically, Roth and colleagues demonstrated that *in vitro* THC treatment was able to inhibit monocyte differentiation into mature dendritic cells, ultimately decreasing monocyte-mediated activation of T cells (262). Using specific CB2 agonists and antagonists, CB2 was shown to be the predominant CB responsible for decreased cAMP levels by THC in purified monocytes, implicating CB2 as the major CB receptor responsible for THC modulation of monocyte function (262). Another report revealed that *in vitro* THC decreased monocyte susceptibility to HIV infection during differentiation into a macrophage, with CB2 implicated as the receptor involved (265). The proposed mechanism of THC was suggested to be through reduction in expression of HIV co-receptors, CD4, CCR5 and CXCR4. Furthermore, THC, albeit at a high concentration (30μM), reduced monocyte expression of CD16 and CD163 during differentiation (265). Lastly, *in vitro* treatment with JWH-015 or JWH-133 impaired human monocyte

chemotaxis in response to CCL2 and CCL3, with the suspected mechanism being a CB2-mediated reduction in the chemokine receptors, CCR2 and CCR1, and the cell adhesion molecule, intercellular adhesion molecule 1 (ICAM-1) (264).

There is limited research into the effects of CBD on human monocytes as well as the mechanism of action. Recently, evidence has suggested that part of the anti-inflammatory activity of CBD on monocyte function may be through apoptosis via specific signaling pathways (247). Specifically, Wu and colleagues have investigated the role of CBD on monocyte apoptosis and found that CBD promotes monocyte apoptosis through mitochondrial-derived ROS production (266). Furthermore, apoptosis depended on mitochondrial membrane depolarization, as apoptosis was decreased with the mitochondrial permeability transition pore inhibitor, cylcosporin A (266). The CBD-mediated effects observed on monocytes may be through a GPR55, as one report demonstrated that CBD acted as an antagonist of GPR55 and was able to suppress cytokine production and phagocytosis by monocytes (244).

G. Human astrocytes as a target of immune modulation by cannabinoids

Human astrocytes have been confirmed to express both CB1 and CB2 (242). There are limited studies examining the role of THC and CBD on human astrocyte inflammatory functions. However, one study examined the role of the synthetic cannabinoid, WIN 55,212-2, which is similar to THC (CB1/CB2 agonist), on astrocyte production of inflammatory mediators in response to recombinant IL-1β treatment (242). WIN 55,212-2 was shown to suppress human astrocyte production of nitric oxide (NO), TNFα, IP-10, MCP-1 and CCL5 (242). In addition, CBD treatment was shown to decrease IL-6 production by human astrocytes (267).

IV. Rationale and hypotheses

As cannabis use is common amongst HIV-infected individuals, it is important to evaluate whether cannabis and cannabinoid use affects the chronic immune activation that is present in these individuals. As peripherally-derived $CD16^+$ monocytes have been implicated as a major contributor to HIV-associated neuroinflammation, the first objective of **specific aim (SA) 1** was to determine whether cannabis use was associated with lower levels of $CD16^+$ monocytes and the inflammatory chemokine, IP-10, in HIV-infected donors. With the use of isolated leukocytes from HIV- and HIV+ donor, the second objective was to determine the impact of *in vitro* THC treatment on: (a) the percentage of monocytes expressing CD16 and/or CD163 in response to IFNα; and (b) IFNα-mediated monocyte production of IP-10.

As activated ($CD16^+$) monocytes migrate into the brain during HIV pathogenesis, it is important to evaluate their role on functions of brain-resident cells, including astrocytes secretion of cytokines and chemokines. When dysregulated, astrocyte secretion of cytokines/chemokines can lead to sustained infiltration of immune cells into the CNS, exacerbating neuroinflammation and neuronal damage (24, 25). Due to the presence of HIV in the brain (60, 88, 268), monocytes have the potential to become activated by HIV ssRNA through TLRs 7 and 8 (133, 134, 138) to secrete cytokines (TNFα, IL-6 and IL-1β) (139). In addition, IFNα, has been shown to be elevated in the CSF of HIV patients and correlates with neurocognitive impairment (147-149). The specific activation profile and inflammatory factors produced by monocytes in each circumstance have the potential to promote differing astrocyte secretion profiles. Therefore, the objective of **SA2** was to develop a human co-culture system containing IFNα and the TLR7 agonist, R837, to elucidate the effect of monocytes on astrocyte secretion of specific inflammatory factors (MCP-1, IL-6 and IP-10).

The major objective of **SA3** was to evaluate the impact of THC and CBD on monocyte-mediated astrocyte production of MCP-1 and IL-6 when co-cultured and stimulated with the TLR7 agonist, R837. Furthermore, with the use of monocytes isolated from HIV-negative and HIV-infected donors, we determined the effect of THC and CBD on monocyte-derived IL-1β, as it is a primary factor governing astrocyte secretion of MCP-1 and IL-6. Lastly, the objective of **SA4** was to evaluate the mode of action by which THC suppresses monocyte secretion of IL-1β in response to TLR7 activation.

The **hypotheses** that were developed for the studies presented here were: (a) THC suppresses IFNα-mediated monocyte activation into the $CD16^+$ phenotype and IP-10 production; (b) TLR-activated monocytes promote astrocyte secretion of MCP-1, IL-6 and IP-10; and (c) THC and CBD suppress TLR7-activated monocyte-mediated astrocyte production of MCP-1 and IL-6 through direct action on the monocytes.

MATERIALS AND METHODS

I. Material and methods for specific aim 1

A. HIV-infected donors.

HIV+ male donors were recruited for blood draw under the IRB protocol (IRB# 11-202) by Dr. Peter Gulick and enrolled into the Mid-Michigan HIV consortium (MMHC). Donors received the standard of care and donor information was electronically available through the Research Data Capture (REDcap) (Vanderbilt University), which supports 21 CFR Part 11 compliance for clinical research and trials data and HIPAA guidelines. All HIV+ donors are currently on ART and negative for hepatitis C. Cannabis use was determined by self-reporting and confirmed by plasma detection of THC metabolites using THC ELISA (RTU) Forensic Kit (Neogen Corporation, Lansing, MI). In this study, 4 of the 42 HIV+ donors had a discrepancy between self-reported use and THC metabolite detection and were classified based on results from the THC ELISA (RTU) Forensic Kit for cannabis use. HIV-MJ- donors tested negative for THC metabolites.

B. Collection of plasma and leukocytes from whole blood of HIV-MJ-, HIV+MJ- and HIV+MJ+ donors.

Whole blood was collected from HIV-MJ- (Stanford Blood Center) and HIV+ donors in acid citrate dextrose (ACD) or heparin tubes and either shipped (HIV-MJ- donors) or stored (HIV+ donors) overnight at room temperature. The next day, the number of leukocytes per mL of blood was obtained using a coulter counter. An aliquot of cells from whole blood collected in ACD or heparin tubes was used for cell surface staining. Before surface staining, red blood cells were removed using ACK lysis buffer. For plasma collection, whole blood was collected in heparin tubes only. Plasma was collected and stored at -80°C.

C. Peripheral blood mononuclear cell (PBMC) and CD16$^-$ monocyte isolation for *in vitro* studies.

PBMCs were isolated from human leukocyte packs (Gulf Coast Regional Blood Center, Houston, TX) of HIV-MJ- donors and whole blood of HIV+ (MJ- and MJ+) donors by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Pittsburgh, PA). Purified CD16$^-$ monocytes were isolated by negative selection using Human Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) per manufacturer's direction. The mean (±SD) monocyte purity for donors (N=14) used in this report was 88.4±5.7%. The mean percent of CD16$^-$ cells within the monocyte population was 99.2±0.6% (<1% CD16$^+$ monocytes).

D. Chemicals.

Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) were dissolved in 100% ethanol (National Institute on Drug Abuse, Bethesda, MD). For cell culture experiments, THC and CBD were serially diluted in RPMI 1640. The vehicle concentration for each treatment was 0.03% ethanol.

E. Cell culture and activation.

PBMCs ($5x10^6$ cells/mL) or purified CD16$^-$ monocytes ($1x10^6$ cells/mL) were cultured in media containing RPMI1640 (Gibco^{TM}) supplemented with 5% human AB serum (Sigma-Aldrich, St. Louis, MO) and 100 U/ml Penicillin/100 µg/mL streptomycin (Gibco^{TM}). Leukocytes were stimulated with Universal Type I Interferon Alpha (PBL Assay Science, Piscataway Township, NJ) and incubated at 37°C and 5% CO_2. For experiments involving THC/CBD treatment, cells were incubated at 37°C and 5% CO_2 with the corresponding concentration of THC/CBD for 30 min prior to IFNα addition.

F. Flow cytometry.

FACS buffer (PBS, 1% BSA, 0.1% NaN_3) was used to wash cells in between staining and fixing steps. First, cells were incubated with FACS containing 20% human AB serum to block Fc receptors. Cells were then incubated with antibodies and LIVE/DEAD™ Fixable Near-IR Dead Cell Stain (Thermo Fisher Scientific, Waltham, MA). BD Cytofix™ (BD Biosciences, San Jose, CA) was used to fix cells. For intracellular staining, cells were stained with antibody in PERM wash (BD Biosciences). Fixed cells were analyzed on a FACS BD Canto II™ (BD Biosciences). Antibodies included anti-CD14-Pe-Cy7 (clone: M5E2), anti-CD16-APC (3G8) and anti-CD163-BV421 (GHI/61) from BioLegend (San Diego, CA), and anti-IFNAR2-APC-Vio770 (REA124) from Miltenyi Biotec. Anti-IP-10-PerCP-eFluor710 (4NY8UN) antibody was purchased from eBioscience (San Diego, CA). For intracellular IP-10 staining, a protein transport inhibitor (eBioscience) was added to cell culture 5h prior to experiment takedown. Data analysis was performed using FLOWJO v10 software. The gating strategy for $CD16^+$ monocytes is in Figure 4. A Boolean gate was used to determine the percent of $CD16^+CD163^+$ cells within the monocyte population. For experiments involving purified monocytes, viable monocytes were gated based on SSC and FSC-A and analyzed for CD16 and CD163 expression.

G. Plasma and supernatant IP-10 analysis.

Plasma or supernatants were collected and stored at -80°C. Plasma/supernatants were thawed and IP-10 protein levels were quantified using LEGENDplex™ or ELISAmax™ from BioLegend per manufacturer's direction.

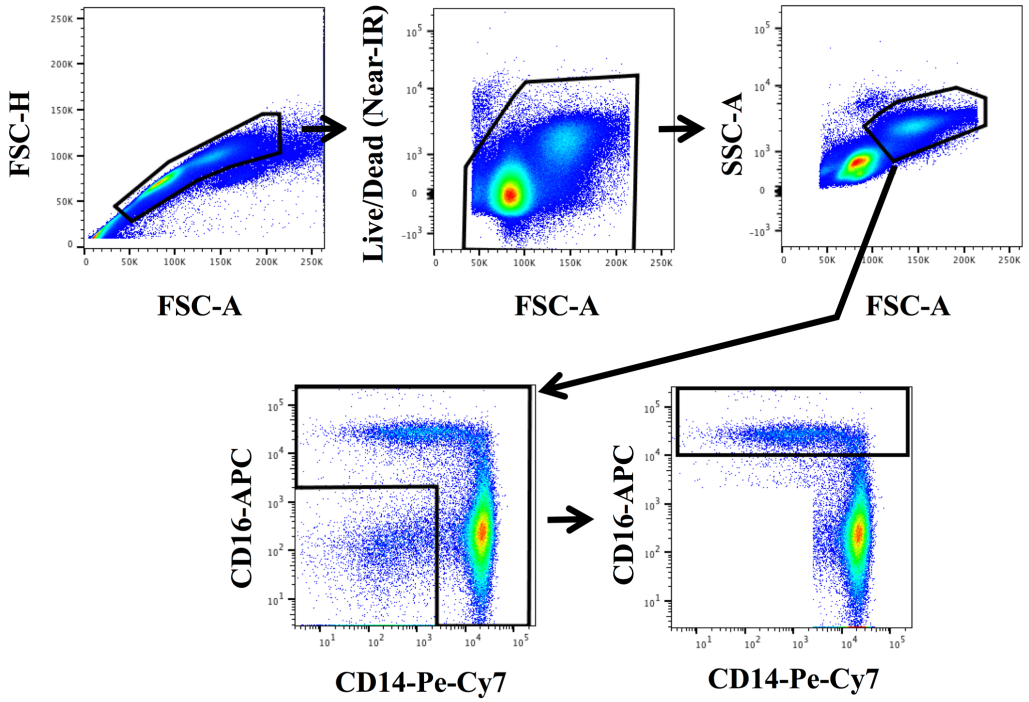

Figure 4. Gating strategy for CD16^{+} monocytes from whole blood or PBMCs. Depicted above is the gating strategy used for identification and quantification of the CD16^{+} monocyte population within whole blood or PBMCs. Within whole blood or PBMCs, viable monocytes were gated based on SSC and FSC-A. Next, the total monocyte population (CD16^{-} and CD16^{+} monocytes) was gated based on CD14 and CD16 expression. Within the monocyte population, cells were gated based on CD16 expression.

H. Statistical analysis.

Statistical analysis was performed using Prism 7 (GraphPad, San Diego, CA). The experimental data was graphed as the mean +/- SEM. The statistical tests performed for each experiment are indicated in the figure legends.

II. Materials and Methods for specific aim 2

A. Reagents.

Dulbecco's Modified Eagle Medium **(**DMEM) (Catalog #: 12100046) and Roswell Park Memorial Institute (RPMI) 1640 (Catalog #: 31800-022) were purchased from Gibco™ by Life Technologies (Carlsbad, CA). DMEM was supplemented with 10% Fetal Bovine Serum (HyClone) (Thermo Fisher Scientific, Waltham, MA), 1% non-essential amino acids (Gibco™), 1mM sodium pyruvate (Gibco™) and 100 U/ml Penicillin/100 µg/mL streptomycin (Gibco™). RPMI1640 was supplemented with 5% human AB serum (Sigma-Aldrich, St. Louis, MO) and 100U/ml Penicillin/100µg/mL streptomycin (Gibco™). Trypsin-EDTA (0.25%) was purchased from Gibco™. Universal Type I Interferon Alpha was purchased from PBL Assay Science (Piscataway Township, NJ). Imiquimod (R837) and ssRNA40 were purchased from InvivoGen (San Diego, CA). Golgi transport inhibitor (Monensin (2µM)/Brefeldin A (3.0 µg/mL) in methanol) was purchased from Thermo Fisher Scientific. Antibodies purchased from BioLegend (San Diego, CA) included anti-CD45-Pacific Blue (clone: HI30, Cat. #: 304029), anti-CD14-Pe-Cy7 (M5E2, 301814), anti-CD16-APC (3G8, 302012), anti-CD56-PerCP (HCD56, 318342), anti-CD57-PerCP/Cy5.5 (HNK-1, 359622), anti-IL-6-APC (MQ2-13A5, 501112), anti-IL-1β-FITC (JK1B-1, 508206) and anti-MCP-1-PE (5D3-F7, 502604). Anti-IP-10-PerCP-eFluor710 (4NY8UN, 46-9744-41) antibody was purchased from Thermo Fisher Scientific. For cytokine neutralization experiments, LEAF™ anti-human MCP-1 (2H5, 505905), anti-human IL-6 (MQ2-

13A5, 501109), anti-human TNFα (MAb1, 502803), armenian hamster IgG (HTK888, 400915), rat IgG1, κ (RTK2071, 400413) and mouse IgG1, κ (MOPC-21, 400123) were purchased from BioLegend. Anti-human IL-1β (4H5, mabg-hil1b-3) and mouse IgG1, κ (T8E5, mabg1-ctrlm) were purchased from Invivogen (San Diego, CA). Recombinant human IL-1β was purchased from BioLegend.

B. U251 and human primary astrocyte cell culture.

The U-251 MG (U251) astrocyte cell line was purchased from Sigma-Aldrich (Cat #: 09063001) and passaged in supplemented DMEM according to manufacturer's direction. U251 cells were used between passages 4 and 10. Fetal-derived normal human astrocytes (NHA) isolated from the cerebral cortex were purchased from Lonza (Basel, Switzerland) and cultured according to manufacturer's direction in astrocyte basal media (ABM™) supplemented with SingleQuots™ Kit (rhEGF, insulin, ascorbic acid, GA-1000, L-glutamine and FBS). Media was changed every other day for passaging and plate seeding. NHA were used up to passage 4. Depending on the experiment, primary astrocytes from two or three donors were used (donor 1-3).

C. HIV-infected donors.

HIV+ male donors were recruited for blood draw under the IRB protocol (IRB# 11-202) by Dr. Peter Gulick and enrolled into the Mid-Michigan HIV consortium (MMHC). Donors received the standard of care and donor information was electronically available through the Research Data Capture (REDcap) (Vanderbilt University), which supports 21 CFR Part 11 compliance for clinical research and trials data and HIPAA guidelines. All HIV+ donors are currently on ART and negative for hepatitis C.

D. PBMC and monocyte isolation.

PBMCs were isolated from human leukocyte packs (Gulf Coast Regional Blood Center, Houston, TX) of healthy donors and whole blood of HIV-infected donors by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Pittsburgh, PA). Pan monocytes were isolated by negative selection (Human Pan Monocyte Isolation Kit from BioLegend or Miltenyi Biotec (Bergisch Gladbach, Germany)) per manufacturer's direction. The monocyte purity was greater than 90%.

E. Monocyte-astrocyte co-culture.

One day prior to co-culture, U251 astrocytes were seeded at a cell density of $7x10^4$ cells/well in a 24-well plate ($3.5x10^4$ cells/cm^2) in supplemented DMEM. The estimated cell density on the day of co-culture was $1x10^5$ cells/well (as determined by optimization experiments). On the day of co-culture with U251 astrocytes, monocytes were purified as described above and resuspended in supplemented RPMI 1640. Normal human astrocytes (NHA) were seeded in supplemented ABM™ 3-6 days prior to co-culture at a cell density (based on doubling time) that would be an estimated $1x10^5$ cells/well on the day of co-culture. For co-culture involving NHA, monocytes were placed in supplemented ABM™. Monocytes were placed at the appropriate concentration to establish a monocyte: astrocyte ratio of 1:20 with the exception of Figure 1, where monocytes were placed at different concentrations for the differing cell ratios. Monocytes were added to the wells containing astrocytes at a volume of 500µl. Overall, the total well volume was 500µl with an astrocyte concentration of $2x10^5$ cells/mL and monocyte concentration of $1x10^4$ cells/mL. When appropriate, IFNα and/or R837 were added to the co-culture at the concentration stated in the figure legends. The co-culture was incubated at 37°C and 5% CO_2 for 20 hours (h) and a Golgi block was added to culture for an additional 4 h.

At 24 h, wells were washed with non-supplemented media and trypsinization was performed to remove astrocytes from plate. The flow cytometry procedure below was performed to measure cell viability and cytokine production. For particular experiments, supernatants were collected at 20 h and stored at -80°C. LEGENDplex™ (BioLegend) technology was utilized to determine cytokine levels in supernatants per manufacturer's direction. Undetectable cytokine levels for specific samples were given a value of 0pg/mL for graphing and statistical analysis. For supernatant MCP-1 and IL-6 levels, ELISAmax™ technology (Biolegend) was used.

F. Transwell assay.

Transwell®-Clear Inserts (24-well plate format) with a 0.4µm pore size (Catalog #: 3470) were purchased from Corning (Corning, NY). The co-culture was performed as above with the exception of the total well volume being 700µl. The lower chamber consisting of astrocytes at a volume of 600µl while the upper chamber contained monocytes at a volume of 100µl. The final cell concentration of monocytes and astrocytes was kept the same (astrocyte: $2x10^5$ cells/mL and monocyte: $1x10^4$ cells/mL).

G. Cytokine measurements in monocyte supernatants.

To determine the cytokines produced by monocytes, the co-culture procedure above was performed in the absence of astrocytes (monocytes only). Supernatants were collected at 20 h and stored at -80°C. LEGENDplex™ (BioLegend) technology was utilized to determine cytokine levels in supernatants per manufacturer's direction. Data analysis was performed using the LEGENDplex™ software. Undetectable cytokine levels for specific samples was given a value of 0 pg/mL for graphing and statistical analysis.

H. Cytokine neutralization assay.

For cytokine neutralization, the co-culture procedure above was performed with the addition of neutralization antibodies targeting human MCP-1, IL-6, TNFα and IL-1β. The neutralization antibodies were used at a concentration of 1μg/mL. The isotype control for each respective neutralization antibody was added to culture at the same concentration (1μg/mL).

I. Intracellular IL-1β production by monocytes.

Purified monocytes ($1x10^6$ cells/mL) were left untreated (NS) or stimulated with IFNα or R837 for 16 h and a Golgi block was added for 4 h. The flow cytometry procedure below was used to measure intracellular IL-1β protein expression.

J. Flow cytometry.

Staining buffer (PBS, 1% BSA, 0.1% NaN_3) was used to wash cells in between staining and fixing steps. Cells were first incubated with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain (Thermo Fisher Scientific) and then with staining buffer containing 20% human AB serum to block Fc receptors. BD Cytofix™ (BD Biosciences, San Jose, CA) was used to fix cells. For intracellular staining, cells were stained with antibody in BD Perm/Wash™ (BD Biosciences). Fixed cells were analyzed on a BD Canto II™ (BD Biosciences). For intracellular staining, a protein transport inhibitor containing Monensin and Brefeldin A (Thermo Fisher Scientific) was added to cell culture 4 h prior to harvesting of cells for analysis. Data analysis was performed using FLOWJO v10 software. Monocyte surface staining to determine purity was performed immediately after isolation using anti-CD14, CD16, CD45, CD56, and CD57. Single cells were first gated based on CD45 expression ($CD45^+$). Within the $CD45^+$ population, monocytes were defined as $CD14^{++}CD16^-$ (classical), $CD14^{++}CD16^+$ (intermediate) and $CD14^{+/-}CD16^+$ (non-classical) but lacking CD56/CD57 expression. For analysis of co-culture experiments, astrocytes

were gated apart from monocytes based on forward-scatter area and height (FSC-A and FSC-H) and optimization experiments verified that the astrocytes were $GFAP^+$ (Fig. 5A-B). An additional optimization experiment was performed using the leukocyte marker CD45 to show no monocyte contamination in the gating strategy utilized (Fig. 5C-D). The percent of MCP-1^+, IL-6^+ or IP-10^+ cells within the astrocyte population was reported in the figures. The mean fluorescent intensity (MFI) within the respective positive cells for the corresponding protein was calculated. The gates set for each protein were based on isotype controls.

K. Recombinant IL-1β treatment of astrocytes.

U251 or primary astrocytes were seeded into 24-well plates as described in co-culture section above. Astrocytes were treated with varying concentrations of recombinant IL-1β (noted in each experiment) for 20 h and a Golgi block was added for 4 h. Additional experiments on astrocytes were performed with IL-1β treatment (20pg/mL) in combination with IFNα or R837. Two independent experiments were performed in triplicates for U251 cells. For primary cells, one independent experiment was performed for two separate biological replicates.

L. Statistical analysis.

Statistical analysis was performed using Prism 7 (GraphPad, San Diego, CA). The experimental data was graphed as the mean +/- SEM. The statistical tests performed for each experiment are indicated in the figure legends. Data sets with increased variability were log transformed prior to performing the statistical test.

III. Materials and Methods for specific aim 3 and 4

A. Reagents.

Imiquimod (R837) and ssRNA40 were purchased from InvivoGen (San Diego, CA). Golgi transport inhibitor (Monensin (2μM)/Brefeldin A (3.0 μg/mL) in methanol) was purchased

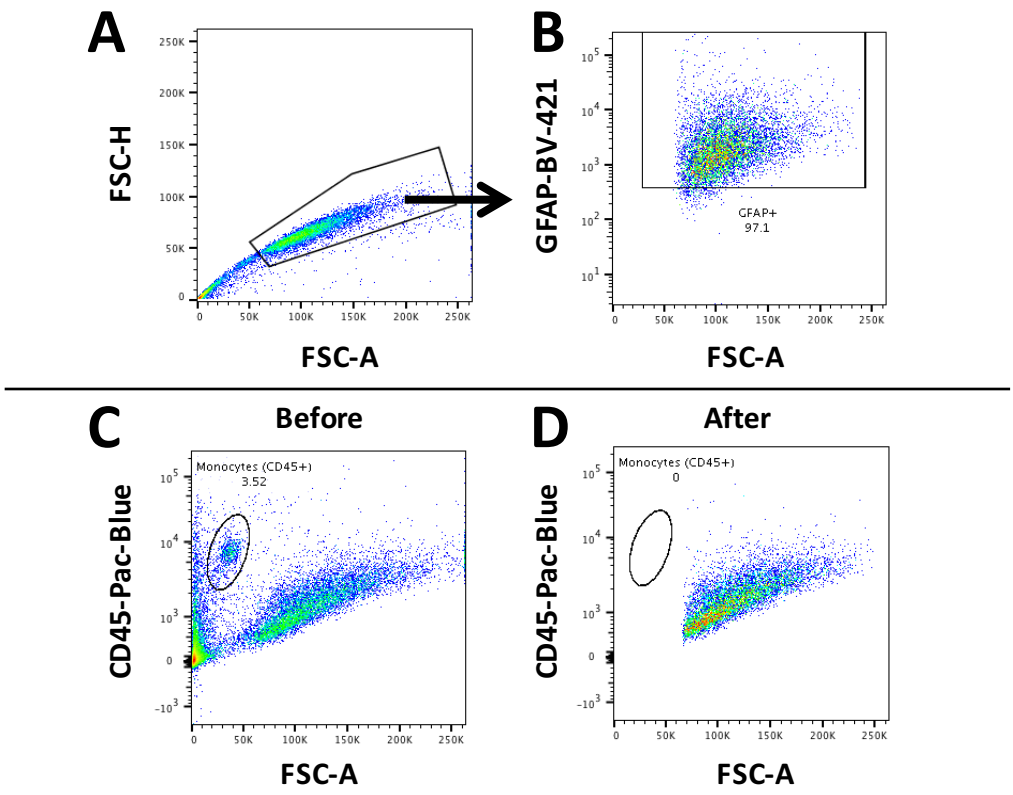

Figure 5: Gating strategy to distinguish astrocytes from monocytes was based on forward scatter height (FSC-H) and area (FSC-A). (A) Representative plot demonstrating the gating strategy to differentiate astrocytes from monocytes. (B) Astrocyte purity ($GFAP^+$) from panel A was determined. An unstained control was used to set the $GFAP^+$ gate. (C-D) Representative plots illustrating no monocyte ($CD45^+$) contamination in the gated astrocyte population. Monocytes were gated on based CD45 expression before (C) and after (D) the addition of the FSC-H and FSC-A gate.

from Thermo Fisher Scientific. Trypsin-EDTA (0.25%) was purchased from Gibco™. Antibodies purchased from BioLegend (San Diego, CA) included anti-CD45-Pacific Blue (clone: HI30, Cat. #: 304029), anti-CD14-Pe-Cy7 (M5E2, 301814), anti-CD16-APC (3G8, 302012), anti-CD56-PerCP (HCD56, 318342), anti-CD57-PerCP/Cy5.5 (HNK-1, 359622), anti-IL-6-APC (MQ2-13A5, 501112), anti-IL-1β-FITC (JK1B-1, 508206) and anti-MCP-1-PE (5D3-F7, 502604). Anti-CREB (pS133)/ATF-1 (pS63)-PE (J151-21, 558436) and anti-NF-κB p65 (pS529)-PE (K10-895.12.50, 558423) were purchased from BD Biosciences (San Jose, CA). Recombinant human IL-1β was purchased from BioLegend.

B. Chemicals.

Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD) were obtained from the National Institute on Drug Abuse (Bethesda, MD) or Cayman Chemical (Ann Arbor, MI) and dissolved in 100% ethanol. The cannabinoid receptor 2 agonist, JWH-015, was purchased from Cayman Chemical and dissolved in 100% ethanol. For cell culture experiments, cannabinoids were serially diluted in supplemented astrocyte media. The vehicle concentration for each treatment was 0.03% ethanol.

C. HIV-infected donors.

HIV+ male donors were recruited for blood draw under the IRB protocol (IRB# 11-202) by Dr. Peter Gulick and enrolled into the Mid-Michigan HIV consortium (MMHC). Donors received the standard of care and donor information was electronically available through the Research Data Capture (REDcap) (Vanderbilt University), which supports 21 CFR Part 11 compliance for clinical research and trials data and HIPAA guidelines. All HIV+ donors are currently on ART and negative for hepatitis C.

D. PBMC and monocyte isolation.

PBMCs were isolated from either human leukocyte packs (Gulf Coast Regional Blood Center, Houston, TX) or whole blood of HIV-infected donors by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Pittsburgh, PA). Pan monocytes were isolated by negative selection (Miltenyi Biotec (Bergisch Gladbach, Germany)) per manufacturer's direction. The monocyte purity was greater than 90%.

E. Human primary astrocyte cell culture.

Fetal-derived normal human astrocytes (NHA) isolated from the cerebral cortex were purchased from Lonza (Basel, Switzerland) and cultured according to manufacturer's direction in astrocyte basal media (ABM™) supplemented with SingleQuots™ Kit (rhEGF, insulin, ascorbic acid, GA-1000, L-glutamine and FBS). Media was changed every other day for passaging and plate seeding. NHA were used up to passage 4.

F. Cannabinoid treatment of monocyte-astrocyte co-culture.

Normal human astrocytes (NHA) were seeded in supplemented ABM™ 3-6 days prior to co-culture at a cell density (based on doubling time) that would be an estimated $2x10^5$ cells/mL on the day of co-culture. On the day of monocyte isolation, astrocytes were treated with cannabinoids (THC and CBD) at concentrations noted in the respective figures. Monocytes were added immediately to the well at a final concentration of $1x10^4$ cells/mL to establish a monocyte: astrocyte ratio of 1:20. Imiquimod/R837 (TLR7 agonist). In a 24-well plate format, there was $1x10^5$ astrocytes and $5x10^3$ monocytes at a total well volume of 500µl. The co-culture was incubated at 37°C and 5% CO_2 for 20 hours (h) and a Golgi block was added to culture for an additional 4 h. Prior to the addition of the Golgi block, an aliquot of supernatant was removed and stored at -80°C. At 24 h, wells were washed with non-supplemented media and

trypsinization was performed to remove astrocytes from plate. The flow cytometry procedure below was performed to measure cell viability and cytokine production.

G. Cannabinoid treatment of TLR7 and TLR8-activated monocyte monocultures.

Purified monocytes ($4x10^5$ cells/mL) were cultured in supplemented ABM™ and treated with THC or CBD. Monocytes were immediately stimulated with R837 (10μg/mL) or ssRNA40 (0.5μg/mL) and incubated at 37°C and 5% CO_2.

H. Cannabinoid treatment of astrocytes monocultures stimulated with recombinant IL-1β and R837.

Primary astrocytes were seeded into 24-well plates as described in co-culture section above. Astrocyte monocultures were treated with cannabinoids and activated using recombinant IL-1β (noted in each experiment) plus R837 (10μg/mL). Astrocytes were incubated for 20 h and a Golgi block was added for 4 h.

I. Supernatant measurements of cytokines and chemokines.

For particular experiments, supernatants were collected at indicated time points and stored at -80°C. For detection of supernatant MCP-1, IL-6 and IL-1β levels, ELISAmax™ (Biolegend) was used.

J. Flow cytometry.

Staining buffer (PBS, 1% BSA, 0.1% NaN_3) was used to wash cells in between staining and fixing steps. Cells were first incubated with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain (Thermo Fisher Scientific) and then with staining buffer containing 20% human AB serum to block Fc receptors. BD Cytofix™ (BD Biosciences) was used to fix cells. For intracellular staining, cells were stained with antibody in BD Perm/Wash™ (BD Biosciences). Fixed cells were analyzed on a BD Canto II™ (BD Biosciences). For intracellular staining, a protein

transport inhibitor containing Monensin and Brefeldin A (Thermo Fisher Scientific) was added to cell culture 4 h prior to harvesting of cells for analysis. Data analysis was performed using FLOWJO v10 software. Monocyte surface staining to determine purity was performed the same as in SA2 ((refer to Materials and Methods for specific aim 2, section J: Flow cytometry). For analysis of co-culture experiments, the procedure in SA2 was utilized to gate apart on only astrocytes (refer to Materials and Methods for specific aim 2, section J: Flow cytometry).

K. Annexin-V and 7-amino-actinomycin D (7-AAD) staining of human monocytes.

Monocytes were removed from tissue culture plates using mini cell scrappers (Biotium, Fremont, CA) at time points stated in the figure legends and transferred to a 96-well U-bottom plates. The Pacific Blue™ Annexin V Apoptosis Detection Kit with 7-AAD (BioLegend) was used per manufacturer's direction. Samples were analyzed via flow cytometry.

L. Caspase-1 activity.

At the time points stated in the figure legends, monocytes were treated with the fluorescent caspase-1 inhibitor probe FAM-YVAD-FMK (FAM-FLICA® Caspase-1 Assay Kit, ImmunoChemistry Technologies, Bloomington, MN) for 30 minutes. Cells were washed once and stained with the viability stain, NEAR-IR, for 10 minutes. Cells were washed twice and fixed (BD Cytofix^{TM}). Cell scraping or trypsin was used to remove monocytes from culture plates. Samples were analyzed via flow cytometry.

M. Gene expression analysis.

Monocyte RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). Reverse transcription polymerase chain reaction (PCR) was performed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Gene expression was

determined by real-time PCR using Taqman probes targeting *IL1B* (Hs01555410_m1, ThermoFisher). The loading control was 18S ribosomal RNA (ThermoFisher).

N. Statistical analysis.

Statistical analysis was performed using Prism 7 (GraphPad, San Diego, CA). The experimental data was graphed as the mean +/- SEM. The statistical tests performed for each experiment are indicated in the figure legends. Data sets with increased variability were log transformed prior to performing the statistical test.

RESULTS

I. Specific aim 1: Determine the effect of cannabis use and Δ^9-THC on peripheral monocyte activation and secretion of IP-10 in HIV-negative and HIV+ individuals.

A. HIV+MJ+ donors possess lower levels of circulating $CD16^+$ monocytes and plasma IP-10 compared to HIV+MJ- donors.

Monocyte expression of CD16 and CD163, and plasma IP-10 was determined in whole blood collected from HIV-MJ-, HIV+MJ- and HIV+MJ+ donors. There were no significant differences in age, body mass index (BMI), CD4 count, CD4/CD8 ratio and years infected with HIV between HIV+MJ- and HIV+MJ+ donors. In addition, there was a similar profile between HIV+MJ- and HIV+MJ+ donors in terms of being on ART, having undetectable viral loads, cigarette use, alcohol and other drugs of abuse (Table 1). When the levels of $CD16^+$ monocytes were compared, HIV+MJ+ donors had a significantly lower level compared to HIV+MJ- donors (Fig. 6A). A lower number of $CD16^+CD163^+$ monocytes was also observed in HIV+MJ+ donors when compared to HIV+MJ- donors but not significant ($p=0.052$) (Fig. 6B). In addition, plasma IP-10 was also significantly lower in HIV+MJ+ donors compared to HIV+MJ- donors (Fig. 6C).

B. IFNα treatment of PBMCs and purified monocytes increases the expression of both CD16 and CD163 on monocytes in HIV-MJ- and HIV+MJ- donors but not HIV+MJ+ donors.

Monocyte transition into the $CD16^+$ phenotype in circulation is a key step prior to monocyte migration into the CNS during HIV infection (88, 269), but the specific mechanism(s) of this monocyte transition remains unclear. Since a type I IFN gene signature has been identified in monocytes from HIV+ individuals (109, 153), we sought to determine the effect of IFNα on monocyte expression of CD16 and CD163.

Table 1. HIV-positive patient demographic and immunologic information.

Patient Information	HIV+MJ-	HIV+MJ- (N)	HIV+MJ+	HIV+MJ+ (N)	P
Age (years)	53.4 (11.7)	27	53.8 (8.93)	13	0.922
BMI	29.1 (7.3)	27	26 (7.9)	13	0.136
CD4 T-cell Count (cells/µL)	609.7 (400.1)	29	500.3 (278.2)	13	0.501
CD4/CD8 Ratio	0.8 (0.55)	29	0.56 (0.43)	13	0.099
Time Infected with HIV (years)	14.0 (9.3)	27	19.2 (11.9)	13	0.139
% on ART	100	29	100	13	-
% with Undetectable Viral Load	86.2	29	76.9	13	-
Cigarette Smoking (%)	31.0	29	30.8	13	
Alcohol Use (%)	51.7	29	61.5	13	
Other Drug Use (%)	0	28	0	12	

BMI: body mass index; CD4: cluster of differentiation 4; CD8: cluster of differentiation 8; HIV: human immunodeficiency virus; ART: antiretroviral therapy. Age, BMI, CD4 T-cell count, CD4/CD8 ratio and time infected with HIV are expressed as a mean (standard deviation - SD) and either an unpaired t-test or a Mann-Whitney test was performed. The sensitivity of detection for viral load was 50 copies/mL. Other drug use includes cocaine, methamphetamine and heroin. Unequal N values present between rows indicate missing patient information.

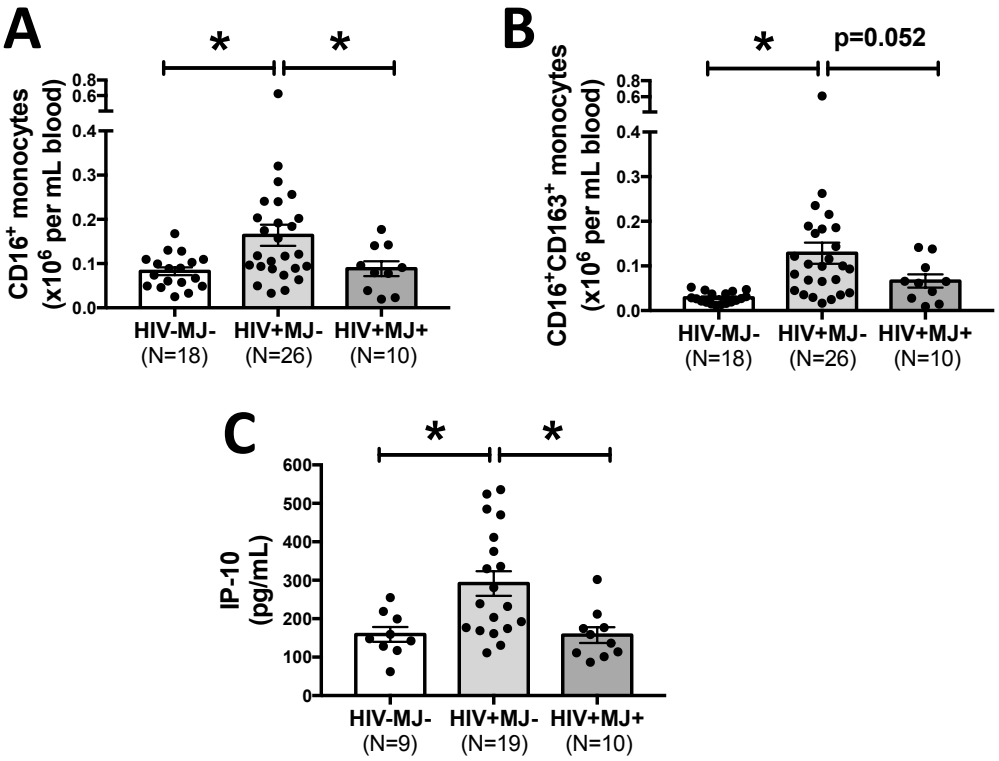

Figure 6. HIV+MJ+ donors display a lower level of circulating $CD16^+$ monocytes, $CD16^+CD163^+$ monocytes and plasma IP-10 compared to HIV+MJ- donors. (A-C) Whole blood and plasma was collected from HIV-MJ-, HIV+MJ- and HIV+MJ+ donors and the number ($x10^6$ per mL of blood) of $CD16^+$ monocytes, $CD16^+CD163^+$ monocytes and serum IP-10 (pg/mL) was measured. For A-C, data was log transformed and a one-way ANOVA with a Dunnett's post-hoc test was performed ($p<0.05$). All graphs are mean +/- SEM.

Human PBMCs isolated from HIV-MJ- donors were stimulated with 50U/ml of IFNα and cells were harvested at 6, 16, 24 and 48h post stimulation. Figure 7 illustrates the effect of IFNα (50U/mL) on monocyte expression (within PBMCs) of CD16 and CD163 at 48h. IFNα treatment for 48h led to a significant increase in the percent of monocytes expressing CD16 (Fig. 8A), while a significant increase in percent of $CD163^+$ monocytes was observed only at 24h (Fig. 8B). A significant increase in the percent of monocytes co-expressing CD16 and CD163 ($CD16^+CD163^+$) was observed at 48h (Fig. 8C). There was a notable increase in the percent of $CD16^+$, $CD163^+$ and $CD16^+CD163^+$ monocytes without IFNα stimulation compared to 0 hr background expression (Fig. 8A-C), which may be due to adherence-mediated activation (270). To determine the range of concentrations by which recombinant IFNα increases CD16 and CD163 expression on monocytes, PBMCs from HIV-MJ- donors were stimulated with IFNα at concentrations ranging from 12.5-200U/mL for 48h. IFNα induced monocyte expression of CD16 at all concentrations tested (Fig. 9A) while only inducing CD163 expression between 12.5 and 50U/mL (Fig. 9B). When observing co-expression of CD16 and CD163 on monocytes, IFNα significantly increased the percent of $CD16^+CD163^+$ monocytes (Fig. 9C).

PBMCs were then isolated from HIV+MJ- and HIV+MJ+ donors to determine if there was a difference in the IFNα-mediated induction of CD16 and CD163 on monocytes from HIV+MJ- and HIV+MJ+ donors. Interestingly, IFNα treatment significantly increased the percentage of monocytes expressing CD16, CD163 and CD16/CD163 in HIV+MJ- donors (Figs. 10A-C), which was similar to that of HIV-MJ- donors. However, IFNα treatment only increased the percentage of $CD163^+$ monocytes and not $CD16^+$ or $CD16^+CD163^+$ monocytes of HIV+MJ+ donors (Fig. 10A-C), suggesting that cannabis use may be suppressing monocyte induction of CD16. To determine if IFNα is having a direct role on monocyte expression of CD16 and

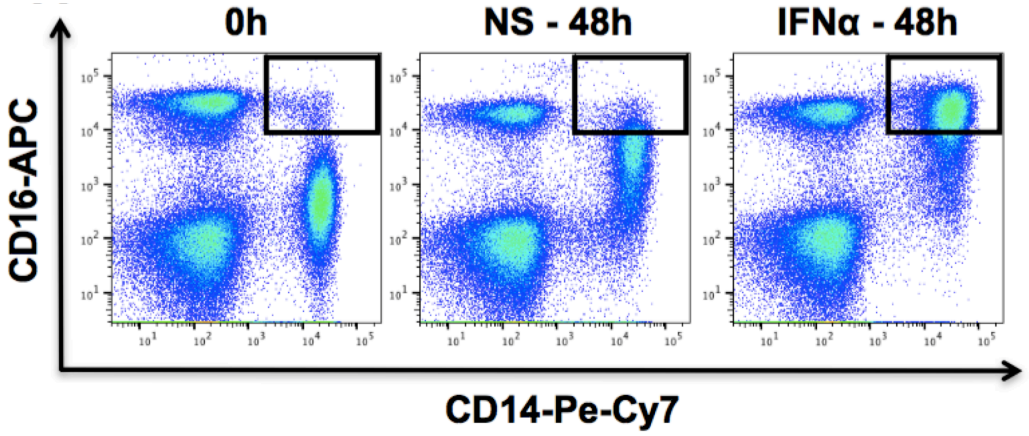

Figure 7. IFNα treatment increased CD16 and CD163 expression on monocytes. PBMCs from HIV-MJ- donors were cultured without stimulation (NS) or with the addition of IFNα (50U/ml) for 48h. Flow cytometry plots represent PBMCs at time 0h, 48h NS and 48h with IFNα. Viable PBMCs were analyzed for CD14 and CD16 expression. The gates set above are monocytes ($CD14^{+}$) expressing CD16 (gate).

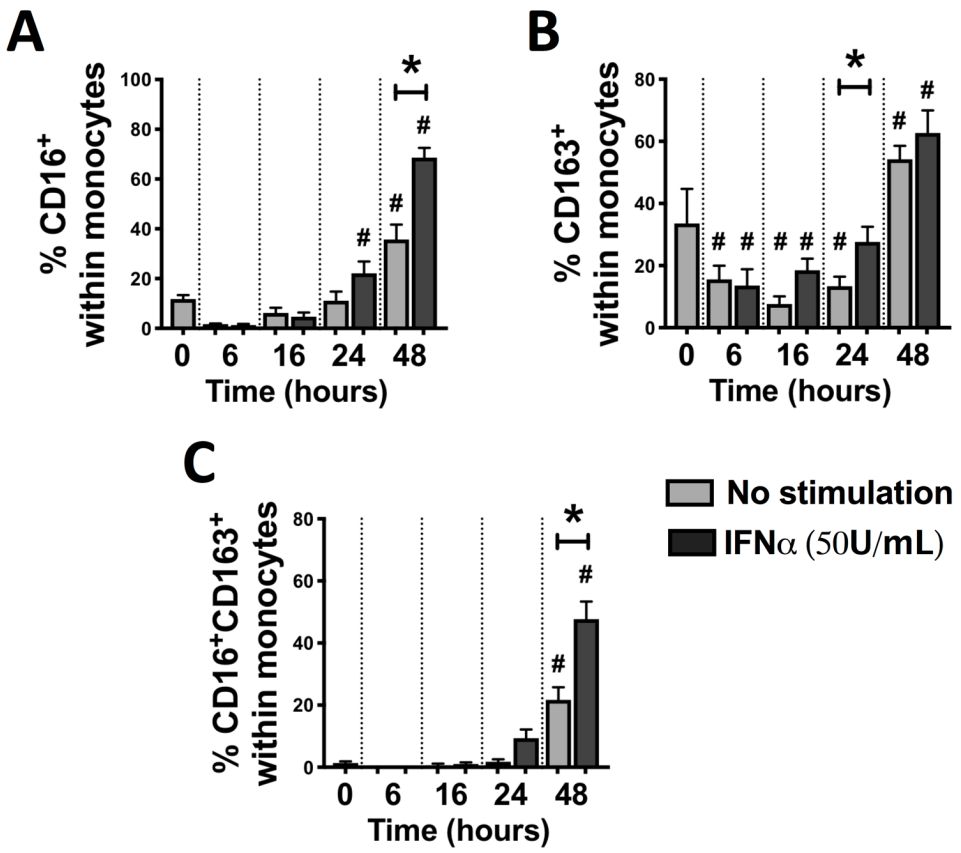

Figure 8. IFNα treatment increased CD16 and CD163 expression on monocytes within PBMCs. (A-C) HIV-MJ- PBMCs (N=7) were treated with IFNα (50U/mL) for 6, 16, 24 and 48h. Flow cytometry was used to measure the percentage of $CD16^+$, $CD163^+$ and $CD16^+CD163^+$ cells within the monocyte population. *Statistically different from non-stimulated control at specific time point and # denotes a statistical difference from 0 hr time point ($p<0.05$, two-way RM ANOVA with a Tukey's multiple comparisons post-test for A-C). Graphs in A-C are mean +/- SEM.

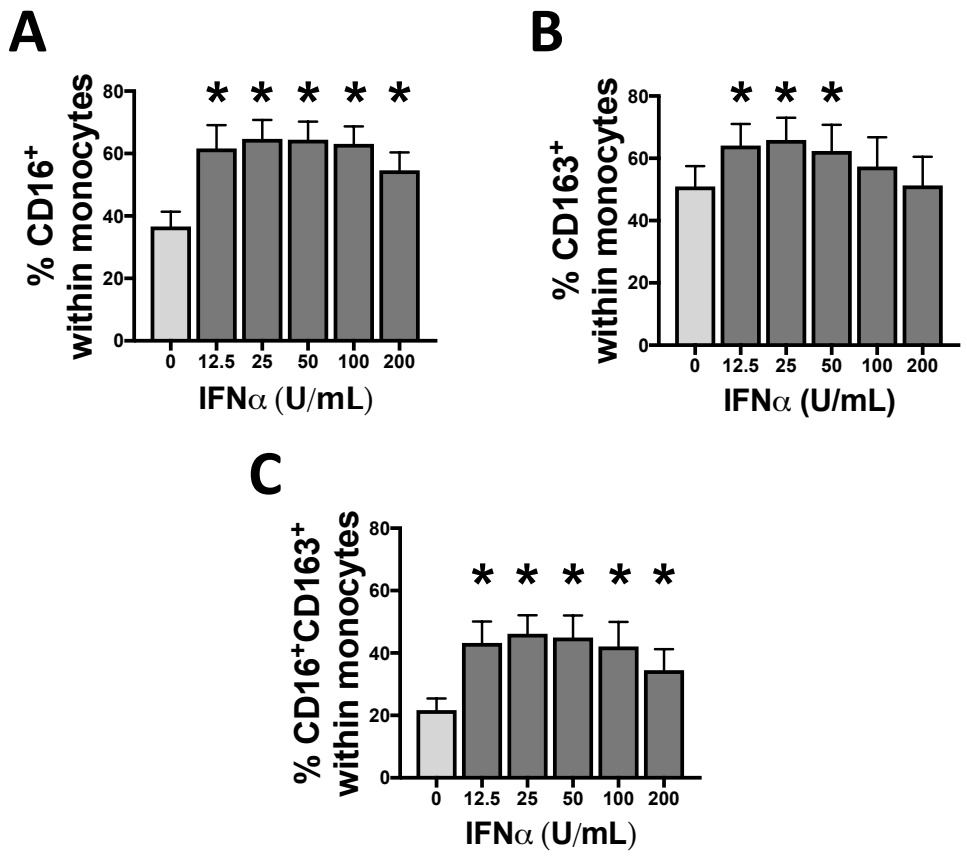

Figure 9. IFNα treatment increased CD16 and CD163 expression on monocytes within PBMCs. (A-C) HIV-MJ- PBMCs (N=7) were treated with varying concentrations of IFNα (0-200U/mL) for 48h. Flow cytometry was used to measure the percentage of $CD16^+$, $CD163^+$ and $CD16^+CD163^+$ cells within the monocyte population. *Statistically different from non-stimulated controls ($p<0.05$, one-way RM ANOVA with a Dunnett's multiple comparisons post-test for A-C). Graphs in A-C are mean +/- SEM.

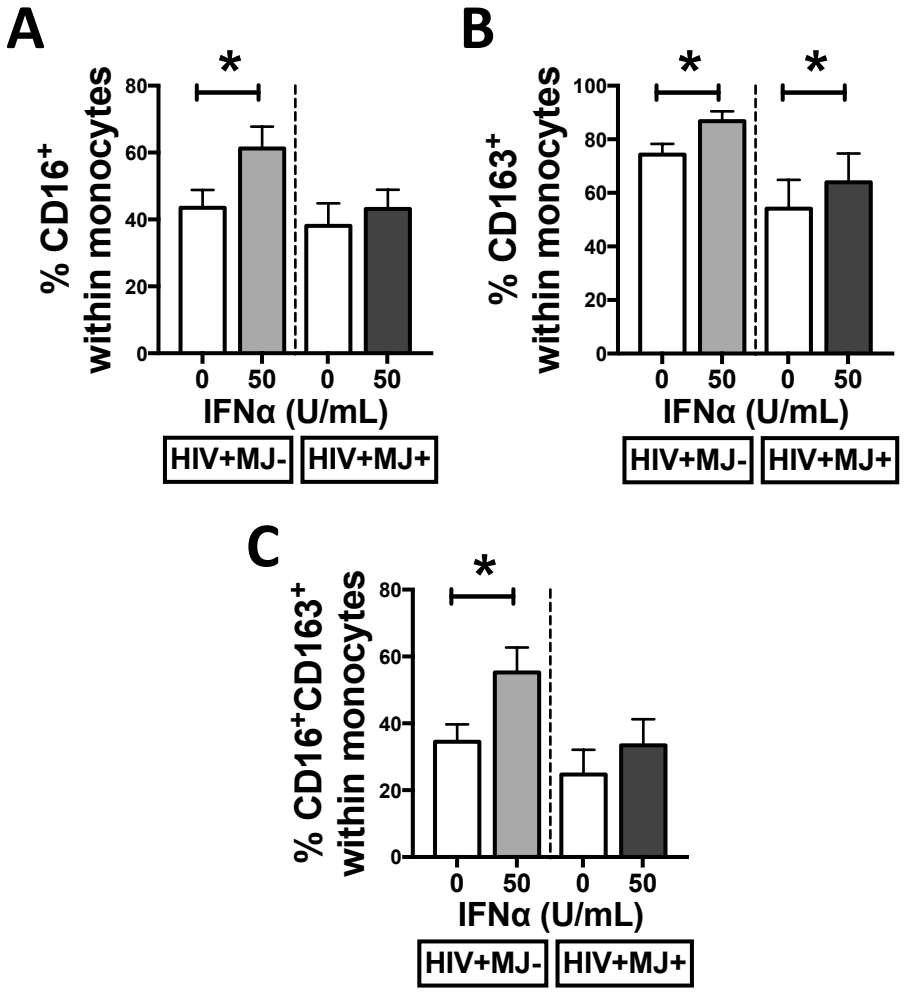

Figure 10. IFNα treatment increased CD16 and CD163 expression on monocytes from HIV+MJ- but not HIV+MJ+ PBMCs. (A-C) HIV+MJ- (N=6) and HIV+MJ+ (N=7) PBMCs were treated with IFNα (50U/mL) for 48h. Flow cytometry was used to measure the percentage of $CD16^+$, $CD163^+$ and $CD16^+CD163^+$ cells within the monocyte population. *Statistically different from non-stimulated controls ($p<0.05$, two-way ANOVA with a Sidak's multiple comparisons test for A-C). Graphs in A-C are mean +/- SEM.

CD163, $CD16^-$ monocytes from HIV-MJ- and HIV+MJ- donors were purified prior to IFNα (50U/ml) treatment. As with PBMCs, IFNα treatment of purified monocytes led to an increased percentage of $CD16^+$, $CD163^+$ and $CD16^+CD163^+$ monocytes for both HIV-MJ- and HIV+MJ- donors (Figs. 11A-C).

C. *In vitro* THC treatment of HIV-MJ- PBMCs and purified monocytes impairs the IFNα-mediated induction of CD16 and CD163 expression on monocytes.

Since HIV+MJ+ donors have lower levels of $CD16^+$ and $CD16^+CD163^+$ monocytes ($p=0.052$) in whole blood compared to HIV+MJ- donors, we sought to determine whether *in vitro* THC treatment influenced monocyte expression of CD16 and CD163 in response to IFNα. PBMCs from HIV-MJ- donors were pre-treated with 1, 5 and 10μM of THC and stimulated with IFNα (50U/mL) for 48h. THC treatment markedly decreased the percentage of $CD16^+$ monocytes in a concentration-dependent manner with significant suppression at 1, 5 and 10μM THC (Fig. 12A). In addition, THC treatment significantly decreased the percentage of $CD163^+$ and $CD16^+CD163^+$ monocytes (Figs. 12B-C). THC treatment had no significant effect on cell viability (>95% for each treatment group). As IFNα modulates cell function through the IFNα/β receptor (IFNAR) (145), we next sought to determine the effect of THC on monocyte expression of IFNAR using the same experimental approach as above. THC at 10μM modestly decreased the percentage of $IFNAR^+$ monocytes after 48h of IFNα treatment (Fig. 12D).

To determine if THC has a direct inhibitory effect on the monocyte population and not influencing monocyte activation via a bystander effect, $CD16^-$ monocytes from HIV-MJ- PBMCs were purified, pre-treated with 0.5, 1, 5 and 10μM of THC and stimulated with IFNα (50U/ml) for 48h. As seen in PBMCs, THC treatment decreased all three monocyte

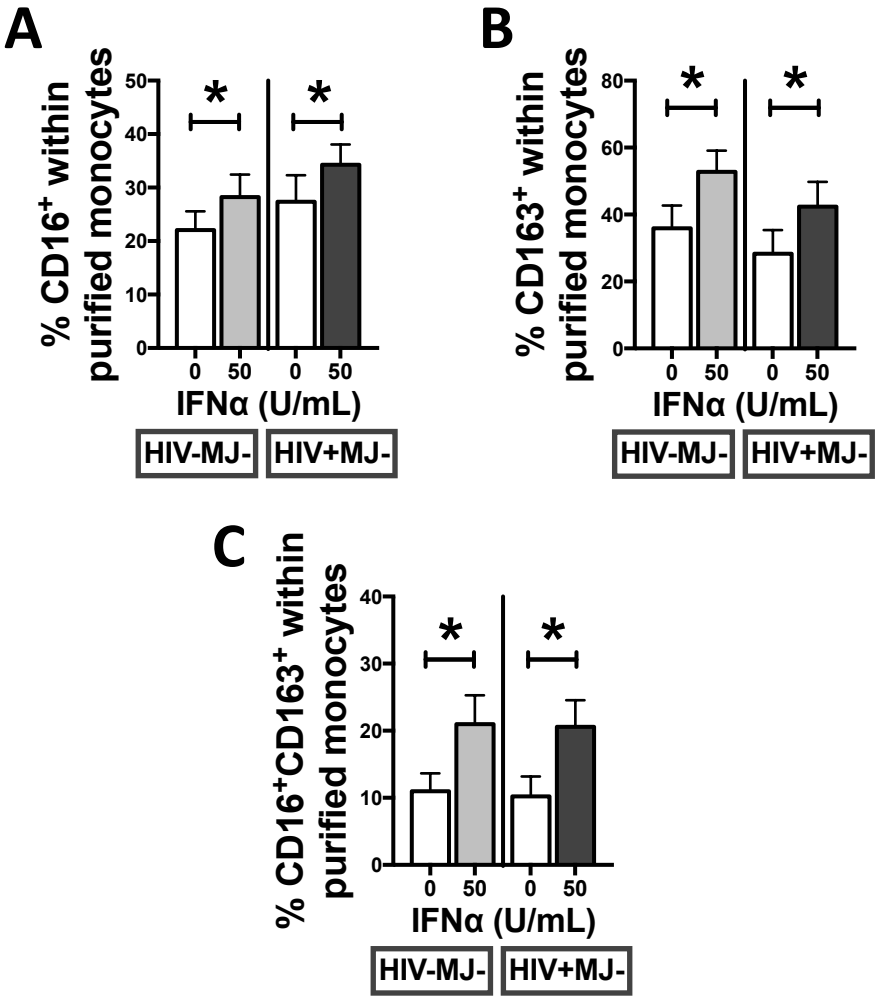

Figure 11. IFNα treatment increased CD16 and CD163 expression on purified monocytes from HIV-MJ- and HIV+MJ- donors. (A-C) Purified $CD16^-$ monocytes from HIV-MJ- (N=7) and HIV+MJ- (N=7) donors were treated with IFNα (50U/mL) for 48h. Flow cytometry was used to measure the percentage of $CD16^+$, $CD163^+$ and $CD16^+CD163^+$ cells within the monocyte population. *Statistically different from non-stimulated controls ($p<0.05$, two-way ANOVA with a Sidak's multiple comparisons test for A-C). Graphs in A-C are mean +/- SEM.

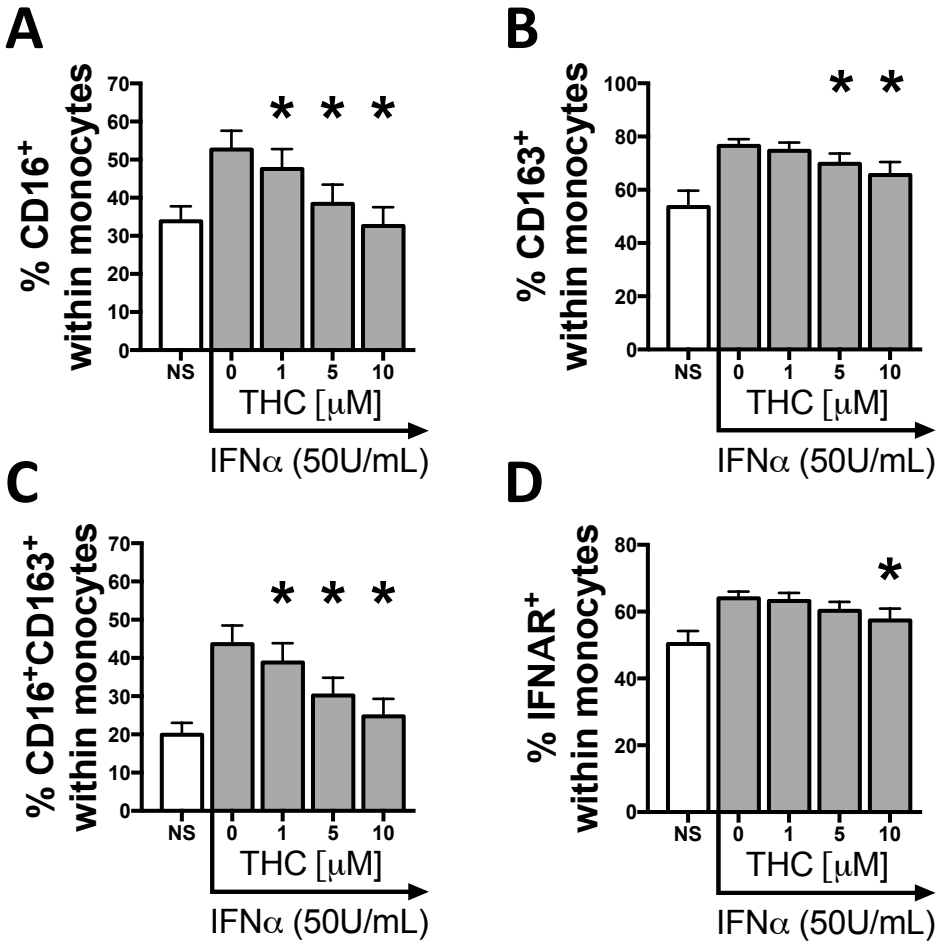

Figure 12. THC treatment decreased the percentage of $CD16^+$, $CD163^+$, $CD16^+CD163^+$ and $IFNAR^+$ cells within the monocyte population of PBMCs. (A-D) PBMCs from HIV-MJ- donors (N=11 for A-C and N=5 for D) were pretreated with 0 (vehicle – 0.03% EtOH), 1, 5 and 10μM of THC for 0.5h and stimulated with IFNα (50U/ml) for 48h. Flow cytometry was used to measure the percentage of $CD16^+$, $CD163^+$, $CD16^+CD163^+$ and $IFNAR^+$ cells within the monocyte population. *Statistically different from vehicle control (50 U/mL IFNα+vehicle) ($p<0.05$, RM one-way ANOVA with a Dunnett's multiple comparisons post-test). NS represents vehicle without IFNα addition. Graphs in A-D are mean +/- SEM.

populations ($CD16^+$, $CD163^+$ and $CD16^+CD163^+$) in a concentration-dependent manner (grey bars in Fig. 13A-C). Next, we confirmed that THC treatment also directly impaired monocyte expression of CD16 and CD163 in purified monocytes of HIV+MJ- donors (black bars in Fig. 13A-C). THC treatment had no effect on cell viability (>95% for each treatment group).

D. CBD does not impair CD16 or CD163 expression in IFNα-stimulated PBMCs from HIV-MJ- donors.

THC has a binding affinity to both CB1 and CB2 with a Ki of 25.1nM and 35.2nM for CB1 and CB2, respectively (228). By contrast, CBD, another cannabinoid present in cannabis, displays high structure similarity to THC but has 80-fold higher Ki to CB1 and CB2 (228). To better understand the role of CB1/CB2 in the THC-mediated impairment of CD16 and CD163 expression on monocytes, PBMCs from HIV-MJ- donors were pre-treated with THC or CBD at 1, 5 and 10μM and stimulated with 50U/mL of IFNα for 48h. As observed in Figure 12, THC significantly decreased the percentage of monocytes expressing of CD16, CD163 and CD16/CD163, while CBD at the same concentrations elicited no significant effects on the percentage of monocytes expressing of CD16, CD163 and CD16/CD163 (Fig. 14A-C).

E. THC treatment of PBMCs and purified monocytes decreased supernatant IP-10 levels from HIV-MJ-, HIV+MJ- and HIV+MJ+ donors.

After observing lower plasma IP-10 in HIV+MJ+ donors, when compared to HIV+MJ- donors, we then examined the impact of *in vitro* THC on monocyte production of IP-10. First, to determine the cellular source of IP-10 in response to IFNα, PBMCs from HIV-MJ- donors were stimulated with IFNα (50U/mL) for 24h and intracellular IP-10 staining was performed. Figure 15A is one representative donor of three, which demonstrates IFNα treatment increases the percent of $IP\text{-}10^+$ cells. Of the $IP\text{-}10^+$ cells, >90% were $CD14^+$ monocytes (Fig. 15A).

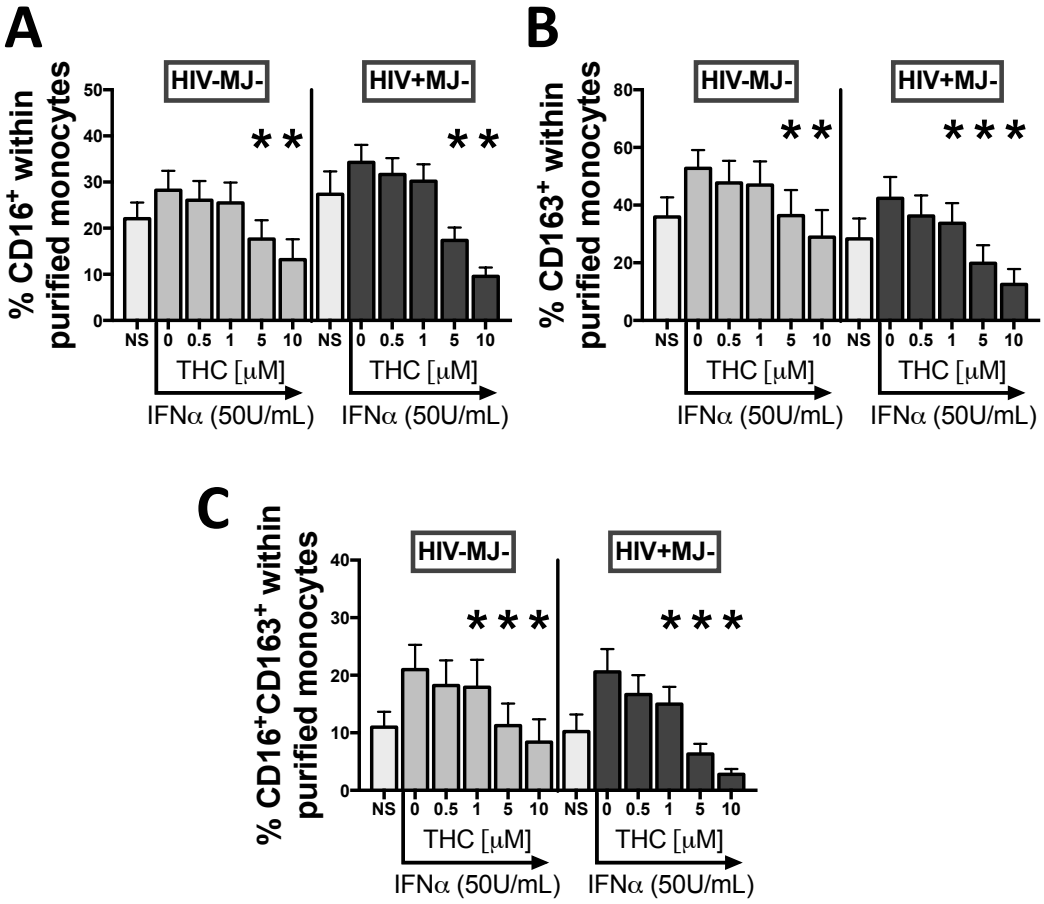

Figure 13. THC treatment decreased the percentage of CD16$^+$, CD163$^+$, and CD16$^+$CD163$^+$ within purified monocytes. (A-C) Purified CD16$^-$ monocytes from HIV-MJ- (N=7) and HIV+MJ- (N=7) donors were pretreated with 0 (vehicle – 0.03% EtOH), 0.5, 1, 5 and 10μM of THC for 0.5h and stimulated with IFNα (50U/ml) for 48h. Flow cytometry was used to measure the percentage of CD16$^+$, CD163$^+$ and CD16$^+$CD163$^+$ cells within the monocyte population. *Statistically different from vehicle control (50 U/mL IFNα+vehicle) ($p<0.05$, RM one-way ANOVA with a Dunnett's multiple comparisons post-test). NS represents vehicle without IFNα addition. Graphs in A-C are mean +/- SEM.

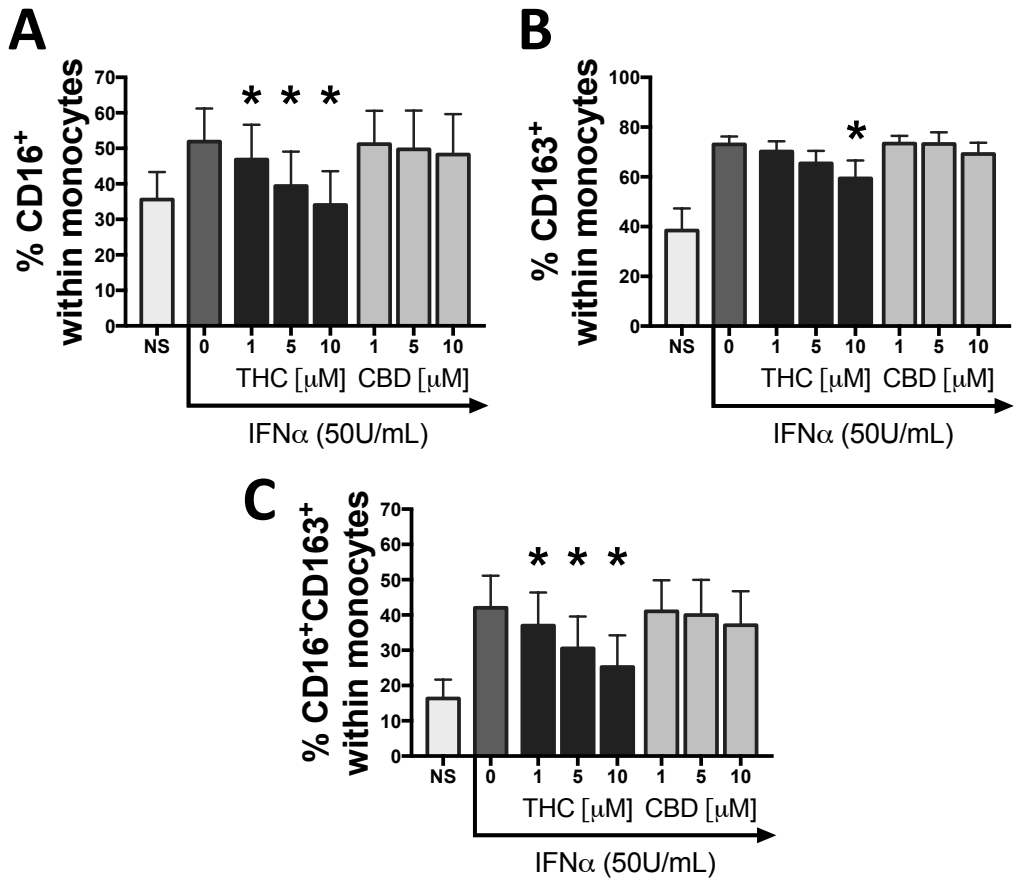

Figure 14. THC but not CBD decreased the percentage of $CD16^+$, $CD163^+$ and $CD16^+CD163^+$ cells within the monocyte population of IFNα-treated PBMCs. PBMCs from HIV-MJ- donors (N=5) were pretreated with 0 (vehicle – 0.03% EtOH), 1, 5 and 10μM of THC or 1, 5 and 10μM of CBD for 0.5h and stimulated with IFNα (50U/ml) for 48h. Flow cytometry was used to measure the percentage of $CD16^+$, $CD163^+$ and $CD16^+CD163^+$ cells within the monocyte population. *Statistically different from vehicle control (50 U/mL IFNα+vehicle) group ($p<0.05$, RM one-way ANOVA with a Dunnett's multiple comparisons post-test). NS represents vehicle without IFNα addition. Graphs in A-C are mean +/- SEM.

Of the IP-10^{+} monocytes in the IFNα treatment group, 73% were CD16^{-} and 27% were CD16^{+} (p=0.126) (Fig. 15B), suggesting that CD16 expression isn't a prerequisite for monocyte production of IP-10. Next, PBMCs from HIV-MJ-, HIV+MJ- and HIV+MJ+ donors were pre-treated with THC (1 and 5μM) and stimulated with IFNα for 48h. IFNα triggered a significant increase in supernatant IP-10 levels in the three donor groups, with no significant differences detected across groups (Fig. 16A). IP-10 levels were suppressed by 1 and 5μM THC in HIV-MJ- donors, while significant suppression was only seen at 5μM THC in HIV+MJ- and HIV+MJ+ donors (Fig. 16B). To determine if THC directly impairs monocyte production of IP-10, monocytes were purified from HIV-MJ- and HIV+MJ- donors, treated with THC (0.5, 1, 5 and 10μM) and stimulated with IFNα for 48h. IFNα stimulation significantly increased supernatant IP-10 in purified monocytes from HIV-MJ- and HIV+MJ- donors (Fig. 17A). THC treatment decreased supernatant IP-10 in a concentration-dependent manner with significant differences observed at 0.5-10μM THC in both HIV-MJ- and HIV+MJ- donors (Fig. 17B).

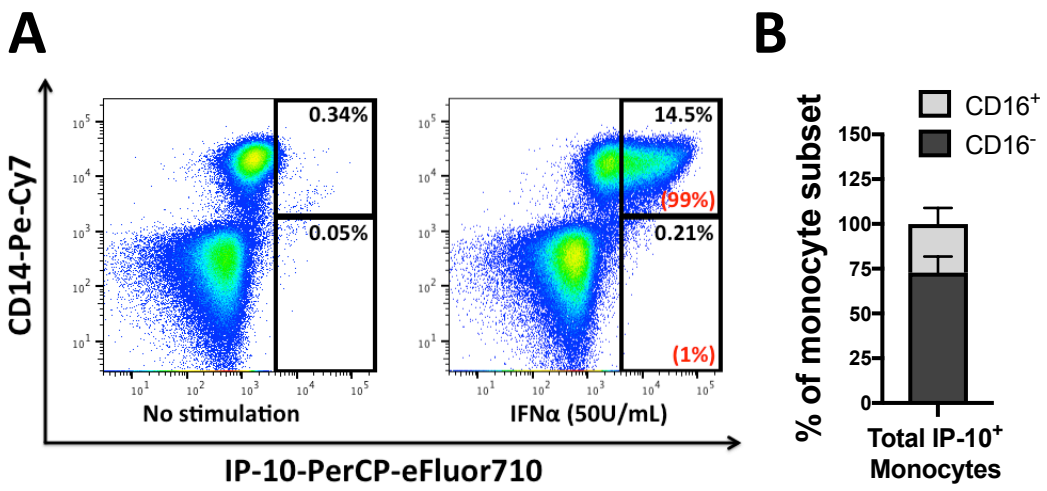

Figure 15. CD16$^-$ monocytes are the major source of IP-10 in PBMCs from HIV-MJ- donors. PBMCs from HIV-MJ- donors (N=3) were stimulated with IFNα (50U/ml) for 24h. Extracellular CD14 and CD16 staining and intracellular IP-10 staining was performed. (A) Flow cytometry plots are from one representative donor. The percentages on the upper right of the gate refer to percent of CD14$^+$IP-10$^+$ or CD14$^-$IP-10$^+$ cells within total PBMCs. The percentages in parentheses display the portion of IP-10$^+$ cells coming from the CD14$^+$ and CD14$^-$ populations. (B) The contribution of each monocyte subset (CD16$^-$ and CD16$^+$) to the total IP-10$^+$ monocyte population was determined. For B, a paired t-test was performed). Graph in B is mean +/- SEM.

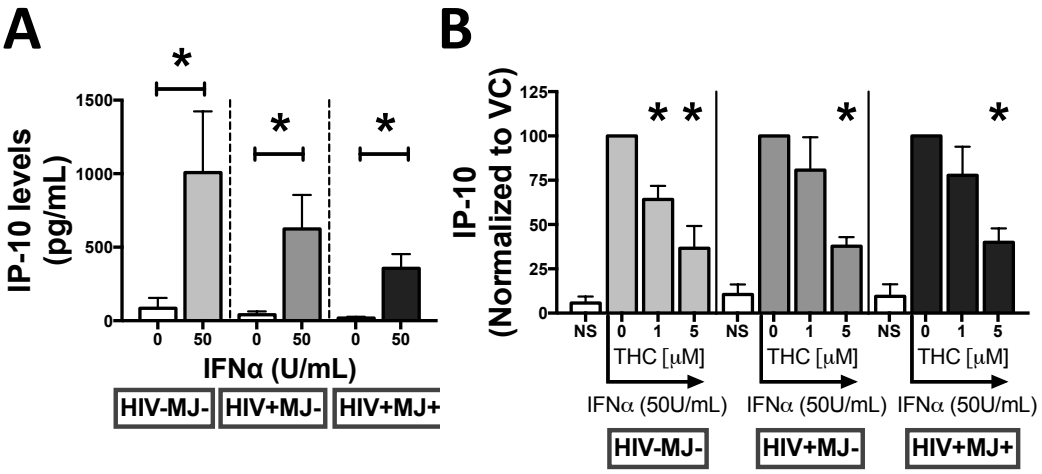

Figure 16. THC decreased supernatant IP-10 levels in IFNα-stimulated PBMCs from HIV-MJ-, HIV+MJ- and HIV+MJ+ donors. PBMCs from HIV-MJ- (N=3), HIV+MJ- (N=5) and HIV+MJ+ (N=5) donors were pre-treated with vehicle (0.03% EtOH) or THC (1 and 5μM) for 0.5h and stimulated with IFNα (50U/ml) for 48h. Supernatants were harvested and LEGENDplex™ was used to quantify IP-10 levels (*$p<0.05$). IFNα induction of IP-10 (pg/mL) is displayed in A and the effect of THC on IP-10 levels in B. For B, IP-10 levels for each THC treatment group was normalized to the donors vehicle control response (i.e., 0μM THC), which served as 100%. For A, * denotes a statistical difference from non-stimulated controls ($p<0.05$, two-way ANOVA with a Sidak's multiple comparisons test). For B, * denotes a statistical difference from vehicle control (50U/mL IFNα+vehicle) group ($p<0.05$) (RM one-way ANOVA with a Dunnett's multiple comparisons post-test). Graphs are mean +/- SEM.

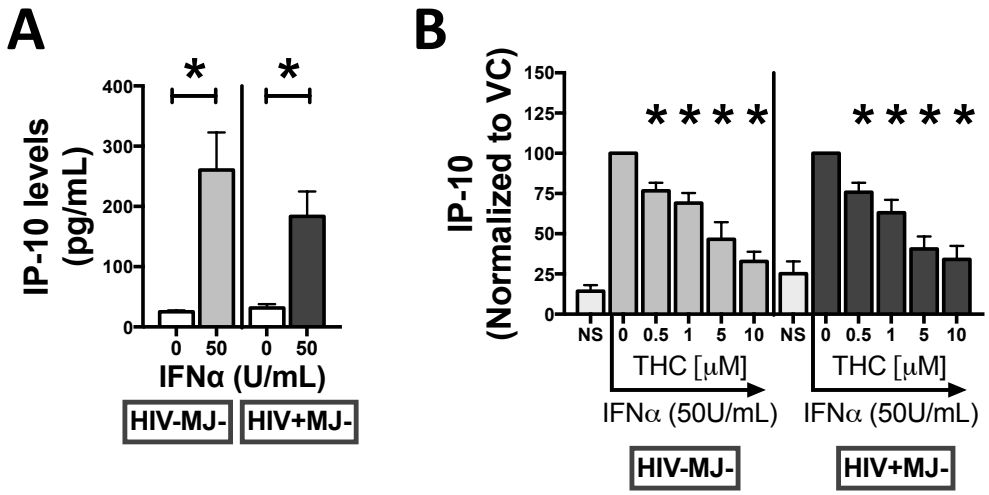

Figure 17. THC decreased supernatant IP-10 levels in IFNα-stimulated purified monocytes from HIV-MJ- and HIV+MJ- donors. (A) Purified $CD16^-$ monocytes from HIV-MJ- (N=7) and HIV+MJ- (N=7) donors were pre-treated with vehicle (0.03% EtOH), 0.5, 1 and 5 and 10μM of THC for 0.5h and stimulated with IFNα (50U/ml) for 48h. IFNα induction of IP-10 (pg/mL) is displayed in A and the effect of THC on IP-10 levels in B. For B, IP-10 levels for each THC treatment group was normalized to the donors vehicle control response (i.e., 0μM THC), which served as 100%. For A, * denotes a statistical difference from non-stimulated controls ($p<0.05$, two-way ANOVA with a Sidak's multiple comparisons test). For B, * denotes a statistical difference from vehicle control (50U/mL IFNα+vehicle) group ($p<0.05$) (RM one-way ANOVA with a Dunnett's multiple comparisons post-test). Graphs in B and C are mean +/- SEM.

II. Specific aim 2: Determine the effect of human primary monocytes, in combination with IFNα and/or TLR7 agonist (R837) on astrocyte production of MCP-1, IL-6 and IP-10 in a co-culture system.

A. Human primary monocytes induce human U251 and primary astrocytes to produce MCP-1 and IL-6.

To determine whether monocytes were able to promote astrocyte production of specific inflammatory factors, purified monocytes from human donors were cultured with U251 or primary astrocytes at cell ratios (monocyte:astrocyte) ranging from 1:100 to 1:5, and astrocytes were measured for intracellular MCP-1, IL-6 and IP-10. As shown in flow cytometry plots in Figures 18A, monocytes at a 1:5 ratio (monocyte:astrocyte) strongly increased the percent of astrocytes producing MCP-1 compared to astrocytes cultured alone (0:1 ratio). Furthermore, the astrocyte MCP-1 response increased in a monocyte-dependent manner, with comparable responses observed between U251 and primary astrocytes (Fig. 18B-C). Interestingly, MCP-1 production by astrocytes (U251 and primary) was significantly increased at the lowest ratio (1:100) tested compared to astrocytes alone (0:1 ratio) (Fig. 18B-C). The percent of astrocytes producing IL-6 also increased in a monocyte-dependent manner, with a significant increase in IL-6^+ astrocytes detected at a 1:50 ratio for U251 and 1:20 for primary astrocytes when compared to astrocytes alone (0:1 ratio) (Fig. 19A-C). Astrocytes from a second donor were used to verify these findings and similar trends were observed in MCP-1 and IL-6 production; however, the percent of MCP-1^+ astrocytes were significantly increased at the 1:20 ratio when compared to astrocytes alone (Fig. 20A-B). IP-10 production by astrocytes was not induced by monocytes (Fig. 21).

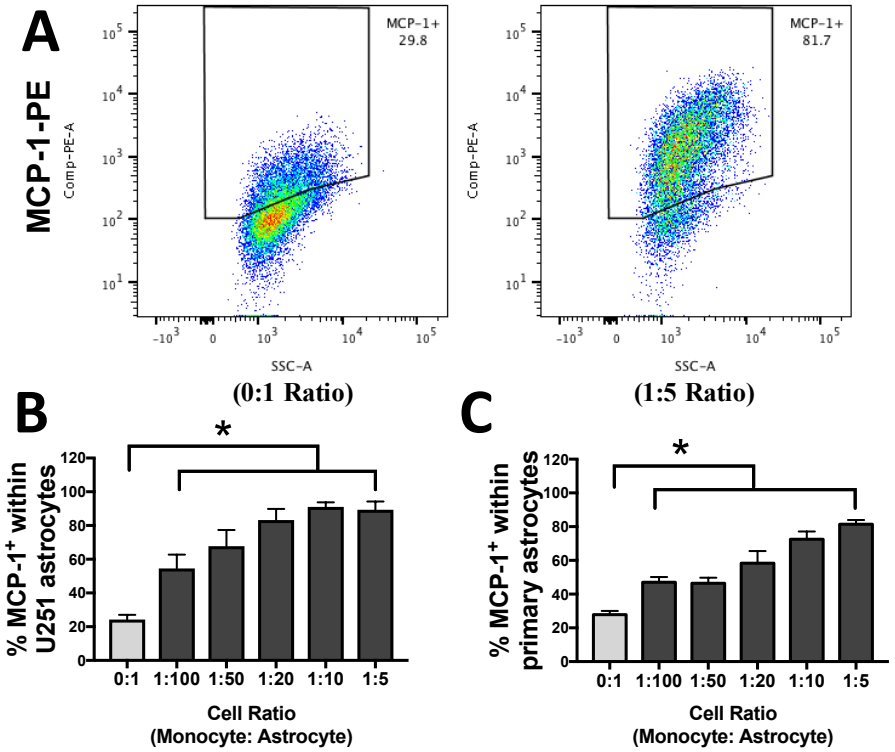

Figure 18. Human primary monocytes induce human U251 and primary astrocytes to produce MCP-1. Human primary monocytes (N=5 for panel B and N=5 for panel C) were purified from healthy donors and co-cultured with U251 (panel B) or primary astrocytes (donor 1 – panel C). Astrocytes were cultured at $2x10^5$ cells/mL and monocytes were added at various concentrations to establish monocyte: astrocyte ratios. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine the percent of astrocytes producing MCP-1. FSC-A and FSC-H were used to separate the astrocyte population from the monocytes. Panels A-B are flow cytometry plots of primary astrocyte production of MCP-1 comparing astrocytes alone (0:1 ratio) to a monocyte to astrocyte ratio of 1:5. For statistical analysis, a repeated measures (RM) analysis of variance (ANOVA) with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from 0:1 ratio group ($p<0.05$). Graphs are mean +/- SEM.

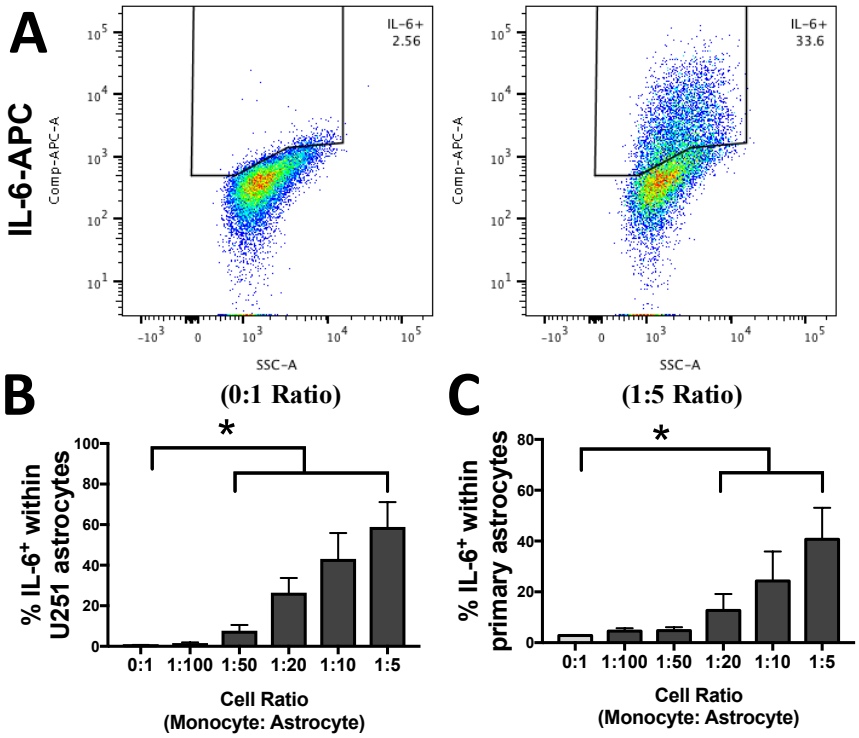

Figure 19. Human primary monocytes induce human U251 and primary astrocytes to produce IL-6. Human primary monocytes (N=5 for panel B and N=5 for panel C) were purified from healthy donors and co-cultured with U251 (panel B) or primary astrocytes (donor 1 – panel C). Astrocytes were cultured at $2x10^5$ cells/mL and monocytes were added at various concentrations to establish monocyte: astrocyte ratios. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine the percent of astrocytes producing IL-6. FSC-A and FSC-H were used to separate the astrocyte population from the monocytes. Panels A-B are flow cytometry plots of astrocyte production of IL-6 comparing astrocytes alone (0:1 ratio) to a monocyte to astrocyte ratio of 1:5. For statistical analysis, a repeated measures (RM) analysis of variance (ANOVA) with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from 0:1 ratio group ($p<0.05$). Graphs are mean +/- SEM.

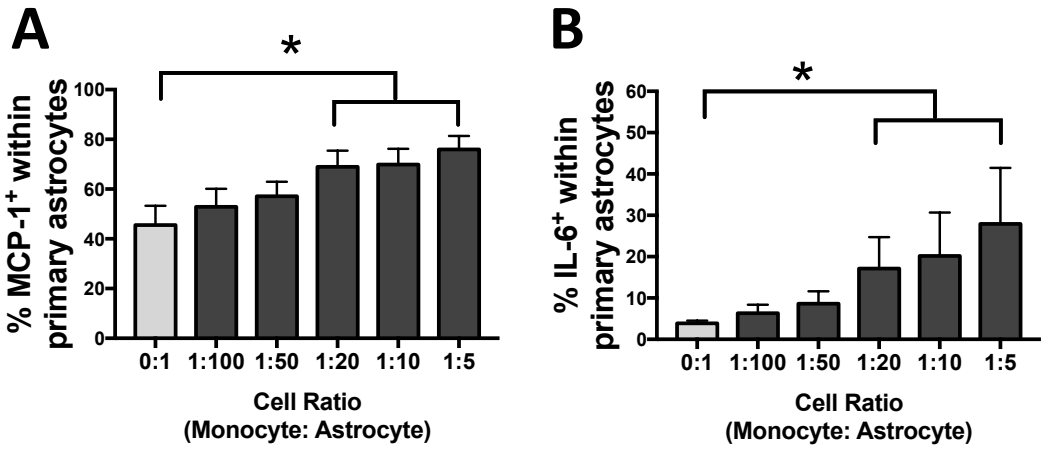

Figure 20. Human primary monocytes induce human U251 and primary astrocytes to produce MCP-1 and IL-6. Human primary monocytes (N=6) were purified from healthy donors and co-cultured with primary astrocytes (donor 2). Astrocytes were cultured at $2x10^5$ cells/mL and monocytes were added at various concentrations to establish monocyte: astrocyte ratios. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine the percent of astrocytes producing MCP-1 (panel A) or IL-6 (panel B). FSC-A and FSC-H were used to separate the astrocyte population from the monocytes. For statistical analysis, a RM ANOVA with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from 0:1 ratio group ($p<0.05$). Graphs are mean +/- SEM.

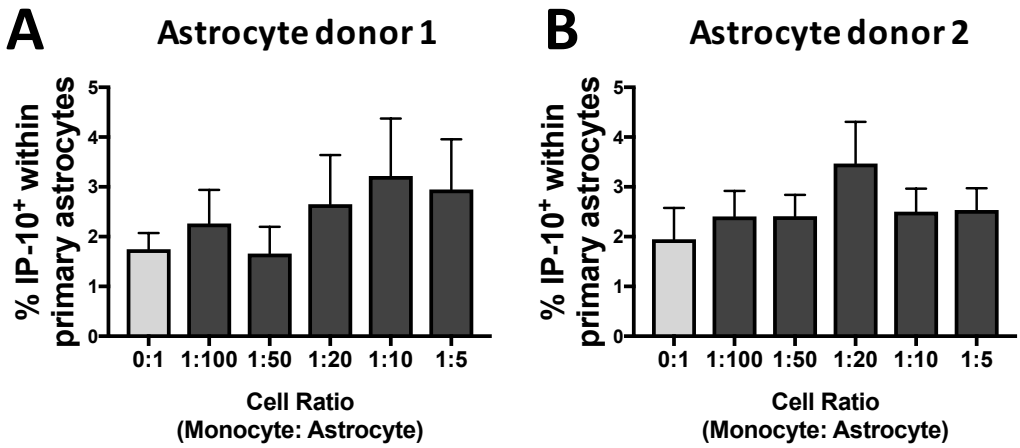

Figure 21. Human primary monocytes do not modulate primary astrocyte production of IP-10. Human primary monocytes (N=5 for A and N=6 for B) were purified from healthy donors and co-cultured with primary astrocytes. Astrocytes were cultured at $2x10^5$ cells/mL and monocytes were added at various concentrations to establish monocyte: astrocyte ratios. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine the percent of astrocytes producing IP-10. For statistical analysis, a RM ANOVA with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from 0:1 ratio group ($p<0.05$). Graphs are mean +/- SEM.

B. Imiquimod/R837 treatment alone and with IFNα, enhances monocyte-mediated astrocyte production of MCP-1, IL-6 and IP-10.

The next objective was to determine whether imiquimod/R837 and IFNα treatment of the co-culture affected monocyte-mediated astrocyte production of MCP-1, IL-6 and/or IP-10. R837 treatment of the co-culture containing U251 or primary astrocytes increased the percent of MCP-1^+ astrocytes compared to co-culture without stimulation (NS) (Fig. 22A and C). This observation was paralleled with an increase in supernatant MCP-1 in the R837-stimulated co-culture (Fig. 22B and D). IL-6 production by astrocytes was also augmented by R837-treatment of the co-culture, as evidenced by an increase in IL-6^+ astrocytes as well as elevated supernatant IL-6 compared to the NS-co-culture (Fig. 23). It is notable that R837 treatment of astrocytes alone displayed a trending increase in the percent of MCP-1^+ and IL-6^+ cells (two technical replicates) (Fig. 22C and 23C), suggesting that R837 is capable of directly stimulating primary astrocyte production of these factors. The R837-mediated effects observed in Figures 22 and 23 were also evident in co-cultures containing primary astrocytes from two additional donors (Fig. 25A-D and 26A-D). R837 had minimal to no effect on astrocyte production of IP-10 in the co-culture, which was observed with both U251 and primary astrocytes (Fig. 24, 25E-F and 26E-F). After extending the co-culture period to 48 and 72 h, R837 promoted a minor, but statistically significant increase in the percent of astrocyte expressing IP-10 at the 48 h time point (Fig. 27A). Furthermore, a similar effect was observed on supernatant IP-10 levels, as a minor increase was observed at 48 as well as 72 h (Fig. 27B).

Recombinant IFNα treatment, in combination with monocytes, had no effect on the percent of astrocytes expressing MCP-1 or supernatant MCP-1 in the U251 co-culture compared to co-culture without stimulation (NS) (Fig. 22A-B). However, IFNα did augment the production

of IL-6 and IP-10 (% and supernatant) in U251 containing co-cultures (Fig. 23A-B and 24A-B). In co-cultures containing primary astrocytes from three separate biological donors, IFNα induced MCP-1, IL-6 and IP-10 production by astrocytes compared to the control (NS) co-culture, evidenced by an increase in the percent positive astrocytes as well as supernatant levels of these factors (Fig. 22C-D, 23C-D, 24C-D, 25A-F and 26A-F). It is noteworthy that although not statistically significant, the IFNα treated co-culture using a third astrocyte donor also displayed increases in MCP-1 and IL-6 (Fig. 25A-D). Interestingly, direct addition of IFNα addition to astrocytes alone (0:1 ratio) resulted in increased supernatant IP-10 levels of primary astrocyte co-cultures (Fig. 24D, 25F and 26F).

To determine the combined impact of R837 and IFNα, the co-culture was treated with R837 (1μg/mL) and IFNα (100U/mL) alone, and in combination. In co-cultures containing either U251 or primary astrocytes from three separate donors, there was an additive effect on the percent of astrocytes expressing IL-6 with R837 and IFNα together, compared to when treated individually (Fig. 28B, 28E, 28H and 28K). No additive effect was observed on the percent of MCP-1^+ or IP-10^+ astrocytes in co-culture containing U251 cells or for two of the three primary astrocyte donors (Fig. 28A, 28C-D, 28F-G, 28I-J and 28L). Overall, monocytes, in combination with R837 and IFNα, augment astrocyte production of MCP-1 and IL-6, while inducing IP-10 compared to the NS-co-culture (Fig. 28).

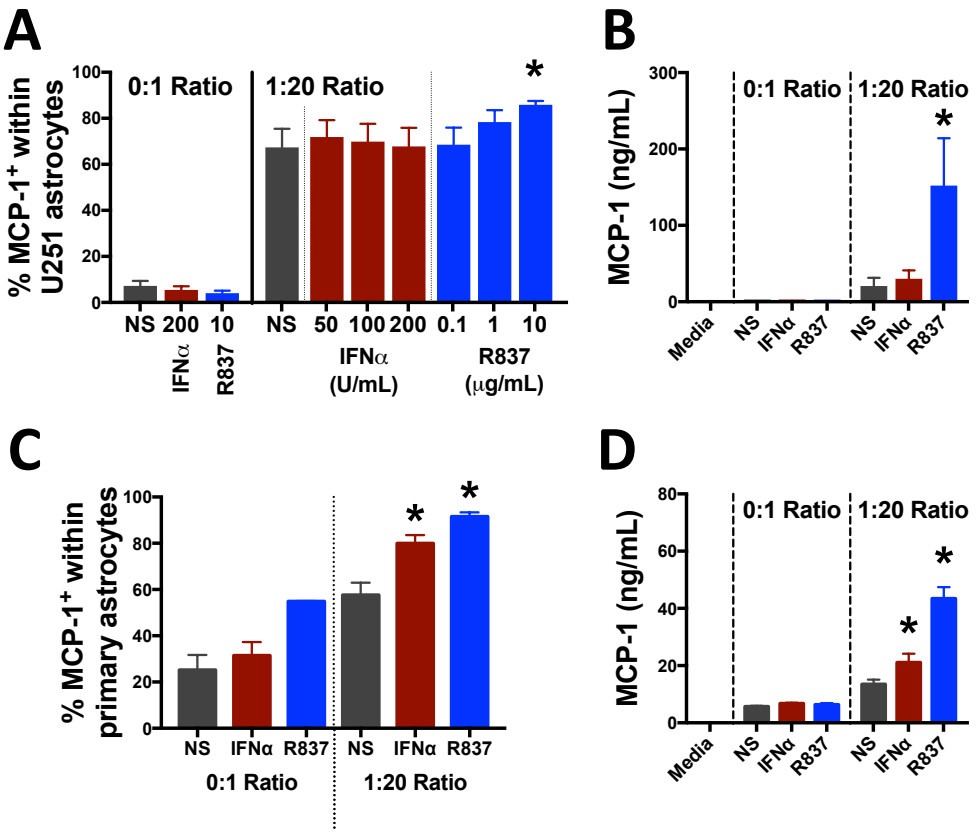

Figure 22. Imiquimod/R837 treatment enhances monocyte-mediated astrocyte production of MCP-1. Human primary monocytes (N=9 for A, N=3 for B and N=5 for C-D) were co-cultured with U251 or primary astrocytes (donor 1) at a ratio of 1:20 (monocyte: astrocyte). (A) Co-culture was stimulated with IFNα (50, 100 and 200U/mL) or R837 (0.1, 1 and 10μg/mL). (B-D) Co-culture was stimulated with IFNα (100U/mL) or R837 (10μg/mL). Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. An aliquot of supernatant was collected prior to the addition of the Golgi block. Intracellular staining and flow cytometry were performed to determine the percent of astrocytes producing MCP-1. Panels B and D are supernatant levels of MCP-1 in co-culture measured via ELISAmax™. A RM ANOVA with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from NS of the respective ratio group ($p<0.05$). Graphs are mean +/- SEM.

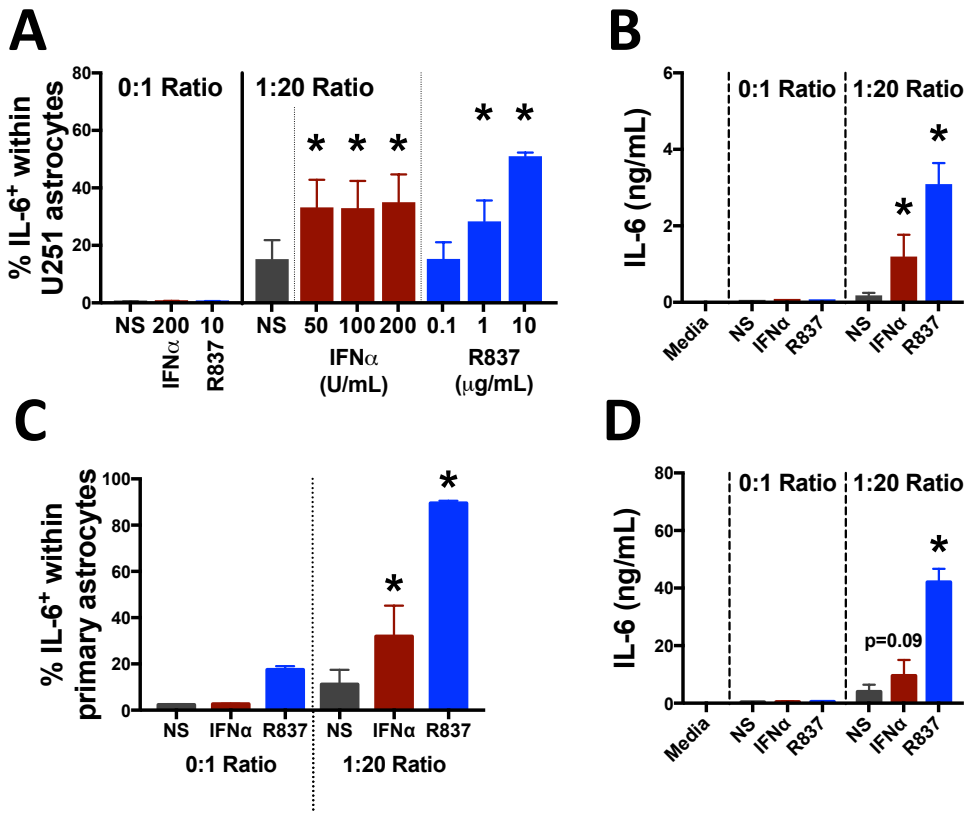

Figure 23. Imiquimod/R837 and IFNα treatment enhances monocyte-mediated astrocyte production of IL-6. Human primary monocytes (N=9 for A, N=3 for B and N=5 for C-D) were co-cultured with U251 or primary astrocytes (donor 1) at a ratio of 1:20 (monocyte: astrocyte). (A) Co-culture was stimulated with IFNα (50, 100 and 200U/mL) or R837 (0.1, 1 and 10μg/mL). (B-D) Co-culture was stimulated with IFNα (100U/mL) or R837 (10μg/mL). Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. An aliquot of supernatant was collected prior to the addition of the Golgi block. Intracellular staining and flow cytometry were performed to determine the percent of astrocytes producing IL-6. Panels B and D are supernatant levels of IL-6 in co-culture measured via LEGENDplex™ or ELISAmax™. A RM ANOVA with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from NS of the respective ratio group ($p<0.05$). Graphs are mean +/- SEM.

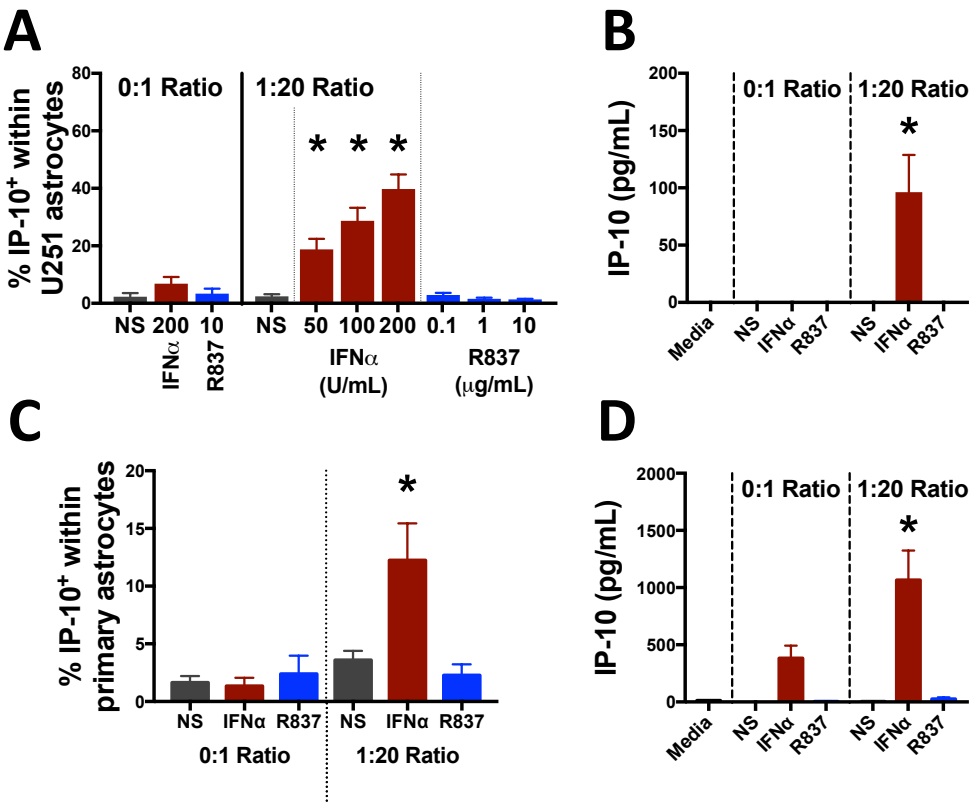

Figure 24. IFNα treatment enhances monocyte-mediated astrocyte production of IP-10. Human primary monocytes (N=9 for A, N=3 for B and N=5 for C-D) were co-cultured with U251 or primary astrocytes (donor 1) at a ratio of 1:20 (monocyte: astrocyte). (A) Co-culture was stimulated with IFNα (50, 100 and 200U/mL) or R837 (0.1, 1 and 10μg/mL). (B-D) Co-culture was stimulated with IFNα (100U/mL) or R837 (10μg/mL). Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. An aliquot of supernatant was collected prior to the addition of the Golgi block. Intracellular staining and flow cytometry were performed to determine the percent of astrocytes producing IP-10. Panels B and D are supernatant levels of IP-10 in co-culture measured via LEGENDplex™. A RM ANOVA with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from NS of the respective ratio group ($p<0.05$). Graphs are mean +/- SEM.

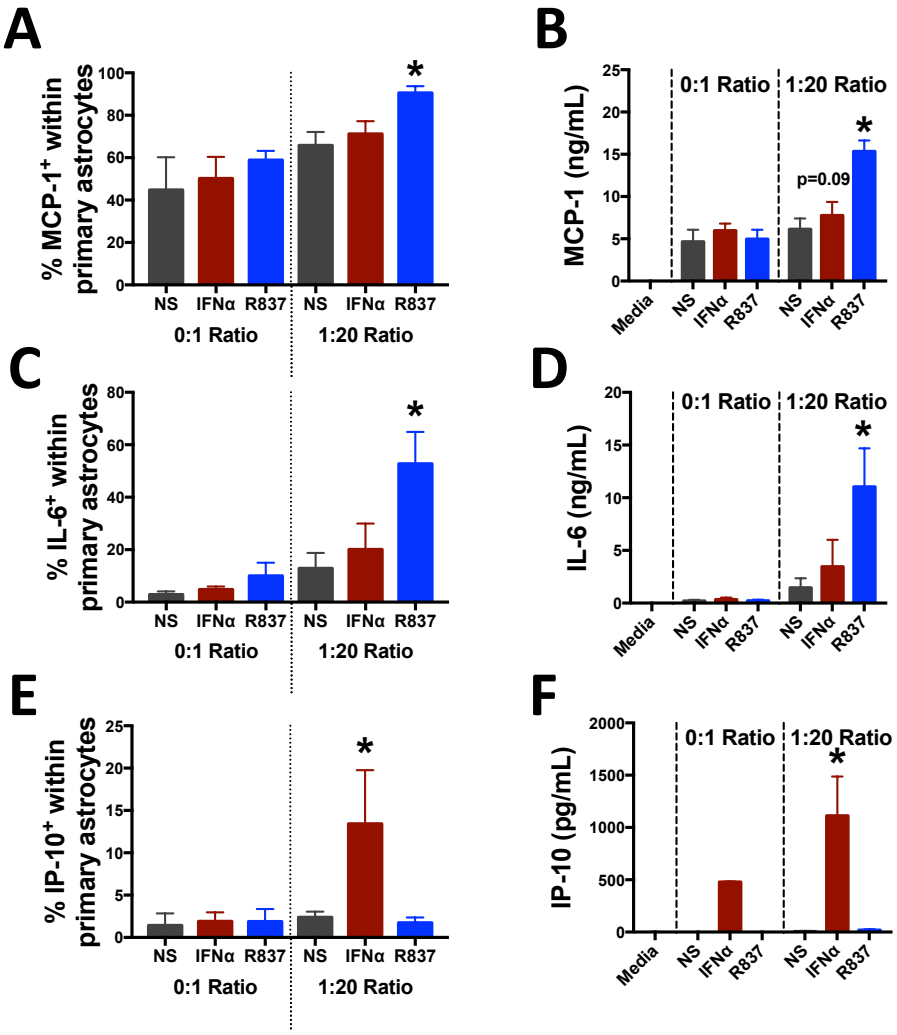

Figure 25. Imiquimod/R837 enhances monocyte-mediated astrocyte production of MCP-1 and IL-6, and IFNα promotes IP-10 production. Human primary monocytes (N=6) were co-cultured with primary astrocytes (donor 2) at a ratio of 1:20 (monocyte: astrocyte) and stimulated with IFNα (100U/mL) or R837 (10μg/mL). Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. An aliquot of supernatant was collected prior to the addition of the Golgi block. Intracellular staining and flow cytometry were performed to determine cytokine production by astrocytes (% positive). Panels B, D and F are supernatant levels of each cytokine in co-culture measured via LEGENDplex™ or ELISAmax™. A RM ANOVA with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from NS of the respective ratio group ($p<0.05$). Graphs are mean +/- SEM.

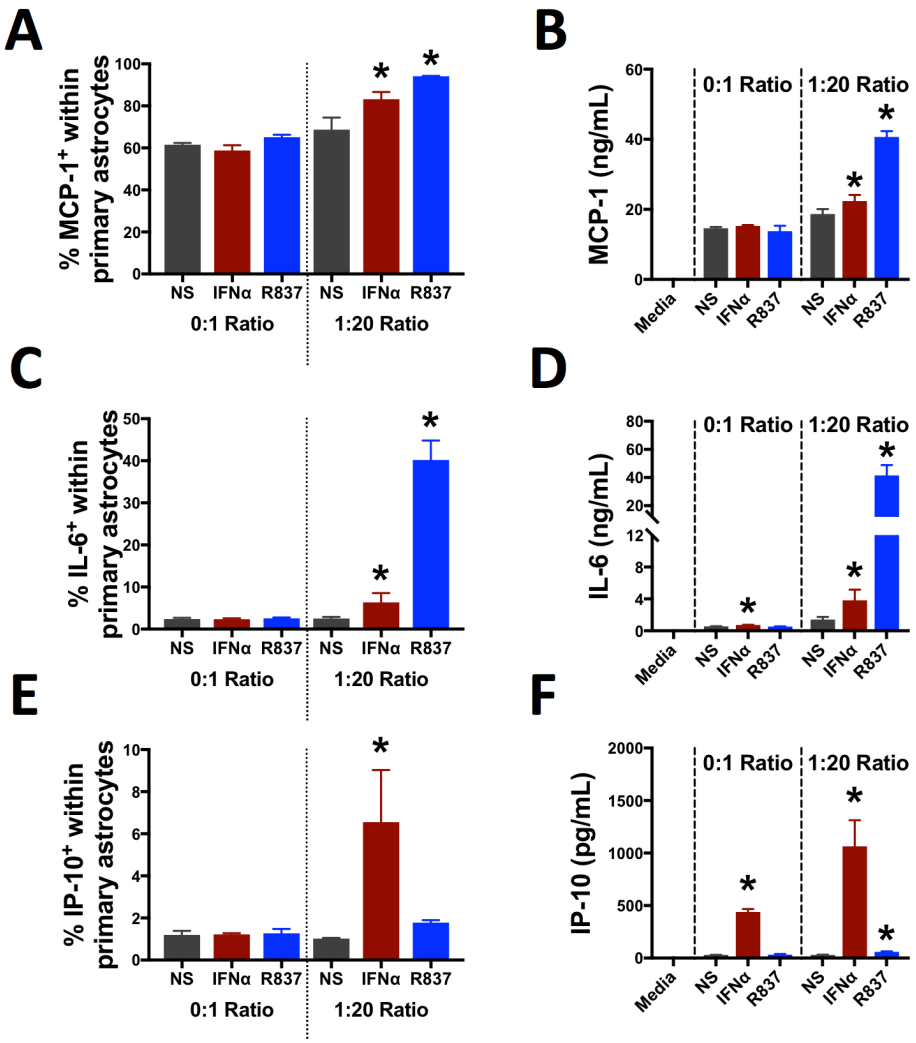

Figure 26. Imiquimod/R837 enhances monocyte-mediated astrocyte production of MCP-1 and IL-6, and IFNα promotes IP-10 production. Human primary monocytes (N=6) were co-cultured with primary astrocytes (donor 3) at a ratio of 1:20 (monocyte: astrocyte) and stimulated with IFNα (100U/mL) or R837 (10μg/mL). Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. An aliquot of supernatant was collected prior to the addition of the Golgi block. Intracellular staining and flow cytometry were performed to determine cytokine production by astrocytes (% positive). Panels B, D and F are supernatant levels of each cytokine in co-culture measured via LEGENDplex™ or ELISAmax™. A RM ANOVA with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from NS of the respective ratio group ($p<0.05$). Graphs are mean +/- SEM.

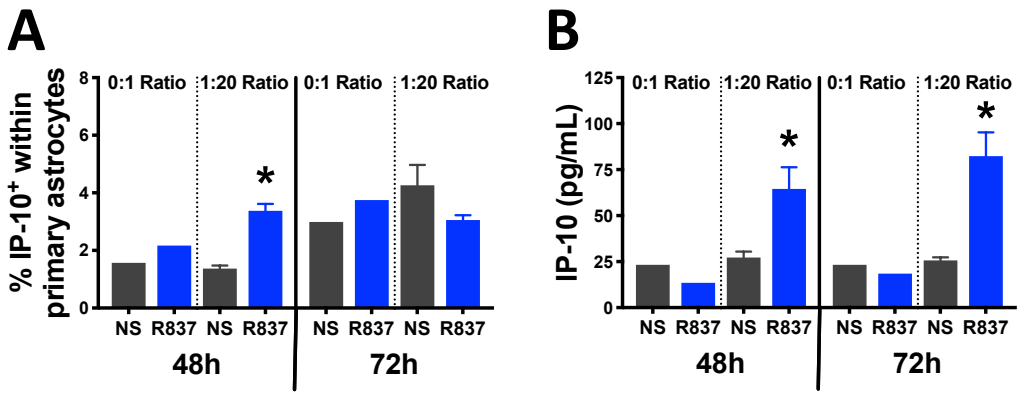

Figure 27. R837 treatment of co-culture results in minimal production of IP-10 at 48 and 72 h. Human primary monocytes (N=6) were co-cultured with primary astrocytes (donor 3) at a ratio of 1:20 (monocyte:astrocyte) and stimulated with IFNα (100U/mL) or R837 (10µg/mL). Cells were co-cultured for 48 and 72 h, with a Golgi block added 4 h before cell harvest. An aliquot of supernatant was collected prior to the addition of the Golgi block. (A) Intracellular staining and flow cytometry were performed to determine the percent of astrocytes producing IP-10. (B) Supernatant IP-10 levels were measured via ELISAmax™. For A-B, dataset was log transformed and a paired t-test was performed between NS and R837 treatment groups for each time point. * denotes a statistical difference from NS (1:20 ratio) treatment group ($p<0.05$). Graphs are mean +/- SEM.

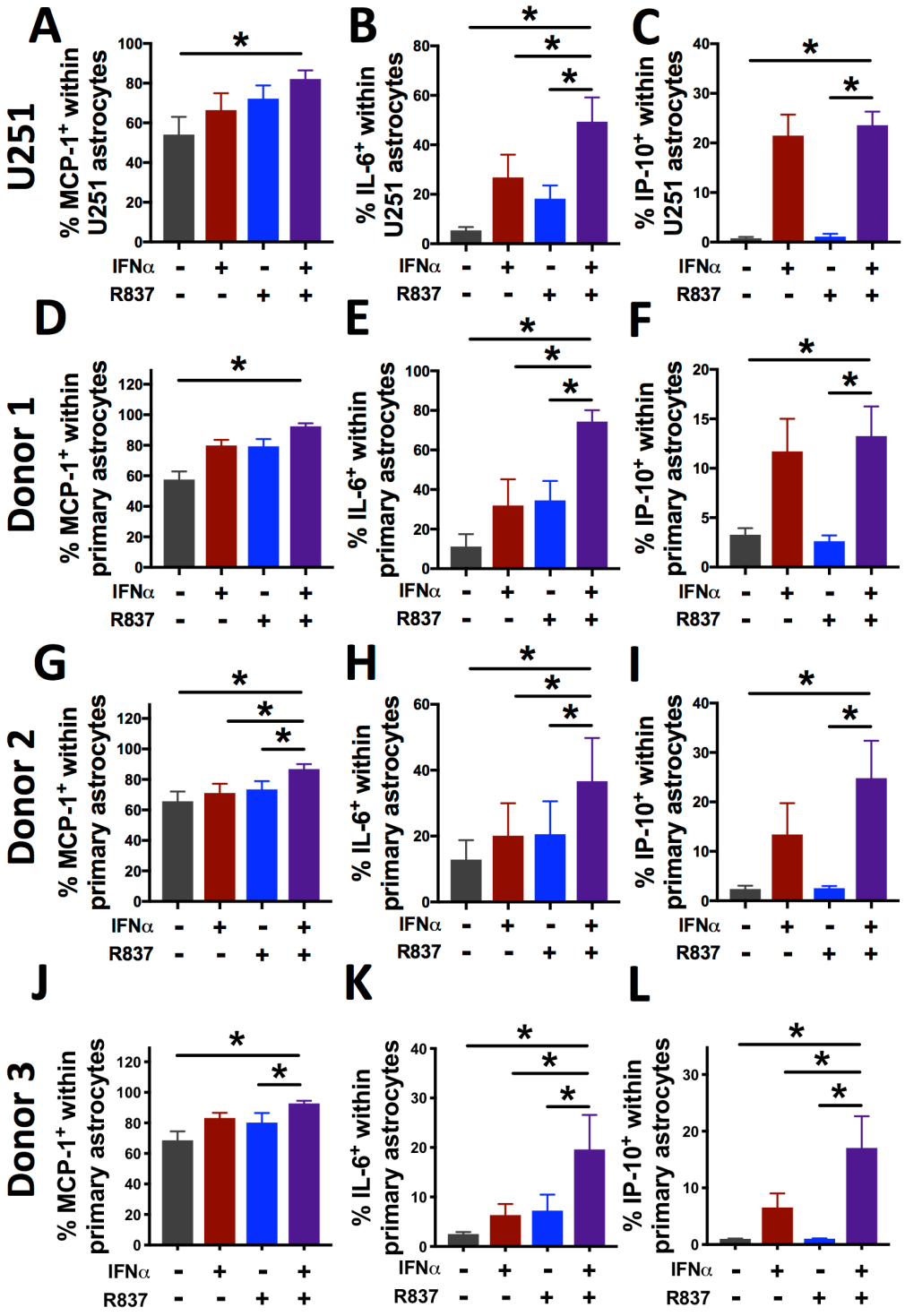

Figure 28. Imiquimod/R837 treatment alone and with IFNα, enhances monocyte-mediated astrocyte production of MCP-1, IL-6 and IP-10.

Figure 28 (cont'd)

IFNα (100U/mL) and R837 (1μg/mL) were added alone and in combination to the co-culture containing monocytes (N=6 for A-C, N=5 for D-F, N=6 for G-I and N=6 for J-L) and astrocytes. U251 astrocytes were used for A-C. Primary astrocytes from three donors were used for D-I. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. The percent of astrocytes producing MCP-1, IL-6 and IP-10 was determined via intracellular staining and flow cytometry. A RM ANOVA with a Tukey's post-test was performed. * denotes a statistical difference from the two groups connected via a line ($p<0.05$). Graphs are mean +/- SEM.

C. Imiquimod/R837 and ssRNA40 (TLR8 agonist) enhance monocyte-mediated astrocyte production of MCP-1 and IL-6 in co-cultures containing monocytes from HIV-infected donors.

To determine whether R837 activation of monocytes isolated from HIV-infected donors could augment astrocyte production of MCP-1 and IL-6, R837-stimulated co-cultures were performed as above; however, monocytes were isolated from HIV-infected donors. As shown in Figure 29, R837-stimulated co-cultures containing monocytes from HIV-infected donors enhanced astrocyte production of MCP-1 (% and MFI) and IL-6 (% and MFI), with significance observed at 10μg/mL of R837. In addition, we determined whether the HIV-relevant TLR8 agonist, ssRNA40, displayed a similar profile as R837. ssRNA40 is a GU-rich ssRNA oligonucleotide derived from the long terminal repeat (LTR) region of the HIV genome and is a known TLR8 agonist. ssRNA40 promoted a similar enhancement profile as R837 on monocyte-mediated astrocyte production of MCP-1 and IL-6, with a significant increase in MCP-1 and IL-6 production (% and MFI) detected at both 0.05 and 0.5μg/mL of ssRNA40 (Fig. 30).

D. Astrocyte production of MCP-1, IL-6 and/or IP-10 in response to monocytes alone and in combination with IFNα or R837 is primarily mediated through soluble factors.

To determine whether monocyte-mediated astrocyte production of MCP-1, IL-6 and IP-10 was driven through cell-cell contact or secreted soluble factors, Transwell®-Clear Inserts with a 0.4μm pore size were utilized to separate the monocytes and astrocytes. Briefly, the co-culture was prepared as above with no stimulation, IFNα (100U/mL) or R837 (10μg/mL) and with and without transwell inserts. U251 astrocytes were in the bottom chamber and monocytes were placed in the upper chamber. As shown in Figure 31A-B, monocyte-mediated astrocyte production of IL-6 and MCP-1, regardless of activation, was similar between normal wells (N)

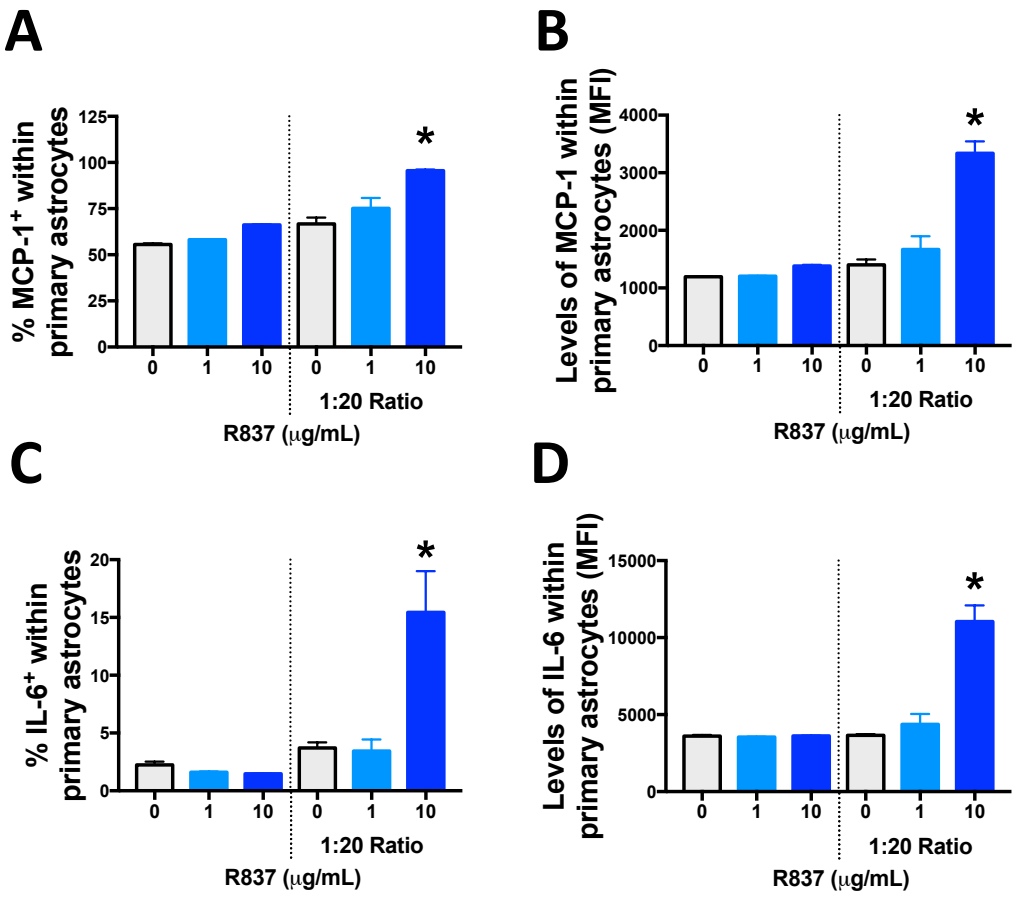

Figure 29. Imiquimod/R837 enhances monocyte-mediated astrocyte production of MCP-1 and IL-6 in co-cultures containing monocytes from HIV-infected donors. Human primary monocytes (N=5) from HIV-infected donors were co-cultured with primary astrocytes (donor 4) at a ratio of 1:20 (monocyte: astrocyte) and stimulated with R837 (1 and 10μg/mL). Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine cytokine production by astrocytes (% positive and mean fluorescence intensity - MFI). A RM ANOVA with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from 0 of the respective ratio group ($p<0.05$). Graphs are mean +/- SEM.

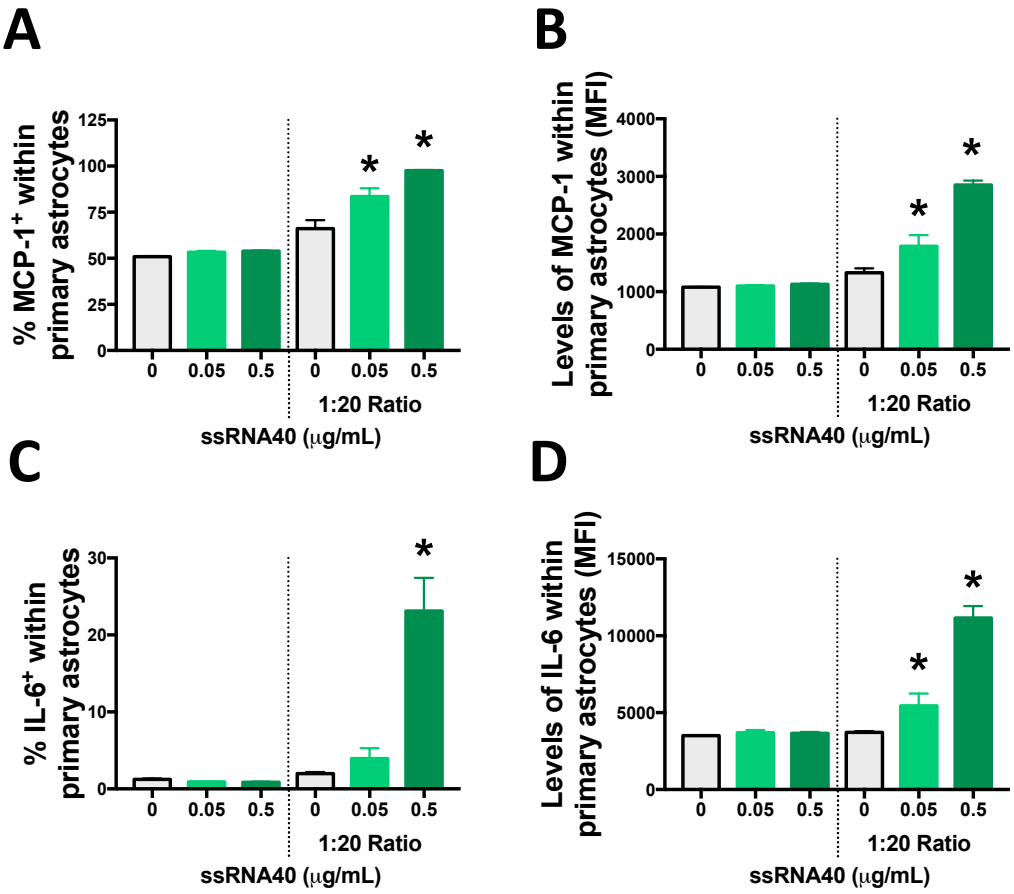

Figure 30. ssRNA40 enhances monocyte-mediated astrocyte production of MCP-1 and IL-6 in co-cultures containing monocytes from HIV-infected donors. Human primary monocytes (N=5) from HIV-infected donors were co-cultured with primary astrocytes (donor 4) at a ratio of 1:20 (monocyte: astrocyte) and stimulated with ssRNA40 (0.05 and 0.5μg/mL). Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine cytokine production by astrocytes (% positive and MFI). A RM ANOVA with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from 0 of the respective ratio group ($p<0.05$). Graphs are mean +/- SEM.

and transwells (T). However, there was a significant decrease in monocyte-mediated astrocyte production of IP-10 (IP-10^{+} cells) when stimulated with IFNα in transwells compared to normal wells (Fig. 31C). Overall, these data demonstrate that soluble factors released from monocytes are promoting astrocyte production of IL-6 and MCP-1, and both soluble factors and cell-cell contact are promoting IP-10 production.

E. Monocytes display differing cytokine/chemokine secretion profiles when cultured without stimulation, with IFNα or R837.

To determine potential monocyte-secreted factors that stimulate astrocyte production of MCP-1, IL-6 and IP-10, monocytes monocultures were treated with IFNα or R837 under the same conditions as the co-culture (monocytes only). Supernatants were collected from NS-, IFNα- and R837-stimulated monocytes at 20 h post-activation and several known monocyte-derived inflammatory factors (MCP-1, TNFα, IL-1β, IFNγ, IL-6 and IP-10) were quantified using LEGENDplex™. Untreated monocytes (NS) produced minimal but detectable amounts of TNFα, IL-1β, IL-6 and MCP-1 compared to media alone (Fig. 32). R837 stimulation enhanced production of IL-1β (5.94-fold), IL-6 (5.78-fold) and TNFα (1.59-fold) compared NS monocytes while IFNα only induced monocyte production of MCP-1 (3.61-fold) (Fig. 32). IFNγ was not induced by monocytes regardless of the stimulation and IP-10 was not detectable (Fig. 32).

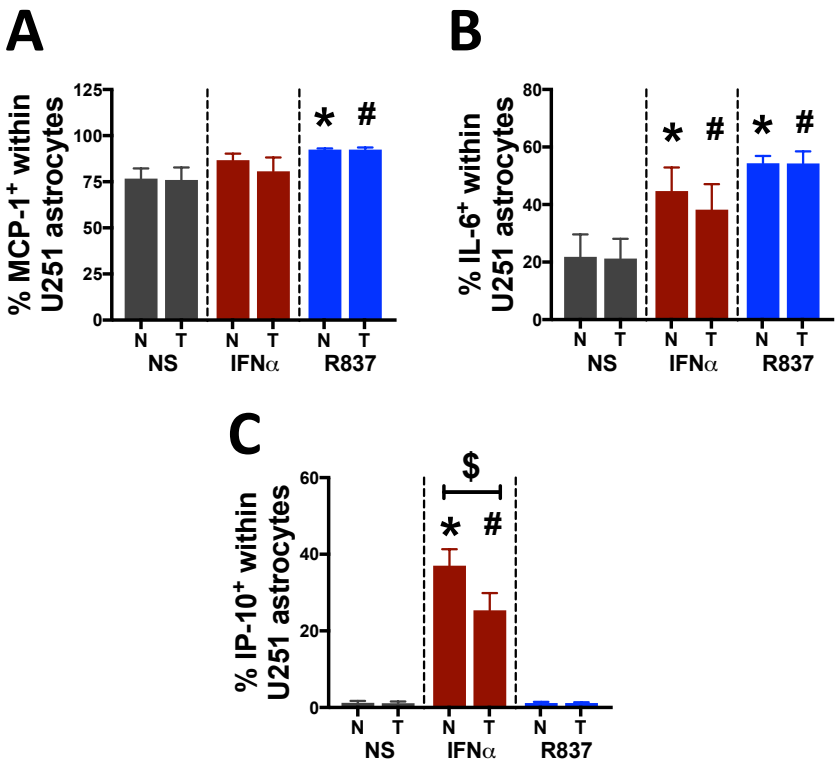

Figure 31. Cell-cell contact is in part, responsible for monocyte-mediated astrocyte production of IP-10 but not IL-6 or MCP-1 in IFNα or R837-stimulated co-cultures. Human monocytes (N=9) were co-cultured with U251 astrocytes at a ratio of 1:20 (monocyte: astrocyte) in either normal wells (N) or 0.4μm transwells (T). Co-culture was stimulated with IFNα (100U/mL) or R837 (10μg/mL). Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1, IL-6 and IP-10 (% positive). A RM two-way ANOVA with a Sidak's multiple comparisons post-test was performed. * denotes a statistical difference from the NS group in normal (N) wells ($p<0.05$) and # denotes a statistical difference from the NS group in transwells (T). $ denotes a statistical difference between normal wells and transwells. Graphs are mean +/- SEM.

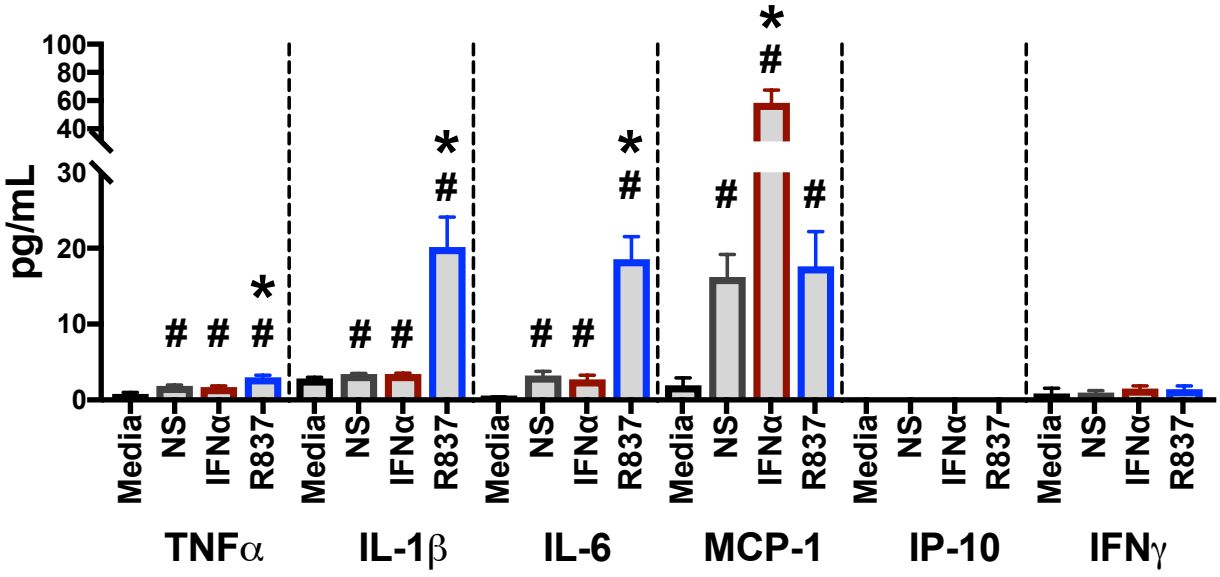

Figure 32. Monocyte production of various cytokines and chemokines when stimulated with IFNα or R837. Human monocytes (N=9) were cultured alone at $1x10^4$ cells/mL. Monocytes were either left untreated (NS), treated with IFNα (100U/mL) or R837 (10μg/mL). Monocytes were cultured for 20 h and supernatants were collected. LEGENDplex™ was used to measure levels of MCP-1, TNFα, IL-1β, IFNγ, IL-6 and IP-10. An unpaired t-test was performed to determine significant differences between media and treatment. A RM ANOVA with a Dunnett's multiple comparisons post-test was performed to determine differences between NS, IFNα and R837-treated monocytes. # denotes a statistical difference between media control. * denotes a statistical difference from the NS ($p<0.05$). Graphs are mean +/- SEM.

F. IL-1β is a critical soluble factor involved in monocyte-mediated astrocyte production of MCP-1, IL-6 and IP-10 in response to R837 treatment.

To determine whether the cytokines (MCP-1, IL-1β, IL-6 and TNFα) produced by monocytes had an impact on monocyte-mediated astrocyte production of MCP-1, IL-6 and IP-10, neutralizing antibodies targeting these factors were added to the co-culture. Briefly, the co-culture was performed as above with R837 (10μg/mL) or IFNα (100U/mL). Prior to the addition of the monocytes and stimulation, astrocytes were treated with neutralizing antibodies (1μg/mL) targeting MCP-1, IL-1β, IL-6 or TNFα or an isotype control. Astrocytes were measured for intracellular production of MCP-1, IL-6 and IP-10. In the R837-stimulated co-cultures containing U251 or primary astrocytes, anti-IL-1β treatment decreased the percent of astrocytes producing MCP-1 and IL-6 and the levels (MFI) of these factors within the positive cells compared to the isotype control (Fig. 33A-D, 34A-D, 35A-D and 36A-D). Interestingly, IL-1β neutralization of U251 cells displayed a greater suppression of MCP-1 and IL-6 compared to primary astrocytes. In the R837-stimulated co-culture, IL-1β neutralization promoted a 47% and 90% decrease in the percent of MCP-1^+ and IL-6^+ U251 astrocytes as compared to 14% (% MCP-1^+) and 55% (% IL-6^+) reduction in primary astrocytes (Fig. 33A, 33C, 34A and 34C). The reduction in levels (MFI) of each protein with IL-1β neutralization was comparable between U251 and primary astrocytes in the R837-stimulated co-culture (Fig. 33B, 33D, 34B and 34D).

Anti-IL-1β treatment of the NS- and IFNα-stimulated co-cultures containing U251 cells resulted in decreased astrocyte production of MCP-1 and IL-6 (% and MFI), while IP-10 production (% and MFI) was decreased specifically in the IFNα-stimulated co-culture (Fig. 33). A similar response was observed in IFNα-stimulated co-cultures containing primary astrocytes, in which anti-IL-1β decreased astrocyte production (% and/or MFI) of MCP-1, IL-6 and IP-10 in

at least two of the three primary astrocyte donors tested (Fig. 34-36). By contrast, anti-IL-1β treatment of non-stimulated co-cultures containing primary astrocytes from three astrocyte donors displayed minimal to no impact on MCP-1 or IL-6, which differs from the observations in the co-cultures containing U251 cells (Fig. 34-36).

TNF-α neutralization resulted in minor yet significant impairment in the percent of MCP-1^+ (NS and IFNα), IL-6^+ (IFNα), and IP-10^+ (IFNα) U251 astrocytes (Fig. 37A-C). However, in primary astrocytes (both donors), anti-TNF-α treatment of either U251 or primary astrocyte co-cultures displayed minimal to no effect on astrocyte production of MCP-1, IL-6 or IP-10 (Fig. 37D-I). Additionally, no inhibition was observed with anti-IL-6 or MCP-1 treatment in any of the three co-culture conditions containing U251 cells (Fig. 38).

To confirm monocyte production of IL-1β (Fig. 32), intracellular staining for IL-1β was performed on control (NS), IFNα and R837-stimulated monocyte monocultures. IL-1β expression was detected, albeit at lower levels, in monocytes left unstimulated (NS) compared to isotype control (Fig. 39A, B, E and F). Monocytes stimulated with IFNα did not induce IL-1β (% and MFI) compared to NS monocytes, while R837 treatment resulted in a robust increase in the percent and levels (MFI) of IL-1β within monocytes (Fig. 39).

To determine whether astrocytes were producing IL-1β, primary astrocytes from both donors were cultured alone and stimulated with R837 or IFNα and supernatant IL-1β was measured. Regardless of stimulation, minimal IL-1β secretion was observed by astrocytes (Fig. 40A). To identify whether monocytes could induce astrocyte production of IL-1β, monocytes were co-cultured with primary astrocytes, stimulated with IFNα or R837 and measured for intracellular IL-1β. In the NS and IFNα-treated co-cultures, only monocytes were positive for IL-1β (26-27% of monocytes) while <1.5% of astrocytes were IL-1β^+ (Fig. 40B).

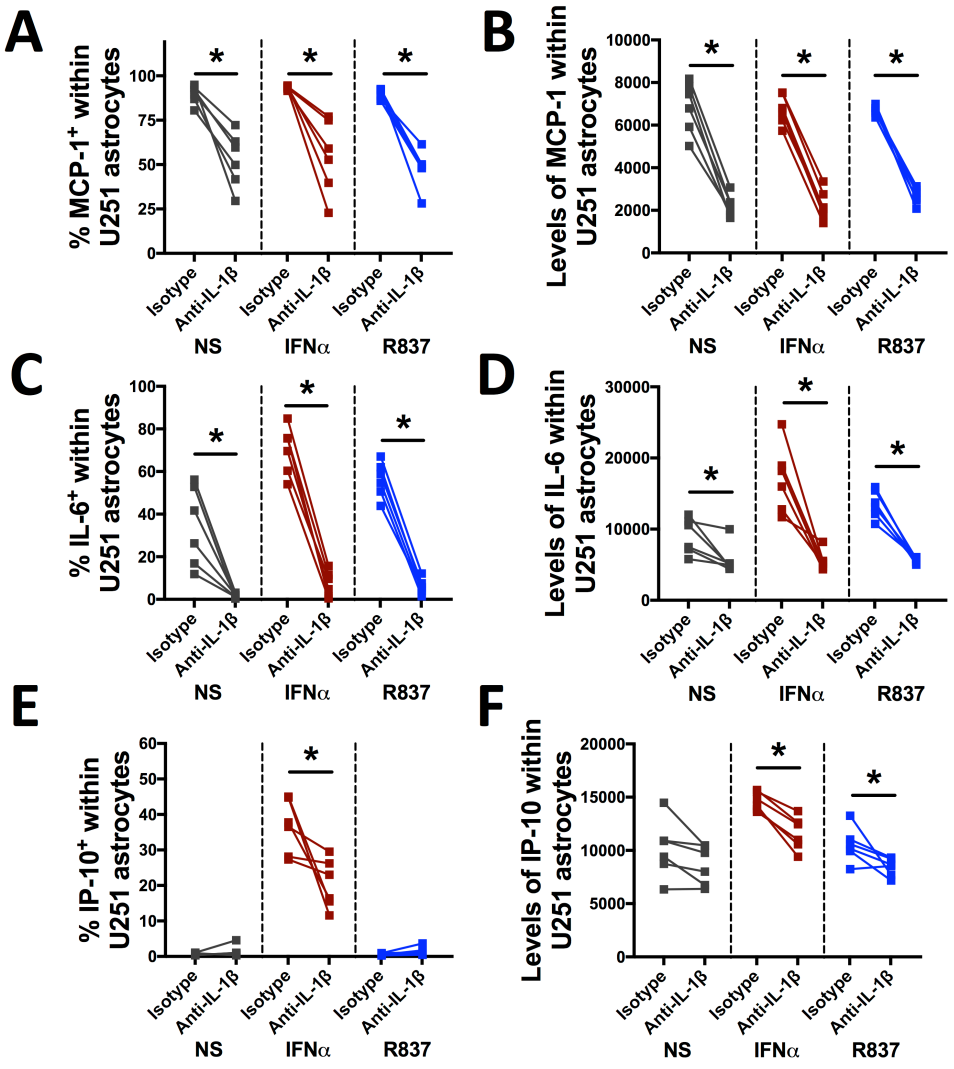

Figure 33. IL-1β is a major factor governing monocyte-mediated U251 astrocyte production of MCP-1, IL-6 and/or IP-10 in response to R837 or IFNα. Human primary monocytes (N=6) were co-cultured with U251 astrocytes at a ratio of 1:20 and stimulated with IFNα (100U/mL) or R837 (10μg/mL). Anti-IL-1β was added to the co-culture at a concentration of 1μg/mL. An isotype (1μg/mL) served as the control. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Astrocyte production of MCP-1, IL-6 and IP-10 was determined via flow cytometry. The percent positive and MFI within positive population was determined. A paired t-test was performed to determine significant differences between isotype control and anti-IL-1β group. * denotes a statistical difference from the isotype control ($p<0.05$). Graphs are mean +/- SEM.

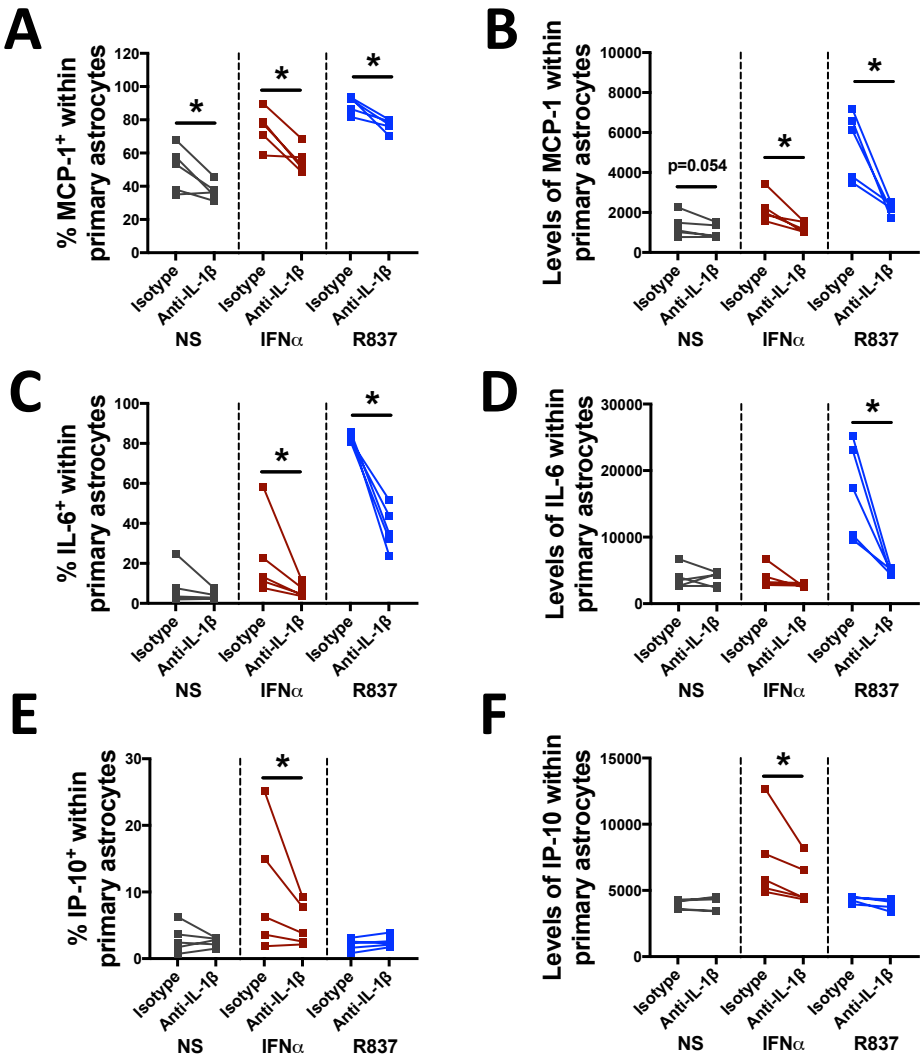

Figure 34. IL-1β is a major factor governing monocyte-mediated primary astrocyte production of MCP-1, IL-6 and/or IP-10 in response to R837 or IFNα. Human primary monocytes (N=6) were co-cultured with primary astrocytes (donor 1) at a ratio of 1:20 and stimulated with IFNα (100U/mL) or R837 (10μg/mL). Anti-IL-1β was added to the co-culture at a concentration of 1μg/mL. An isotype (1μg/mL) served as the control. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Astrocyte production of MCP-1, IL-6 and IP-10 was determined via flow cytometry. The percent positive and MFI within positive population was determined. A paired t-test was performed to determine significant differences between isotype control and anti-IL-1β group. * denotes a statistical difference from the isotype control ($p<0.05$). Graphs are mean +/- SEM.

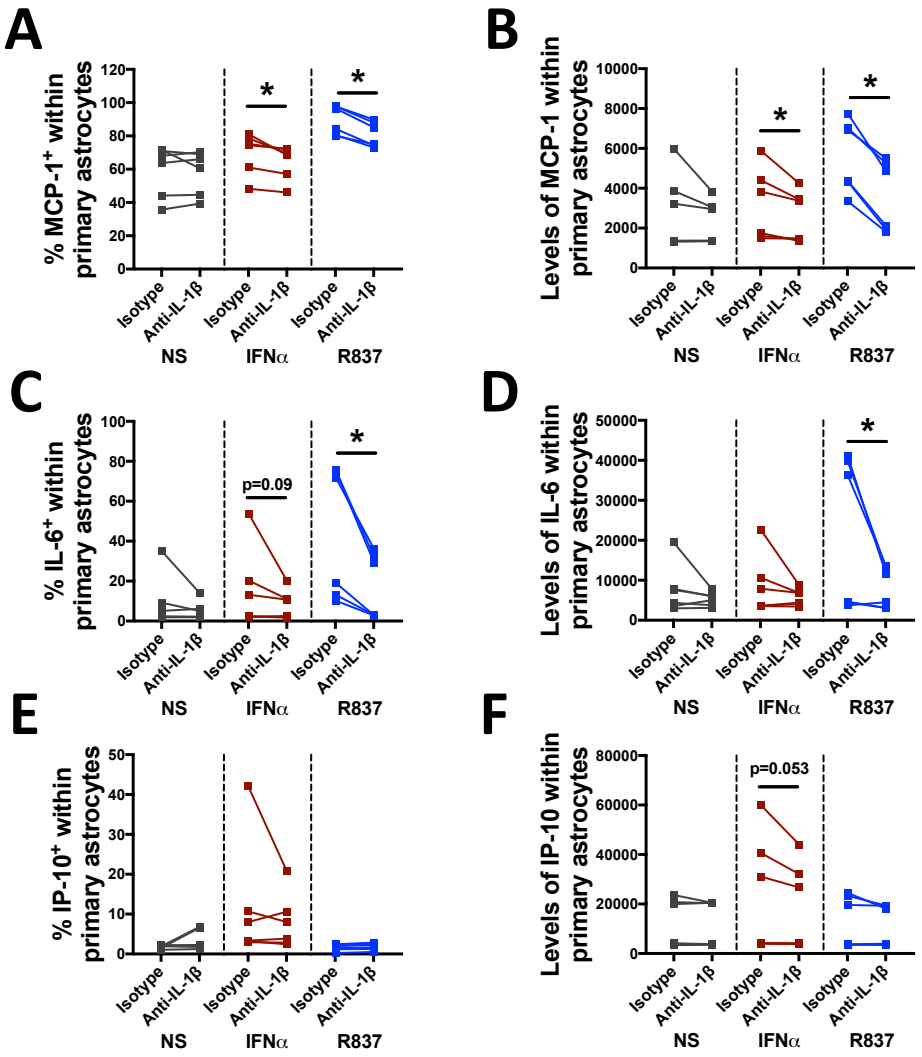

Figure 35. IL-1β is a major factor governing monocyte-mediated primary astrocyte production of MCP-1, IL-6 and/or IP-10 in response to R837 or IFNα. Human primary monocytes (N=6) were co-cultured with primary astrocytes (donor 2) at a ratio of 1:20 and stimulated with IFNα (100U/mL) or R837 (10μg/mL). Anti-IL-1β was added to the co-culture at a concentration of 1μg/mL. An isotype (1μg/mL) served as the control. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Astrocyte production of MCP-1, IL-6 and IP-10 was determined via flow cytometry. The percent positive and MFI within positive population was determined. A paired t-test was performed to determine significant differences between isotype control and anti-IL-1β group. * denotes a statistical difference from the isotype control ($p<0.05$). Graphs are mean +/- SEM.

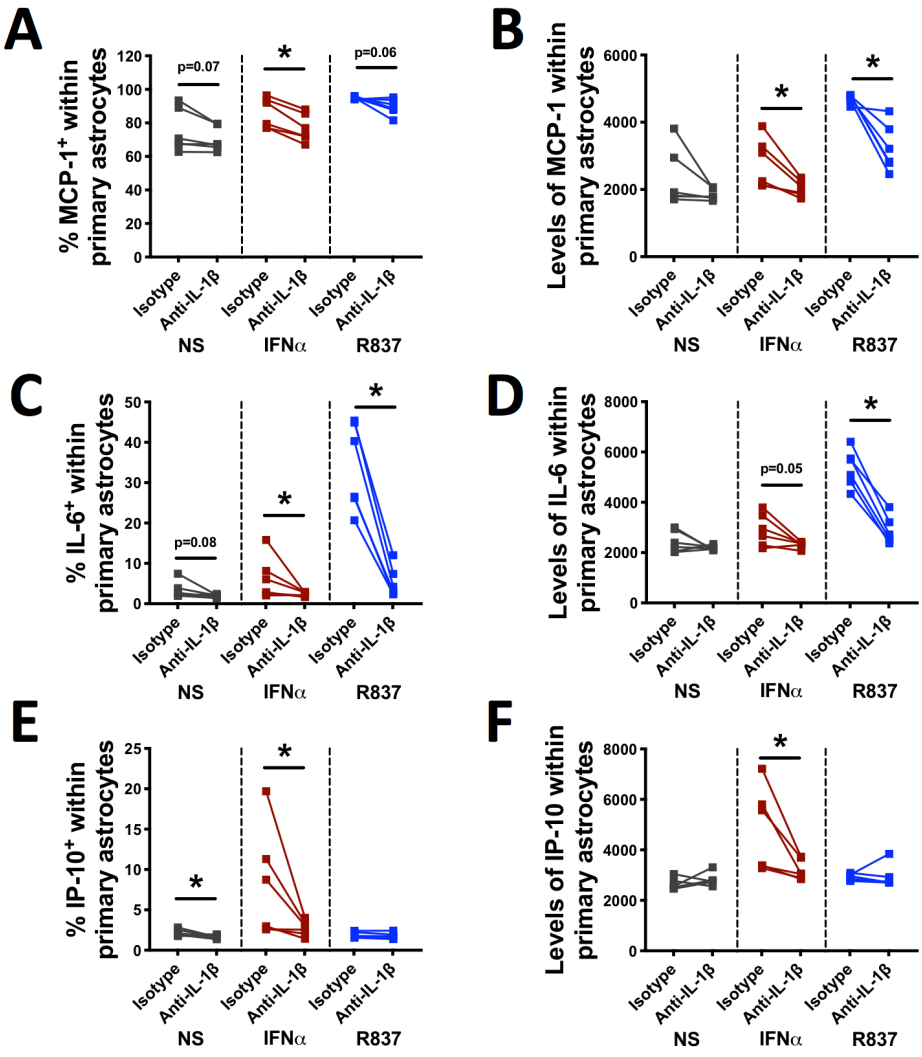

Figure 36. IL-1β is a major factor governing monocyte-mediated primary astrocyte production of MCP-1, IL-6 and/or IP-10 in response to R837 or IFNα. Human primary monocytes (N=6) were co-cultured with primary astrocytes (donor 3) at a ratio of 1:20 and stimulated with IFNα (100U/mL) or R837 (10μg/mL). Anti-IL-1β was added to the co-culture at a concentration of 1μg/mL. An isotype (1μg/mL) served as the control. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Astrocyte production of MCP-1, IL-6 and IP-10 was determined via flow cytometry. The percent positive and MFI within positive population was determined. A paired t-test was performed to determine significant differences between isotype control and anti-IL-1β group. * denotes a statistical difference from the isotype control ($p<0.05$). Graphs are mean +/- SEM.

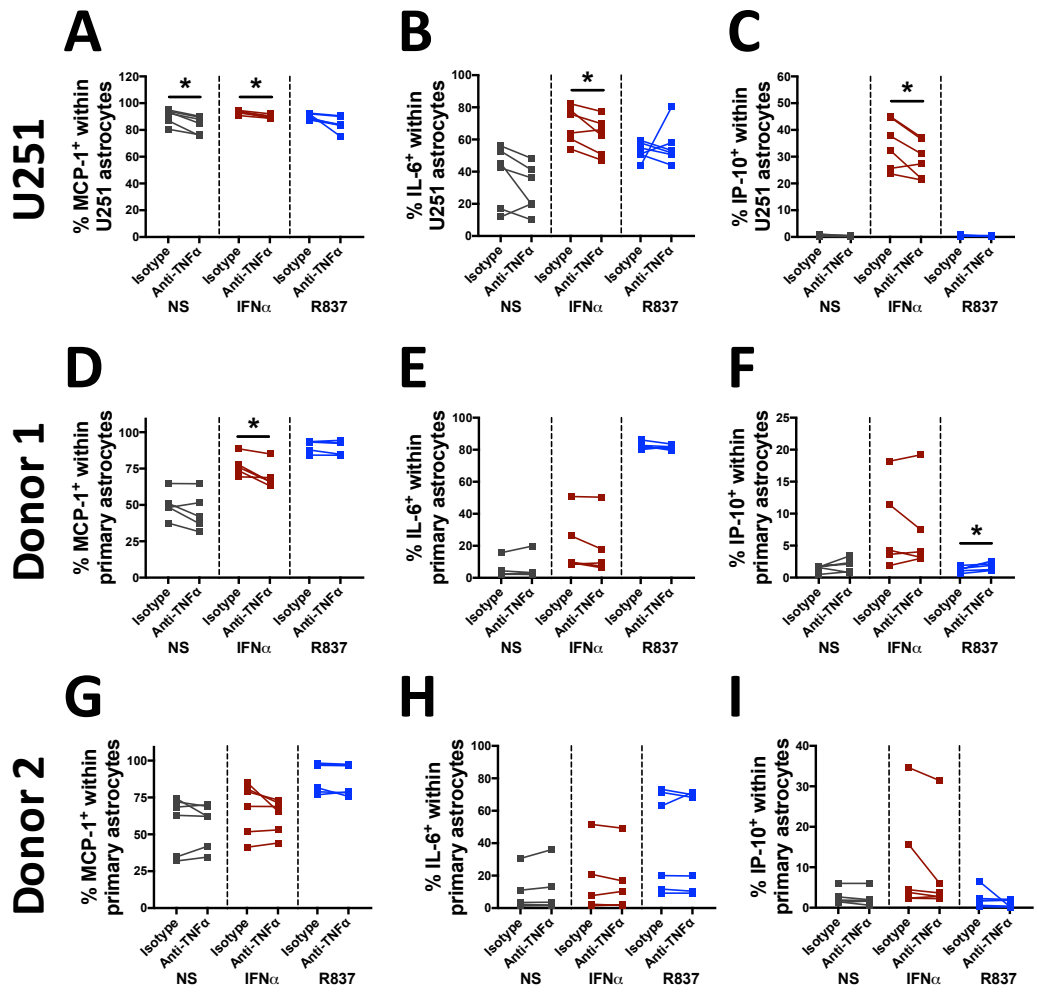

Figure 37. TNFα neutralization of the co-culture has minimal effect on astrocyte production of MCP-1, IL-6 and/or IP-10. Human primary monocytes (N=6 for A-C and N=5 for D-F) were co-cultured with astrocytes (U251 or primary) at a ratio of 1:20 and stimulated with IFNα (100U/mL) or R837 (10µg/mL). Anti-TNFα was added to the co-culture at a concentration of 1µg/mL prior to stimulation. An isotype (1µg/mL) served as the control. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1, IL-6 and IP-10 (% positive). A paired t-test was performed to determine significant differences between isotype control and anti-TNFα group. A log transformation was performed for D-I prior to the paired t-test. * denotes a statistical difference from the isotype control ($p<0.05$). Graphs are mean +/- SEM.

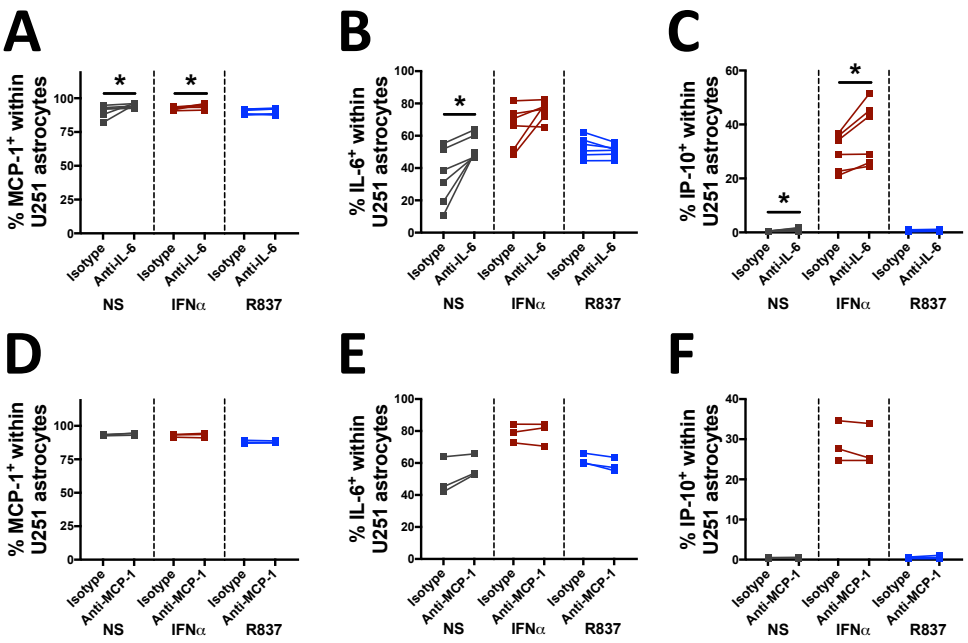

Figure 38. IL-6 and MCP-1 neutralization of the co-culture has minimal effect on astrocyte production of MCP-1, IL-6 and/or IP-10. Human primary monocytes (N=6 for A-C and N=3 for D-F) were co-cultured with U251 astrocytes at a ratio of 1:20 and stimulated with IFNα (100U/mL) or R837 (10µg/mL). Anti-IL-6 or anti-MCP-1 were added to the co-culture at a concentration of 1µg/mL prior to stimulation. An isotype (1µg/mL) served as the control. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1, IL-6 and IP-10 (% positive). A paired t-test was performed to determine significant differences between isotype control and anti-cytokine group. * denotes a statistical difference from the isotype control ($p<0.05$). Graphs are mean +/- SEM.

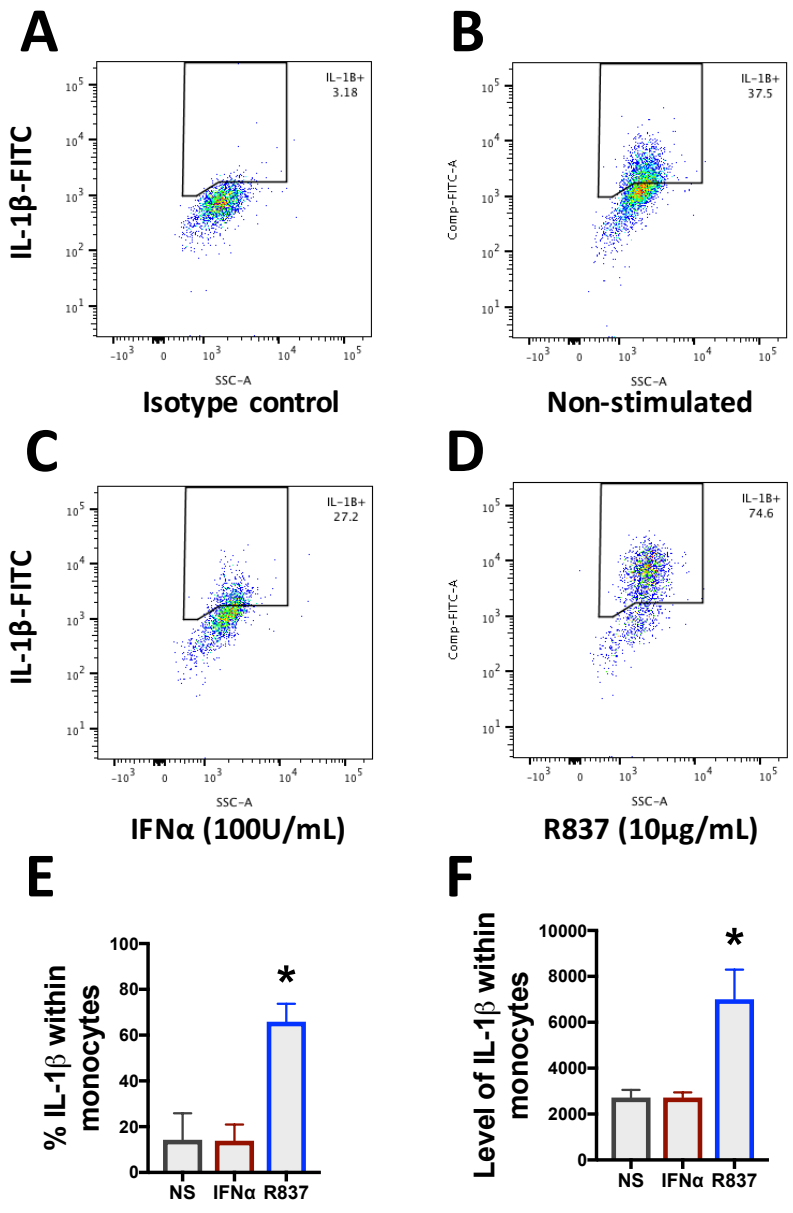

Figure 39. R837 but not IFNα, induces intracellular expression of IL-1β in monocytes. (A-F) Primary monocytes (N=3) were left untreated (NS) or stimulated with IFNα (100U/mL) and R837 (10µg/mL) for 20 h and a Golgi block was added for 4 h. Intracellular staining and flow cytometry were used to measure IL-1β (% positive and MFI within positive population). (A-D) Flow cytometry plots from one representative donor demonstrating monocyte production of IL-1β when left without stimulation (NS) or stimulated with IFNα or R837. A RM ANOVA with a Dunnett's multiple comparisons post-test was performed for F and I. * denotes a statistical difference from NS. Graphs are mean +/- SEM.

For the R837-treated co-culture, both populations had detectable IL-1β, with roughly 70% of the monocytes and 10% of the astrocytes expressing IL-1β (Fig. 40B).

G. Replacement of monocytes with recombinant IL-1β in the control, R837 and IFNα cultures, promotes a similar profile of MCP-1, IL-6 and IP-10 production by astrocytes.

To determine if IL-1β alone (at concentrations comparable to that observed in Fig. 32) could promote astrocyte production of MCP-1, IL-6 and IP-10, U251 and primary astrocytes were treated with recombinant IL-1β at various concentrations (0.5, 1, 5, 10, 20, 50 and 100pg/mL) and intracellular MCP-1, IL-6 and IP-10 production was measured. Recombinant IL-1β induced astrocyte production of MCP-1 and IL-6 in a concentration-dependent manner (Fig. 41A-B and 41D-E). A significant increase in the percent of MCP-1$^+$ astrocytes was detected as low as 0.5 and 1pg/mL of IL-1β for U251 and primary astrocytes, respectively, when compared to 0pg/mL (Fig. 41A and D). A detectable increase in IL-6 production was observed starting at 5pg/mL of IL-1β for both U251 and primary astrocytes (Fig. 41B and E). IL-1β had no effect on IP-10 production for both U251 and primary astrocytes (Fig. 41C and F). Similar trends were observed with the second primary astrocyte donor (Fig. 42A-C). One notable difference between astrocyte donors was the percent of astrocytes producing IL-6 in response to IL-1β. For instance, the percent of astrocytes producing IL-6 in response to 100pg/mL of IL-1β in astrocyte donor 1 was 56% (Fig. 41E) versus 28% in the second donor (Fig. 42B).

To determine whether IL-1β treatment could mimic the actions of monocytes in the IFNα- and R837-stimulated co-cultures, astrocyte monocultures were treated with recombinant IL-1β in combination with R837 or IFNα. The concentration of IL-1β added (20pg/mL) to culture was selected based on its comparable profile to monocytes (1:20 ratio) at promoting astrocyte production of MCP-1 and IL-6. As noted in Figure 22C, direct stimulation of primary

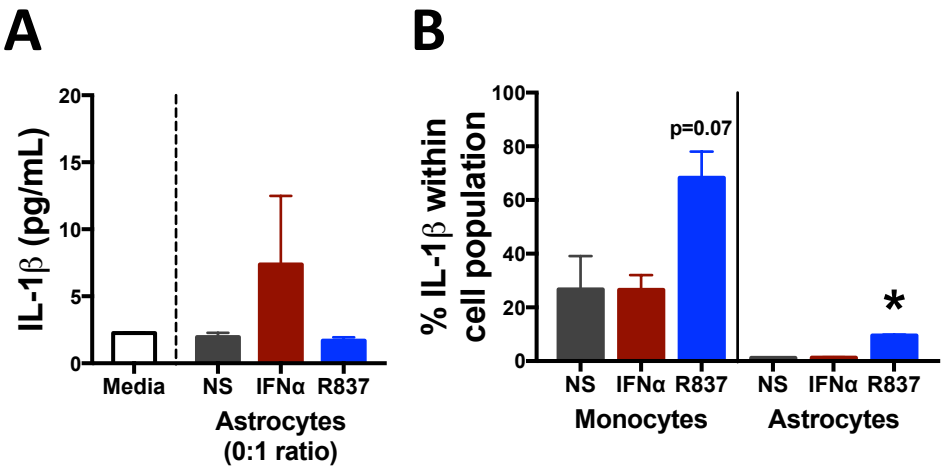

Figure 40. Primary astrocytes produce minimal to no IL-1β when cultured alone or with monocytes. (A) Primary astrocyte monocultures were left untreated (NS), treated with IFNα (100U/mL) or R837 (10µg/mL). Cells were incubated for 20 h and supernatants were collected. LEGENDplex™ was used to measure supernatant IL-1β. (B) Human primary monocytes (N=3) were co-cultured with primary astrocytes (donor 2) at a ratio of 1:5 (monocyte:astrocyte) and stimulated with IFNα (100U/mL) or R837 (10µg/mL). Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine monocyte and astrocyte production of IL-1β (% positive). Monocytes were identified based on CD45 expression. For B, dataset was log transformed and a RM ANOVA with a Dunnett's multiple comparisons post-test was performed. * denotes a statistical difference from NS. Graphs are mean +/- SEM.

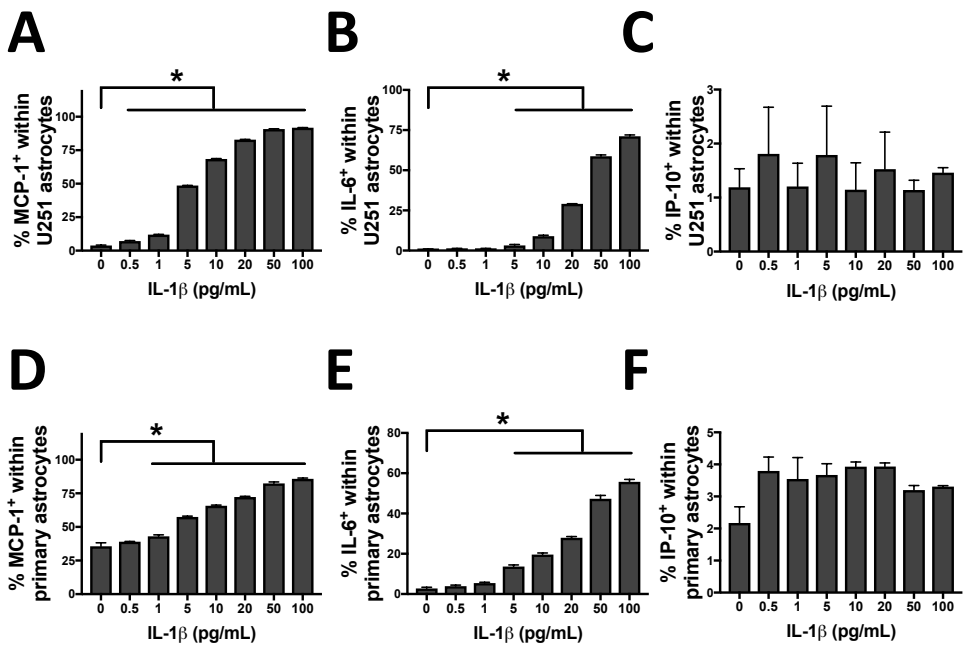

Figure 41. Recombinant IL-1β induces a similar astrocyte response compared to monocytes. (A-F) Astrocytes (U251 – panels A-C or primary donor 1 – panels D-F) were treated with varying concentrations of recombinant IL-1β (0, 0.5, 1, 5, 10, 20, 50 and 100pg/mL). Cells were incubated for 20 h and a Golgi block was added for 4 h. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1, IL-6 and IP-10 (% positive). A RM ANOVA with a Dunnett's multiple comparisons post-test was performed for A-F. * denotes a statistical difference from 0pg/mL. Graphs are mean +/- SEM.

astrocytes with R837 promotes a minor, but significant increase in the percent of astrocytes expressing MCP-1 compared to control (NS) astrocytes (Fig. 42D, 42G and 43D), demonstrating direct activity of R837 on the primary astrocytes. Treatment of U251 cells in combination with both IL-1β and R837 decreased the percent of MCP-1^+ astrocytes compared to IL-1β alone, while having no effect on IL-6 or IP-10 production (Fig. 43A-C). By contrast, IL-1β+R837 treatment of primary astrocytes increased the percent of IL-6^+ astrocytes compared to IL-1β alone, which was observed in 2 of 3 astrocyte donors examined (Fig. 42E, 42H and 43E). IL-1β+R837 treatment of primary astrocytes had minimal to no effect on MCP-1 and IP-10 production compared to IL-1β alone (Fig. 42D, 42F, 42G, 42I, 43D and 43F).

IL-1β+IFNα treatment enhanced IL-6 production (%) by astrocytes (U251 and primary) compared to IL-1β alone, and IP-10 production was induced (Fig. 42E, 42H, 43B and 43E), showing similarity to the IFNα-treated co-cultures. Minor effects were observed with IFNα on the percent of MCP-1^+ astrocytes (U251 and primary astrocytes) compared to IL-1β alone (Fig. 42D, 42F, 42G, 42I, 43A, 43C, 43D and 43F).

H. The intermediate monocyte population positively associates with U251 astrocyte production of IL-6.

As there are three subsets of human monocytes (classical, intermediate and non-classical) (4), we determined whether there was an association between specific monocyte subsets and astrocyte production (U251) of IL-6, IP-10 or MCP-1. Figure 44A demonstrates the flow cytometry gating strategy used to separate each monocyte subset. The percent of each subset was determined within the monocyte population. Then the overall percent within the whole monocyte preparation was determined, which took into account the overall purity of the sample.

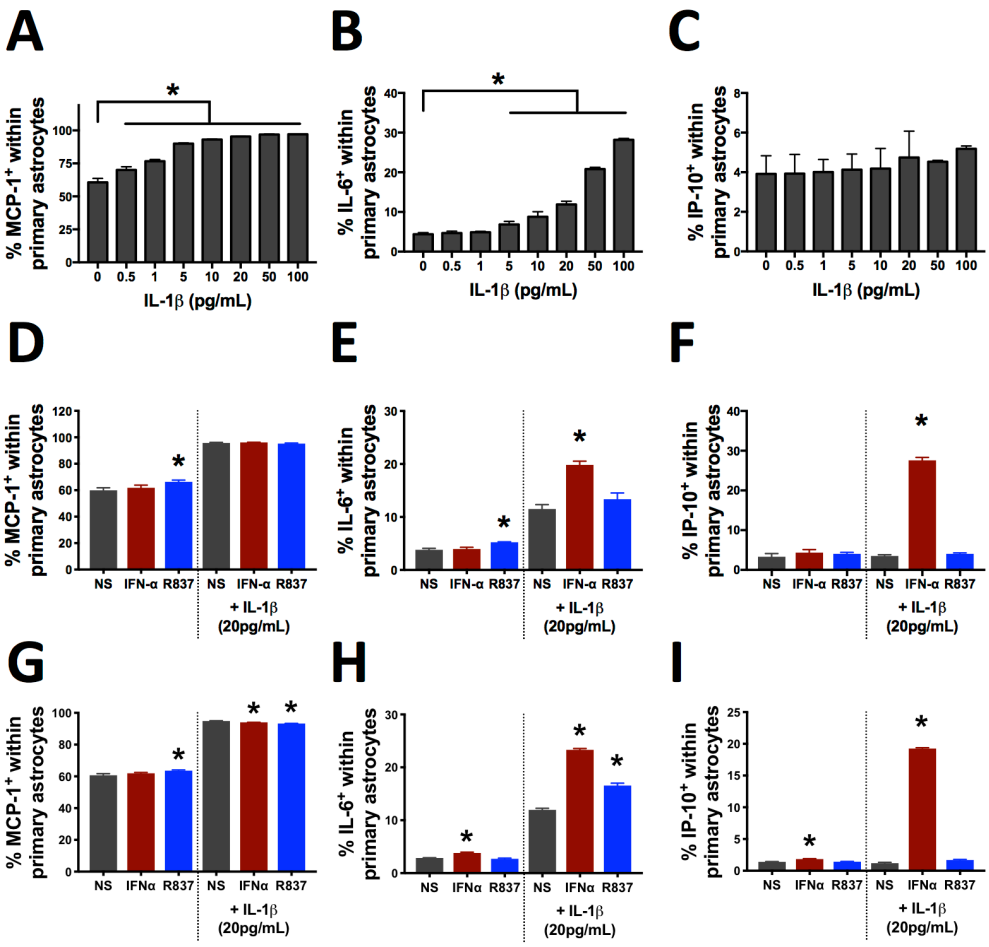

Figure 42. Recombinant IL-1β alone, and with IFNα or R837, induces a similar astrocyte response compared to monocytes. (A-C) Primary astrocytes (donor 2) were treated with varying concentrations of recombinant IL-1β (0, 0.5, 1, 5, 10, 20, 50 and 100pg/mL). (D-F) Primary astrocytes (donor 2 for D-F and donor 3 for G-I) were left untreated (NS), treated with IFNα (100U/mL) or R837 (10µg/mL) alone or in combination with IL-1β (20pg/mL). Cells were incubated for 20 h and a Golgi block was added for 4 h. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1, IL-6 and IP-10 (% positive). Graphs displayed above are from one of the independent experiments. A RM ANOVA with a Dunnett's multiple comparisons post-test was performed for A-F. * denotes a statistical difference from 0pg/mL for A-C and NS + IL-1β for D-I. Graphs are mean +/- SEM.

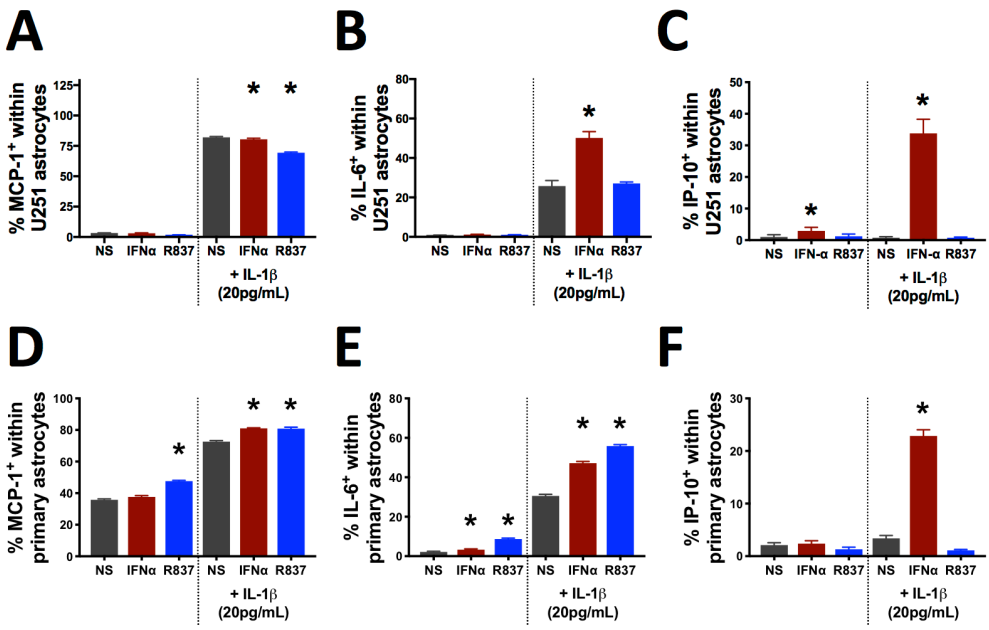

Figure 43. Recombinant IL-1β in combination with IFNα or R837, induces a similar astrocyte response compared to monocytes. (A-F) Astrocytes (U251 or primary donor 1) were left untreated (NS), treated with IFNα (100U/mL) or R837 (10μg/mL) alone or in combination with IL-1β (20pg/mL). Cells were incubated for 20 h and a Golgi block was added for 4 h. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1, IL-6 and IP-10 (% positive). A RM ANOVA with a Dunnett's multiple comparisons post-test was performed for A-F. * denotes a statistical difference from NS + IL-1β. Graphs are mean +/- SEM.

Interestingly, the percent of intermediate monocytes exhibited a significant positive correlation with the percentage of IL-6^{+} astrocytes in the NS ($r=0.54$, $p=0.032$) and IFNα-treated ($r=0.61$, $p=0.013$) co-cultures, while a trending correlation ($r=0.44$, $p=0.09$) was observed with R837 treatment (Fig. 44B-D).

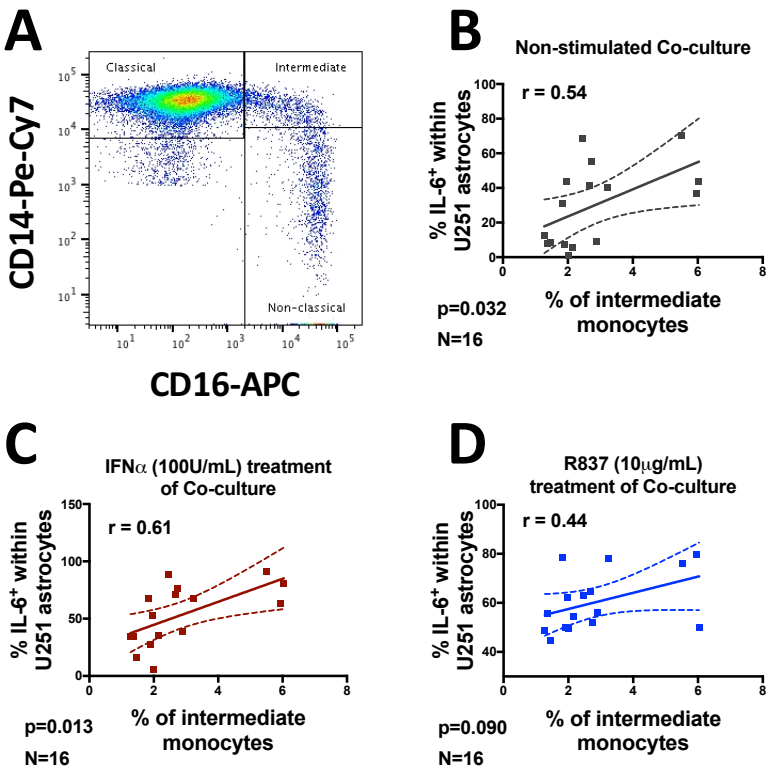

Figure 44. The intermediate monocyte population positively associates with U251 astrocyte production of IL-6. Human primary monocytes (N=16) were co-cultured with U251 astrocytes at a ratio of 1:20 and stimulated with IFNα (100U/mL) or R837 (10μg/mL). Cells were co-cultured for 24 h, with a Golgi blocker added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1, IL-6 and IP-10 (% positive). Extracellular staining of CD14 and CD16 was performed on an aliquot of monocytes prior to co-culture. The percent of each monocyte population was determined based on the gating strategy in panel A. A correlation analysis (Prism 7, GraphPad) was performed between monocyte subsets and U251 astrocyte production of MCP-1, IL-6 or IP-10.

III. Specific aim 3: Determine the effect of Δ^9-THC and CBD on monocyte-mediated astrocyte production of MCP-1 and IL-6 when stimulated with the TLR7 agonist, R837.

A. THC treatment of the TLR7-stimulated co-culture resulted in decreased astrocyte production of MCP-1 and IL-6, while CBD increased IL-6 and had no effect on MCP-1.

To determine the effect of THC and CBD on monocyte-meditated astrocyte secretion of MCP-1 and IL-6 in response to R837, monocytes and astrocytes were co-cultured at a 1:20 ratio, treated with THC (1, 5 and 10μM) or CBD (1, 5 and 10μM) and stimulated with R837 (10μg/mL) for 20 hours. A Golgi block was added for 4 hours and astrocytes were measured for intracellular MCP-1 and IL-6 via flow cytometry. Prior to the addition of the Golgi block, an aliquot of supernatant was collected and an ELISA was performed to quantify supernatant levels of MCP-1 and IL-6. THC treatment at the highest concentration examined (10μM) slightly decreased the percent of astrocytes expressing MCP-1 while CBD treatment displayed no significant effects. (Fig. 45A). However, when the levels (MFI) of MCP-1 within MCP-1^+ astrocytes were evaluated, THC was shown to decrease MCP-1 levels in a concentration-dependent manner, with significance reached at both 5 and 10μM (Fig. 45B). Furthermore, THC decreased supernatant MCP-1 levels in a similar manner as the MCP-1 levels (MFI) (Fig. 45C). CBD treatment of the co-culture displayed no significant effects on the levels (MFI) of MCP-1 within MCP-1^+ astrocytes, while promoting a slight decrease in supernatant MCP-1 in a non-concentration-dependent manner at 1 and 10μM (Fig. 45B and 45C).

THC was identified to decrease the percent of IL-6^+ astrocytes and IL-6 levels (MFI) in a concentration-dependent manner, with significant suppression in the percent of IL-6^+ astrocytes observed at 5 and 10μM, and levels (MFI) at 10μM (Fig. 46A and 46B). Furthermore, the

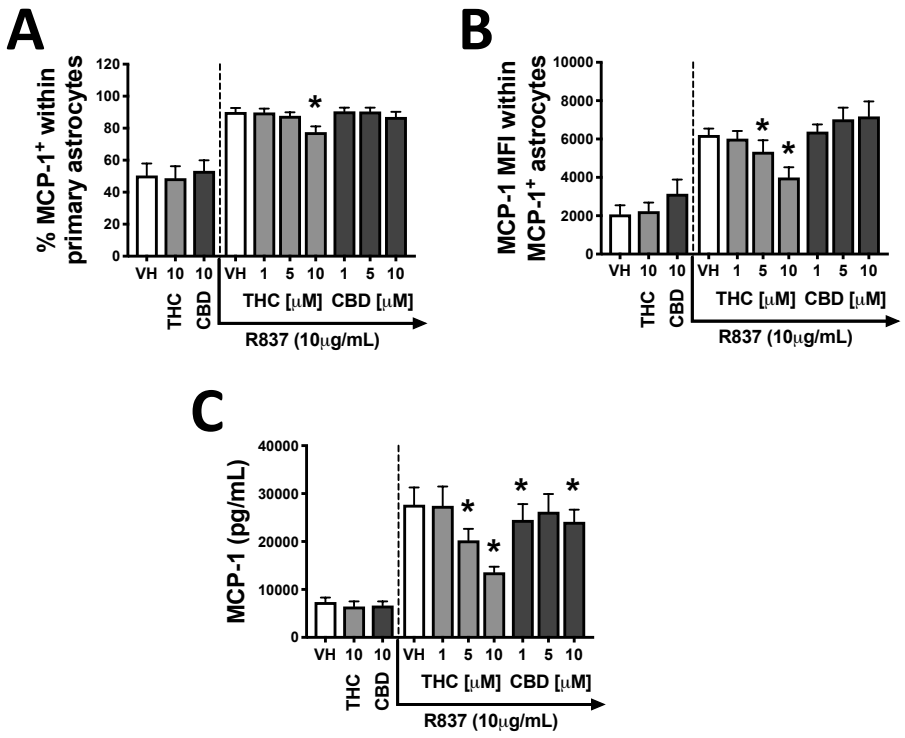

Figure 45. THC treatment of the TLR7-stimulated co-culture resulted in decreased astrocyte production of MCP-1, while CBD had a minimal effect on MCP-1. Human primary monocytes (HIV-negative) (N=9) were co-cultured with primary astrocytes at a ratio of 1:20 (monocyte:astrocyte), treated with vehicle (0.03% EtOH), THC or CBD (1, 5 and 10μM) and stimulated with R837 (10μg/mL). Monocyte and astrocyte cell concentrations were $1x10^4$ and $2x10^5$ cells/mL, respectively. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. An aliquot of supernatant was collected prior to the addition of the Golgi block. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1 (% positive and MFI within positive population). ELISAmax™ technology from Biolegend was used to determine supernatant levels of MCP-1. A repeated measures (RM) ANOVA with a Dunnett's post-hoc test was performed. *Statistically different from vehicle control (R837+vehicle) ($p<0.05$). All graphs are mean +/- SEM.

amount of secreted IL-6 in the supernatants in response to THC treatment was similar to the intracellular IL-6 measurements, with 5 and 10μM THC promoting a significant decrease (Fig. 46C). However, CBD treatment of the R837-stimulated co-culture resulted in an augmentation in IL-6 production by astrocytes. Each IL-6 measurement (percent, MFI and supernatant) displayed a concentration-dependent increase with CBD treatment (Fig. 46A-C). Significance was detected at 10μM CBD for the percent of IL-6$^+$ astrocytes (Fig. 46A), and 5 and 10μM for levels (MFI) within IL-6$^+$ astrocytes as well as supernatant IL-6 (Fig. 46B-C). THC and CBD treatment had no observable effects on astrocyte viability (data not shown).

B. THC and CBD treatment of the TLR7+IL-1β-stimulated astrocyte monoculture promoted a decrease in astrocytes expressing MCP-1 and IL-6.

To determine the direct effect of THC and CBD on astrocyte production of MCP-1 and IL-6, astrocyte monocultures from two separate donors were treated with varying concentrations of THC or CBD and stimulated with IL-1β+R837 for 20 hours. A Golgi block was added for 4 hours and astrocytes were measured for intracellular MCP-1 and IL-6 via flow cytometry. For both astrocyte donors, THC treatment decreased the percent of astrocytes expressing MCP-1 and IL-6 (Figs. 47A, 47C, 48A and 48C). In addition, THC had no effect on the levels of MCP-1 (MFI) in both donors but decreased astrocyte levels of IL-6 (Fig. 47B, 47D, 48B and 48D). CBD was shown to decrease the percent of astrocytes expressing MCP-1 while displaying a mixed effect on MCP-1 levels (MFI), as one donor displayed a slight increase and the other a decrease (Fig. 47A-D). This is in contrast to the co-culture observation in which CBD had minimal impact on MCP-1. Furthermore, CBD treatment had a mixed effect on the percent of IL-6$^+$ astrocytes, with one donor displaying a concentration-dependent suppression (donor 1) and

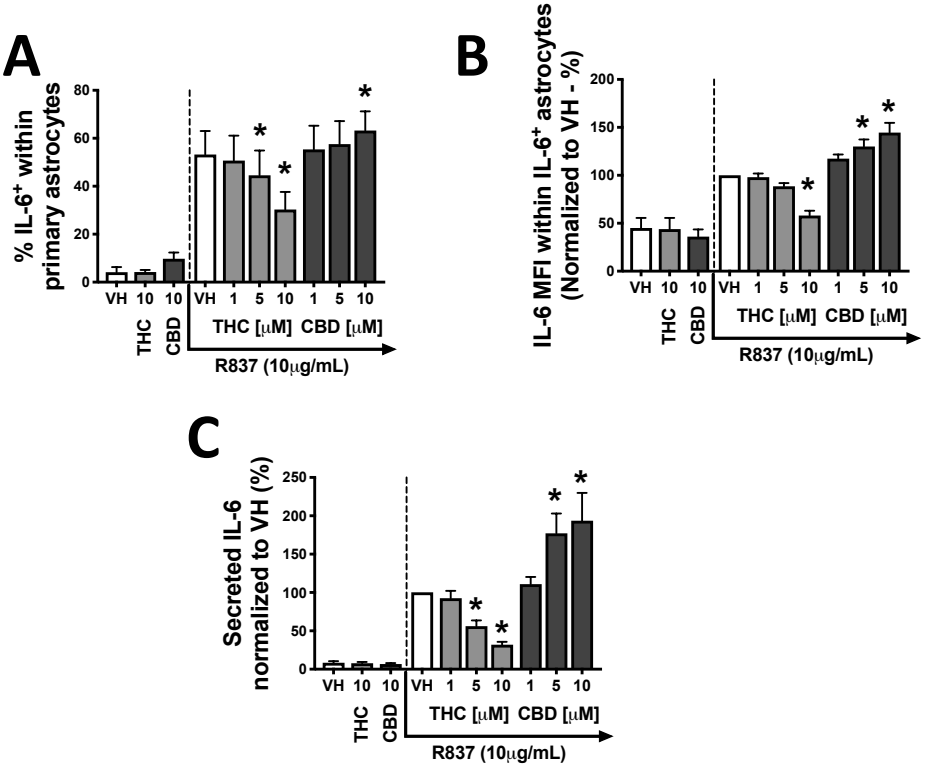

Figure 46. THC treatment of the TLR7-stimulated co-culture resulted in decreased astrocyte production of IL-6, while CBD increased IL-6. Human primary monocytes (HIV-negative) (N=9) were co-cultured with primary astrocytes at a ratio of 1:20 (monocyte:astrocyte), treated with vehicle (0.03% EtOH), THC or CBD (1, 5 and 10μM) and stimulated with R837 (10μg/mL). Monocyte and astrocyte cell concentrations were $1x10^4$ and $2x10^5$ cells/mL, respectively. Cells were co-cultured for 24 h, with a Golgi block added 4 h before cell harvest. An aliquot of supernatant was collected prior to the addition of the Golgi block. Intracellular staining and flow cytometry were performed to determine astrocyte production of IL-6 (% positive and MFI within positive population). ELISAmax™ technology from Biolegend was used to determine supernatant levels of IL-6. The supernatant level of IL-6 of the vehicle + R837 in panel C was 59.7ng/mL. A repeated measures (RM) ANOVA with a Dunnett's post-hoc test was performed. *Statistically different from vehicle control (R837+vehicle) ($p<0.05$). All graphs are mean +/- SEM.

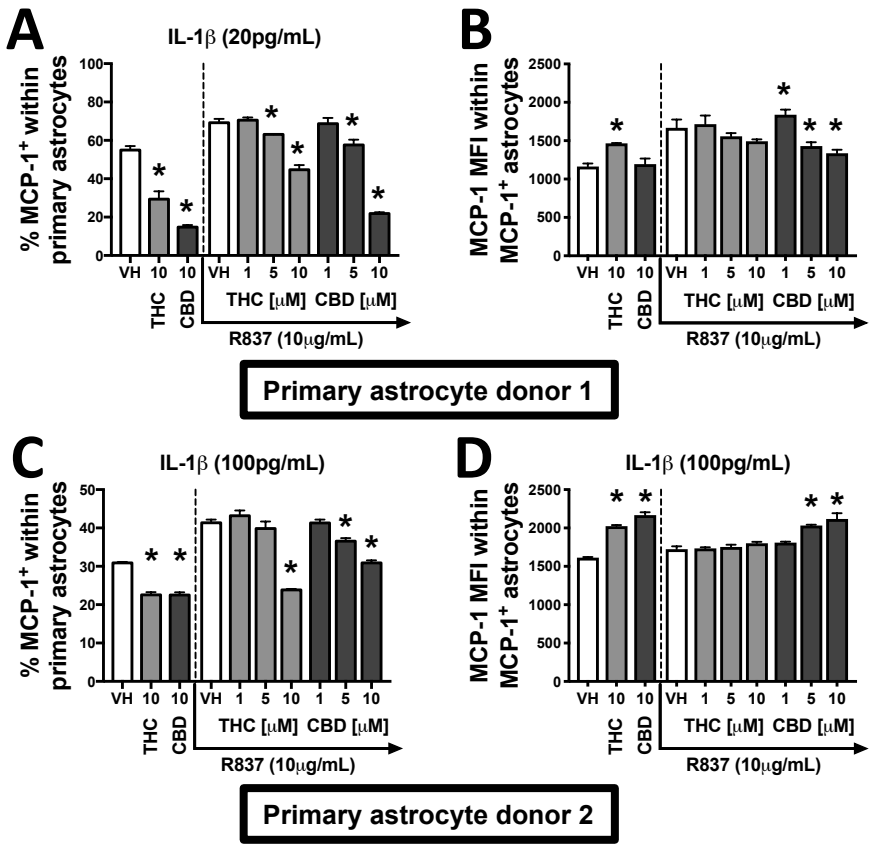

Figure 47. THC and CBD treatment of the TLR7+IL-1β-stimulated astrocyte monoculture promoted a decrease in astrocytes expressing MCP-1. Primary astrocytes were treated with vehicle (0.03% EtOH), THC or CBD (1, 5 and 10μM) and stimulated with both IL-1β (20 or 100pg/mL) and R837 (10μg/mL). Cells were cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine astrocyte production of MCP-1 (% positive and MFI within positive population). For each astrocyte donor, experimental wells were in triplicate. A RM ANOVA with a Dunnett's post-hoc test was performed. *Statistically different from the respective vehicle control ($p<0.05$). All graphs are mean +/- SEM.

the second donor showing no concentration-dependent effects (Figs. 48A and 48C). The same trend was observed with the levels (MFI) of IL-6 within IL-6^+ astrocytes (Fig. 48B and 48D). This observation differs in comparison to the effects observed with CBD in the co-cultures, as an augmentation in the astrocyte response was observed with IL-6. Overall, THC and CBD tended to have a direct inhibitory effect on astrocyte production of MCP-1 and IL-6 when stimulated with recombinant IL-1β and R837, which, in some instances (e.g., CBD treatment), differs from the observations in the co-culture. As in the co-culture, THC and CBD treatment had no observable effect on astrocyte viability (data not shown).

C. THC treatment of co-culture and monocyte monoculture stimulated with R837 resulted in decreased monocyte production of IL-1β, while CBD augmented IL-1β.

As monocyte-derived IL-1β is a critical factor governing astrocyte secretion of MCP-1 and IL-6 in the R837-stimulated co-culture, the impact of THC and CBD on TLR7-mediated monocyte secretion of IL-1β was examined. First, we determined the effect of THC and CBD on IL-1β levels in the R837-stimulated co-culture supernatants (same co-cultures as displayed in Figure 45-46). Figure 49A demonstrates a significant increase in the amount of supernatant IL-1β in the R837-stimulated co-culture compared to the non-stimulated (NS) co-culture. Furthermore, THC treatment led to decreased supernatant IL-1β in concentration-dependent manner with significance detected at the lowest concentration measured (1μM) (Fig. 49B). Conversely, CBD increased IL-1β levels in the R837-stimulated co-culture at 5 and 10μM (Fig. 49B), which was similar to that of supernatant IL-6 (Fig. 46).

To determine if THC and CBD had a direct effect on monocyte production of IL-1β, monocyte monocultures were treated with THC or CBD and stimulated with R837 for 20 hours. As with the co-culture, R837 stimulation of monocytes promoted a significant increase in IL-1β

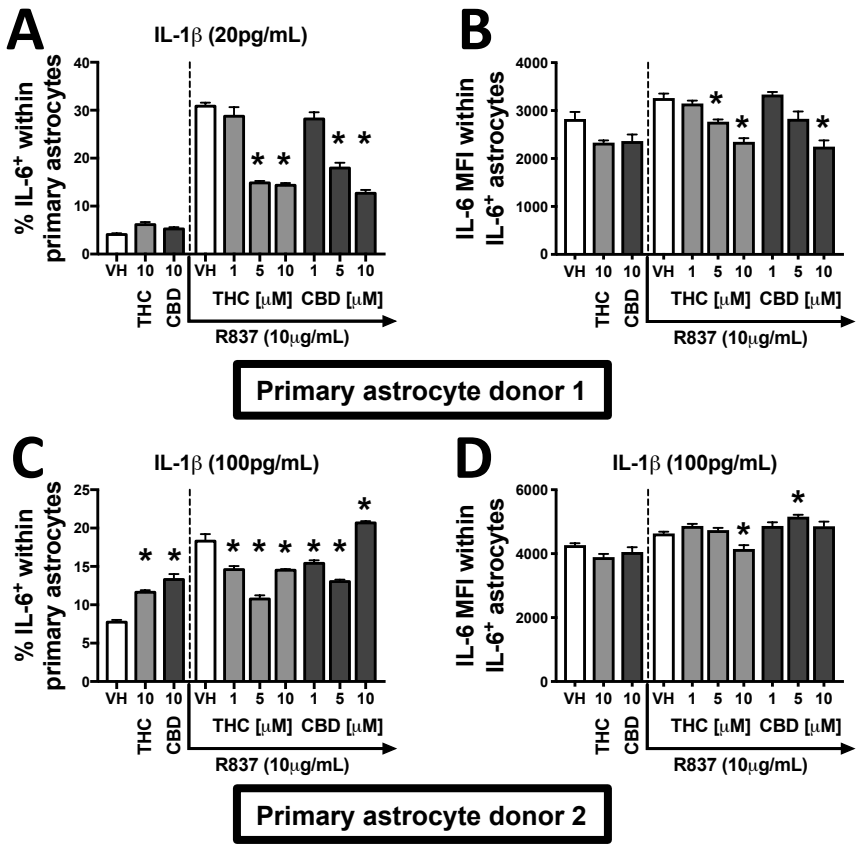

Figure 48. THC and CBD treatment of the TLR7+IL-1β-stimulated astrocyte monoculture promoted a decrease in astrocytes expressing IL-6. Primary astrocytes were treated with vehicle (0.03% EtOH), THC or CBD (1, 5 and 10μM) and stimulated with both IL-1β (20 or 100pg/mL) and R837 (10μg/mL). Cells were cultured for 24 h, with a Golgi block added 4 h before cell harvest. Intracellular staining and flow cytometry were performed to determine astrocyte production of IL-6 (% positive and MFI within positive population). For each astrocyte donor, experimental wells were in triplicate. A RM ANOVA with a Dunnett's post-hoc test was performed. A RM ANOVA with a Dunnett's post-hoc test was performed. *Statistically different from the respective vehicle control ($p<0.05$). All graphs are mean +/- SEM.

levels in monocyte monocultures (Fig. 49C). The differences in IL-1β levels between the co-culture and monocyte monoculture was due to the increased cell concentration of monocytes in the monocultures ($4x10^5$ vs $1x10^4$ cells/mL). THC and CBD were shown to have similar effects as in the co-culture, with a concentration-dependent decrease in secreted IL-1β with THC and an increase with 10μM CBD (Fig. 49D). However, there was no significant activity with 5μM CBD, which is in contrast with the effects on IL-1β in the co-culture (Fig. 49D). In addition, the percent increase of secreted IL-1β over the vehicle control with 10μM CBD was higher in the co-culture (516%) (Fig. 49B) as compared to monocyte monocultures (196%) (Fig. 49D). As 1μM THC decreased IL-1β by 30% compared to the vehicle control (Fig. 49D), we determined whether lower concentrations of THC (0.1 and 0.5μM) could suppress monocyte-derived IL-1β in response to R837. As shown in Figure 49E, THC as low as 0.5μM promoted a significant decrease in IL-1β levels. To determine if THC was selectively suppressing IL-1β or promoting a generalized suppression of monocyte secretion of cytokines, the supernatants from Figure 49E were quantified for IL-6. THC treatment had no effect on monocyte secretion of IL-6 in response to R837 (Fig. 49F), suggesting that THC is specifically suppressing IL-1β levels.

D. THC and CBD at 10μM promoted an apoptotic phenotype in response to TLR7 activation.

To determine whether the THC or CBD treatment was inducing apoptosis and/or cell death, monocyte monocultures were treated with THC or CBD and stimulated with R837 for 20 h. Furthermore, additional cannabinoid compounds were tested including JWH-015 (CB2 agonist), THC-COOH (non-active towards CB1 or CB2) and cannabinol (CBN). Cells were harvested and stained for Annexin-V (marker of apoptosis) and 7-Aminoactinomycin D (7-AAD) (marker of cell death). The gating strategy in Figure 50A-C was used to identify cells that:

(a) viable (7-AAD$^-$Annexin-V$^-$); (b) viable but apoptotic (7-AAD$^-$Annexin-V$^+$); (c) dead and apoptotic (7-AAD$^+$Annexin-V$^+$); and (d) dead but not apoptotic (7-AAD$^+$Annexin-V$^-$). THC and CBD at the highest concentration used (10μM) was shown to increase the percent of dead/apoptotic monocytes and viable/apoptotic monocytes (Fig. 50A-E). However, THC and CBD at concentrations ranging from 0.1-5μM and 1-5μM, respectively, had no significant effects on the percent of dead/apoptotic monocytes and viable/apoptotic monocytes (Figure 50D-E). Furthermore, JWH-015 and THC-COOH displayed minimal to no effect on viability or apoptosis (Figure 50D-E), suggesting that THC and CBD at 10μM are promoting apoptosis through a specific mechanism that is unrelated to the CB2-receptor but not due to non-specific lipophilic action. Interestingly, cannabinol (CBN), which is similar in structure to THC and CBD but displays lower binding activity for CB1/2 compared to THC (5-20 fold) (228), demonstrated a similar apoptotic/viability profile as THC and CBD (Figs. 50D-E). Specifically, CBN at 10μM promoted a significant increase in the percent of dead/apoptotic cells and a non-significant ($p=0.08$) increase in live/apoptotic monocytes (Fig. 50D-E). An additional experiment was performed with THC and CBD using concentrations of 1, 5 and 10μM and the same observations on cell viability and apoptosis were observed with the 10μM concentration for both compounds (Fig. 51). In addition, a minor increase in the percent of dead/apoptotic cells was observed with 5μM CBD treatment (Fig. 51A).

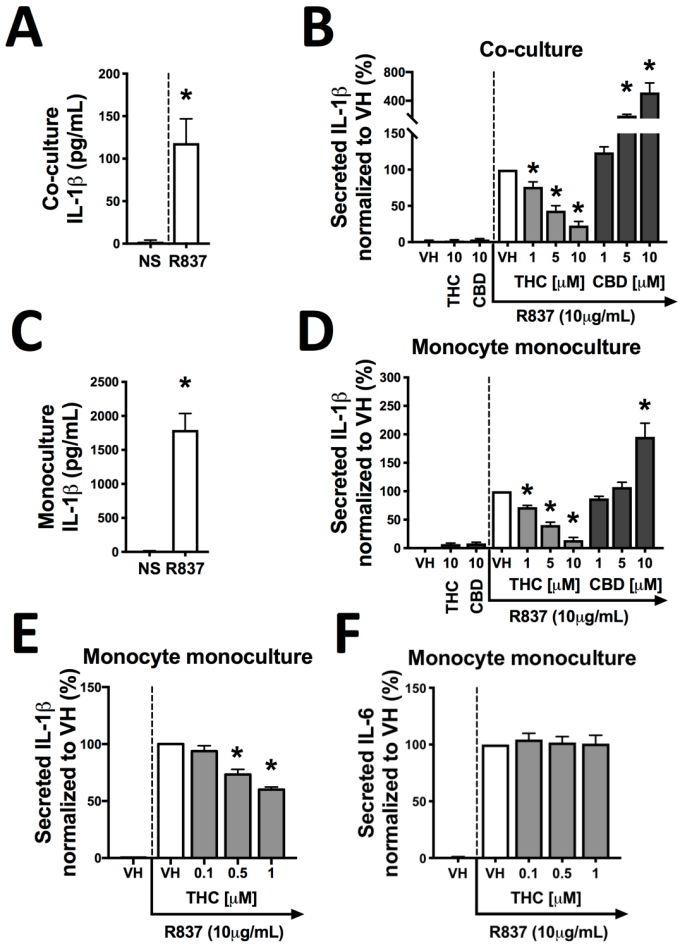

Figure 49. THC treatment of co-culture and monocyte monoculture stimulated with R837 resulted in decreased monocyte production of IL-1β, while CBD augmented IL-1β. (A-B) Human monocytes (HIV-negative) (N=9) ($1x10^4$ cells/mL) were co-cultured with primary astrocytes at a ratio of 1:20, treated with vehicle (0.03% EtOH), THC or CBD (1, 5 and 10μM) and stimulated with R837 (10μg/mL). (C-D) Human monocyte monocultures (N=21) ($4x10^5$ cells/mL) were treated with vehicle, THC or CBD (1, 5 and 10μM) and stimulated with R837. (E-F) Human monocyte monocultures (N=8) ($4x10^5$ cells/mL) were treated with vehicle (0.03% EtOH) or THC (0.1, 0.5 and 1μM) and stimulated with R837. Supernatants were harvested at 20 h post activation for IL-1β and IL-6 quantification via ELISA. The level of IL-6 in the R837-stimulated vehicle in panel F was 23.9ng/mL. Panels A and C demonstrate the raw levels of IL-1β produced. For panels B, D, E and F, IL-1β or IL-6 levels were normalized to the R837-stimulated vehicle control, which serves as 100%. For A and C, a paired t-test was performed. For B, D, E and F, a RM ANOVA with a Dunnett's post-hoc test was performed. *Statistically different from vehicle control (R837+vehicle) or between non-stimulated (NS) and R837 treatment groups ($p<0.05$). All graphs are mean +/- SEM.

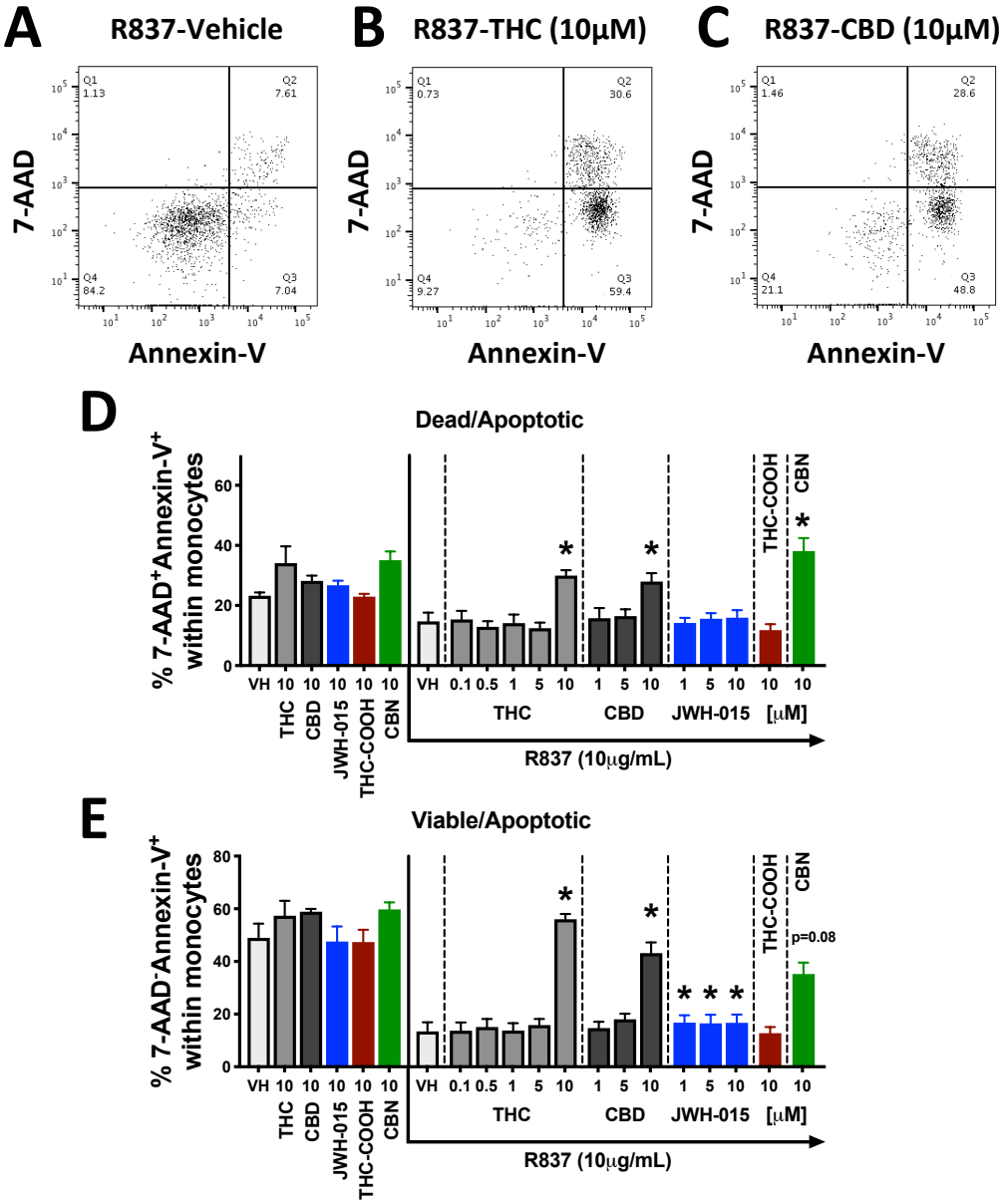

Figure 50. THC and CBD at 10µM promoted monocyte apoptosis in response to TLR7 activation. Human monocytes (HIV-negative) (N=4) were treated with vehicle (0.03% EtOH), THC, CBD, JWH-015, THC-COOH or CBN and stimulated with R837 (10µg/mL). Monocytes were harvested at 20 h post activation and an Annexin-V/7-AAD kit was used to measure apoptosis and viability. (A-C) Flow cytometry plots from one representative donor showing the gating strategy to determine the percent of dead/apoptotic (7-AAD$^+$/Annexin-V$^+$ - panel D) and live/apoptotic (7-AAD$^-$/Annexin-V$^+$ - panel E) cells within the R837-stimulated monocyte population as well as the effect of THC (10µM) and CBD (10µM) compared to vehicle control. A RM ANOVA with a Dunnett's post-hoc test was performed. *Statistically different from vehicle control (R837+vehicle) ($p<0.05$). All graphs are mean +/- SEM.

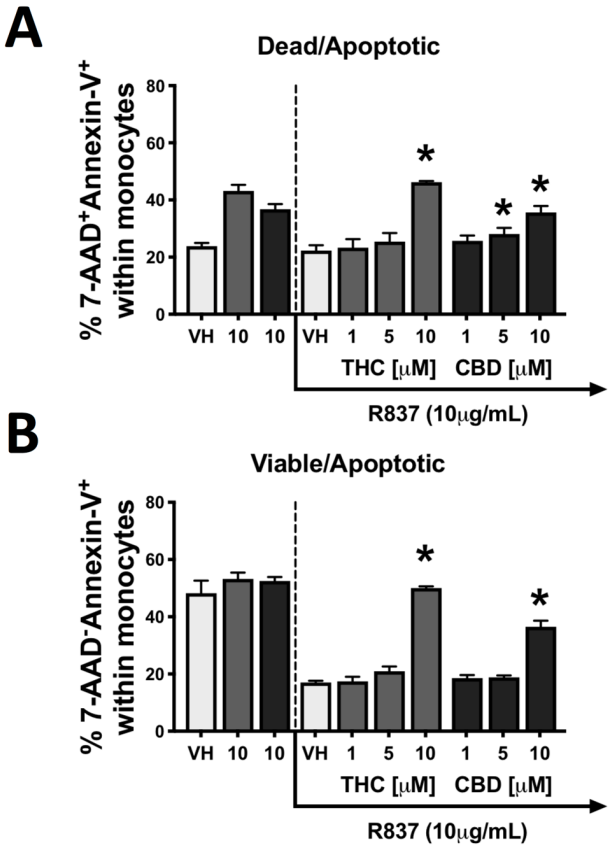

Figure 51. THC and CBD at 10μM induced monocyte apoptosis in response to TLR7 activation. Human monocytes (HIV-negative) (N=4) were treated with vehicle (0.03% EtOH), THC or CBD and stimulated with R837 (10μg/mL). Monocytes were harvested at 20 h post activation and an Annexin-V/7-AAD kit was used to measure apoptosis and viability. The gating strategy in Figure 51 was used to determine the percent of live/apoptotic (7-AAD$^-$/Annexin-V$^+$ - panel E) cells within the R837-stimulated monocyte population A RM ANOVA with a Dunnett's post-hoc test was performed ($p<0.05$). All graphs are mean +/- SEM.

E. THC and CBD treatment of TLR7-stimulated monocytes from HIV+ donors promoted a similar IL-1β secretion profile compared to HIV-negative donors.

We determined whether the above activity of THC and CBD on monocyte-derived IL-1β occurs in monocytes from HIV+ donors. Monocytes isolated from HIV+ donors were treated with THC or CBD and stimulated with R837 for 20 hours. Supernatant was collected and measured for IL-1β. As shown in Figure 52, THC exhibited similar effects on IL-1β secretion by TLR7-stimulated monocytes compared to the HIV-negative donors utilized in the above sections. However, significant suppression of IL-1β was detected with only 5μM THC as compared to 0.5μM in HIV-negative donors (Fig. 52B). This sensitivity difference seems to be due to 2 of the 6 donors displaying minimal responsiveness to THC treatment with the other 4 donors showing a similar profile to HIV-negative donors. Conversely, CBD showed a very similar profile to HIV-negative donors, with significant augmentation of IL-1β production at the 10μM concentration (Fig. 52B). However, the percent increase of secreted IL-1β over the vehicle control with 10μM CBD tended to be higher in the monocyte cultures derived from HIV-infected donors (289%) (Fig. 52B) as compared to monocytes from HIV-negative donors (196%) (Fig. 49D).

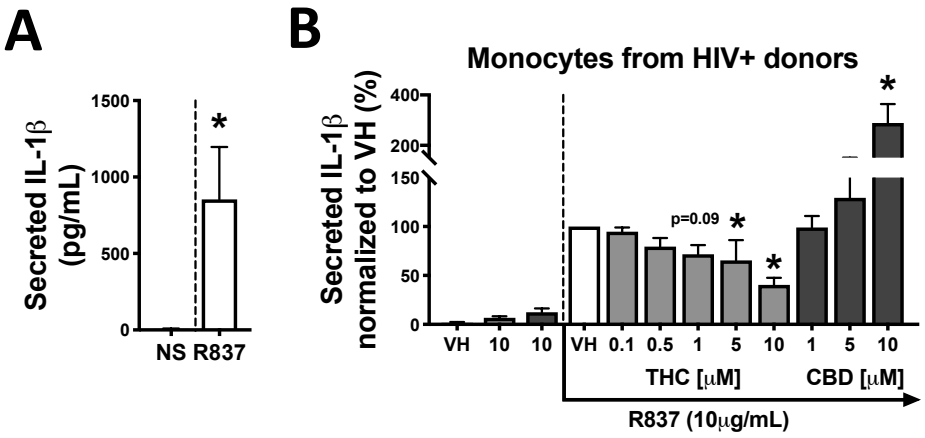

Figure 52. THC and CBD treatment of TLR7-stimulated monocytes from HIV+ donors resulted in a similar IL-1β secretion profile compared to HIV-negative donors. Human monocytes (HIV+ donors) (N=6) were treated with vehicle (0.03% EtOH), THC or CBD and stimulated with R837 (10μg/mL). Supernatants were harvested at 20 h post activation and IL-1β was measured. Panel A shows the raw levels of IL-1β produced. For panel B, IL-1β levels were normalized to the R837-stimulated vehicle control, which serves as 100%. For A, a paired t-test was performed. For B, a RM ANOVA with a Dunnett's post-hoc test was performed. *Statistically different from vehicle control (R837+vehicle) or between non-stimulated (NS) and R837 treatment groups ($p<0.05$). All graphs are mean +/- SEM.

F. Both THC and CBD treatment of TLR8-stimulated monocytes isolated from HIV+ donors resulted in decreased IL-1β secretion.

In addition to R837, monocytes isolated from HIV-negative and HIV+ donors were also activated with the TLR8 agonist, ssRNA40. Specifically, monocytes isolated from either HIV-negative or HIV+ donors were treated with THC or CBD and stimulated with R837 for 20 hours. Supernatant was collected and measured for IL-1β. Similar to R837, THC was also shown to decrease monocyte-derived IL-1β in a concentration-dependent manner in response to ssRNA40 activation for both HIV-negative and HIV+ donors, with significance detected at 1 and 0.5μM, respectively (Fig. 53A-D). Interestingly, CBD displayed differing effects on ssRNA40-stimulated monocyte monocultures as compared to R837. CBD had no effect on IL-1β production by ssRNA40-stimulated monocytes from HIV-negative donors while promoting a significant decrease in IL-1β in HIV+ donors (Fig. 53A-D). This is in stark contrast to the augmentation observed with CBD in R837-stimulated monocytes isolated from HIV-negative and HIV+ donors observed above (Fig. 49D and 52B).

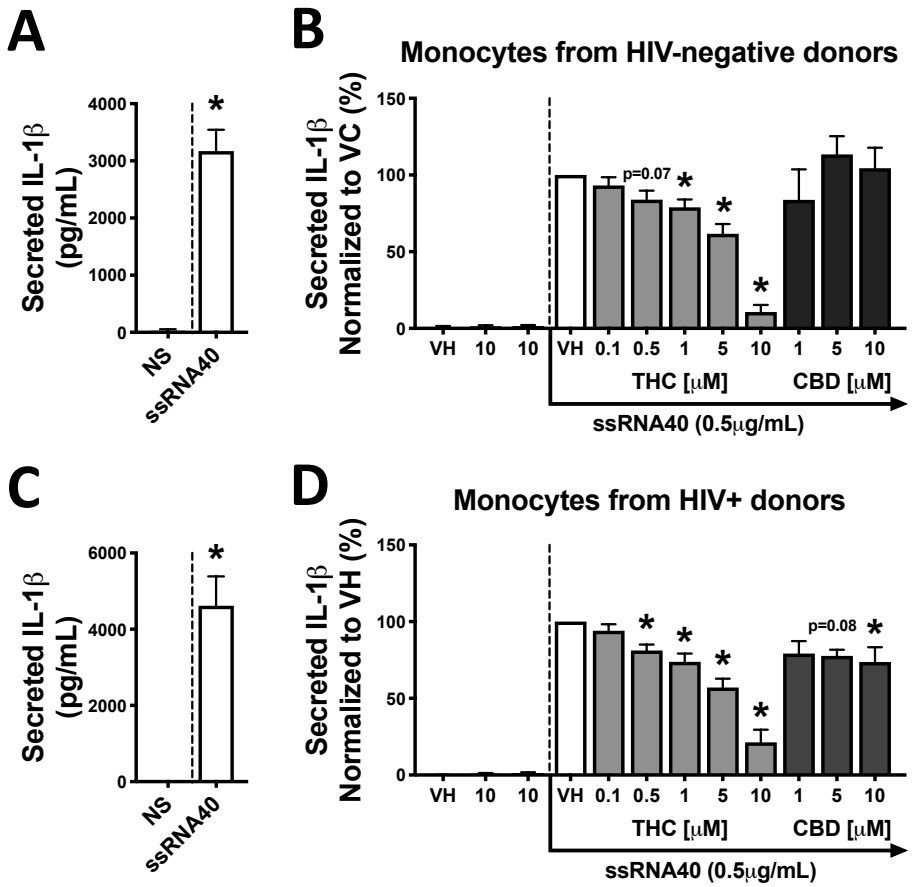

Figure 53. Both THC and CBD treatment of TLR8-stimulated monocytes isolated from HIV+ donors resulted in decreased IL-1β secretion. Human monocytes (HIV-negative and HIV+ donors) (N=6 each) were treated with vehicle (0.03% EtOH), THC or CBD and stimulated with ssRNA40 (0.5μg/mL). Supernatants were harvested at 20 h post activation and IL-1β was measured. Panels A and C shows the raw levels of IL-1β produced. For panels B and D, IL-1β levels were normalized to the ssRNA40-stimulated vehicle control, which serves as 100%. For A and C, a paired t-test was performed. For B and D, a RM ANOVA with a Dunnett's post-hoc test was performed. *Statistically different from vehicle control (R837+vehicle) or between non-stimulated (NS) and R837 treatment groups ($p<0.05$). All graphs are mean +/- SEM.

IV. Specific aim 4: Determine the mode of action by which THC suppresses monocyte-derived IL-1β in response to the TLR7 agonist, R837.

A. The CB2 agonist, JWH-015, suppressed TLR7-mediated monocyte secretion of IL-1β similar to that of THC.

As THC is known to modulate immune cell activity, in part, through CB1 and CB2 (224, 225), we determined whether the CB2 agonist, JWH-015, impaired R837-mediated monocyte secretion of IL-1β similar to that of THC. Since the Ki for CB2 of JWH-015 (13.8nM) and THC (35nM) are within 2-3 fold (228, 271), the same *in vitro* concentrations of both chemicals were used. Specifically, monocyte monocultures were treated with either THC (1, 5 and 10μM) or JWH-015 (1, 5 and 10μM) and stimulated with R837 for 20 hours. JWH-015 decreased monocyte-derived IL-1β in a concentration-dependent manner with significant suppression observed at 5 and 10μM (Fig. 54A). The suppression with JWH-015 was similar to that of THC, suggesting that THC is acting in part, through CB2. However, some distinctions were observed including sensitivity differences between the two compounds, as 1μM THC but not JWH-015 promoted a significant decrease in IL-1β (Fig 54A). In addition, THC at 10μM promoted a greater decrease in IL-1β (88%) compared to JWH-105 (57%) (Fig. 54A). These results suggest that THC is also acting through additional mechanisms/receptors. To examine for potential CB-receptor independent mechanisms, R837-stimuated monocytes were treated with the THC metabolite, 11-nor-9-carboxy-THC (THC-COOH) (210), and measured for IL-1β. THC-COOH (10μM) had no effect on monocyte secretion of IL-1β as compared to the potent suppression observed with the equivalent concentration of THC (Fig. 54B).

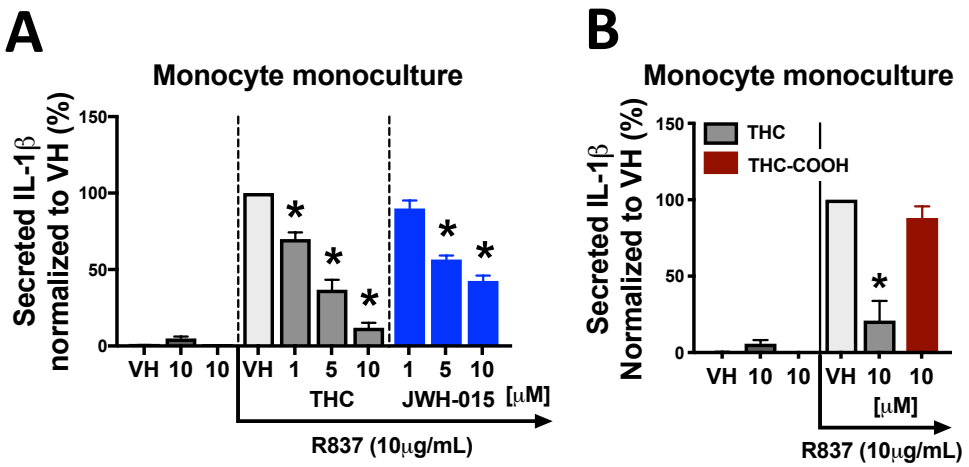

Figure 54. The CB2 agonist, JWH-015, suppressed TLR7-mediated monocyte secretion of IL-1β similar to that of THC. Human monocytes (N=12 for A and N=8 for B) were treated with vehicle (0.03% EtOH), THC, JWH-015 or THC-COOH (11-Nor-9-carboxy-THC) and stimulated with R837. Supernatants were harvested at 20 h post activation and IL-1β was measured via ELISA. The datasets in panels A-B are normalized to the R837-stimulated vehicle control, which serves as 100%. (A) A RM ANOVA with a Dunnett's post-hoc test was performed ($p<0.05$). (B) A paired t-test was performed between activated (R837) vehicle control (VH) and activated treatment group (THC or THC-COOH). * denotes a statistical difference from the VH + R837 control ($p<0.05$). All graphs are mean +/- SEM.

B. THC modulates early monocyte signaling events, including decreasing *IL1B* mRNA levels, when stimulated with R837.

To evaluate the mode of action of THC on impairment of IL-1β secretion by monocytes, we first determined the time point at which suppression was observed with THC treatment. Isolated monocytes were treated with THC (5μM) and stimulated with R837 (10μg/mL). Supernatants were harvested at 2.5, 5, 10, 15, 20 and 25 h and IL-1β was quantified. As observed in Figure 55, significant suppression of IL-1β by THC was observed as early as 5 h and suppression was sustained over the duration of time course (25 h), suggesting that THC was suppressing early cellular events. As transcriptional induction of *IL1B* mRNA is an early event that is critical for IL-1β production and secretion, we determined the impact of THC on *IL1B* mRNA levels. Monocyte monocultures were treated with THC and stimulated with R837 for 3 and 5 h. RNA was isolated and RT-qPCR (Taqman) was used to quantify monocyte expression of *IL1B*. The time points of 3 and 5 h were chosen based on Figure 55 and a previous report that identified the peak *IL1B* mRNA expression by human monocytes occurred between 3-6 h post activation with the TLR7/8 agonist, R848 (272). The level of *IL1B* mRNA within monocytes was significantly lower with THC treatment (5 and 10μM) at both the 3 and 5 h time points (Fig. 56A-B). However, there was no significant effect on *IL1B* levels at 1μM THC despite the 30% suppression observed on supernatant IL-1β at this concentration (Fig. 56A), suggesting THC may also be affecting other pathways involved in the production and/or secretion of IL-1β.

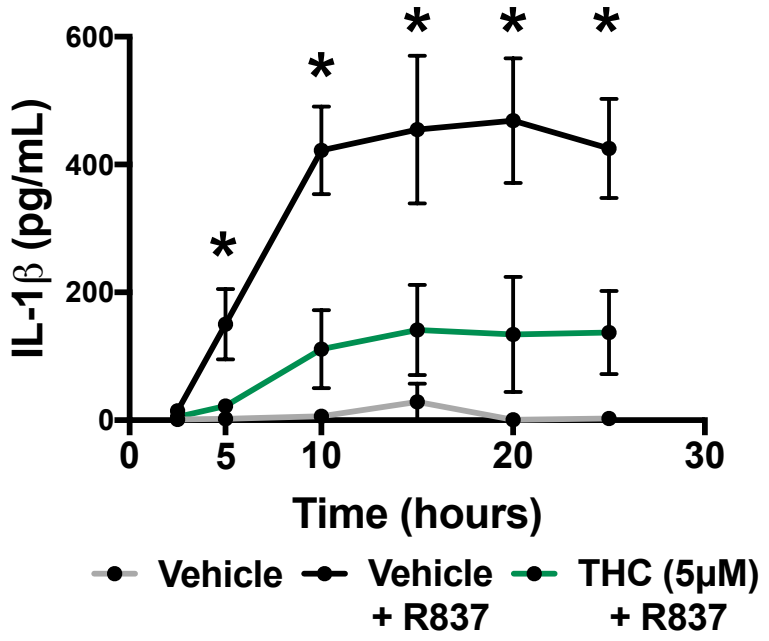

Figure 55. THC decreased TLR7-mediated monocyte secretion of IL-1β as early as 5 hours post-activation. Human monocytes (N=4) were treated with vehicle (0.03% EtOH) or THC (5μM) and stimulated with R837 (10μg/mL) for 2.5, 5, 10, 15, 20 and 25 h. Supernatants were collected at each time point and IL-1β levels were measured. A two-way RM ANOVA with a Sidak's post-hoc test was performed. * denotes a statistical difference between the VH + R837 control and THC + R837 treatment group ($p<0.05$). All graphs are mean +/- SEM.

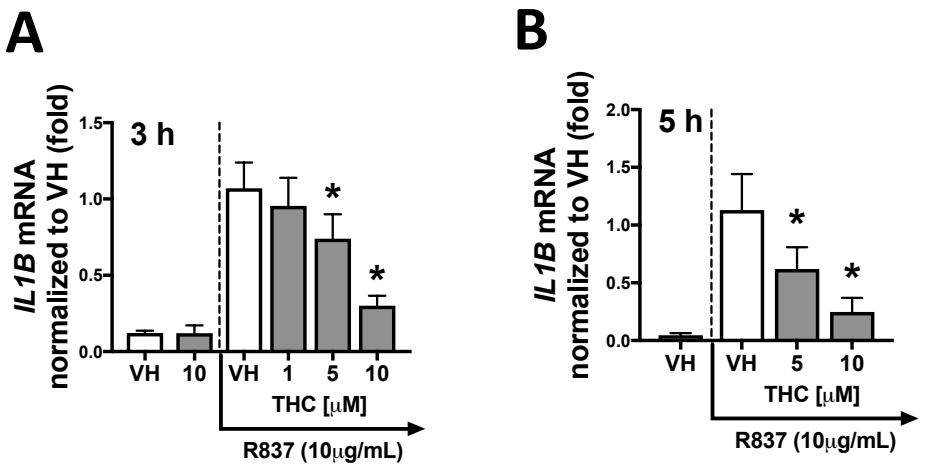

Figure 56. THC decreased *IL1B* mRNA levels in monocytes when stimulated with R837. (A) Human monocytes (N=6) were treated with vehicle (0.03% EtOH) or THC (1, 5 and 10μM) and stimulated with R837 (10μg/mL) for 3 h. (B) Human monocytes (N=4) were treated with vehicle or THC (5 and 10μM) and stimulated with R837 (10μg/mL) for 5 h. (B-C) Monocytes were harvested and *IL1B* mRNA was quantified. *IL1B* mRNA levels were normalized to the R837-stimulated vehicle control (VH). A RM ANOVA with a Dunnett's post-hoc test was performed. * denotes a statistical difference from the VH + R837 control ($p<0.05$). All graphs are mean +/- SEM.

C. THC decreased caspase-1 activity within R837-stimulated monocytes resulting in accumulation of intracellular IL-1β.

We next examined additional pathways involved in IL-1β production and secretion that may be altered by THC. It is well established that IL-1β secretion requires both transcription/translation of the pro-form (pro-IL-1β) and inflammasome-mediated caspase cleavage of pro-IL-1β into the mature form (177, 273). The major caspase involved in cleavage of pro-IL-1β in monocytes is caspase-1, which becomes activated with inflammasome formation and activation (273, 274). To determine whether THC modulates the activity of caspase-1, we first determined the time point at which monocyte caspase-1 activity was significantly increased with R837 compared to non-stimulated cells. A fluorescence caspase-1 inhibitor probe (FAM-YVAD-FMK), which labels active caspase-1 molecules, was used to measure caspase-1 activity within monocytes. First, isolated monocytes were stimulated with R837 (10μg/mL) and monocytes were harvested at 5, 15, and 20 h to determine the time point at which caspase-1 activity is induced. R837 treatment significantly induces caspase-1 activity (MFI) at the 5 and 15 h time points (Fig. 57). The time point selected for THC evaluation of caspase-1 activity was 15 h, as it parallels with peak levels of IL-1β (Fig. 55). Figures 58A-C are representative flow cytometry plots from one donor demonstrating the effect caspase-1 activity at 15 h in response to R837 activation and the impact of THC (5μM). R837 activation elicited approximately a 2-fold increase in caspase-1 activity levels (MFI) within the monocytes population, which was decreased by THC in a concentration-dependent manner, with significance detected at 0.5μM (Fig. 58D).

As caspase-1 activity is important for cleavage of pro-IL-1β into the mature form (273, 274), the suppression in caspase-1 activity observed with THC may result in an accumulation of

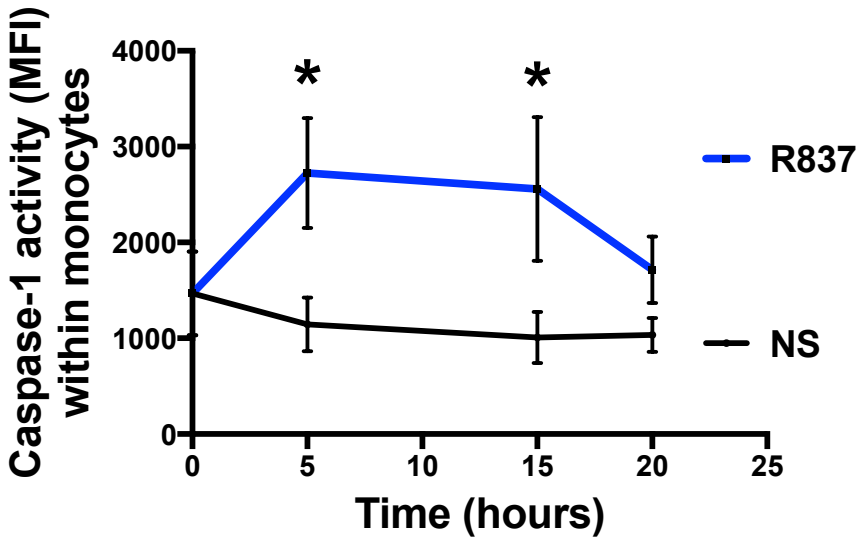

Figure 57. Caspase-1 activity within monocytes is induced by R837 at 5 and 15 hours post-activation. Human monocytes (N=3) were stimulated with R837 (10μg/mL) for 5, 15, and 20 h. Cells were harvested and quantified for caspase-1 activity (MFI) via flow cytometry. Dataset was log transformed and a two-way RM ANOVA with a Sidak's post-hoc test was performed. * denotes a statistical difference between the R837-treated and non-stimulated (NS) monocytes ($p<0.05$). All graphs are mean +/- SEM.

non-cleaved pro-IL-1β in the cell. To address this, monocytes were treated with THC (0.1-5μM) and stimulated with R837 for 20 h. Monocytes were harvested and intracellular IL-1β was examined via intracellular flow cytometry. It is noteworthy that the anti-IL-1β clone used here detects total IL-1β and does not distinguish the pro- and mature forms. Figure 58E-G are flow cytometry plots from the same representative donor as in the caspase activity analysis in Figure 58A-C. Interestingly, THC at 5μM, promoted an increase in intracellular IL-1β expression (MFI) (Fig. 58E-H), matching the inhibitory effects observed on caspase-1 activity (Fig. 58A-D).

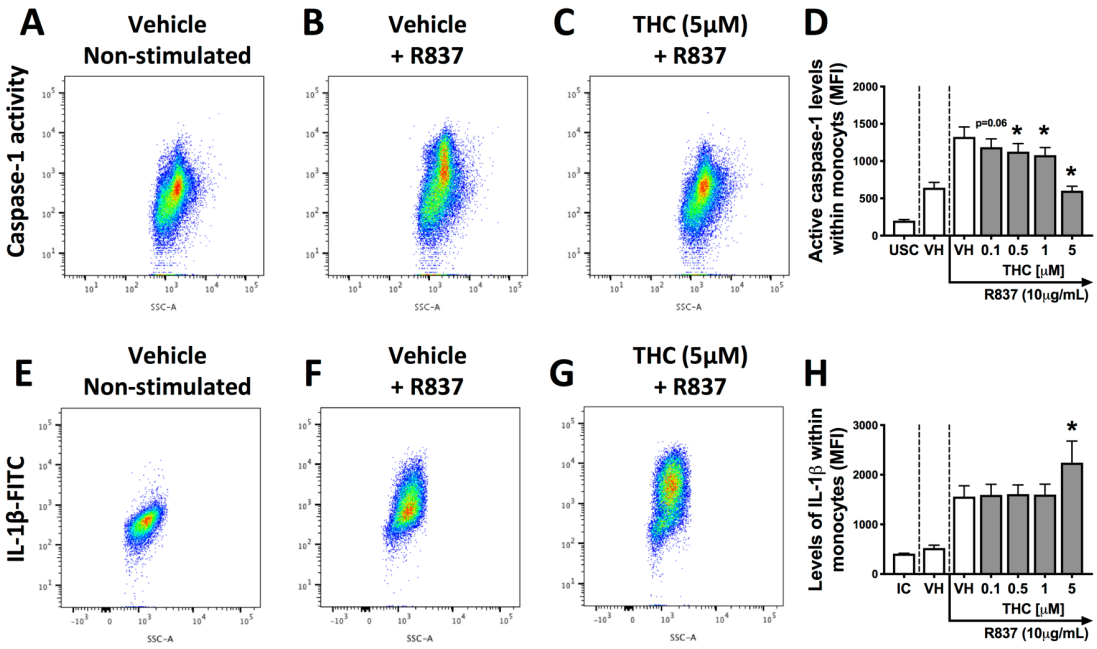

Figure 58. THC decreased caspase-1 activity within R837-stimulated monocytes resulting in increased intracellular accumulation of IL-1β. (A-D) Human monocytes (N=7) were treated with vehicle (0.03% EtOH) or THC (0.1, 0.5, 1 and 5μM) and stimulated with R837. Cells were harvested at 15 h post-activation and quantified for caspase-1 activity (MFI) via flow cytometry. (E-H) Human monocytes (N=7) were treated with vehicle (0.03% EtOH) or THC (0.1, 0.5, 1 and 5μM) and stimulated with R837. Cells were harvested at 20 h and quantified for intracellular IL-1β expression (MFI within total monocyte population) via flow cytometry. Flow cytometry plots in panels A-C and E-G are from the same representative donor. A RM ANOVA with a Dunnett's post-hoc test was performed ($p<0.05$). * denotes a statistical difference from the vehicle + R837 control ($p<0.05$). All graphs are mean +/- SEM.

DISCUSSION

I. The effect of cannabis use and Δ^9-THC on peripheral monocyte activation and secretion of IP-10 in HIV-negative and HIV+ individuals.

In aim 1 (156), we show that IFNα treatment of PBMCs and purified monocytes isolated from HIV-MJ- donors promotes monocyte transition into the $CD16^+$ phenotype as well as increases the percent of $CD163^+$ and $CD16^+CD163^+$ monocytes. These findings coincide with previous studies reporting that monocytes from HIV-infected individuals display a type I interferon gene signature (109, 153). Similarly, *in vivo* IFNα therapy promoted an increase in the percent of $CD16^+$ monocytes (154). Taken together, these observations strongly support IFNα as an inflammatory factor that increases the frequency of $CD16^+$ and $CD16^+CD163^+$ monocytes during HIV infection. These findings are noteworthy since circulating $CD16^+$/$CD16^+CD163^+$ monocytes traffic to the brain during HIV infection promoting viral entry as well as secretion of inflammatory and neurotoxic factors (74, 76, 88, 91). Interestingly, the IFNα-mediated monocyte transition to $CD16^+$ was only observed in HIV+MJ- donors and not HIV+MJ+ donors, suggesting that cannabis use may impair the induction of CD16. Future studies investigating the differences in monocytes expression of IFNAR and key downstream signaling molecules between HIV+MJ- and HIV+MJ+ donors will provide insights into the lack of CD16 induction observed in HIV+MJ+ donors.

After the initial observation that HIV+MJ+ donors displayed lower circulating $CD16^+$ and $CD16^+CD163^+$ ($p=0.052$) monocytes compared to HIV+MJ- donors, we demonstrated that *in vitro* THC treatment decreased the percent of monocytes expressing CD16, CD163 and CD16/CD163 in IFNα-treated PBMCs isolated from HIV-MJ- donors. Furthermore, THC treatment impaired monocyte expression of IFNAR in HIV-MJ- donors; however, the

impairment was modest and only observed at the highest concentration of THC (10μM). With significant impairment in CD16 expression seen as low as 1μM THC, these findings suggest that THC is impairing IFNAR-mediated signaling. THC impairment of CD16 and CD163 expression on monocytes was also observed in purified $CD16^-$ monocytes demonstrating that THC acts directly on the monocyte population and not through a bystander mechanism. Furthermore, treatment with the low affinity CB1/CB2 agonist, CBD, yielded no significant effect on CD16 or CD163 expression, suggesting that THC is modulating monocyte activity through a cannabinoid receptor (CB1/CB2)-dependent mechanism. As HIV-infected individuals have chronic immune activation (120, 275) and CB1/CB2 expression may change with monocyte/macrophage activation status (224), the sensitivity of immune cells to THC treatment may vary between HIV-MJ- and HIV+MJ- donors. Therefore, we performed experiments using monocytes from HIV+MJ- donors, which demonstrated that monocytes isolated from HIV+MJ- donors displayed similar impairment by THC on CD16 and CD163 expression to that of HIV-MJ- donors. Overall, these findings suggest that the THC present in cannabis may be a significant contributor to the decreased levels of $CD16^+$ monocytes observed in HIV+MJ+ donors.

Another interesting observation in this study is that plasma IP-10 levels were lower in HIV+MJ+ donors compared to HIV+MJ- donors. IP-10 has been shown to be elevated in the CSF of patients with cognitive impairment and is thought to be an important contributor to neuroinflammation during HIV infection (276). Furthermore, IP-10 has been shown to stimulate HIV replication in monocyte-derived macrophages and promote neuronal apoptosis *in vitro* (81, 277). Using intracellular IP-10 staining, we report that the monocyte population is the primary cell type within the PBMCs of HIV-MJ- donors secreting IP-10 in response to IFNα and monocyte expression of CD16 isn't necessary for IP-10 production. This is in agreement with a

previous report showing the monocyte population is a major source of IP-10 when stimulated with TLR7/8 ligands (136). When comparing the IFNα-mediated induction of IP-10 between HIV-MJ-, HIV+MJ- and HIV+MJ+ donors, similar induction profiles were observed. THC treatment was shown to decrease IP-10 in all three groups, with HIV-MJ- donors showing a slight increase in sensitivity to THC. Using purified monocytes from HIV-MJ- and HIV+MJ- donors, we demonstrate that THC has a direct effect on the monocytes resulting in decreased IP-10 levels. Furthermore, THC at a concentration of 0.5μM significantly decreased IP-10 levels, which is within the concentration range observed in blood of individuals smoking cannabis (209).

There were limitations in the cross-sectional design comparing blood $CD16^+$ monocytes and plasma IP-10 in HIV-MJ-, HIV+MJ- and HIV+MJ+ donors (Fig. 1). First, the absence of HIV-MJ+ donors hindered our ability to make comparisons between HIV-MJ+ and HIV+MJ+ donors. However, the HIV-MJ- donors served as a comparator to show the increased levels of inflammatory markers observed in HIV+MJ- donors. The central focus was to identify potential differences in the number of monocytes expressing CD16/CD163 and plasma IP-10 between HIV+MJ- and HIV+MJ+ donors. Second, the exposure level of cannabis in the HIV+MJ+ population could not be quantified due to many variables. This remains a systemic limitation in studies investigating cannabis use, as exposure levels can be influenced by multiple variables (206). However, we could confirm cannabis use and whether respondents were accurate in stating cannabis use in the patient questionnaire by assaying blood samples for the presence of THC metabolites. Lastly, the HIV-MJ- donors in this study were from different geographical locations compared to the HIV+MJ- and HIV+MJ+ donors. Importantly, all HIV+ donors, HIV+MJ- and HIV+MJ+, were from the Mid-Michigan area.

Overall, the results presented here show that *in vitro* THC treatment promotes anti-inflammatory effects on monocyte processes that are implicated in HIV-associated neuroinflammation, including monocyte transition into the $CD16^+$ phenotype and secretion of IP-10. With these results, it is tempting to speculate that THC is one of the major components of cannabis that elicits the decrease in circulating $CD16^+$ monocytes and plasma IP-10 that was observed in HIV+MJ+ donors. However, the *in vivo* effects of THC when inhaled through cannabis use may be different than that observed *in vitro* due to the additional 100-plus cannabinoids that are present in cannabis as well as other plant-derived constituents (e.g., terpenes) (216). Therefore, cannabinoids in combination with other plant-associated compounds may contribute to the observed anti-inflammatory actions. In addition, cannabis use could have indirect immune modulating effects, such as through stress reduction, which can have an impact on inflammation (278, 279).

II. The effect of human primary monocytes, in combination with IFNα and/or TLR7 agonist (R837) on astrocyte production of MCP-1, IL-6 and IP-10 in a co-culture system.

As circulating monocytes have the potential to exacerbate several neurological conditions, including HAND (74, 280-282), it is important to investigate the role of activated monocytes on the brain-resident cell inflammatory responses. TLR7 agonist (imiquimod) activation of the immune system, which attempts to mimic aspects of HIV ssRNA-mediated immune activation, has been identified to promote a neuroinflammatory response in animal models with similar features to HIV-associated neuroinflammation. Specifically, monocyte infiltration into the brain, along with increased astrocyte reactivity and cytokine production has been observed during *in vivo* imiquimod (TLR7)-induced neuroinflammation (167-170). Several inflammatory factors were induced in the brain of imiquimod-treated mice, including MCP-1,

IL-6 and IP-10, which were observed across multiple studies (167-170). IL-6, MCP-1 and IP-10 are notable as they are also increased in the plasma and/or CSF during HIV infection and associated with neuronal injury and/or cognitive impairment (96-98, 109, 112, 155-157). The potential for monocytes to induce astrocyte secretion of these factors could facilitate a positive feedback loop of immune cell infiltration, including monocytes (MCP-1) and T cells (IP-10) (24, 170, 283, 284), which would exacerbate neuroinflammation and contribute to HAND. Here, we utilized a human co-culture system to determine whether the TLR7 agonist, imiquimod/R837, and IFNα enhance monocyte-mediated astrocyte secretion of MCP-1, IL-6 and IP-10.

In SA2 (285), non-stimulated monocytes were able to promote astrocyte production of MCP-1 and IL-6 at cell ratios as low as 1:100 and 1:20 (monocyte:astrocyte), respectively. Furthermore, imiquimod/R837 activation of monocytes from HIV-negative donors enhanced astrocyte secretion of MCP-1 and IL-6. Interestingly, R837 had a minimal impact on IP-10 production in the co-culture, which contrasts with the induction observed *in vivo* (167-170). However, IFNα, which is produced in response to TLR7 stimulation (286, 287), was in fact, able to induce astrocyte production of IP-10, as well as MCP-1 and IL-6. When combined, R837 and IFNα promoted an additive response on IL-6 production by astrocytes, suggesting an exacerbation of the astrocyte inflammatory response when the stimuli are present together. With the use of monocytes from HIV-infected donors, TLR7 (R837) and TLR8 (ssRNA40) stimulation of the co-culture resulted in enhanced production of MCP-1 and IL-6 by astrocytes, which matched the observations with co-cultures containing monocytes from HIV-negative donors. It is tempting to speculate that ratios lower than 1:100 in combination with R837 or ssRNA40 would induce significant monocyte-mediated astrocyte responses. Overall, the findings presented here suggest that a low-level of TLR-activated monocytes into the CNS can

likely promote astrocyte secretion of pro-inflammatory factors; highlighting a potential mechanism of astrocyte dysfunction and neuroinflammation during HIV pathogenesis.

As the biological mechanism of monocyte-induced astrocyte activation may be a critical target for therapeutic development for the treatment of HAND, we determined the mechanism by which R837-stimulated monocytes provoked astrocyte production of MCP-1 and IL-6. The mechanism was first shown to be through soluble factors as cell-cell contact was not required. This finding differs from a previous study that showed cell-cell contact was necessary for monocyte-mediated astrocyte production of MCP-1 (158). This discrepancy may be due to different astrocyte sources used for the transwell assay, as U251 cells were used in this study as compared to primary astrocytes. In addition, other notable differences between studies include differing monocyte isolation techniques, monocyte:astrocyte ratios, monocyte stimulation (e.g., R837), time points and endpoint measurements (e.g., intracellular vs. supernatant). However, IFNα-induced astrocyte production of IP-10 was mediated, in part, through cell-cell contact.

Monocyte-derived IL-1β was identified to be a critical soluble factor responsible for astrocyte production of MCP-1, IL-6 and IP-10, as anti-IL-1β neutralization of the R837-stimulated co-culture strongly suppressed astrocyte production of MCP-1 and IL-6. This is in agreement with a previous study that showed the importance of IL-1β in monocyte-astrocyte interactions (158). Interestingly, IL-1β was shown to be significantly elevated in the brain of imiquimod-treated mice and has been implicated as a key factor involved in neuronal injury during HIV-associated dementia (82, 168, 169). However, we did not observe substantial involvement of TNFα in the co-culture, which was also reported (158). In addition, primary astrocytes were not as sensitive to IL-1β neutralization as compared to the cell-line suggesting

that other soluble factors released by monocytes, such as IL-1α (288), or direct effects of the stimuli on the astrocytes are contributing to the response.

The importance of monocyte-derived IL-1β was further supported when astrocyte monocultures treated with recombinant IL-1β induced MCP-1 and IL-6, mimicking the actions of the monocytes in the co-culture. This is consistent with the current literature of IL-1β stimulating astrocyte production of MCP-1 and IL-6 (83, 158); however, a notable observation was the sensitivity of human astrocytes to IL-1β. IL-1β levels as low as 1 and 5pg/mL induced astrocyte production of MCP-1 and IL-6, respectively, which is significant as it may be challenging to detect such low concentrations in the CSF or plasma samples with standard approaches (bead-based or ELISA assays). Consequently, difficulties may arise when attempting to associate plasma or CSF levels of IL-1β with markers of neuronal injury and/or cognitive decline in diseases involving chronic neuroinflammation.

R837 in combination with recombinant IL-1β had differing effects on IL-6 or MCP-1 production in astrocyte monocultures, which seemed to depend on the astrocyte source. R837 in combination with IL-1β augmented IL-6 production compared to IL-1β alone in two of three primary astrocyte donors while having no effect on U251 cells and a second astrocyte donor. The findings from the co-culture and astrocyte monocultures suggest that R837 is primarily enhancing astrocyte production of MCP-1 and IL-6 by inducing monocyte secretion of IL-1β. In addition, IFNα seems to be directly acting on the astrocyte, plausibly through binding to the IFNAR (289), and when in concert with monocyte-derived IL-1β, induces the production of IP-10 and IL-6.

Correlation analysis of monocyte subsets and astrocyte production of MCP-1, IL-6 and IP-10 identified an association between the percent of intermediate monocytes and astrocyte

production of IL-6, with significant correlations found in the NS and IFNα-stimulated co-cultures and a trending correlation ($p=0.09$) in the R837-treated co-culture. These findings hint at potential involvement of intermediate monocytes on astrocyte secretion of IL-6 through the release of IL-1β. This observation is concordant with the current body of literature that demonstrates that monocyte subsets expressing CD16 contribute to HIV-associated neuroinflammation and HAND (74, 88, 93, 290). However, R837 treatment of monocytes, which displayed enhanced IL-1β secretion compared to control and IFNα, had a weaker association between intermediate monocytes and astrocyte production of IL-6. This may be due to R837 promoting IL-1β production from intermediate monocytes as well as other subsets (e.g., classical/non-classical), which has been observed with TLR7-stimulation of human monocytes (139). Activating other monocyte subsets would plausibly decrease the contribution from the intermediate population and thus lower the association between intermediate monocytes and astrocyte production of IL-6. Future experiments comparing specific monocyte populations in the co-culture need to be performed to address the role of each monocyte subset on the astrocyte inflammatory response.

For the majority of endpoints in this study, all three of the primary astrocyte donors responded similarly, especially in the R837-stimulated co-culture. However, a few differences were observed. For example, IFNα stimulation of co-cultures containing primary astrocytes from two of the three donors displayed an augmentation in MCP-1 and IL-6, while the second donor exhibited a non-significant increase in these factors. The differences observed here may be attributed to factors such as astrocyte heterogeneity (291, 292).

There were limitations in this section, including the source of primary astrocytes used, which were fetal-derived, as adult primary astrocytes were not accessible. There is a possibility

for varying inflammatory responses between fetal and adult astrocytes in terms of monocyte and IL-1β activation. However, a previous study demonstrated IL-1β-mediated activation of adult human astrocytes to release pro-inflammatory factors (29), suggesting that adult astrocyte responses to monocytes and IL-1β would be similar to the fetal astrocyte responses reported here. Also, as physiologically relevant ratios of monocyte:astrocytes aren't well established during viral-mediated neuroinflammation and is likely dependent on a number of factors, the ratios utilized in this report may not precisely reflect what is observed *in vivo*. In addition, the co-culture presented here consisted of only monocytes and astrocytes, despite other cell types, including microglia, pericytes, brain microvascular endothelial cells (BMVECs) and differentiated macrophages, being present during *in vivo* neuroinflammation and interacting with monocytes (68, 293). In fact, IL-1β has been shown to induce inflammatory responses in pericytes and BMVECs (294, 295), suggesting that monocyte-derived IL-1β also impacts the function of these cell types. Future studies will be needed to evaluate the role of imiquimod/R837 on other cell-cell interactions. Lastly, despite being freshly isolated human monocytes, once present in the *in vitro* co-culture conditions, these cells have the potential to shift to a macrophage phenotype due to increased basal activation (270).

III. The effect of Δ^9-THC and CBD on monocyte-mediated astrocyte production of MCP-1 and IL-6 when stimulated with the TLR7 agonist, R837.

In aim 3, we evaluated the impact of THC, as well as CBD, on monocyte-mediated astrocyte inflammatory responses in the context of viral (TLR7)-mediated neuroinflammation. With the use of the TLR7-stimulated co-culture system developed in aim 2, we demonstrate that THC suppresses monocyte-induced astrocyte production of MCP-1 and IL-6, suggesting that THC has the potential to alter leukocyte trafficking into the brain. Interestingly, the anti-

inflammatory actions of THC on monocyte-astrocyte inflammation observed here may be beneficial in the context of HIV-associated neuroinflammation, as it would potentially suppress leukocyte recruitment as well as monocyte secretion of IL-1β that exists due to activated monocytes that have entered the brain (74, 76). In fact, animal models that induce neuroinflammation have demonstrated beneficial anti-inflammatory actions by THC and various agonists for CB1/CB2 (219, 296, 297). However, under different circumstances, THC may be detrimental as it may impair monocyte-astrocyte inflammatory responses that may be necessary to clear viruses such as influenza that have seeded in the brain (298, 299). THC has been noted to impair the peripheral inflammatory response to influenza in a mouse model (215). In addition, THC could have detrimental anti-inflammatory effects on different immune cell populations (e.g., $CD4^+$ T cells) in HIV-infected individuals that are necessary to elicit effective immune responses to common pathogens. This would especially be true for HIV-infected individuals that don't respond well to ART and remain immune compromised (i.e., low level of $CD4^+$ T cells). Conversely, CBD treatment enhanced astrocyte production of IL-6 in the TLR7-stimulated co-culture, which is in contrast to the current body of literature demonstrating CBD as having anti-inflammatory activity (217). However, one report noted that CBD enhances LPS-induced pulmonary inflammation *in vivo*, suggesting the possibility of pro-inflammatory actions of CBD (223). As cannabis strains, on average, contain 12% THC and 0.2% CBD (207), the data presented here would suggest that cannabis would display an overall anti-inflammatory effect; however, future studies will be needed to investigate this hypothesis. In the context of HIV infection and HAND, cannabis use may be beneficial in decelerating monocyte-mediated astrocyte neuroinflammatory processes. However, cannabis strains and/or oils containing

primarily CBD may augment neuroinflammation, which may be detrimental in the context of HAND.

When the co-culture was separated into astrocyte and monocyte monocultures, THC and CBD were shown to modulate both cell types, albeit to varying degrees. Specifically, THC was shown to suppress MCP-1 and IL-6 production in IL-1β/R837-treated astrocytes, however, the magnitude of suppression was modest compared to the inhibitory effects observed in the co-culture. The direct suppressive effects of THC on astrocyte production of MCP-1 and IL-6 are in agreement with the findings by Sheng et al 2004, in which the synthetic cannabinoid receptor agonist, WIN55,212-2, suppressed astrocyte production of several inflammatory factors (e.g., MCP-1) (242). Furthermore, THC has been identified to suppress inflammatory processes in other glial populations, including IL-6 and IL-1β production by LPS-activated microglia (BV-2 cell line) (243). Interestingly, CBD displayed immune suppressive effects on MCP-1 and IL-6 production in astrocyte monocultures, which is in opposition to the co-culture, in which no effects were observed on MCP-1 and IL-6 production was enhanced with CBD. This would suggest that CBD is stimulating pro-inflammatory processes by monocytes in the co-culture system. At this point in time, there aren't any reports that have investigated the role of CBD on human astrocyte secretion of inflammatory factors. However, CBD has been shown to suppress IL-6 and IL-1β production by LPS-activated BV-2 microglia (243).

When observing the supernatant level of IL-1β in both the TLR7-stimulated co-cultures and monocyte monocultures, THC was identified to suppress IL-1β while CBD enhanced IL-1β, matching the observations in astrocyte production of MCP-1 and IL-6 in the co-culture. Thus, the effects of THC and CBD on monocyte secretion of IL-1β seem to be more critical in the overall co-culture response compared to the direct effects of cannabinoids on the astrocyte population.

Interestingly, THC as low as 0.5μM in R837-stimulated monocyte monocultures was able to suppress IL-1β secretion. This is of importance as 0.5μM is within the range of peak plasma concentration of THC (0.24-0.87μM) after inhalation of a cannabis cigarette containing 3.55% THC (209). In addition, present day cannabis material contains roughly 12% THC (207), which is almost 4-fold higher than the report above, which would suggest that concentrations of 1μM used in this study are within the observable ranges *in vivo*.

The THC-mediated suppression of IL-1β observed here is similar to a report that demonstrated the suppressive effect of THC on LPS-induced monocyte secretion of IL-1β (300), suggesting a common mode of action by THC. Interestingly, this same report mentioned that CBD augmented LPS-mediated IL-1β secretion by monocytes; however, the authors did not show the data in the report (300). Albeit not in human monocytes, there have been a few additional studies that have reported immunomodulatory effects of THC and CBD on IL-1β in monocyte/macrophage and microglia cell lines. Specifically, Shivers and colleagues utilized monocyte cell lines (THP-1 and U937) to demonstrate the immunomodulatory activity of THC on IL-1β, and determined that depending on the cell line type and activation, THC either enhanced or suppressed IL-1β activity (301). Furthermore, THC and CBD have been shown to suppress IL-1β production from LPS-stimulated BV-2 microglia (243), which for CBD, is in contrast to the findings presented with TLR7-stimulated monocytes.

The current body of literature has indicated that cannabinoids at higher concentrations (>10μM) can induce apoptosis in various immune cell populations as a pathway to immunosuppression (247, 248, 250, 251, 254, 266, 302). We demonstrate that THC and CBD, only at 10μM, induce monocyte apoptosis when stimulated through TLR7 and is through a non-CB2 receptor pathway, as JWH-015 had minimal to no effect on monocyte apoptosis.

Furthermore, THC-COOH at 10μM had no effect on monocyte apoptosis, suggesting that THC and CBD aren't promoting monocyte apoptosis due to non-specific alterations of the plasma membrane. As cannabinol (CBN) also induced a similar apoptotic profile in monocytes, this would suggest that THC, CBD and CBN might be promoting apoptosis through a similar non-CB2-mediated mechanism. However, no apoptotic effects were observed with THC concentrations of 5μM and below, suggesting that the mechanism by which THC (<5μM) is suppressing IL-1β is through targeting specific mechanisms of the TLR7-induced IL-1β signaling pathway and not apoptosis. Conversely, the enhancement in monocyte-derived IL-1β observed with CBD was only significant at the 10μM, suggesting that CBD-induced apoptosis may be, in part, leading to the augmentation in IL-1β.

Finally, we showed that the effect of THC and CBD on TLR7-induced monocyte-derived IL-1β from HIV-negative donors also occurs in HIV-infected donors. Furthermore, THC suppressed monocyte secretion of IL-1β when stimulated with the TLR8 agonist, ssRNA40, in both HIV-negative and HIV-infected donors, suggesting that THC is suppressing common aspects of the TLR signaling and/or inflammasome pathways. In contrast, CBD displayed differing effects on TLR8-stimulated monocytes compared to TLR7. In monocytes isolated from both HIV-negative and HIV+ donors, CBD did not enhance IL-1β secretion when stimulated through TLR8, highlighting differential effects of CBD on TLR 7 and 8 signaling. In fact, CBD promoted a decrease in TLR8-induced monocyte-derived IL-1β from HIV-infected donors, which was not seen in HIV-negative donors. As the receptors and/or cellular targets by which CBD modulates leukocyte activity aren't elucidated, it remains difficult to develop hypotheses to address these novel observations.

A limitation in this specific aim is whether the concentrations of THC used in this study are relevant to the brain concentrations observed *in vivo* of cannabis users. As it is not feasible to quantify brain concentrations of THC immediately after cannabis use in humans, we based the *in vitro* concentrations used in this report on the peak plasma concentrations, which range from 0.24-0.87μM after inhalation of a cannabis cigarette containing 3.55% THC (209). As noted above, this concentration range has the potential to be elevated due to the increased THC content in present day cannabis preparations (207). Secondly, we did not have access to effective CB1 agonists, which didn't allow us to examine the role of CB1 in IL-1β secretion by TLR7-activated monocytes. Lastly, the co-culture used in this study only examines the role of THC on monocyte-mediated astrocyte inflammation; however, other cell types (e.g., microglia, pericytes and endothelial cells) are present during *in vivo* neuroinflammation. Thus, the effect of THC on other cell-cell interactions and inflammatory processes may be different than the observations between monocytes and astrocytes.

IV. The mode of action by which THC suppresses monocyte-derived IL-1β in response to the TLR7 agonist, R837.

As TLR7-activated monocyte-derived IL-1β is a critical factor in HIV-associated neuroinflammation and notably sensitive to THC treatment, we explored the mode of action by which THC suppresses IL-1β secretion. First, we identified that the CB2 agonist, JWH-015, decreased monocyte-derived IL-1β in response to TLR7 activation, suggesting that THC is, in part, acting through the CB2 receptor, which is consistent with the current body of literature on THC-mediated modulation of monocyte function (262, 265). The non-active CB receptor metabolite, THC-COOH, had no effect on monocyte secretion of IL-1β, which further supports a CB receptor-dependent and not a non-specific mechanism. In addition, the anti-inflammatory

effects observed with JWH-015 on monocyte secretion of IL-1β provide supportive evidence for CB2 as a therapeutic target for the treatment of inflammatory conditions, which has garnered substantial interest in the context of neuroinflammation (224, 241, 303).

When evaluating the mode of action, THC was identified to suppress aspects of both major pathways for IL-1β secretion: (1) transcription/translation of pro-IL-1β; and (2) inflammasome-mediated caspase-1 activation (177, 273). Specifically, we identified that THC treatment resulted in decreased *IL-1B* mRNA levels in TLR7-stimulated monocytes. In addition to THC-mediated impairment in *IL-1B* levels, THC was also shown to suppress inflammasome activation, evidenced by inhibition of caspase-1 activity and subsequent accumulation of intracellular IL-1β within activated monocytes. Interestingly, the suppression of caspase-1 activity was observed starting at 0.5μM THC as compared to 5μM THC for *IL1B* mRNA. These observations would suggest that suppression of inflammasome activation by THC is primarily responsible for the suppression of IL-1β secretion at lower *in vitro* concentrations of THC.

A limitation when addressing the mode of action of THC is that we could not determine the involvement of CB1 in THC-mediated suppression of IL-1β secretion due to the unavailability of reliable CB1 selective agonists. Future studies will be needed to determine the role of CB1 in monocyte secretion of IL-1β.

V. Concluding remarks and future directions.

The overall conclusion based on the results presented from this dissertation is that THC suppresses specific human monocyte inflammatory processes implicated in HAND; including peripheral IFNα-mediated monocyte activation and TLR-activated monocyte-mediated astrocyte production of cytokines and chemokines (Fig. 59). In this specific context, cannabis use by the HIV-infected population may be beneficial. However, whether cannabis use is beneficial as a

whole in the HIV-infected population remains unclear, as cannabis use has the potential to affect other immune cell types and immune functions as well as functions elsewhere in the body. For example, THC has been identified to suppress functions of other immune cells in HIV-infected individuals including plasmacytoid dendritic cell secretion of IFNα and T cell proliferation (222, 236). Suppression of IFNα during chronic stages of infection may be beneficial due to its role in sustaining chronic immune activation. However, as IFNα is important for proper host defense during acute HIV infection or infection by other viruses (e.g., influenza), suppression by cannabinoids may be detrimental. In addition, cannabinoid-mediated suppression of $CD4^+$ T cell proliferation may be detrimental, as it could impair the adaptive immune response to HIV and opportunistic pathogens. This could be particularly concerning for HIV-infected individuals that don't respond well to ART and remain immune compromised (e.g., low level of circulating $CD4^+$ T cells). Finally, as mentioned in the literature review, there are other major concerns with cannabis use by HIV-infected individuals that are unrelated to the immune system including medication adherence and direct effects on cognition (e.g., impaired memory). Therefore, more research needs to be performed to gain a better understanding on whether cannabis use by HIV-infected individuals is beneficial or detrimental to immune function as well as elsewhere in the body.

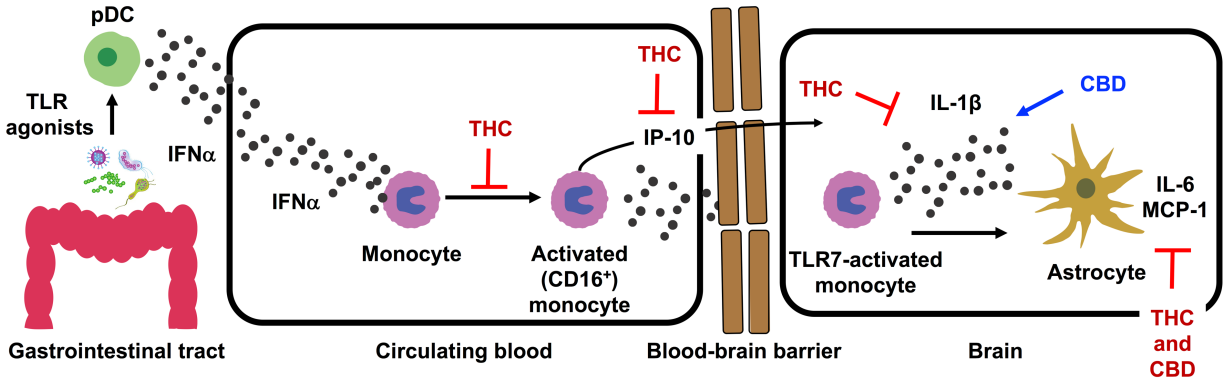

Figure 59. Schematic diagram of the observed effects of THC and CBD on monocyte activation and monocyte-mediated astrocyte secretion of inflammatory factors. The layout of the figure represents specific chronic immune activation and inflammatory events that occur in the gastrointestinal tract, circulating blood and brain during HIV infection. The proposed inflammatory cascade originates at the gut in which plasmacytoid dendritic cells (pDC) respond to microbial translocation (TLR agonists) and secrete IFNα resulting in monocyte transition to the $CD16^+$ phenotype in circulating blood. Activated ($CD16^+$) monocytes traffic across the blood-brain barrier into the brain and can become stimulated by HIV through TLR7 ligation. TLR7 ligation and activation in monocytes results in secretion of IL-1β, which promotes chronic secretion of pro-inflammatory factors (MCP-1 and IL-6) by astrocytes. *In vitro* THC (but not CBD) treatment was shown to suppress monocyte transition to the $CD16^+$ phenotype as well as secretion of IP-10 in response to IFNα stimulation. THC was also shown to impair TLR7-mediated monocyte secretion of IL-1β, which is an important factor driving astrocyte secretion of MCP-1 and IL-6. *In vitro* CBD treatment augmented monocyte secretion of IL-1β in response to TLR7 stimulation resulting in enhanced astrocyte production of IL-6 in the co-culture. THC and CBD exhibited direct inhibitory effects on MCP-1 and IL-6 production in TLR7 and IL-1β-stimulated astrocyte monocultures, suggesting immune suppressive effects on the astrocyte population.

In addition, the results presented within this dissertation show that the CB2 agonist, JWH-015, suppressed monocyte secretion of IL-1β, which provides further evidence for CB2 as a therapeutic target (224, 303). Further research will be necessary to determine the role of CB2 in other monocyte processes (e.g., monocyte transition to $CD16^{+}$) as well as its effects on other inflammatory processes implicated in neuroinflammation. In addition, experiments utilizing CB2 antagonists or CB2 knockout cells will be needed to confirm that the immunological effects observed with CB2 agonists are in fact working through CB2 ligation. Lastly, the evidence presented here on CBD promoting a pro-inflammatory enhancement on monocyte secretion of IL-1β when stimulated through TLR7 would suggest detrimental effects of recreational and/or medical use of CBD on mechanisms of chronic viral-mediated neuroinflammation. These findings with CBD warrant further investigation into the safety of CBD, especially as its use is becoming increasingly popular.

Each of the four specific aims addressed several key questions related to monocyte-dependent processes implicated in HAND and cannabis use including: (a) the role of IFNα on monocytes induction of the specific activation markers (e.g., CD16 and CD163) and chemokines (e.g., IP-10) that have been associated with HAND; (b) the role of IFNα and TLR activation on monocyte-mediated astrocyte inflammatory processes; and (c) the impact of the cannabinoids, THC and CBD, on these inflammatory processes. As a result of the advancement in our current understanding of specific monocyte inflammatory processes implicated in HAND and modulation by cannabinoids, several new questions have surfaced.

We were the first to identify that HIV-infected individuals using cannabis displayed a lower level of circulating activated ($CD16^{+}$) monocytes and plasma IP-10 compared to HIV-infected non-users (156). After publishing these findings, a group from the University of

Washington reported similar findings, in which heavy cannabis users (HIV-infected) had a lower frequency of both $CD16^+$ monocyte subsets (intermediate and non-classical) compared to HIV-infected non-users (304). Furthermore, HIV-infected cannabis users displayed a lower frequency of activated $CD4^+$ and $CD8^+$ T cell subsets as well as a decrease in inflammatory cytokine (IL-23 and TNFα)-producing antigen presenting cells (304). Together, the two reports suggest an association between cannabis use and a beneficial decrease in systemic inflammation in the HIV-infected population. Furthermore, these findings would also suggest that specific components of cannabis are promoting anti-inflammatory activity, especially on the monocyte population.

In addition to the *ex vivo* comparisons between cannabis and non-cannabis using HIV-infected individuals, we also presented *in vitro* results supporting THC as a major cannabinoid providing anti-inflammatory effects on IFNα-mediated monocyte induction of CD16 and IP-10 production. As the mechanism of monocyte induction of CD16 during HIV infection is not well understood, we also provide a putative novel role for IFNα in inducing CD16 expression on monocytes, which corresponds to the IFN gene signature observed in monocytes from HIV-infected individuals (109, 153). Based on these *in vitro* findings, new objectives have arisen, including determining: (a) the putative role of IFNα on priming monocyte secretion of pro-inflammatory cytokines and chemokines in response to microbial products; (b) the role of IFNα on the expression of key chemokine receptors and cell adhesion molecules responsible for monocyte crossing the BBB; and (c) the impact of THC and CBD on these putative mechanisms.

As gut microbial translocation and chronic IFNα production are key mechanisms promoting chronic immune activation during HIV infection (114-116), it is important to evaluate the potential for IFNα to prime monocytes to become more responsive to pattern-associated molecular patterns (PAMPs) that arise from gut-derived microbes. Prior research performed

elsewhere has demonstrated that IFNα does have the capability of priming monocytes to become more responsive to specific bacterial products (175), suggesting that IFNα may be potentiating monocyte immune responses during chronic HIV infection. Therefore, the impact of IFNα on priming monocytes from HIV-infected donors to become more responsive to both HIV-related and bacterial PAMPs should be investigated. Notable functions to be measured would include cytokine and chemokine secretion as well as the production of reactive species. As monocyte crossing the BBB is an important step implicated in HAND, the impact of IFNα alone, and in combination with PAMPs, on monocyte expression of brain-related chemokine receptors (e.g., CCR2) and cell adhesion molecules (e.g., JAM-A and ALCAM) should also be investigated. After characterizing the role of IFNα on PAMP-mediated monocyte inflammatory processes, the effect of THC and CBD should be examined. Based on the results presented here with THC suppressing IFNα-induced IP-10 production and TLR7/8-mediated IL-1β secretion by monocytes, it would be hypothesized that THC would suppress monocyte production of specific cytokines. In addition to evaluating THC and CBD, other notable constituents of cannabis, such as other cannabinoids (e.g., CBN) and terpene compounds, should be examined to gain an understanding of the putative anti-inflammatory role of these compounds. Furthermore, selective CB2 agonists should be explored to determine if CB2 ligation promotes similar actions as THC, which would provide critical insights into whether the effects observed with THC are CB2-mediated.

The second specific aim demonstrated that TLR7 activation of monocytes, in combination with IFNα, induce astrocyte production of MCP-1, IL-6 and IP-10, which are key factors involved in monocyte recruitment during HIV-associated neuroinflammation. In addition, monocyte-derived IL-1β was identified to be a critical factor governing the astrocyte response,

highlighting a potential mechanism of astrocyte dysfunction during HAND. Furthermore, the HIV-related TLR8 agonist, ssRNA40, induced a similar astrocyte production profile of MCP-1 and IL-6 in the co-culture containing monocytes from HIV-infected donors. This specific aim also demonstrates the effectiveness of the astrocyte line, U251, as a surrogate for primary astrocytes in this co-culture model, as similar conclusions could be drawn between the two.

After identifying the pro-inflammatory impact of monocytes on astrocyte secretion of specific cytokines and chemokines in a human co-culture system, several new objectives have arisen including, (a) examining the effect of HIV-related viral proteins (e.g., gp120) on monocyte-mediated astrocyte inflammation and function; (b) determining the effect of activated monocytes on other astrocyte functions (e.g., glutamate regulation); and (c) the impact of activated monocytes on other cell types in the brain (e.g., microglia).

As HIV viral proteins are thought to be a major contributor to neuroinflammation, neuronal injury and HAND (61), the impact of the viral proteins, gp120 and Tat, on monocyte-mediated astrocyte secretion of pro-inflammatory cytokines and chemokines is important to address. As gp120 and Tat have been identified to induce IL-1β production by human primary macrophages (305, 306), it would be predicted that gp120 and Tat treatment of the co-culture would promote an induction of MCP-1 and IL-6 due to monocyte induction of IL-1β; however, gp120 and Tat may have additional effects on the monocytes and/or astrocyte that could induce secretion of other cytokines and chemokines (e.g., TNFα) (142, 143, 307).

As noted in the literature review, astrocytes have several important functions in the brain including neurotransmitter homeostasis, which includes glutamate regulation (21, 24, 25). Impaired glutamate uptake is speculated to be a major mechanism promoting neuronal dysfunction and HAND (77), as excess extracellular glutamate can promote neuronal

excitotoxicity. Evaluating the effect of activated monocytes from HIV-infected donors on astrocyte-mediated glutamate uptake will be important to address, as it will help identify the extent at which monocytes alter other astrocyte functions besides astrocyte inflammation. Interestingly, recombinant IL-1β has been shown to directly inhibit glutamate uptake by human astrocytes (308), suggesting that monocytes, through IL-1β secretion, contribute to impaired glutamate control.

Numerous cell types present in the brain have the potential to be impacted by monocytes during neuroinflammation, including microglia and brain microvascular endothelial cells (BMVECs). As microglia and BMVECs are immune competent cells that have an array of immune functions, it is highly probable that monocytes alter the functions of these cells. Interestingly, there have been studies to support this notion. For example, IL-1β has been shown to induce BMVECs to secrete an array of cytokines as well as induce expression of cell adhesion molecules (e.g., ICAM-1) (295). Therefore, it would be informative to evaluate the impact of monocytes activated via HIV-related PAMPs on the function of additional cell types present within the brain.

Specific aim 3 determined that THC and CBD promoted differing effects on monocyte-mediated astrocyte production of MCP-1 and IL-6 in the R837-stimulated co-cultures containing monocytes from HIV-negative donors. THC suppressed astrocyte secretion of MCP-1 and IL-6 while CBD augmented IL-6 and had a minimal effect on MCP-1. With the use of separate monocyte and astrocyte monocultures, THC and CBD were shown to directly target both cell types. To our surprise, THC and CBD were both shown to decrease the percent of astrocytes producing IL-6 and MCP-1, which for THC, is concordant with the co-culture observation. However, CBD promoted dichotomous effects between the co-culture (increase) and astrocyte

monocultures (decrease). Our findings were explained when THC and CBD were shown to suppress and augment monocyte production of IL-1β, respectively, which was observed in monocytes isolated from both HIV-negative and HIV-infected subjects. Furthermore, THC was shown to suppress TLR8-mediated monocyte secretion of IL-1β similar to that observed with TLR7 stimulation, By contrast, CBD suppressed secretion of IL-1β in response to TLR8 stimulation, which was contradictory to the augmentation observed in TLR7-activated monocytes. Based on these results, the following areas of investigation should be examined: (a) determine whether THC and CBD promote similar effects on astrocyte production of MCP-1, IP-10 and IL-6 when co-cultures are stimulated with the aforementioned stimuli (i.e., gp120 and Tat); (b) determine if the CB2 agonist, JWH-015, suppresses the co-culture response in a similar manner as THC; (c) examine the effect of THC on monocyte secretion of IL-1β in response to other modes of activation, including HIV viral proteins (e.g., gp120 and Tat) and other PRRs (e.g., RIG-I); and (d) determining the molecular mechanism by which CBD augments R837-mediated monocyte secretion of IL-1β.

It is important to determine whether the effects observed with THC and CBD on R837-stimulated co-culture responses occurs with other stimuli such as HIV viral proteins. Therefore, the impact of THC and CBD on astrocyte production of pro-inflammatory factors in co-cultures stimulated with ssRN40 as well as HIV viral proteins should be examined. Furthermore, determining the effect of JWH-015 on astrocyte production of MCP-1 and IL-6 in the stimulated co-cultures would help to clarify whether THC is modulating cellular function through a CB2-dependent mechanism. Based on the direct inhibitory effect of JWH-015 on monocyte secretion of IL-1β in R837-stimulated monocultures in SA4, it would be predicted that JWH-015 would suppress IL-1β production and the subsequent decrease in astrocyte production of MCP-1 and

IL-6 in the R837-stimulated co-culture. In addition, if CB2 selective agonists are shown to promote anti-inflammatory effects on co-culture and monocyte-specific immune responses, it may open new areas of investigation into whether targeting CB2 could have therapeutic value in treating HIV-associated neuroinflammation.

As THC was shown to suppress monocyte secretion of IL-1β in response to TLR 7 and 8 activation and TLR4 (300), it is probable that THC modulates common aspects of the TLR-mediated signaling pathway responsible for monocyte secretion of IL-1β. A major question that arises is whether THC impairs IL-1β secretion when monocytes are stimulated via other modes of action, including HIV viral proteins (e.g., gp120 and Tat) and other PRRs (e.g., RIG-I) (305, 306, 309). Addressing this question would be informative, as it would determine whether THC suppression of IL-1β is specific to TLRs or impacting IL-1β production through a broader mechanism that is common across multiple receptor-mediated signaling pathways.

As CBD was shown to specifically augment monocyte secretion of IL-1β in response to TLR7 stimulation but not TLR8, the mechanism by which CBD promotes these effects should be investigated. Since the molecular target(s) by which CBD modulates immune function isn't well established, it will be difficult to evaluate the specific molecular mechanism. Nonetheless, determining the effect of CBD on key elements of the pathways (e.g., transcription and caspase-1 activity) involved in TLR7-mediated IL-1β secretion will still be highly informative. The CBD-modulated targets observed should be examined in the TLR8-stimulated monocytes to confirm that these targets are unique to TLR7. In addition, other modes of activation, including additional TLRs as well as viral protein activation, should be examined to identify whether the stimulatory effect of CBD on monocyte secretion of IL-1β is unique to TLR7.

In specific aim 4, THC was shown to impair monocyte-derived IL-1β in response to R837 stimulation through decreasing *IL1B* mRNA levels and caspase-1 activation, suggesting that THC is affecting both signal 1 (transcription/translation) and signal 2 (inflammasome-mediated caspase activation) in the IL-1β production and maturation pathway. Furthermore, the CB2 agonist was shown to suppress monocyte-derived IL-1β similar to that of THC, suggesting that THC is, in part, modulating monocyte function through a CB2-dependent mechanism. As the purpose of SA4 was to determine the mode of action of THC, future studies should be employed to examine the specific molecular events that are modulated by THC. In terms of signal 1, identifying the transcription factors that are involved and modulated by THC should be examined, including NFκB, CREB and AP-1. After determining which transcription factors are modulated, identifying upstream events (e.g., MAPK and PKA) that contribute to activation of the transcription factor under focus should be examined to find the initiating event modulated by THC. In terms of signal 2 (e.g., inflammasome-mediated maturation of IL-1β), the focus should be on: (a) examining the impact of THC on the expression level of the proteins comprising the inflammasome (i.e., NLRP3, ASC and pro-caspase-1); and (b) the major signaling pathway(s) for inflammasome activation (e.g., ATP release and K^+ efflux).

To conclude, the results presented within this thesis suggest that cannabinoid use, specifically, THC, by the HIV-infected population may be beneficial as an anti-inflammatory acting to impair monocyte processes implicated in contributing to neuroinflammation and HAND. Future investigations examining cannabis and/or cannabinoid use in an *in vivo* setting need to be performed to determine whether the *in vitro* observations identified here are in fact occurring *in vivo*. These future studies could utilize the SIV model to examine whether cannabinoids provide anti-inflammatory effects. In fact, recent studies have emerged that

demonstrate that *in vivo* THC administration attenuates intestinal inflammation during SIV infection (310, 311). Finally, a protocol has been published for a 12-week *in vivo* pilot study investigating the effects of THC/CBD, at various ratios, on safety, tolerability and chronic immune activation in ART-treated HIV-infected individuals (312).

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