The Effect of Cannabinoids in Myeloid Inflammation in the Context of HIV Infection

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Abstract

Background

Despite the efficacy of antiretroviral treatment (ART) to suppress human immunodeficiency virus (HIV) replication, people living with HIV (PLWH) still experience persistent immune activation and chronic inflammation that contribute to a variety of comorbidities. Macrophages are highly activated in PLWH because of the persistent antigen stimulation due to the microbial translocation secondary to increased gut permeability and intestinal immune dysregulation. These activated macrophages harbored a pro-inflammatory phenotype (M1) and secrete a high volume of inflammatory mediators, thereby sustaining the persistent activation of the immune system. Therefore, inducing a shift from this pro- inflammatory M1 phenotype to an anti- inflammatory (M2a) macrophage phenotype may be beneficial in reducing chronic inflammation in ART-treated PLWH. Phytocannabinoids cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (THC) exhibit anti-inflammatory properties, and are also widely used by PLWH, making CBD and THC interesting therapeutic strategies. Thus, herein we aim to assess the potential anti-inflammatory effects of CBD and THC on the *in vitro* differentiation of fresh monocytes into monocyte-derived macrophages (MDM), and the polarization of MDM into a pro- or anti-inflammatory subset. Additionally, we will replicate these experiments in monocyte cell lines with and without latent HIV infection. We hypothesize that the anti-inflammatory properties of cannabinoids can induce an anti-inflammatory phenotype of macrophages and total PBMC, which could result in reduced systemic inflammation in PLWH.

Methods

Monocytes of healthy donors were treated with macrophage colony-stimulating factor (MCSF) and allowed to differentiate into macrophages for six days. During this time, the cells were treated with a low (1μg/ml) and high (2.5μg/mL) dose of CBD or THC, or a low dose of CBD

and THC combined. To assess the effect of cannabinoids on macrophage polarization, after six days of differentiation with MCSF treatment, macrophages were induced from MDM into a pro-inflammatory M1 phenotype using IFN- γ and LPS, or anti-inflammatory M2a phenotype using IL-4, in the presence of high dose CBD or THC treatment. Flow cytometry was then used to immunophenotype the resulting macrophages by examining various markers of inflammation and macrophage function. The effects of cannabinoids were further explored in the U1 monocyte cell line. U1 cells were treated with Phorbol 12-myristate 13-acetate (PMA) for 48 hours, promoting their differentiation into macrophages. The cells were then treated with a high dose of CBD and THC for 24 hours, followed by characterization using flow cytometry and the HIV viral titer was determined.

Results

Cannabinoid treatment during macrophage differentiation resulted in phenotypic changes, as determined by flow cytometry. Notably, expression of cell surface markers CB2 and CCR5 both decreased with treatment with a high dose of CBD or THC. We found that there were decreased levels of TLR4, CD14, CD163 in cells treated with CBD or THC and CD16 with THC alone. CD206 expression increased with CBD treatment. There were no significant differences in the effect of CBD and THC on the polarization of MDMs to a pro-inflammatory M1 phenotype, or anti-inflammatory M2a phenotype. However, we did observe non-significant increases in CD14, CD11b and decreases of CD163 associated with THC treatment in M1 cells and decreased CD16 expression associated with THC treatment in M2a cells. We observed decreased p24 production in U1 cells treated with CBD and THC, but no associated phenotypic changes.

Conclusion

The effect of CBD and THC on myeloid cells, in terms of *in vitro* monocyte differentiation and the polarization of MDMs suggests a shift to a more anti-inflammatory phenotype. Additionally, decreased production of HIV by differentiated U1 cells suggests potential benefits for PLWH although this finding will need to be confirmed in clinical trials. Future studies should examine changes in levels of pro and anti-inflammatory cytokines to expand upon these observations.

Résumé

Malgré l'efficacité du traitement antirétroviral (TARV) qui permet de supprimer la réplication du virus de l'immunodéficience humaine (VIH), les personnes vivant avec le VIH (PVVIH) présentent toujours une activation immunitaire persistante qui contribuent à l'apparition de diverses comorbidités. Les macrophages sont fortement activés et d'un phénotype pro-inflammatoire de type M1. Par conséquent, l'induction d'un passage d'un phénotype de macrophage M1 pro-inflammatoire à un phénotype de macrophage anti-inflammatoire M2a pourrait être bénéfique pour réduire l'inflammation chronique chez les PVVIH sous TARV. CBD et THC présentent des propriétés anti-inflammatoires et nous visons ici à évaluer les effets anti-inflammatoires sur la différenciation in vitro des monocytes en macrophages dérivés des monocytes (MDM) et la polarisation de ces MDM en un sous-type pro- ou anti-inflammatoire. Nous reproduirons ces expériences sur des lignées cellulaires de monocytes avec ou sans infection latente par le VIH. Cette recherche contribuera à la compréhension générale de de l'impact des cannabinoïdes dans le contexte de l'activation immunitaire persistante et soutiendra potentiellement le développement de leur utilisation comme stratégie d'intervention pour l'inflammation chronique associée au VIH en conjonction avec le TARV.

Méthodes

Les monocytes de donneurs sains ont été traités avec le facteur de stimulation des colonies de macrophages afin d'induire leur différenciation en macrophages. Les cellules ont été traitées avec des concentrations croissantes de CBD ou de THC, ou encore avec une combinaison de CBD et THC, tous deux à faible concentration. Afin, d'évaluer l'effet des cannabinoïdes sur la polarisation des macrophages en M1ou M2a, après traitement au MCSF, des macrophages ont été induits, à partir de MDM, à l'aide de cytokines, vers un M1 ou M2a, en présence de forte dose de CBD ou

de THC. La cytométrie en flux a ensuite été utilisée pour phénotyper les macrophages obtenus, en examinant divers marqueurs de l'inflammation et de la fonction des macrophages. Les effets des cannabinoïdes ont été explorés plus en détail sur les lignées cellulaires de monocytes U1. U1 ont été traitées avec du PMA, favorisant leur différenciation en macrophages et été traitées avec une forte dose de CBD et de THC, suivies d'une caractérisation par cytométrie en flux. Un test ELISA visant la capside p24 du VIH a été utilisé pour déterminer l'effet des cannabinoïdes sur le titre viral.

Résultats

Le traitement par les cannabinoïdes pendant la différenciation des macrophages nous avons observé une importante réduction de l'expression des marqueurs CB2 et CCR5 avec le traitement de CBD et de THC. De même, l'expression de TLR4, CD14 et CD163 a également été réduite à la surface des cellules traitées avec une combinaison de THC et de CBD, et l'expression de CD16 a été réduite sur les cellules traitées exclusivement par du THC. Le traitement par le CBD a également induit une augmentation de CD206. D'autre part, nous n'avons observé aucune différence significative entre le traitement par le CBD et THC, sur la polarisation des MDM vers un phénotype M1 ou M2a. Bien que non statistiquement significative, nous avons tout de même observé une augmentation de CD14 et CD11b et une réduction de CD163 sur les cellules M1, ainsi qu'une réduction de CD16 sur les cellules M2a, induite par le THC. Nous avons observé une réduction de la production de p24 par les cellules U1 traitées par le CBD et le THC, mais sans être associée à des changements phénotypiques.

Conclusion

L'effet du CBD et du THC sur les cellules myéloïdes, en termes de différenciation in vitro des monocytes et de polarisation des MDM suggère une transition vers un phénotype plus anti-

inflammatoire. La réduction de la production de VIH par les cellules U1 différenciées induite par le traitement par les cannabinoïdes suggère que leur utilisation pourraient être potentiellement bénéfique chez les PVVIH.

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List of Abbreviations

2-AG: 2-arachidonoylglycerol

AEA: Anandamide; N -arachidonoylethanolamine

AIDS: Acquired Immunodeficiency Syndrome

APCs: Antigen presenting cells

ART: Antiretroviral Therapy

CBD: Cannabidiol

CB_1: Cannabinoid receptor 1

CB_2: Cannabinoid receptor 2

CDC: Center of Disease Control and Prevention

DAA: Direct acting antivirals

DAMPs: Damage-associated molecular patterns

DCs: Dendritic cells

DNA: Deoxyribonucleic acid

DHEA: N- docosahexaenoylehtanolamine

ELISA: Enzyme-linked immunosorbent assay

EV: Extracellular vesicle

FBS: Fetal bovine serum

FDA: U.S Food and Drug Administration

GALT: Gut associated lymphoid tissue

HAART: highly active antiretroviral therapy

HAND: HIV-associated neurocognitive disorder

Hb: Hemoglobin

HCV: Hepatitis C Virus

HEK: Human embryonic kidney cells

Hp: Haptoglobin

HSCs: Hematopoietic stem cells

HIV: Human Immunodeficiency Virus

IL: Interleukin

IP: Interferon γ-induced protein

PAMPs: Pathogen-associated molecular patterns

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate-buffered saline

PLWH: People living with HIV

PMA: Phorbol 12-myristate 13-acetate

LEA: N-linoleoylethanolamine

LPS: Lipopolysaccharide

LSM: Lymphocyte separation media

LXA_4: Lipoxin A

MCSF: Macrophage-colony stimulating factor

MDM: Monocyte-derived macrophages; macrophages dérivés des monocytes (fr)

MFI : Median FLourescent Intensity

mRNA : messager RNA

miRNA : microRNA

MUHC: McGill University Health Centre

NADA : N-arachidonoyl dopamine

Noladin Ether: 2-arachidonyl glyceryl ether

qPCR: quantitative polymerase chain reaction

RNA: Ribonucleic acid

SIV : Simian immunodeficiency virus

SVR: Sustained virological response

TARV: traitement antirétroviral

THC : Δ^9-tetrahydrocannabinol

TNF: Tumor necrosis factor

VIH: Virus de l'immunodéficience humaine (fr)

Virodhamine: O-arachidonoyl-ethanolamine

List of Tables and Figures

Preface

This thesis conforms to the McGill University Graduate and Post-Doctoral Studies' guidelines for thesis preparation.

Author contribution for Chapter 1 Literature Review:

This chapter was written by Sarah Grech, with advice and suggestions from Dr. Cecilia Costiniuk.

Author contribution for Chapter 2 Methods:

The methods described in this chapter were designed by Sarah Grech, Edwin Caballero, Dr. Ralph-Sydney Mboumba Bouassa, Dr. Franck Dupuy, Dr. Suzanne Samarani, Dr. Cecilia Costiniuk and Dr. Mohammad-Ali Jenabian.

Author contribution for Chapter 3 Results:

The experiments described in this chapter were performed by Sarah Grech, Dr. Ralph-Sydney Mboumba Bouassa, MariaLuisa Vigano, Edwin Caballero and Dr. Franck Dupuy.

Data analyses were performed by Sarah Grech, Dr. Ralph-Sydney Mboumba Bouassa and Dr. Cecilia Costiniuk.

Author contribution for Chapter 4 Discussion:

This chapter was written by Sarah Grech, with advice and suggestions from Dr. Cecilia Costiniuk.

Introduction

Myeloid cells are a key component of the innate immune system, but when consistently activated, are large contributors to chronic inflammation. In the context of HIV, myeloid cells are significant to the development of non-AIDS morbidities, particularly in the gut, which is a major source of HIV replication and pathogenesis. Dysregulation of gut mucosal integrity and gut immunity allows for the translocation of microbes and microbial products, such as lipopolysaccharide (LPS) from the lumen of the gut into peripheral circulation, resulting in systemic inflammation. During an inflammatory response, monocytes are recruited to the site of inflammation and differentiate into macrophages. Cannabinoids such as CBD and THC are known for their anti-inflammatory properties and are used widely by PLWH. Thus, it is critical to understand the effect of CBD and THC on myeloid cells, especially during this monocyte to macrophage differentiation step. Furthermore, understanding how cannabinoids impact macrophages as they are polarized into more or less inflammatory phenotypes is crucial when considering that most tissue-resident macrophages are embryonically derived and not a result of monocyte replenishment. Finally, to elucidate the effect of CBD and THC in the context of HIV, the latently HIV-infected monocyte cell line, U1, will be differentiated into macrophages, followed by treatment with CBD and THC. This will allow us to address the differences in response to CBD and THC between HIV-infected and uninfected macrophages.

Hypothesis

Cannabinoids will promote a shift in myeloid cells towards an anti-inflammatory phenotype, resulting in reduced systemic inflammation in the context of HIV.

Objectives

1) To assess the effect of cannabinoid treatment on *in vitro* differentiation of monocytes to macrophages as determined by cell surface markers.
2) To determine the effect of cannabinoid treatment on *in vitro* polarization of monocyte derived macrophages into a pro- or anti-inflammatory macrophage subset.
3) To confirm the results observed in fresh monocytes in a monocytic cell line with latent HIV infection.

Chapter 1: Literature Review

1.1 Human Immunodeficiency Virus (HIV) Origins

In June 1981, a puzzling report from the Center of Disease Control and Prevention (CDC) profiled a cluster of Kaposi's sarcomas and Pneumocystis carinii pneumonia in previously health gay men[1]. This new and infectious acquired immunodeficiency syndrome was rapidly spreading in gay men, intravenous drug users and other marginalized groups, resulting in severe stigmatization. In 1983, the causative agent of the disease was identified by Dr. Barre-Sinoussi, Dr. Luc Montagnier and Dr. Robert Charles Gallo as a lentivirus termed Human Immunodeficiency Virus[2,3]. Despite the abrupt onset and rapid spread of HIV as an epidemic in America in the 1980's, the origins of the HIV epidemic arose much earlier and across the globe. Supercomputers estimated that HIV entered the human population between 1915 and 1945 through zoonotic crossover of simian immunodeficiency virus (SIV) from non-human primates[4]. The earliest viral HIV sequence to date has been identified in a 1959 plasma sample from the Democratic Republic of Congo[5].

While HIV is a relatively new virus to humans, it is genetically diverse. The broad term HIV refers to two separate virus types, HIV-1, and HIV-2. HIV-1 is the most common form of HIV and is most closely related to SIVcpz, a strain of HIV infecting chimpanzees[4]. HIV-1 can be further broken down into M, N, O, and P subtypes[6]. HIV-1 group M is the makes up most of the worldwide cases of HIV, and includes subtypes A, B, C, D, F, G, H, J, K[6]. Clade B is the most common strain found in Europe and North America, with clades A and C common in southern and eastern Africa[7]. HIV-2 is originated from SIVsm, found in sooty mangabeys, and is largely restricted to West Africa[8,9]. HIV-2 differs from HIV-1 in that mortality and transmission rates are much lower[10]. Due to the prominence of HIV-1 worldwide, and especially in a Canadian context, the focus of this thesis will be HIV-1.

1.2 HIV Epidemiology

HIV is a retrovirus that infects and may lead to the death of immune cells, most notably CD4+ helper T cells, but also myeloid cells including macrophages and dendritic cells. This results in immune suppression and the development of acquired immunodeficiency syndrome (AIDS), and potentially death, if left untreated. With over 38 million PLWH worldwide and 63,000 in Canada, HIV constitutes a large global disease burden (Figure 1)[11,12]. HIV is spread through bodily fluids, including blood, semen, breastmilk, vaginal fluids, and rectal secretions[13]. Estimation of per act transmission places risk of HIV infection highest with blood transfusions, followed by vertical transmission, mother-to-child, and receptive anal intercourse[14]. Other means of transmission include the sharing of needles during injection drug use, needlestick injuries, and other sexual activities[14].

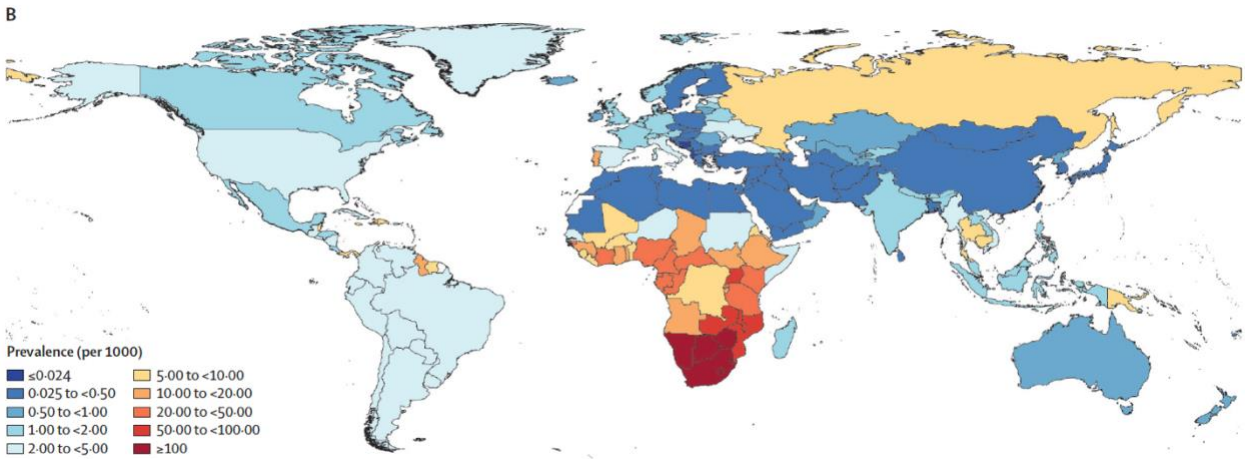


Figure 1. Global prevalence of HIV. Incidence per 1000 people based on data from 2017[15].

Antiretroviral therapy, which is used to suppress HIV replication and prevent progression to AIDS, has dramatically increased the life expectancy of PLWH to be nearly on par with the life expectancy of uninfected individuals[15,16]. As of 2020, 28.2 million people, or 73% of PLWH were able to access ART[17]. However, while ART acts as a treatment for those already infected, it is not able to cure HIV infection. The inability of ART to cure HIV is a consequence of the virus' ability

to form a latent reservoir in infected cells, which persists and then rebounds when ART is stopped. This is because the main classes of antiretrovirals target various stages of the viral life cycle. The stages of the viral life cycle are 1) docking of the virus onto a target cell, 2) the viral single-stranded RN Ais released into the cell cytoplasm, 3) transcription of RNA to DNA with reverse transcriptase, 4) integration of viral DNA into host DNA within the nucleus, 5) transcription of viral mRNA by the host, 6) protein translation and post-translational cleavage facilitated by HIV proteases, 7) viral maturation and budding (Figure 2)[18]. Fusion or entry inhibitors, such as Fuzeon and Maraviroc, prevent this initial step of HIV docking to the target cell[18]. Two classes of drugs, nucleoside reverse transcriptase inhibitors (NRTIs) and non- nucleoside reverse transcriptase inhibitors (NNRTIs) target this second step, preventing effective reverse transcription of HIV RNA into DNA[18]. Integrase inhibitors prevent the integration of viral DNA into the host DNA at step 4. Finally, protease inhibitors prevent the cleavage of viral proteins that is necessary for viral protein maturation[18].

To prevent mutations of HIV that would lead to viral resistance of these medications, as was seen in early treatment plans, multiple medications from various viral classes are used in combination. This combination therapy is often referred to as highly active antiretroviral therapy (HAART), as it is unlikely that the virus will be able to develop resistance to various medications that target different stages of the viral lifecycle. Current treatment guidelines recommend beginning treatment with two NRTIs and in combination with a drug of one of the other classes[19].

ART has the additional benefit of effectively preventing the spread of HIV during sex if complete viral suppression in the blood is achieved[20-22]. Even in the ART era, with effective HIV prevention strategies, approximately 2,100 new infections occur annually in Canada[23]. Still, Moreover, despite effective ART treatment, PLWH still experience chronic immune activation

and persistent inflammation, resulting in a high burden of chronic diseases, including non-AIDS-related malignancies, such as colon and lung cancer, cardiovascular diseases, and neurocognitive disorders[24,25]. Thus, there is a need for therapies to prevent the chronic inflammation that contributes to these non-AIDS morbidities and mortality.

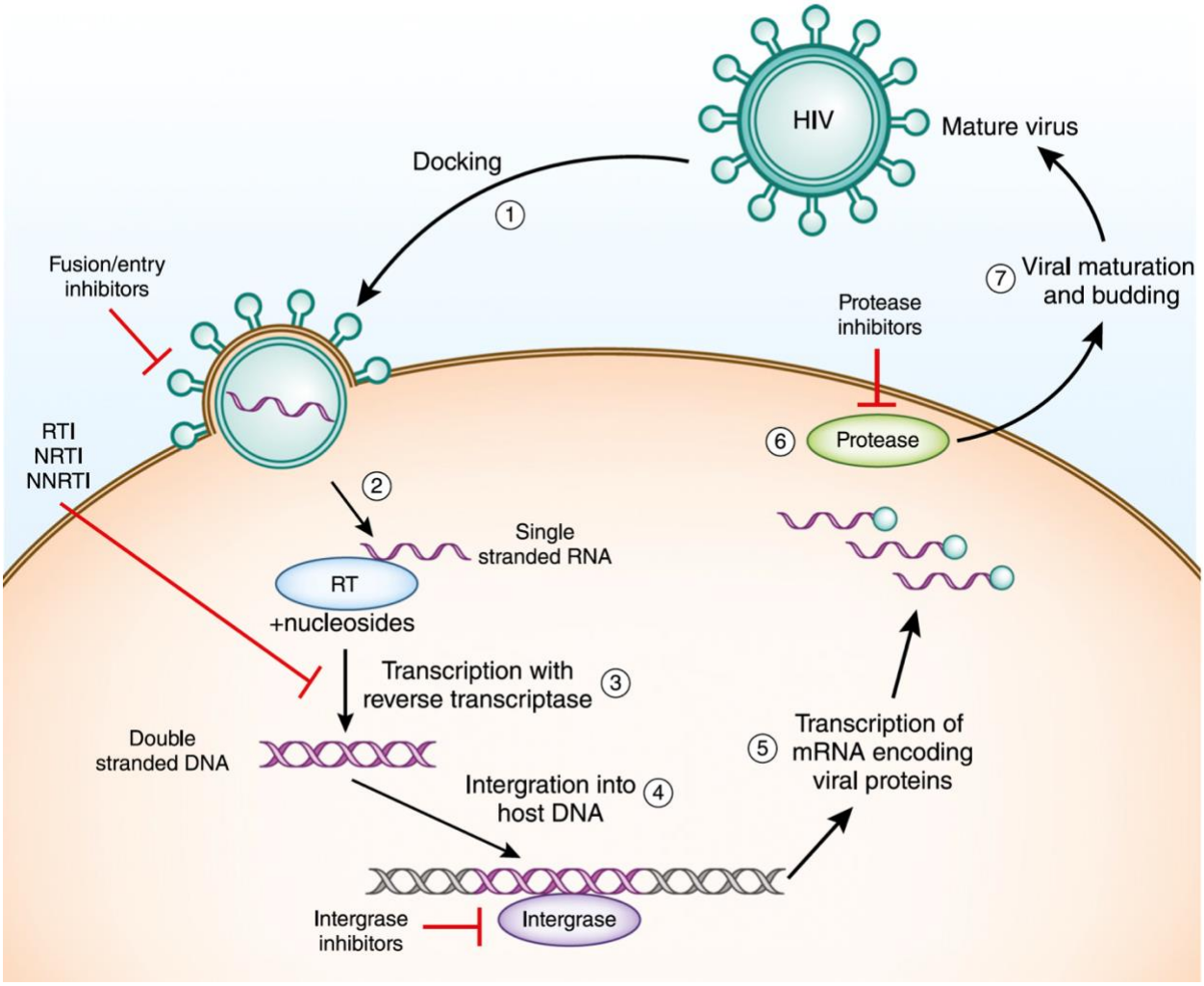


Figure 2. Therapeutic targets of antiretrovirals. The seven stages of the HIV viral lifecycle are outlined, with the drug classes that inhibit viral replication at that stage showcased[18].

1.3 Chronic Inflammation During HIV Infection

The non-AIDS morbidities that often inflict PLWH, including cardiovascular disease, chronic obstructive pulmonary disease, and diabetes, are a result of the interaction of multiple

factors. These factors include the toxicity of antiviral drugs and high rates of comorbidities such as substance abuse, obesity, and hypertension[26]. Another major contributing factor is chronic inflammation and immune dysregulation. This chronic inflammation is evident through persistently increased immune activation markers, such as interferon γ-induced protein (IP)-10, interleukin (IL)-6, MCP-1, and sCTLA-4 in PLWH, despite ART treatment[27,28]. Furthermore, sCD14, an indicator of the monocyte response to lipopolysaccharide (LPS), was determined to be a predictor of mortality during untreated HIV infection[29]. Additionally, sCD14 levels and IL-6 levels were correlated during HIV infection, suggesting that the microbial products that lead to monocyte activation also led to inflammatory markers[29]. ART treatment has been shown to reduce or normalize some signs of inflammation associated with HIV infection, that are dependent on the ART regimen used, amongst other factors. In most cases, incomplete immune restoration of the GALT is found following ART, however, there are some scenarios in which near-complete restoration of gut immunity have been described[30]. This incomplete immune restoration is often characterized by heightened levels of inflammatory factors, lowered CD4+ T cell and dysregulated CD8+ T cell levels in comparison to an uninfected individual[30]. For example, elvitegravir/cobicistat/emtricitabine/ tenofovir disoproxil fumarate has been shown to decrease sCD14 levels more effectively than efavirenz/emtricitabine/tenofovir disoproxil fumarate treatment[31]. In the context of monocyte inflammation, a cocktail of raltegravir/ emtricitibine/tenofovir resulted in reduced inflammatory markers CD86 and HLA-DR, and increased chemokine receptors CCR2 and CXCR1[32].

The current paradigm highlights several potential causes of increased inflammation during HIV infection. Firstly, HIV infection itself both directly activates HIV-specific CD8+ T cells and indirectly activates CD8+ T cells[33]. This may be a result of the cytokine-mediated bystander effect,

which may also be dependent on antigen exposure[34]. Secondly, other pathogens, such as cytomegalovirus, contribute to this chronic inflammation in the context of HIV through higher levels of CD4+ and CD8+ T cells than seen in controls without HIV infection[25,36]. T cells were more highly activated during chronic infection compared to recent infection, and the T cell response was found to be accentuated following ART treatment[37]. Thirdly, in HIV infection, viral replication largely takes place in gut associated lymphoid tissue (GALT), leading to CD4+ T cell depletion and enteropathy[38-40]. Combined, this results in a disruption of the endothelium, allows for the translocation of microbes and microbial products, such as LPS, from the lumen of the gut into peripheral circulation (Figure 3) [41,42]. Monocytes and macrophages are activated by LPS through interactions with CD14 and TLR4, initiating a pro-inflammatory cytokine cascade which, in turn, promotes viral replication and further immune activation[43,44]. Impaired immunoregulatory may contribute to chronic inflammation[26]. Furthermore, gut damage has the negative effect of decreasing oral ART absorption, further contributing to inflammation[45]. A systemic review of relevant literature found that during HIV infection, greater microbiome diversity correlated negatively with markers of microbial translocation, such as sCD14 and LPS[46]. However, overall, there is a decrease in the mean species diversity of the gut[47]. The composition of the microbiome also changes in HIV-infected individuals, with increases in species of Bacteroidetes, Actinobacteria, Proteobacteria, and Firmicutes and decreases in *Prevotella*[46]. In a metagenome analysis of the functional genes in the microbiomes of ART-treated PLWH in comparison to uninfected individuals, there was an increase in genes involved with inflammatory pathways such as LPS synthesis and bacterial translocation decreases in genes involved in amino acid metabolism and energy processes[48].

One of the complications of HIV infection is the latent reservoirs that develop early on in acute infection. These reservoirs harbour virus, largely in memory CD4+ T cells and macrophages, allowing for viral rebound if ART is interrupted[49]. Common viral reservoirs are the lymph nodes, gut, lungs, brain, and testes in males[49]. This may, in part, be due to the difficulties of many antiretrovirals to penetrate tissues, such as rectal tissues, and its ability to enter immune privileged sites, such as crossing the blood-brain barrier to enter the brain[50,51]. Much viral replication occurs in the GALT due to it being an initial site of HIV infection, as well as the high proportion of HIV target cells, including T cell and macrophages[52]. This low-level viral replication can result in viral shedding, even in the context of effective ART treatment, though this shedding is unlikely to contribute to HIV transmission[20-22]. Research of which I co-authored, however, shows that low-level anorectal shedding of HIV during effective ART treatment is not associated with the parameters we measured, including epithelial integrity, T cell activation, neutrophil infiltration, and cytokine secretion[53]. Additionally, bacterial load was lowered in those shedding HIV, with no differences in bacterial composition[53].

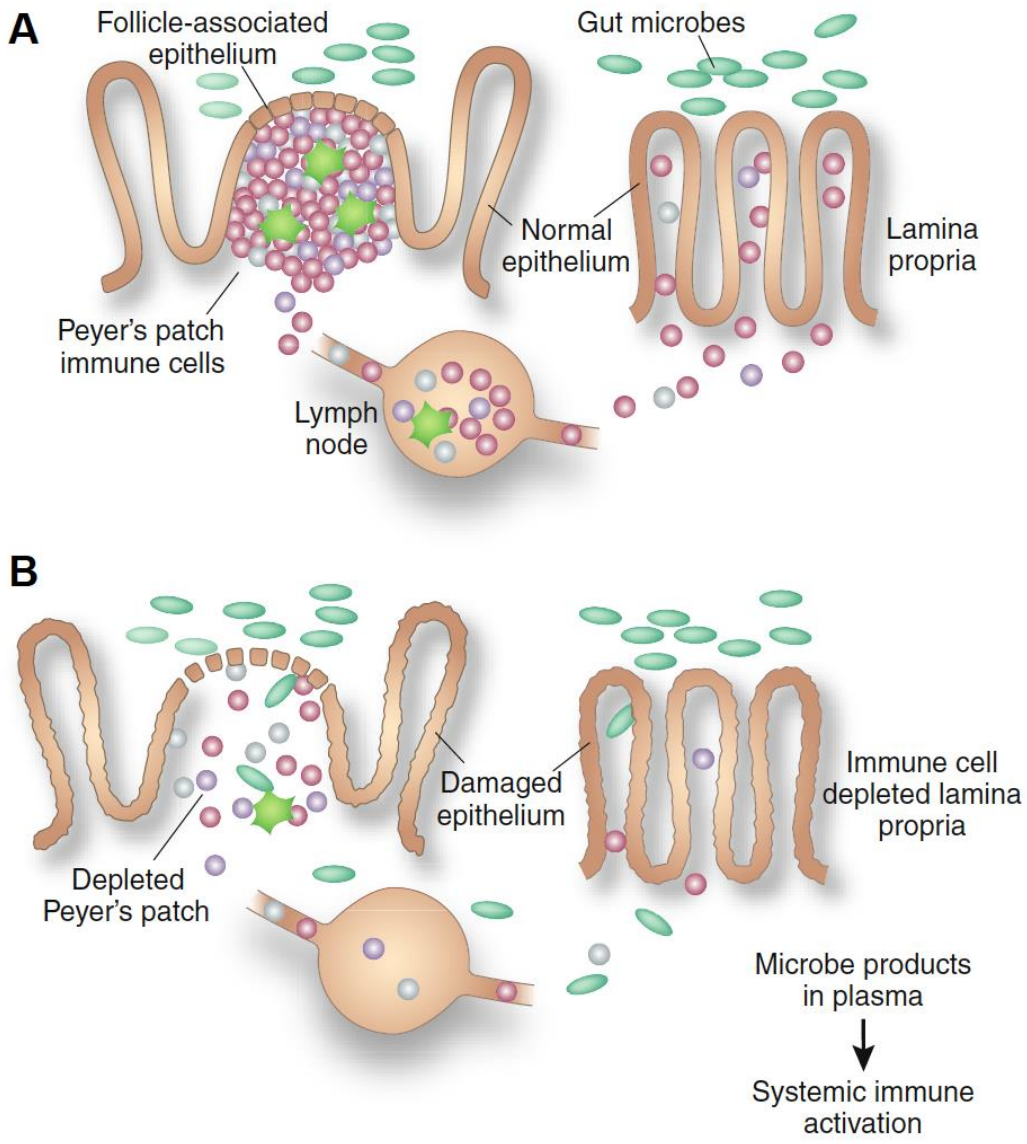


Figure 3. The effect of HIV infection on the gut endothelium. (A) The endothelium of a health individual. Microbes do not cross the epithelium and there are abundant immune cells. (B) The endothelium during HIV infection characterized by immune cell depletion, epithelial breakdown, and microbial translocation. This results in microbial products such as LPS entering circulation and subsequent system immune activation[41].

1.4 Macrophage Origins

Macrophages are a type of tissue-resident myeloid cell that specialize in the detection, phagocytosis, and destruction of dead cells, bacteria, debris, and other foreign material. Macrophages play a crucial role in the innate immune system through their phagocytic abilities,

but also communicate with components of the adaptive immune system, such as T cells, through antigen presentation to adaptive immune cells, such as T cells.

Macrophages have distinct developmental origins, occurring prenatally and postnatally (Figure 4). Postnatally, circulating monocytes derived from hematopoietic stem cells (HSCs) are attracted to sites of infection and differentiate into macrophages. Prenatally, embryonic macrophage development begins very early in fetal development, in the blood islands of the extraembryonic yolk sac[54]. These cells then disseminate once blood circulation has been established[55]. Macrophages are crucial during fetal development to clear debris from tissue remodeling and play a role in vascularization[56,57]. Additionally, aorta-gonad-mesonephros-derived HSCs colonize the liver, resulting in the generation of hematopoietic cells, including monocytes[54]. These monocytes infiltrate peripheral tissues, resulting in tissue-resident macrophages such as alveolar macrophages in the lungs, microglia in the brain, Kupffer cells in the liver and Langerhans cells in the skin[58]. Tissue-resident macrophages are characterized by their long lifespan and capacity for self-renewal[58]. Thus, replenishment of tissue-resident macrophages by MDMs is minimal, as shown in sophisticated fate-mapping studies[59].

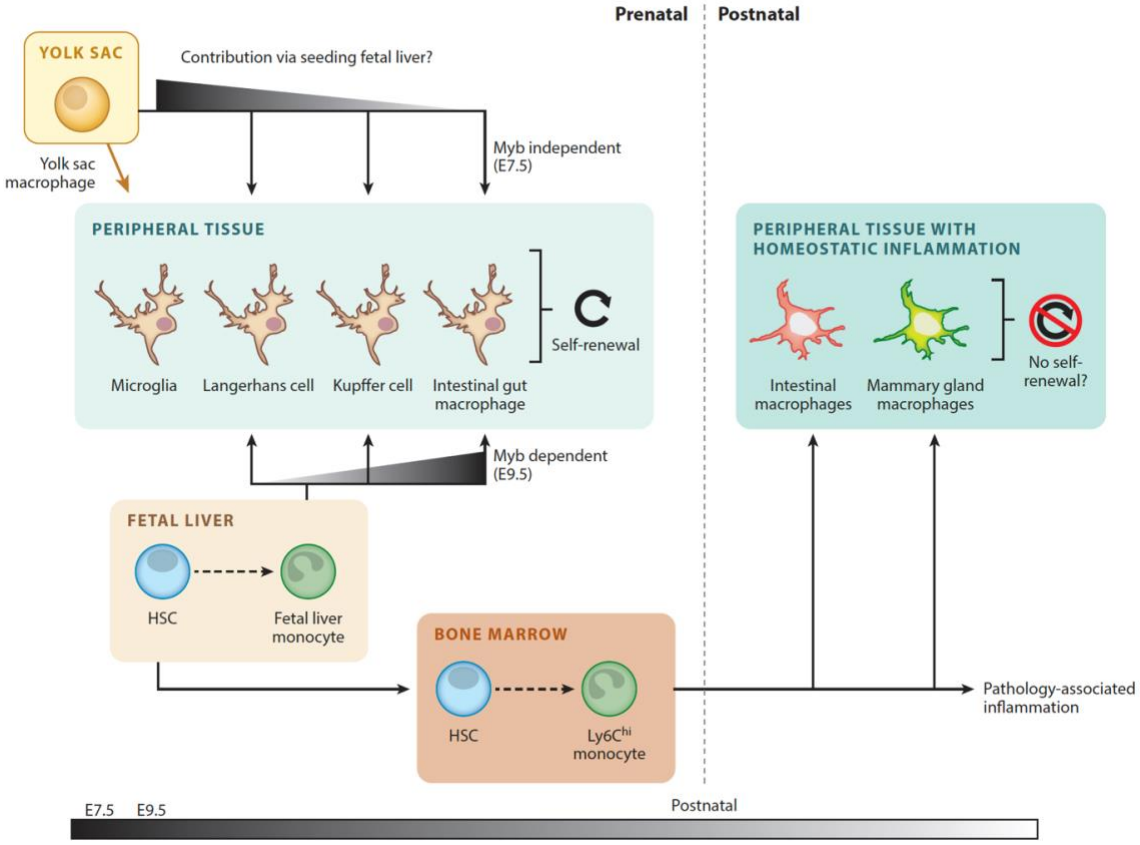


Figure 4. The developmental origins of tissue-resident macrophages[54].

1.5 Macrophage Biology

Macrophages can be classified as pro-inflammatory macrophages, often known as M1 macrophages, and anti-inflammatory or M2 macrophages (Figure 5). M1 macrophages are classically activated macrophages and are critical to host defense against pathogens through phagocytosis and release of pro-inflammatory cytokines including IL-6, tumor necrosis factor (TNF)-α and IL-1[60]. M2, or alternatively activated macrophages, are critical to the resolution of the immune response, tissue repair, remodeling, and wound healing[61].

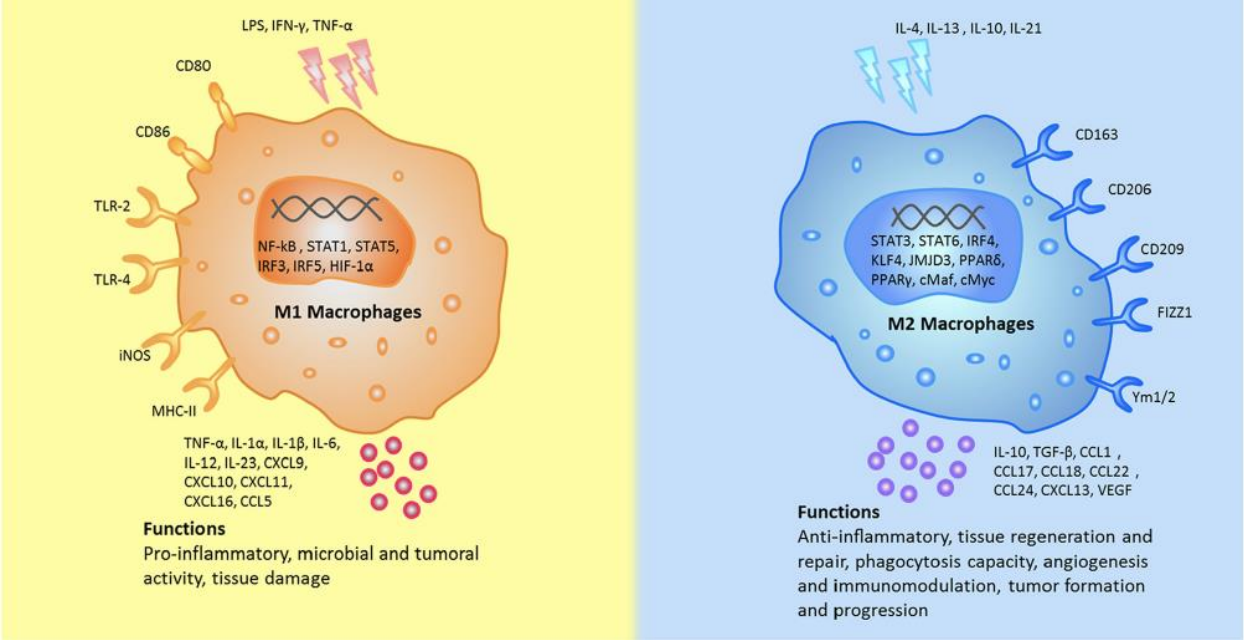


Figure 5. A summary of the physiological functions, surface markers, stimuli and secreted cytokines of macrophage M1 versus M2 macrophage subsets[60].

M2 macrophages can be further broken down into M2a, M2b, M2c and M2d macrophages that all produce anti-inflammatory cytokines including IL-10 (Figure 6)[60]. Although it is convenient to categorize M1 and M2 macrophages as pro- and anti- inflammatory, it must be noted that this is an oversimplification of macrophage diversity. The differentiation of M1 and M2 macrophages is often considered as a spectrum[62]. For example, M2 macrophages also express pro-inflammatory cytokines such as IL-6, TNF-α and IL-1[60].

M2a macrophages are critical to tissue repair and promote cell growth, endocytic activity[60]. M2b macrophages regulate the immune response[48]. M2c macrophages are responsible for the phagocytosis of apoptotic cells[64]. M2d macrophages lead to angiogenesis and contribute to the progression of tumors[60]. It is important to consider that M1 and M2 macrophages do not work in isolation from each other. Removal of damaged tissue and dead cells associated with M1 killing and tissue restoration is performed by M2 macrophages[65].

For the purposes of this study, the research focus will be on M1 and M2a macrophages. While the M2a phenotype is not representative of all M2 macrophages, M1 and M2a macrophages mediate Th1 and Th2 responses, respectively[66]. This is of particular interest in our context as Th1 dominance over the Th2 response is associated with HIV resistance and decreased progression to AIDS[67]. For example, HIV controllers, people who maintain viral control without the use of ART, show a bias towards the Th1 response[68]. Thus, in this study, M1 macrophages will be used as a pro-inflammatory mediator of the Th1 response and M2a macrophages will be used as an anti-inflammatory mediator of the Th2 response.

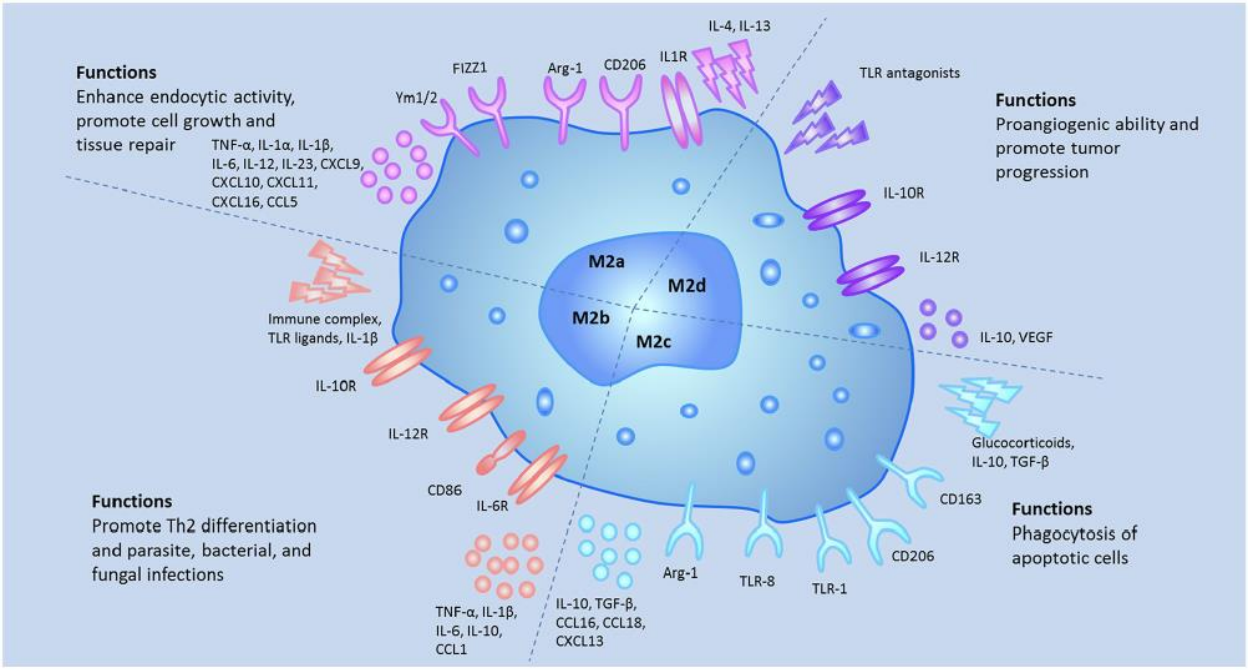


Figure 6. A summary of the physiological functions, surface markers, stimuli, and secreted cytokines of macrophage M2 subsets[60].

1.6 The Pro-Inflammatory Role of Macrophages

During an inflammatory response, typically due to tissue injury, cells of the innate immune system, mainly neutrophils, tissue resident macrophages, and monocytes are recruited to the site of injury. Recruitment is mediated by pathogen-associated molecular patterns (PAMPs), damage-

associated molecular patterns (DAMPs), and chemokines, including CCL2[69,70]. At the site of infection, these monocytes differentiate into M1 macrophages and secrete pro-inflammatory cytokines, including IL-12, IL-23 and Il-1β (Figure 7a)[69]. These cytokines activate other immune cells, including the Th1 and Th17 pro-inflammatory responses[69]. Macrophages further assist in CD4+ helper T cell proliferation, by acting as antigen presenting cells (APCs)[71]. Uptake of necrotic neutrophils, which often occurs at the site of inflammation, enhances the antigen presenting activities of macrophages[71].

1.7 The Anti-inflammatory Role of Macrophages

Once the source of inflammation has been cleared, macrophages play a critical role in the resolution of inflammation. Macrophages are responsible for the clearance of any dead cells and debris resulting from inflammatory response through phagocytosis. The clearance of apoptotic neutrophils by macrophages, known as efferocytosis, is especially important to prevent secondary necrosis and further inflammation[72]. The process of apoptosis is highly regulated to ensure that further inflammation is not induced through the release of intracellular contents, as in the case of necrosis[73]. Accordingly, apoptotic cells release soluble and membrane-bound vesicular "find-me" signals that act as chemoattractants for macrophages, including sphigosine-1-phosphate, CX3CL1, and nucleotides ATP and UTP[74-76]. At the site of apoptosis, macrophages recognize "eat-me" signals, such as phosphatidylserine and annexin-1, and living cells express "don't eat me" signals[69].

During the resolution of inflammation, the macrophage phenotype switches from a pro- to an anti-inflammatory state[77]. This switch has been illustrated in a mouse model of skeletal muscle regeneration, in which inflammatory monocytes were recruited to the site of tissue damage and differentiate into pro-inflammatory MDMs[78]. These pro-inflammatory MDMs quickly convert to

an anti-inflammatory phenotype, that in this context contribute to tissue repair through stimulating myogenesis and fiber growth[78]. In a model of murine inflammatory bowel disease, an M1 to M2 switch was induced, eliminating the symptoms of the conditions[79]. This underscores the significance of macrophages in both driving and resolving inflammation, particularly in the gut. These anti-inflammatory M2 macrophages have a variety of roles including producing lipoxin A (LXA_4), which reduces neutrophil recruitment to the site, lessening the Th1 and Th17 responses, producing anti-inflammatory mediators, and re-establishing the epithelial barrier (Figure 7b)[69,80]. In our study, we are interested in investigating the role of cannabinoids in inducing an M1 to M2 shift in macrophages, and the applications of such a shift in the chronic inflammation associated with HIV infection.

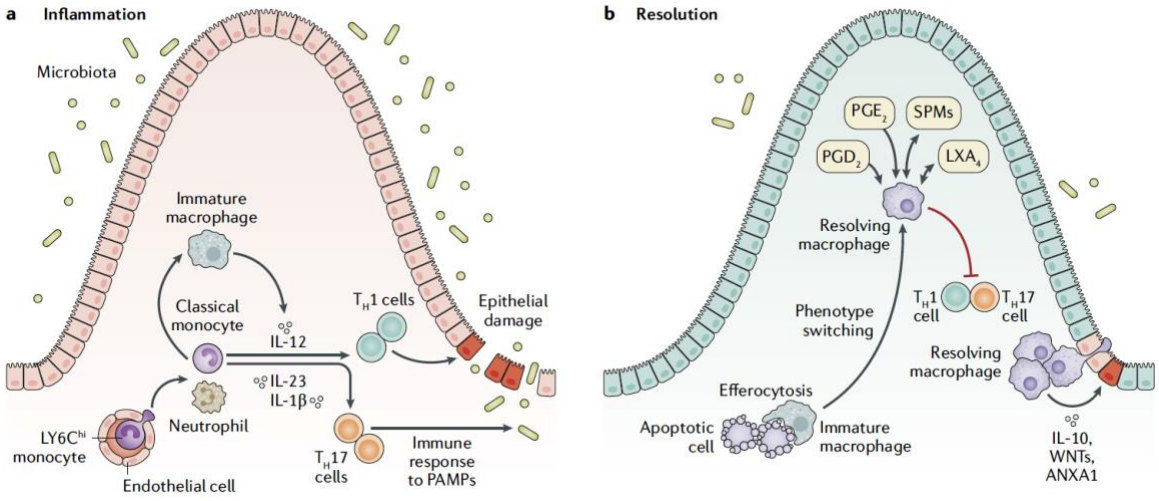


Figure 7. Macrophage function in intestinal (a) inflammation and (b) resolution. Macrophages have distinct roles during and after an inflammatory response[69].

1.8 The Role Myeloid Cells During Chronic Inflammation

Myeloid mediated immune activation is a major driver of chronic inflammation and has tissue specific effects, non-AIDS-related malignancies, such as colon and lung cancer, cardiovascular diseases, and neurocognitive disorders. For example, in the context of

atherosclerosis, LPS and macrophage activation marker sCD14 are predictors of progression of subclinical atherosclerosis[81]. sCD14 is known to bind LPS, delivering it to vascular endothelial cells, which are subsequently activated by the bound LPS, resulting in further inflammation[26]. The likelihood of developing atherosclerosis is increased for PLWH, with an increase in associated monocyte activation markers such as sCD14 observed in men with HIV and atherosclerosis[82]. Macrophages are also associated with the development of HIV-associated neurological diseases (HAND). sCD14 has been correlated with neurocognitive disease development and severity as well[83,84]. An early paper on the study of AIDS dementia complex found that in mild cases, brains of dementia patients with HIV contained increasing amounts of macrophages correlating with disease severity[85]. The presence of abundant macrophages in HAND is now known to facilitate HIV reservoir formation, causing continued inflammation[86]. Potential mechanisms include the HIV directed shift of proteasomes to immunoproteasomes thus disrupting cellular homeostasis[87]. Inflammation caused by microbial translocation may also be due to microglial priming from circulating microbial products, or from immune reconstitution inflammatory syndrome[87]. Overall, in the context of HIV infection, myeloid cells, specifically macrophages and monocytes, are drivers of chronic inflammation, which can result in a large range of pathologies across the body's organs and systems.

1.9 History of Cannabis

Cannabis, and its active medicinal components, cannabinoids, are derived from a flowering plant from the *Cannabaceae* family. The cannabis plant was used in prehistory largely for its fibres in the production of various textiles[88,89]. The origins of the first uses of cannabis for its pharmacological properties have been largely debated; however, its usage has spread across the globe[90].

Early medical uses of cannabis exploited the plant's sedative, relaxant, anxiolytic, anti-biotic, anti-inflammatory, and anti-convulsant properties[89]. For example, Ancient Persians used cannabis to treat migraines, uterine pain, prevent miscarriages and as a muscle relaxant[90]. An Ancient Egyptian medical text, known as the Ebers Papyrus, describes the use of cannabis in the treatment of gynecological disorders ("to cool the uterus") and for the treatment of parasitic infections of the toes[90].

Medical research into the pharmacological use of cannabis occurred in the nineteenth century in Europe. However, interest into the medical properties of cannabis experienced a significant decline in the first half of the twentieth century[89]. Due to the development of pure opioids, such as cocaine and morphine, the cannabis plant was seen as too variable in composition, unpredictable in its effects, and had a shorter shelf life[89]. These issues, compounded by legal barriers arising at the same time, resulted in very little research into the pharmacological uses of cannabis.

Currently, cannabis research is undergoing a revival fueled by the legalization of recreational cannabis in many jurisdictions including several American states and Canada. Cannabinoid treatment has proven successful in many clinical trials. The focus of many successful clinical trials into the use of cannabinoids has been pain associated with a wide array of conditions including diabetic peripheral neuropathy pain, fibromyalgia, and peripheral neuropathic pain associated with allodynia[91-93]. Despite many successful clinical trials, the U.S Food and Drug Administration (FDA) currently only has one approved CBD product[94]. Epidiolex is an oral solution that is used to treat severe seizures for people with Lennox Gastaut syndrome, Dravet syndrome, or tuberous sclerosis complex[95].

In the context of PLWH, cannabis has been historically used to self-treat nausea, pain, anorexia and wasting syndrome caused by HIV itself, and later caused by secondary effects of some ART regimens[96]. Today, many PLWH use cannabis for management of chronic pain, stress, anxiety, and anorexia in addition to recreational usage[97]. Cannabis use is common among PLWH, with over 75% of PLWH reporting lifetime cannabis use, in contrast with 45% of uninfected individuals studied in an American cohort[98].

1.10 Active Compounds in Cannabis

Over 550 unique compounds have been isolated from the resin of the cannabis plant, including 120 terpenes and 113 phytocannabinoids[99]. Other compounds include hydrocarbons, nitrogen-containing compounds, carbohydrates, flavonoids, fatty acids, non-cannabinoid phenols, alcohols, and esters[99].

Terpenes are compounds that contribute to the characteristic smell and taste of the cannabis plant. These include α-pinene, myrcene, limonene, β-caryophyllene, and linalool[99]. Some terpenoids found in cannabis plants have been shown to perform a variety of pharmacological functions including, but not limited to, diminishing inflammation, relaxing muscles, decreasing pain, and reducing anxiety[100-104]. While often not the focus of cannabis research, which often focuses on isolated and purified cannabinoids or synthetic cannabinoids, terpenes have been suggested to contribute to a synergistic phytocannabinoid-terpenoid entourage effect[105]. This could be a factor contributing to the traditional belief that botanical cannabis is more efficacious than synthetic or isolated cannabinoids[106].

Phytocannabinoids are terpenophenolic lipids with a characteristic phenol ring (Figure 8)[106]. Of the cannabinoids derived from the cannabis plant, THC and CBD are the focus of much research due to their known medicinal properties. THC is the main psychoactive compound in cannabis. CBD does not exert a psychoactive effect but has been associated with many of the medicinal properties of the plant, including reducing inflammation, pain, and anxiety. Other "minor cannabinoids" such as tetrahydrocannabivarin, cannabigerol and cannabichromene have proven therapeutic benefits[104].

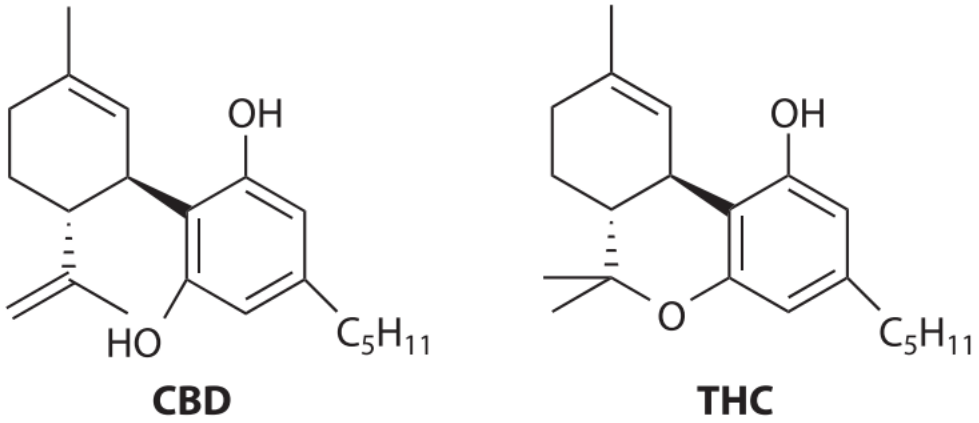


Figure 8. The chemical structures of Δ^9-tetrahydrocannabinol (Δ^9-THC) and cannabidiol (CBD) [107].

1.11 The Endocannabcostinoid System

The endocannabinoid system is a ubiquitous lipid signalling system, composed of endocannabinoids, the G-protein coupled cannabinoid receptors cannabinoid receptor type 1 (CB_1) and CB_2, and enzymes to degrade or synthesize cannabinoids[104,108]. The endocannabinoid system appeared early in evolution, having been identified in invertebrates such as sea urchins and mollusks[109]. Cannabinoid receptors have been highly preserved throughout evolution, with the human, rat, and mouse CB_1 receptor containing 97-99% matched amino acids[108]. This high level of preservation in both vertebrates and invertebrates underscores the importance of the

endocannabinoid system in the regulation of cognition, pain, sensation, mood, memory, locomotion, motivation, hunger signals, motility, gut permeability, and inflammation[96].

Endocannabinoids have been found in all tissues, organs, and bodily fluids[110]. Key endocannabinoids include anandamide (AEA; N -arachidonoylethanolamine), 2-arachidonoylglycerol (2-AG), 2-arachidonyl glyceryl ether (noladin ether), N-arachidonoyl dopamine (NADA), and O-arachidonoyl-ethanolamine (virodhamine)[111]. The function of these signalling molecules largely depends on the location of the cannabinoid receptor to which it binds. Similarly to the phytocannabinoid-terpenoid interactions in the context of the cannabis plant, an entourage effect between endocannabinoids and other metabolites is hypothesized to increase the activity of endocannabinoids[112]. Work by Ben-Shabat *et al.* shows that 2-AG activity was increased by endogenous 2-acyl-glycerols, 2-linoleoyl-glycerol and 2-palmitoyl-glycerol, esters that had no biological effect alone[113].

CB_1 receptors are found mainly in the central nervous system, resulting in psychoactive effects of cannabis, but is also found in the liver, kidneys, lungs, and gut (Figure 9)[96]. Within the brain, CB_1 is found at high levels in cerebral cortex, hippocampus, lateral caudate putamen, substantia nigra pars reticulata, globus pallidus, entopeduncular nucleus and the molecular layer of the cerebellum[114]. Thus, the function of CB_1 agonism is dependent on its location, resulting in both inhibitory and excitatory functions[114]. CB_2 is found predominantly on immune cells. In order of decreasing mRNA (messenger ribonucleic acid) levels, CB_2 is found on B cells, natural killer cells, monocytes, neutrophils, CD4+ T cells, and CD8+ T cells[115]. Macrophages highly express CB_2 on the cell surface[116]. This expression has been found to vary, depending on the activation state of the cell, with higher CB_2 expression during periods of inflammation, such as in

inflammatory bowel disease[116,117]. Due to its presence on immune cells, CB_2 plays a significant role in inflammation.

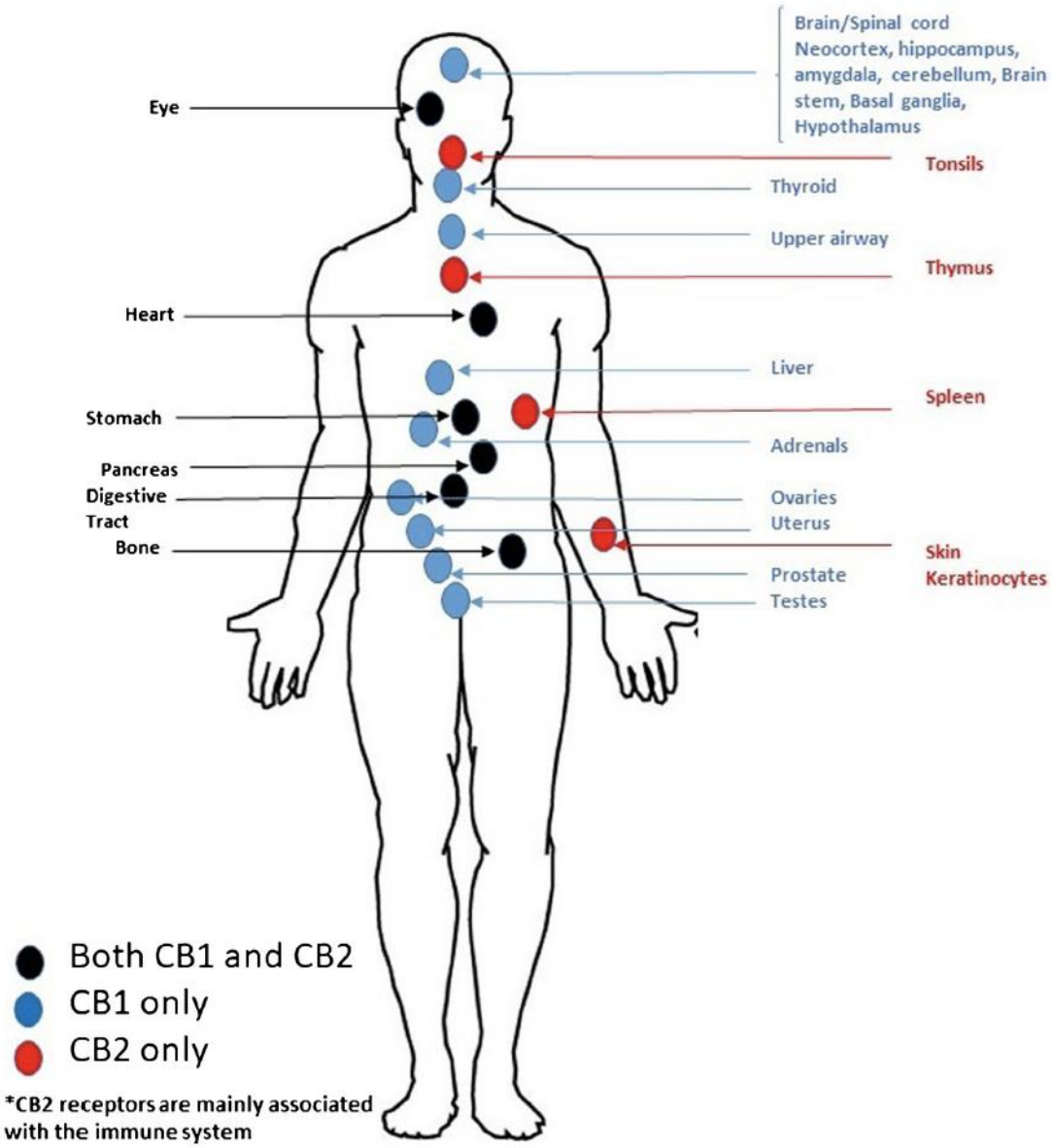


Figure 9. Distribution of CB_1 and CB_2 receptors within the human body. The widespread distribution of cannabinoid receptors contributes to the widespread physiological impact of cannabinoids[118].

1.12 The Effects of Cannabinoids on Myeloid Cells

As previously mentioned, cannabinoids have been associated with anti-inflammatory properties. In U937 cells, a monocyte cell line, CBD has been shown to attenuate LPS-induced release of IL-8 and MCP-1 through the NF-ϰB pathway[119]. Similar suppression of inflammatory cytokines, such as TNF- α and IL-1β were seen in a THP-1 monocyte cell line that had been differentiated into macrophages with CBD treatment[120]. This anti-inflammatory effect likely occurs through several different mechanisms, including anti-inflammasome activity and induction of autophagy[120,121]. Furthermore, CBD is shown to have pro-apoptotic effects on monocytes, further supporting its anti-inflammatory effects[122]. THC is associated with decreased macrophage activation as evident through decreased macrophage spread, phagocytic abilities, and IL-1 production[123.124]. In the context of human monocyte-derived dendritic cells (DCs), THC altered features characteristic of DC differentiation, including promoting less effective antigen uptake and decreased surface receptors including CD11c and HLA-DR[125].

One limitation of these *in vitro* studies is that much of the research on the effects of cannabinoids on myeloid cells is that CBD and THC are studied independently. While this is beneficial in that the direct impact of each compound can be elucidated, most cannabis use includes both CBD and THC. Additionally, in the case of smoked cannabis, or other methods of consuming the cannabis plant as opposed to isolated CBD or THC compounds, the entourage effect of terpenes and other components of the cannabis plant may additionally exert an effect on macrophages that cannot be determined by these studies. Furthermore, understanding the effect of cannabinoids during the key step of monocyte differentiation into macrophages is critical to understanding the impact to the inflammatory response.

1.13 The Effects of Cannabinoids on Lymphoid Cells

While the evidence suggests that cannabinoids exert an anti-inflammatory effect on myeloid cells, the data on the impact of cannabinoids on lymphoid cells is less certain. In Jurkat cell lines, a model of both CD4+ and CD8+ T cells, THC was shown to decrease T cell activation by inhibiting T cell receptor signalling[126]. THC has been shown to decrease human T cell function by decreasing CD40L expression and the deoxyribonucleic acid (DNA)-binding of NFAT and NFkB to the CD40L promoter[127]. With respect to CD8+ T cell effector function, it is impacted by reduced IFN- γ secretion and cytolytic degranulation[128]. Interestingly, suppression of CD8+ T cell function was shown to be independent of CB_1 and CB_2 in $CB_1^{-/-}$ $CB_2^{-/-}$ mice[129]. Cannabinoids have been shown to activate other receptors including transient receptor potential vanilloid 1 (TRPV1), the orphan G-coupled receptor GPR55, the 5-HT1α receptor, and the α3 and α1 glycine receptors[130-132]. Additionally, CBD has been shown to induce a Th1 to Th2 shift, as determined through mRNA expression[133]. This is significant, as M1 and M2 mediate the Th1 and Th2 responses. Thus, a switch to Th2 dominated T cell expression indicates a M2 dominated macrophage population.

There is some evidence, however, suggesting a pro-inflammatory role of cannabinoids on lymphoid cells. For example, in HUT-78 T cells both CBD and THC treatment inhibited production of the anti-inflammatory cytokine, IL-10[134]. Additionally, in human tonsillar B cells, THC has been shown to promote dose-dependent proliferation[135]. Taken together, the impact of cannabinoids on lymphoid cells is inconclusive in terms of pro- or anti- inflammatory effects.

1.14 The Role of Cannabinoids in the Context of HIV Infection

Due to the potential anti-inflammatory roles of cannabinoids, and the chronic inflammation characteristic of PLWH, the role of cannabis in the context of HIV *in vitro* models, during SIV infection of non-human primates and human studies have been well explored.

1.14.1 In vitro *studies*

Similarly to our study, Williams *et al.* set out to understand the effect of THC in monocyte differentiation in regards to susceptibility to HIV infection. Crucially, this paper reported decreased HIV infection of macrophages treated with THC during differentiation, due to a reduction of HIV receptors CCR5, CXCR4, and CD4 on the cell surface[136]. Along these lines, agonism of CB_2 has been shown to decrease cell-to-cell spread of HIV as well as attenuate viral replication[137,138]. Cytokines RANTES, MIP-1a, and MIP-1B have been identified as HIV-suppressive factors[139]. In SRIK-NKL, an NK cell line, THC was shown to reduce production of these HIV-suppressive cytokines[134]. CBD was also shown to reduce production of these cytokines in SRIH-B (ATL), a B cell line[134]. Regarding cellular function, THC has been shown to attenuate IFN-α mediated T cell activation, both in peripheral blood mononuclear cells (PBMCs) from PLWH and healthy controls[140]. Overall, these *in vitro* studies underscore a positive effect of cannabinoids, with a focus on THC, in the context of HIV infection, both in terms of reducing the ability of the virus to infect cells and through reduced immune activation.

1.14.2 *Animal Studies*

Non-human primates, specifically rhesus macaque models of SIV infection are commonly used to study HIV pathogenesis as there are no other natural immune competent animals that can be infected with HIV[141]. Primate models are attractive due to the similarities between primates and humans, as well as the existence of natural SIV infection. These studies are limited in that SIV,

and HIV are genetically different viruses and may present differently pathologically[142]. Additionally, primates have long lifespans, making these experiments lengthy and costly. Alternatively, "humanized" mouse models are created by grafting human immune cells into immunodeficient mice, allowing for HIV replication in human CD4+ T cells or myeloid cells[142]. However, many strains of humanized mice are prone to graft-versus-host disease and the development of spontaneous thyroid lymphomas[143-146].

Seminal work by Kumar *et al.* studied the effect of 0.18mg/kg of THC twice daily, beginning 4 weeks prior to infection with SIV on the gut of SIV-infected rhesus macaques [44]. The dose was increased to 0.32mg/kg of THC over a period of two weeks, once tolerance to the lower dose had been developed[44]. In the THC treated macaques the percentage of anti-inflammatory CD163+ macrophages increased, in combination with a decrease in the absolute number of CD8+ T cells and markers of T cell proliferation and activation, Ki67, HLA-DR, and PD-1[44]. Importantly in the context of SIV infection, which severely depletes CD4+ T cells, no change was observed in CD4+ T cell levels[44]. Furthermore, expression of tight junction markers occludin and claudin-3, epithelial regeneration marker PROM1 and stress protector keratin-8 were all increased with the THC treatment[44]. Taken together, this evidence supports a model of decreased inflammation during SIV infection, following THC treatment, that is driven by gut restoration and homeostasis. This model is supported by Molina *et al.* who showed that in duodenal tissues, there was a shift in cytokine expression to a more Th2 phenotype, including increased levels of IL-4, IL-5, IL-6, and IL-13 as well as shifts in gene expression that indicates an anti-apoptotic and regenerative effect[146]. Furthermore, microRNA (miRNA) modulates pro-inflammatory molecules through the upregulation of mRNAs including miR-10a, miR-24, miR-99b, miR-145, miR-149, miR-187, miR-204[44,147]. miRNAs are a group of non-coding RNA that typically bind complementary RNA

sequences, leading to degradation as a form of post-transcriptional regulation[147]. The anti-inflammatory targets of these miRNA include MMP-8, a matrix-degrading collagenase targeted by miR-204 and NOX4, a known reactive oxygen species targeted by miR-99b (Figure 10) [44,148].

Despite the apparent benefits of THC on SIV-infected rhesus macaques, there is a potential sex difference, with THC treatment protecting male macaques from early death due to SIV, but no impact observed in a female cohort[149,150]. While the exact cause of this potential sex difference in clinical outcomes is unknown, sex-specific differences should be considered in further research on cannabinoids in HIV as women account for nearly half of new infections globally and one quarter of new infections in Canada[11,12]. In a mouse model, cannabinoids were shown to regulate HIV_{gp120}-specific T cell responses, with the impact dependent on the stimulation, potentially explaining how the same cannabinoid can result in differential responses[141].

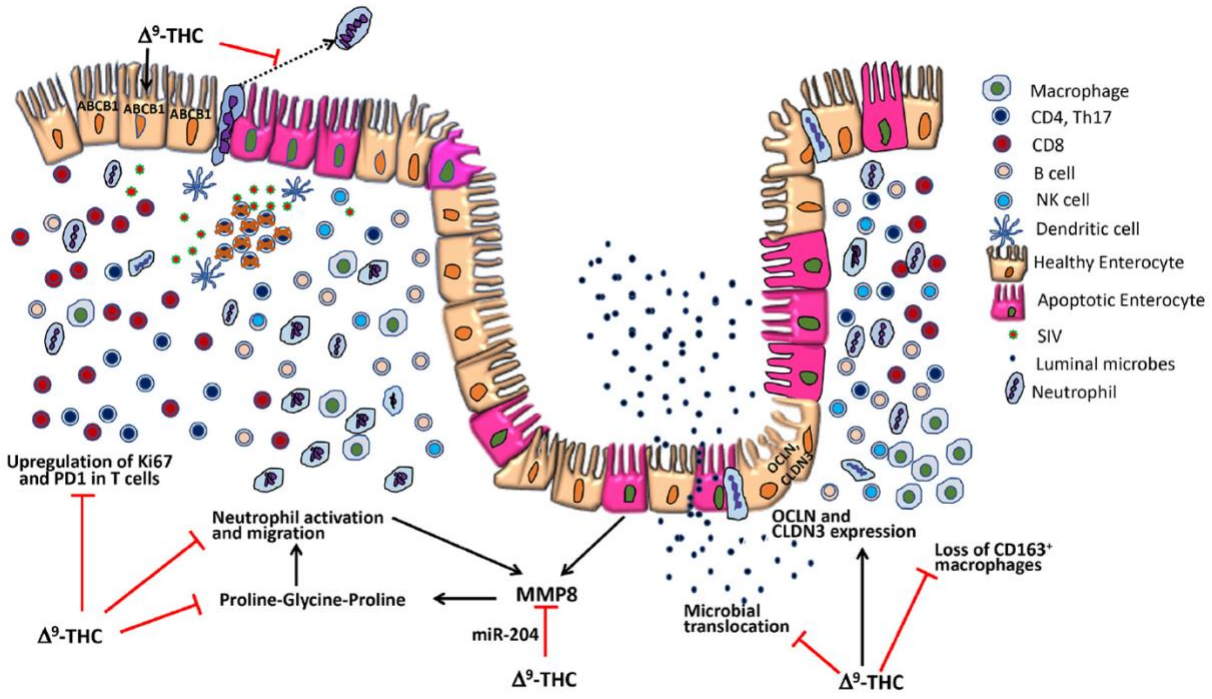


Figure 10. A model of the proposed impact of cannabis on the gut during SIV infection. THC has proposed effects on gut integrity, microbial translocation, inhibiting activation of immune cells including neutrophils and T cells, and preventing loss of anti-inflammatory CD163+ macrophages[44].

1.14.3 *Human Studies*

Much of the research into the effect of cannabinoids on human subjects are observational studies. These observational studies by design have the major disadvantage of being unable to control for numerous potential confounders. Much of this research has been based on self-reported cannabis usage, which is subject to self-report bias, which occurs when a person either does not know the correct answer or wants to answer in a way that is socially desirable. Further, much cannabis sold for recreational purposes has significantly higher levels of THC than CBD. Smoking of the grinded cannabis plant is the most common mode of administration of cannabis recreationally[151]. Plants contain thousands of molecules including terpenes and flavonoids, which exert an "entourage effect" and change the effects produced when THC and CBD are administered alone[104]. In these studies, it is difficult to control for CBD and THC concentrations which, as explored throughout this thesis, can exert very different physiological effects. It is also impossible to elucidate causation from observational studies, only association. The findings of key studies are summarized in Table 1.

Table 1. Summary of human studies on the role of cannabinoids during HIV infection.

Reference	Population	Study design	Key Findings
Keen *et al.* 2019[152]	Black adults from Florida, USA. Half females. 39% of participants had detectable viral load.	Observational study.	Participants who test positive for THC had higher CD4+ and CD8+ counts than those who tested negative for THC
Bon-Miller *et al.* 2014[153]	Adults from California, USA. 20% female. ART treated.	Observational study. Cannabis use self-reported	Participants who were dependant on cannabis reported more severe symptoms of HIV/ART and lower ART adherence.
Rizzo *et al.* 2018[154]	Adult males from Michigan, USA. ART treated. 76% of cannabis users and 86% of cannabis non-	Observational study.	Cannabis use was associated with decreased levels of IP-10 and monocytes in peripheral circulation.

	users had undetectable viral loads.		
de Oliveria Feitosa de Castro *et al.* 2019[155]	Adults from Brazil on ART. Mostly males. Undetectable viral loads.	Observational study.	Cannabis use was associated with reduced levels of inflammatory monocytes and increased sCD14 levels.
Adams *et al.* 2018[156]	American adult males.	Long-term observational study.	Cannabis usage showed no impact on mortality over a 5-year period.
Manuzak *et al.* 2018[157]	Adults from California, USA. Mostly male. ART treated. Viral load less than 75 $\log_{10}$ copies/mL.	Observational study.	Heavy cannabis use was associated with decreased activated T cells and intermediate and non-classical monocytes.
Vidot *et al.* 2019[158]	Adult men from California, USA. ART treated, undetectable viral load.	Observational study.	Heavy cannabis use was associated with higher levels of sCD14 than non-users and non-hazardous users.
Bredt *et al.* 2002[159]	Adults from California, USA. ART treated. Mostly male. Must have stable viral load (less than threefold change over 16-week period).	Randomized control trial. Participants provided with pre-rolled marijuana cigarettes containing 3.95% THC. Dronabinol (cannabinoid used to treat nausea) and placebo arms also included.	No significant changes in immune cell phenotype or function noted over the 21-day period.
Abrams *et al.* 2003[160]	Adults from California, USA. ART treated. Mostly male. Must have stable viral load (less than threefold change over 16-week period).	Randomized control trial. Participants provided with pre-rolled marijuana cigarettes containing 3.95% THC. Dronabinol (cannabinoid used to treat nausea) and placebo arms also included.	No significant changes in HIV RNA or protease inhibitor levels, or CD4+ and CD8+ T cell counts noted over the 21-day period.

In an observational study of HIV-infected cannabis users on ART in Brazil, cannabis use was associated with reduced levels of inflammatory, intermediate, non-classical, activated-classic,

and activated-inflammatory monocytes, as well as lower levels of monocyte activation spontaneously or following LPS stimulation[155,157]. Counterintuitively, increased levels of sCD14, a marker of monocyte activation and indirectly, a marker of microbial translocation have also been observed[155,158]. CD16+ monocyte levels were also seen to be reduced with cannabis use along with IP-10 levels, a proinflammatory molecule produced by monocytes, and a driver of neuroinflammation[154]. In regard to T cells, higher levels of CD4+ and CD8+ T cells, but lower levels of activated CD4+ and CD8+ T cells have been reported with cannabis use[153,157]. Significantly, people who reported to be dependent on cannabis, as opposed to people who use cannabis but were not dependent, saw decreased adherence to ART, a higher viral load and thus, more severe symptoms[153]. This is significant to our understanding of other observational studies as ART adherence has known impacts on inflammation including increased levels of inflammatory markers IL-6, sCD14[159,160]. Over a five-year period, however, cannabis use in PLWH had no effect on mortality[156].

Two older articles were published on the same randomized controlled trial, which was undertaken to assess the impact of cannabis on PLWH. While the randomized control overcomes many of the limitations discussed regarding controlling variables, the short 21-day duration of the study limits our understanding of prolonged cannabis usage[161,162]. Additionally, these studies were limited in breadth of immune function assessed, noting no significant changes in HIV RNA levels, T cell counts, protease inhibitor levels, NK cell function[161,162]. No data was shown on monocyte phenotypes, count, or functionality.

Chapter 2: Methods

2.1 Study Population

Donors were recruited from the Glen site of the McGill University Health Centre (MUHC). Donors were healthy adults, both males and females, who reported no chronic or acute medical conditions. The sex and age of the donors is reported in Table 2.

Table 2. Demographics of study population.

Participant ID	Age	Sex
1	43	F
2	55	F
3	42	M
4	34	M
5	21	F
6	39	F

2.2 Isolation of PBMCs from Whole Blood

Fresh whole blood was collected in lavender top EDTA Vacutainer tubes. The Vacutainer tubes were centrifuged at 300xg for 10 minutes to separate and remove the plasma. The remaining blood was diluted with equal parts RPMI 1640, layered over lymphocyte separation media (LSM) (Wisent Bioproducts, Saint-Jean-Baptiste, QC, Cat# 305-010-CL) and centrifuged at 400xg for 30 minutes with the break off. The interface between the plasma/RPMI layer and the LSM layer, which contains the PBMCs, was aspirated. If the cells were to be used downstream with the EasySep™ Human Monocyte Enrichment Kit without CD16 Depletion (StemCell Technologies, Vancouver, BC, Cat # 19058), then an additional 120xg spin with no break was added to remove platelets. In the case that red blood cells were visually evident in the pelleted PBMCs, 500uL red blood cell lysing buffer (BD Biosciences, San Jose, CA cat#555899) was added for 5 minutes,

then washed off with phosphate-buffered saline (PBS) (Wisent Bioproducts, Saint-Jean-Baptiste, QC, Cat # 311-010-CL). The PBMCs were resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS) (Wisent Bioproducts, Saint-Jean-Baptiste, QC Cat# 080-150) and 1% penicillin/streptomycin (Wisent Bioproducts, Saint-Jean-Baptiste, QC, Cat# 450-201-EL) for downstream use (Figure 11).

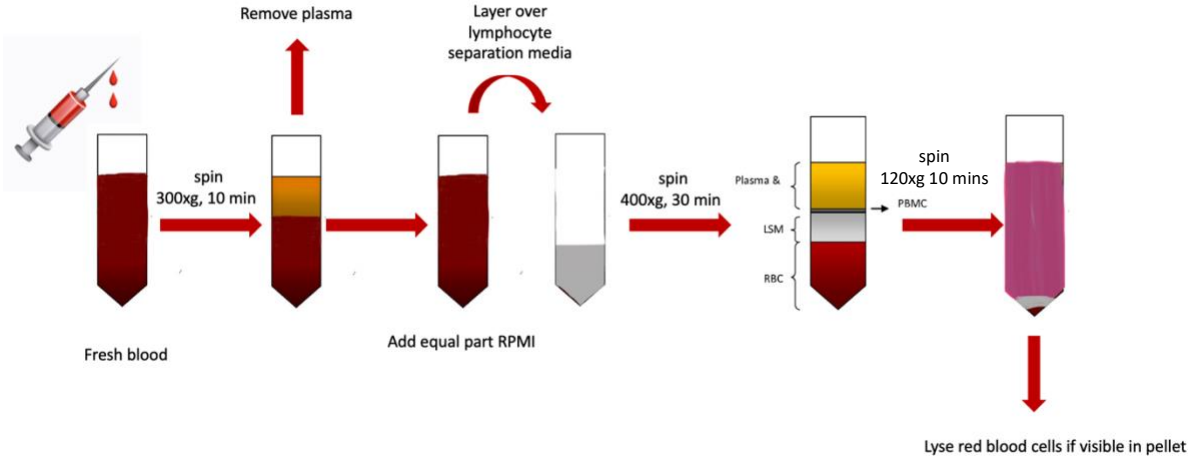


Figure 11. Isolation of PBMCs from fresh blood. Fresh blood from healthy individuals was obtained and PBMCs were isolated using lymphocytes separation media.

2.3 Monocyte Differentiation to Monocyte-Derived Macrophages (MDMs)

To address aim 1, which is to assess the effect of cannabinoid treatment on *in vitro* differentiation of monocytes to macrophages, monocytes isolated from PBMCs were differentiated *ex vivo* into MDMs. Fresh PBMC isolation was completed as described above. Monocytes were isolated in one of two ways. Method one, the adherence method, relied on the adherent nature of monocytes in comparison to other PBMCs. Method two involved using the EasySep™ Human Monocyte Enrichment Kit without CD16 Depletion as described by the manufacturer. Method one was used for preliminary experiments, including our initial classification of MDM and for one

sample included in the phenotypic characterization of MDM for aim 1. Following optimization, method two was subsequently used as it is quicker, easier, and allows for more consistency between wells on a plate. PBMCs were isolated as described above and plated at a concentration of 1 million cells/well on a 12 well plate in RPMI. Cells were allowed to adhere for 2 hours at 37ºC, 5% CO_2. Non-adherent cells were removed, and adherent cells were washed twice with PBS. The cells were then treated with RPMI containing 10% FBS and 1% penicillin/streptomycin and 10ng/mL recombinant human macrophage-colony stimulating factor (MCSF) (R&D Systems, Minneapolis, MN, cat #216-MC-025-CF). Treatments of 1µg/mL and 2.5µg/mL of CBD, THC or MeOH or 1µg/mL of both CBD and THC in combination were added at this time. Concentrations of CBD and THC used had been determined by us to be non-toxic to cells. Lower levels of CBD and THC were selected to be used in combination to ensure we were elucidating the effect of the compounds in combination without the confounding factor of increased cytotoxicity. The half-life of cannabinoids varies dramatically in human studies, largely dependent on method of administration, amongst other factors[163-165]. For example, the half-life of THC following inhalation ranges from 1-3 days for occasional users to 5-13 days for chronic users[164,166]. The half-life for CBD is shorter, ranging from 18-32 hours[167,168]. Cells were allowed to differentiate at 37ºC, 5% CO_2 for 6 days, with half the media in each well replaced on day 3, including MCSF and any treatment. Following differentiation MDMs could be kept in culture for further downstream experimentation. On day 6, MDMs destined for flow cytometry were detached with Accutase (StemCell, Vancouver, BC, cat # 07922) treatment at 37ºC, 5% CO_2 for 45 minutes, and washed twice with PBS to ensure complete detachment of cells. The cells are now ready for Flow Cytometry analysis (Figure 12).

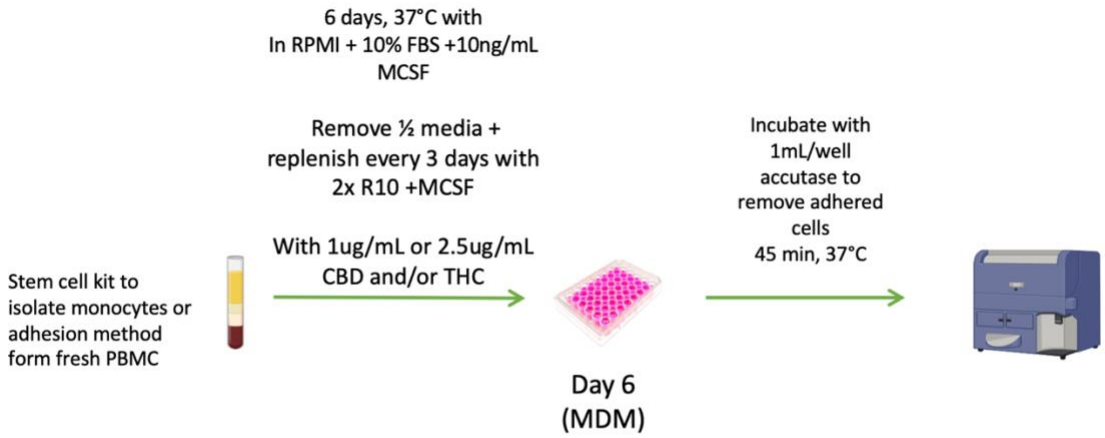


Figure 12. Workflow for differentiation of monocytes to MDMs with cannabinoid treatment. Isolated monocytes were plated in the presence of MCSF and various concentrations of cannabinoids for 6 days, during which they differentiate into macrophages. The cells are then characterized using flow cytometry.

2.4 Polarization of MDMs

MDMs differentiated from fresh monocytes as described above, without cannabinoid treatment, were then used to address aim 2; to determine the effect of cannabinoid treatment on *in vitro* polarization of monocyte derived macrophages into a pro- or anti-inflammatory macrophage subset. On day 6, media was replaced with fresh RPMI containing 10% FBS and 1% penicillin/streptomycin. The media contained 20ng/mL Recombinant human (rh) IFN-γ (Invitrogen, Eugene, OR, cat# VB2938721) and 1μg/mL lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO cat# L2630) to induce an M1 phenotype and 20ng/mL rhIL-4 (R&D, Systems, Minneapolis, MN, cat # 204-IL) to induce a M2a phenotype. During initial macrophage phenotyping experiments the following cytokines were used for differentiation: 20ng/mL rhIFN-γ and 1μg/mL lipopolysaccharide (LPS) to induce an M1 phenotype, 20ng/mL rhIL-4 to induce a M2a phenotype, 1μg/mL LPS and 10ng/mL rhIL-1β (R&D, Systems, Minneapolis, MN, cat # 201-LB) for M2b, and 10ng/mL rhIL-10 for M2c (R&D, Systems, Minneapolis, MN, cat # 217-IL).

During polarization, cells were treated with 2.5µg/mL of CBD, THC or MeOH. Cells were allowed to polarize at 37ºC, 5% CO_2 for 2 days, after which they were detached with Accutase treatment at 37ºC, 5% CO_2 for 45 minutes and washed twice with PBS to ensure complete detachment of cells for Flow Cytometry analysis (Figure 13).

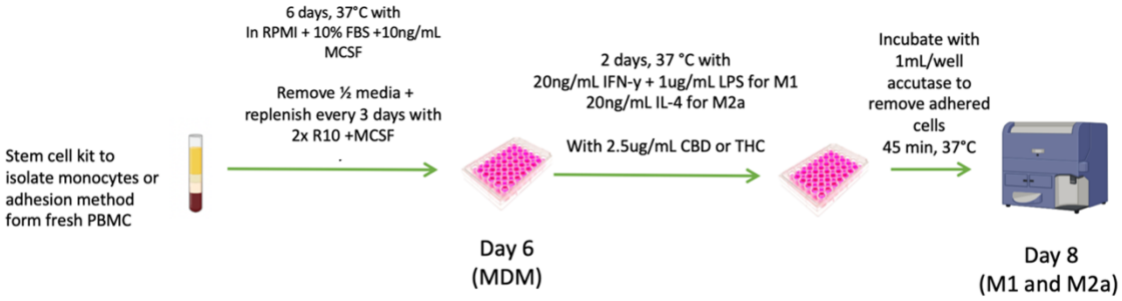


Figure 13. Workflow for the polarization of MDMs into a pro- or anti-inflammatory subset during cannabinoid treatment. Isolated monocytes were plated in the presence of MCSF and various concentrations of cannabinoids for 6 days, during which they differentiate into macrophages. For two days, the MDMs are treated with cytokines to induce an M1 or M2a phenotype in the presence of CBD or THC. The cells are then characterized using flow cytometry.

2.5 Cannabinoid Cytotoxicity Tests

Prior to any experiments with CBD or THC, the toxicity of these compounds on PBMCs was determined. We performed the cytotoxicity tests on whole PBMCs instead of isolated monocytes as during optimization we considered repeating similar experiments on lymphocytes. Cryopreserved PBMCs from a healthy donor were treated with 0, 0.16, 0.32, 0.64, 1.25, 2.5, 5, and 10µg/mL of CBD (Cayman Chemical Company, Ann Arbor, MI, cat # ISO60156), THC (Cayman Chemical Company, Ann Arbor, MI, cat# ISO60157), and the vehicle control, methanol (MeOH). After 24 hours, the cells were stained for flow cytometry using the panel outlined in Table 4. Annexin V and Live/Dead staining was used in combination to identify dead and dying cells and monocytes, T cells, CD4+ T cells and CD8+ T cells were analyzed independently.

Table 4. Immunophenotyping markers for cannabinoid toxicity tests.

Marker	Fluorochrome	Clone	Cat #	Company	Description
Live/Dead Fixable Aqua Stain	405nm excitation	N/A	L34957	Invitrogen, Eugene, OR	A cell viability marker that reacts with amines in dead cells with a permeabilized membrane
Annexin V	APC	N/A	550474	BD Biosciences, San Jose, CA	Detects apoptotic cells by binding to phosphatidylserine
CD3	FITC	HIT3a	555339	BD Biosciences, San Jose, CA	T cell marker
CD4	PE-Cy5	RPA-T4	555348	BD Biosciences, San Jose, CA	Marker of T helper cells
CD8	APC-H7	SK1	560179	BD Biosciences, San Jose, CA	Marker of cytotoxic T cells
CD14	BV786	M5E2	563698	BD Biosciences, San Jose, CA	Detects bacteria by binding LPS; expressed on classical monocytes
CD16	Alexa Fluor 700	3G8	557920	BD Biosciences, San Jose, CA	Fc receptor; expressed on non-classical monocytes

2.6 Immunophenotyping

To assess markers of inflammation and macrophage function, cells were stained with the antibodies outlined in Table 3. CB_1 (PE, clone 368302) (R&D Systems, Minneapolis, MN, cat # FAB3834P) was used in initial macrophage characterization experiments but removed from the final panel. Prior to staining, cells were blocked with Fc Block (Miltenyi Biotec, Bergisch Gladbach, Germany, cat# 130-159-901) for 20 minutes. Antibodies were incubated with the cells for 30 minutes, following which, the cells were fixed with Cytofix (BD Biosciences, San Diego, CA, cat # BDB554655) for 15 minutes. Following staining, cells were acquired on the BD Fortessa

(BD Biosciences, San Jose, CA) and results were analyzed on FlowJo version 10.8.1 (FlowJo LLC, Ashland, OR).

Table 3. Immunophenotyping markers for MDM.

Marker	Fluorochrome	Clone	Cat #	Company	Description
Live/ Dead Fixable Aqua Stain	405nm excitation	N/A	L34957	Invitrogen, Eugene, OR	A cell viability marker that reacts with amines in dead cells with a permeabilized membrane
CD11b[169]	APC	ICRF44	561015	BD Biosciences, San Jose, CA	Pan Myeloid Marker
CD14[170]	BV786	M5E2	563698	BD Biosciences, San Jose, CA	Detects bacteria by binding LPS; sCD14 is upregulated in the context of inflammation
CD16[169]	Alexa Fluor 700	3G8	557920	BD Biosciences, San Jose, CA	Fc receptor
CCR5[171, 172]	BV650	3A9	564999	BD Biosciences, San Jose, CA	HIV co-receptor; Increases upon differentiation of monocytes to macrophages
CD86[170]	Percp-Cy5-5	2331 (FUN-1)	561129	BD Biosciences, San Jose, CA	Provides co-stimulation for T cell activation
CD206[169]	PE-CF594	19.2	564063	BD Biosciences, San Jose, CA	Mannose receptor; plays a role in antigen uptake; upregulated in M2 cells
CD163[169,170, 172]	APC-Cy7	GHI/61	333621	BioLegend, San Diego, CA	Scavenge receptor for hemoglobin-haptoglobin

CD71[169]	BV711	HTA125	563767	BD Biosciences, San Jose, CA	Transferrin receptor
TLR4[170]	BV421	HTA125	312811	BioLegend, San Diego, CA	Pattern Recognition Receptor; Recognizes LPS
CB_2 [173]	Alexa Fluor 488	352110	FAB36551 G	R&D Systems, Minneapolis, MN	Cannabinoid Receptor 2

2.7 Description of U1 Cells

The U1 cell line is a subclone of the monocyte cell line U937, which has been infected with HIV-1[174]. The original U937 cells were obtained from the lungs of a Caucasian child with diffuse histiocytic lymphoma[175]. A mutation of the *tat* protein, a protein critical for the efficiency of HIV viral transcription, results in minimal constitutive expression of HIV-1[175]. This makes U1 cells a good model of HIV latency. Nevertheless, HIV-1 production can be induced with PMA treatment.

2.8 Activation of U1 Cells

To address aim 3, to evaluate the effect of cannabinoids on a HIV-1 infected macrophage cell line, U1 cells were first cultured in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin. For differentiation of this monocytic cell line into macrophages, in addition to activation of the latent HIV-1 infection, 10ng/mL of PMA (Sigma-Aldrich, St. Louis, MO, Cat# P1585) were added to the U1 cells that were plated on a 12-well plate at 500,000 cells/mL. PMA is a known stimulator of macrophage differentiation. It activates protein kinase C, which leads to a downstream cascade of activating various transcription factors including NFκB and AP-1[176]. Cells were cultured for 48 hours at 37ºC, 5% CO_2. The media was aspirated and washed twice with RPMI 1640. The cells were left to rest overnight in RPMI 1640 containing 10% FBS and 1% penicillin/streptomycin at 37ºC, 5% CO_2. Cells were then treated with 2.5µg/mL of CBD or THC for 24 hours. The

supernatant was preserved for HIV-1 quantification, and cells were detached by pipetting for Flow Cytometry. The cells were evaluated for the expression of known macrophage markers CD14 and CD16, CD206 (APC) (BD Biosciences, San Jose, CA, cat #550889) and the critical live/dead staining as described above. CB_2 staining was added as described above as an exploratory marker as CB_2 expression on U1 cells is, to the extent of knowledge, unknown. The cells were stained using the same immunophenotyping protocol described above.

2.9 Quantification of HIV-1

An enzyme-linked immunosorbent assay (ELISA) targeting HIV-1 p24 (Frederick National Laboratory for Cancer Research, Frederick, MD), a component of the viral capsid, was used to quantify the virus produced by the activated U1 cells described above. In preparation for the ELISA, the supernatants from the U1 cells were treated with 1% Triton X (Sigma-Aldrich, St. Louis, MO cat# T6878) to inactivate the virus. The ELISA was performed based on the manufacturer's instructions. The plate was read at 450 nm with a reference at 650nm on the Infinite M200 Pro Nano Quant.

2.10 Statistical Analyses

All statistical tests and corresponding graphs were made on GraphPad Prism version 7 (GraphPad Software, La Jolla, CA). For the immunophenotyping data, the Wilcoxon signed rank non-parametric test was used to compare the expression of each marker on each sample treated with cannabinoids or our vehicle control to the untreated control.

Chapter 3: Results

3.1 Cannabinoid Cytotoxicity Test

To determine cannabinoid concentrations that would not exert a cytotoxic effect on cells, we performed cytotoxicity testing on frozen PBMCs using serial dilutions of CBD, THC, and methanol. Analysis was done using the gating strategy outlined in Figure 14.

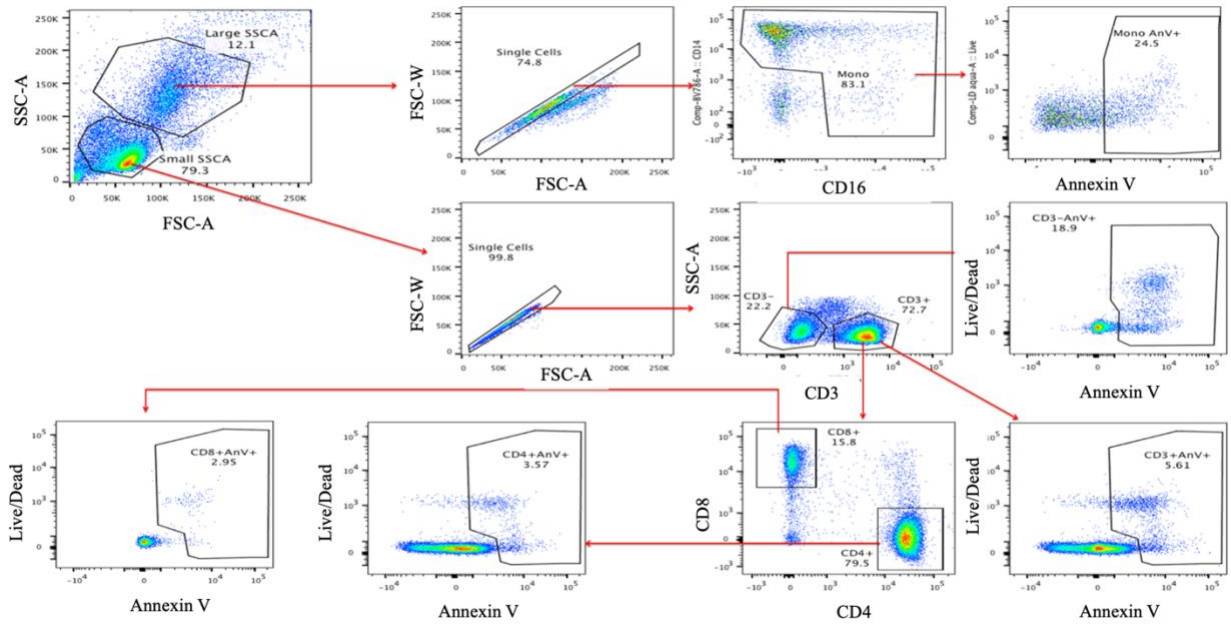


Figure 14. Flow cytometric gating strategy for the toxicity of cannabinoids on PBMCs. Monocytes and lymphocytes were first differentiated based on morphology from the forwards scatter – area (FSC-A) versus side scatter – area (SSC-A) plot. Lymphocytes present with a smaller FSC and SSC whereas monocytes have a larger FSC and SSC. Single cells were then selected for both monocytes and lymphocytes. From there monocytes were identified as CD14+ or CD16+ cells. Lymphocytes were analyzed in terms of both CD3- and CD3+ populations. Within CD3+ cells, CD4+ and CD8+ cells were independently analyzed. From here, dead and dying cells of all groups were measured in percentage of Annexin V+ and Live/Dead+ cells

Of particular interest to this study is the effect of cannabinoids on monocytes, as they would be used for differentiation into macrophages under cannabinoid treatment. We found that monocytes had the highest mortality rate following overnight incubation, followed by CD3- lymphocytes and finally CD3+ lymphocytes, which experienced less than 10% mortality. The high

mortality observed in monocytes may be due to their delicate nature and short life span, in addition to the process of cryopreservation[177]. CD3+ and CD3- lymphocytes all appear unaffected by the concentrations of THC used but show significant mortality when treated with 10µg/mL of CBD. No effect is seen with methanol treatment. In the monocyte population, increased mortality appears around 5µg/mL of both CBD and THC, whereby an equivalent volume methanol exerts no discernable effect (Figure 15). For subsequent experiments on monocytes, we elected to go no higher than 2.5µg/mL, using 1ug/mL as our "low"-dose cannabinoid treatment and 2.5µg/mL as our "high"-dose cannabinoid treatment to identify any potential dose-dependent effects which would not induce mortality and associated inflammation. While these concentrations are within the range used for *in vitro* research, it should be noted that they are higher than physiological concentrations[127,140,178].

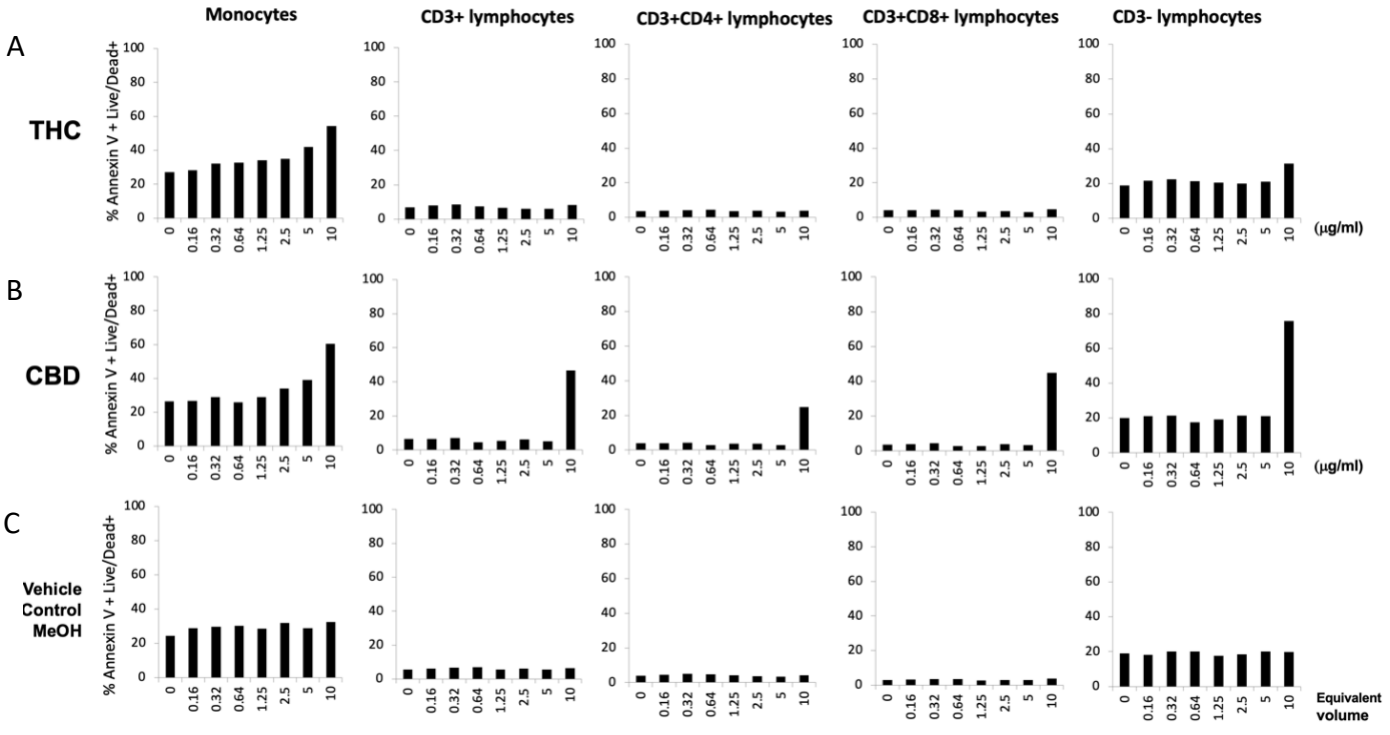


Figure 15. Cytotoxicity of cannabinoids on healthy, cryopreserved PBMCs. Serial dilutions of THC (A), CBD (B) and methanol (vehicle control) (C) were added to PBMCs for 24 hours on one donor. Methanol was added in equivalent volumes to the CBD and THC used, rather than by concentration, to ensure consistency between samples. The toxicity on various cell types were

determined. Monocytes were identified as CD14+ or CD16+. Helper T cells were identified as CD3+CD4+ and cytotoxic T cells were identified as CD3+CD8+. CD3+ and CD3- lymphocytes were also independently assessed. Dead and dying cells were measured in percentage of Annexin V+ and Live/Dead+ cells.

3.2 Morphology of MDMs

Images were taken of MDMs following CBD, THC, and methanol treatment, as well as following polarization into an M1 or M2a subset (Figure 16). Cells were all plated at 1 million cells/mL on 12 well plates. Morphological differences are observed following polarization to M1 and M2a subsets, with M2a cells in particular being highly elongated. Following CBD treatment there appears to be an increase in cell density.

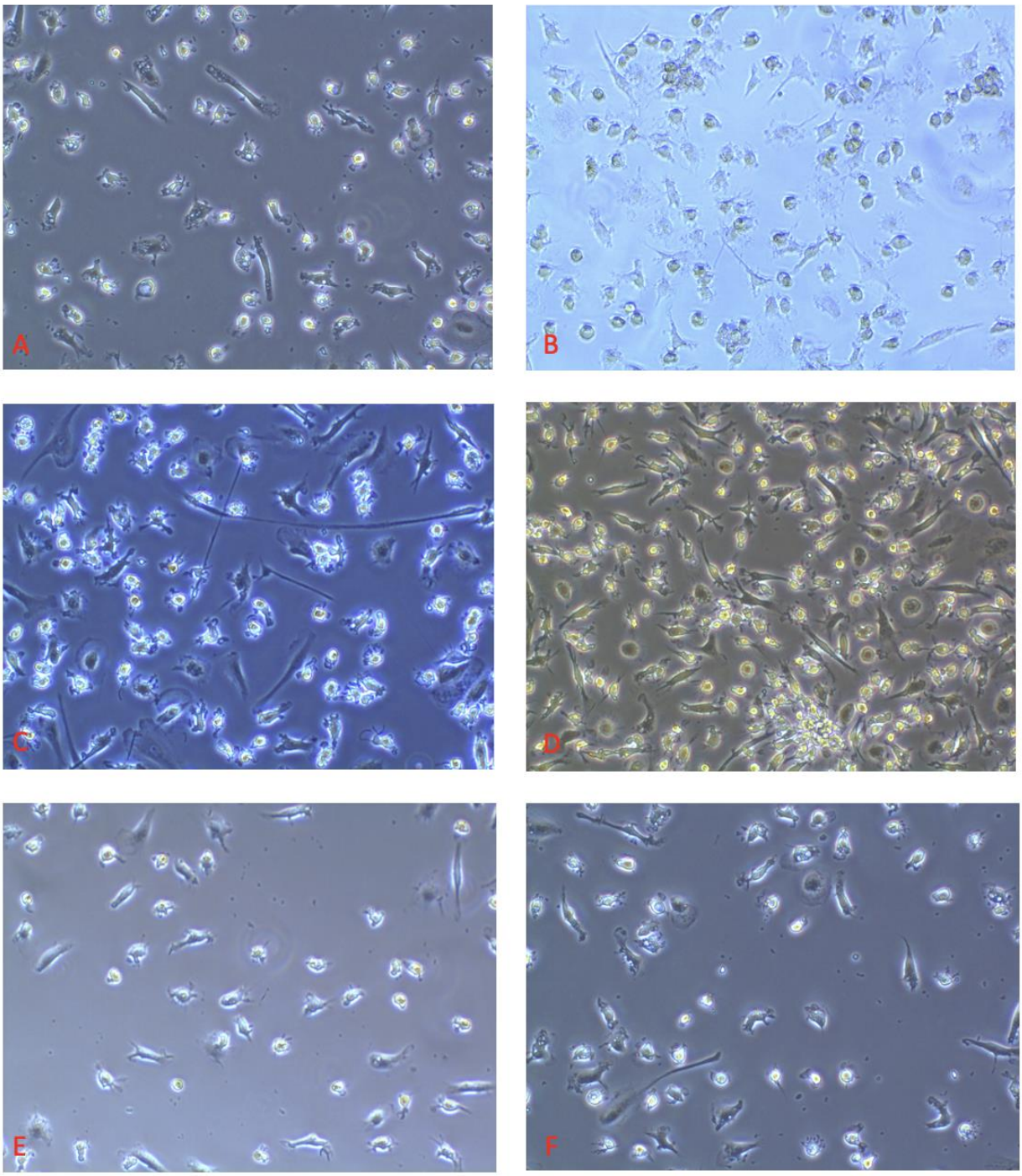


Figure 16. Morphology of MDMs. Images taken at 10X. (A) MDMs (B) M1 cells (C) M2a cells (D) MDMs treated with 2.5µg/mL CBD (E) MDMs treated with 2.5µg/mL THC (F) MDMs differentiated from fresh monocytes isolated using the EasySep™ Human Monocyte Enrichment Kit without CD16 Depletion were treated with 2.5µg/mL methanol. MDM have characteristic protrusions. These protrusions appear especially elongated in M2a cells. While plated at the same

density, MDMs treated with 2.5µg/mL CBD appear to be more confluent. These images are from one donor but are representative of all samples.

3.3 Immunophenotyping

Analysis of MDMs for both aim 1 and aim 2 were analyzed following the gating strategy outlined in Figure 17.

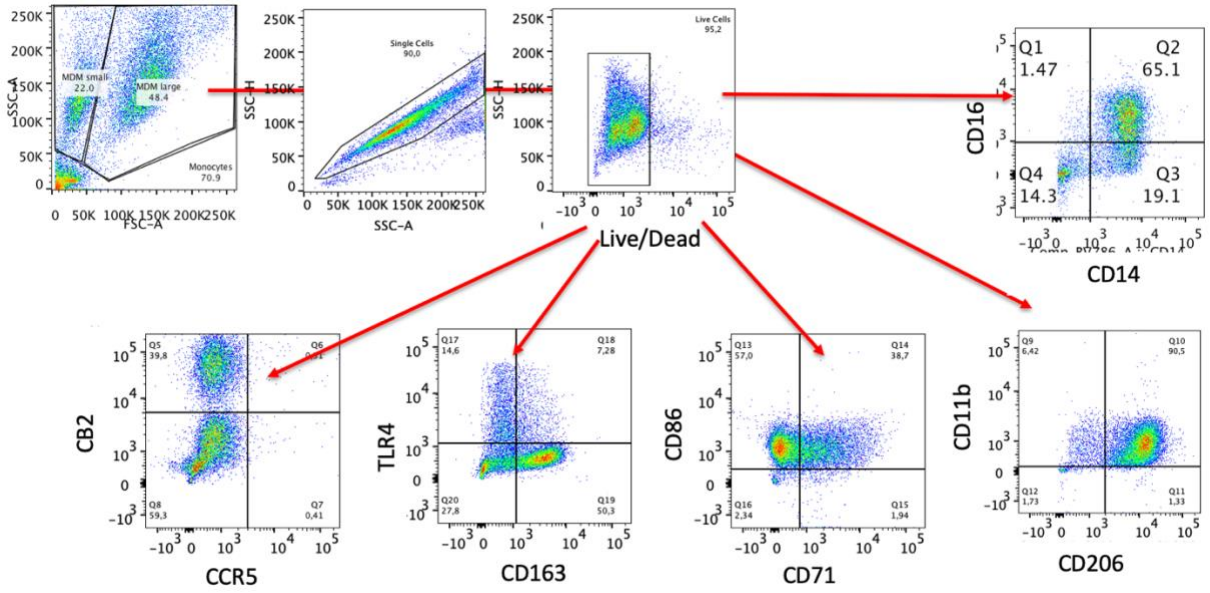


Figure 17. Flow cytometric gating strategy for MDMs. MDMs were defined as the single, live, larger FSC population of cells. The small FSC population is largely composed of undifferentiated monocytes. Within this population, frequencies of CD14, CD16 and CB_2 and median fluorescent intensity (MFI) of CD14, CD16, CB_2, CD163, CCR5, TLR4, CD86, CD206, CD71, and CD11b were quantified.

3.3.1 *Characterization of MDM*

To verify our ability to polarize MDM into M1 and M2 subsets, we first wanted to characterize MDM, M1, M2a, M2b and M2c macrophages following polarization (Figure 18). MDMs were stained in duplicates to ensure consistency with differentiation and staining methodology. The MDM treatment group was run in duplicates, and these replicates show great consistency in MFI expression. CB_1 was not expressed on any of the cells analyzed and was thus removed from further experiments. CB_2 expression was consistently low, although clearly present

when compared to the unstained control. CCR5, an HIV co-receptor, was downregulated in M2a and M2b phenotypes. Significantly, anti-inflammatory markers CD163 and CD206 were upregulated in M2 phenotypes in comparison to M1 phenotypes. Pro-inflammatory markers CD14 and TLR4 were downregulated in M2a and M2b phenotypes.

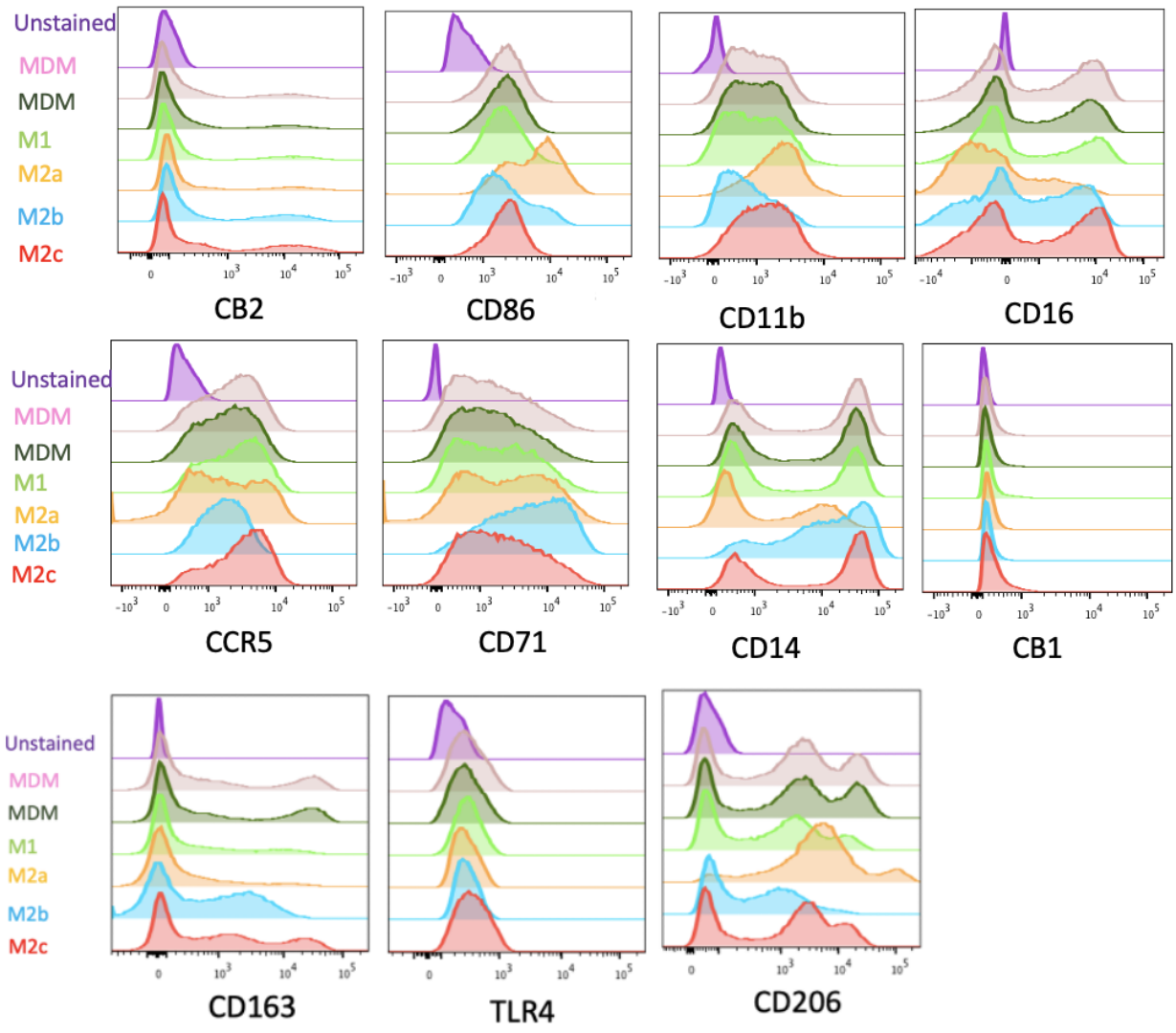


Figure 18. Phenotypic characterization of MDM and associated subtypes. Expression of various myeloid and inflammatory markers, as well as cannabinoid HIV receptors on unstained MDMs (purple), MDMs run in duplicate (pink and dark green), M1 cells (light green), M2a cells (orange), M2b cells (light blue), and M2c cells (red) from one healthy donor. MDMs were polarized using 10ng/mL MCSF in addition to 20ng/mL IFN-γ for M1, 20ng/mL IL-4 for M2a, 1μg/mL LPS and 10ng/mL IL-1β for M2b, 10ng/mL IL-10 for M2c.

3.3.2 *Aim 1*

To assess the effect of cannabinoid treatment on *in vitro* differentiation of monocytes to macrophages we analyzed cell surface markers of myeloid lineage, inflammation, as well as cannabinoid and HIV receptors. Primary cells from six individuals, taken over various days, were analyzed, and only four of six samples were used for the CBD and THC combined conditions. Each coloured dot represents a different participant. This colour coding is consistent across plots.

Firstly, we looked at the percentage of cells in this large FSC category that we performed subsequent analysis on to ensure the cannabinoid treatments were not affecting the differentiation of cells (Figure 19). There was no difference across groups.

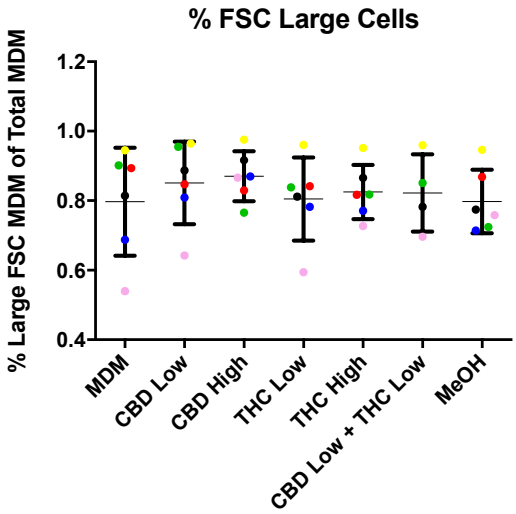


Figure 19. Frequency of fully differentiated MDMs differentiated in the presence of cannabinoids. Frequency of large FSC cells, which are determined to be macrophages that underwent differentiation, and upon which further analysis will be completed. MDMs were differentiated using 10ng/mL of MCSF while being treated with 10ng/mL of MCSF with 1µg/mL CBD (CBD low), 2.5µg/mL CBD (CBD high), 1µg/mL THC (THC low), 2.5µg/mL THC (THC high), 1µg/mL CBD and 1 µg/mL THC (CBD low + THC low) or 2.5µg/ml methanol (MeOH). Statistical analysis was done between the MDM and each condition calculated using Wilcoxon signed rank non-parametric test. p-values < 0.1 noted on graph, * indicates a p-value <0.05. Bars are plotted as mean with standard deviation.

Next, we wanted to assess the expression of CB_2, which is important for the effect of cannabinoids on immune cells (Figure 20). While there were no significant differences in the frequency of CB_2 positive cells following cannabinoid treatment, the MFI of cells that underwent CBD high and THC high treatments during macrophage differentiation was significantly lowered ($p = 0.0312$ and $p = 0.0312$, respectively). CB_2 is the only marker HIV co-receptor CCR5 expression was also analyzed, and MFI was decreased in the cells treated with low and high levels of CBD and high levels of THC ($p = 0.0312$, $p = 0.0312$, and $p = 0.0312$, respectively) (Figure 20).

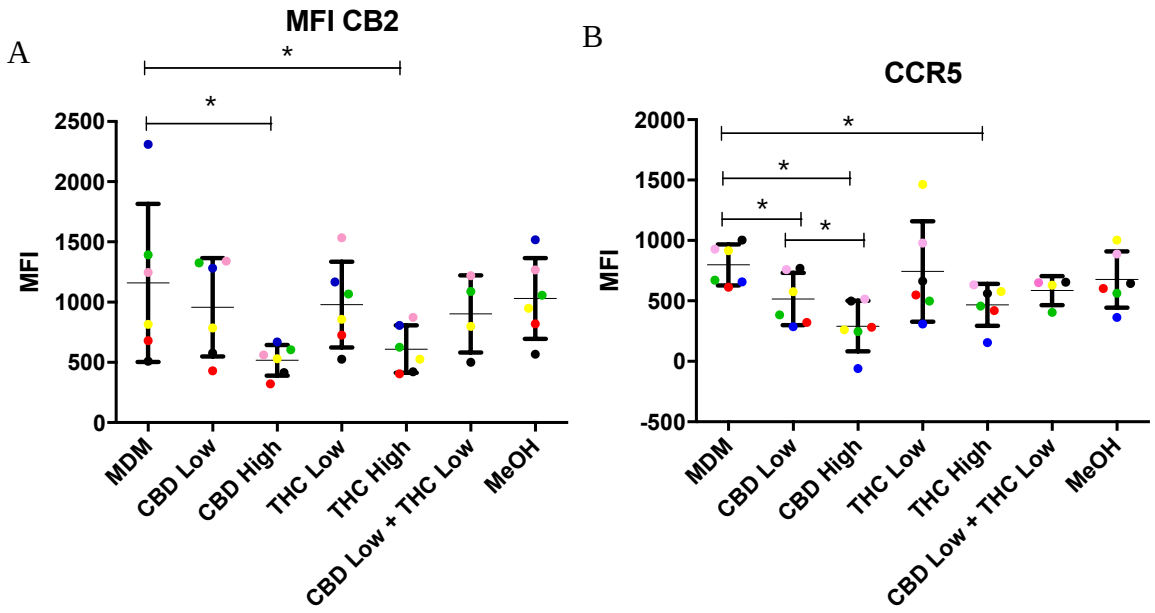


Figure 20. Cannabinoid receptor and HIV co-receptor on MDMs differentiated in the presence of cannabinoids. MFI of CB_2 (A) and CCR5 (B) of on macrophages that underwent differentiation into MDMs using 10ng/mL of MCSF while being treated with 1µg/mL CBD (CBD low), 2.5µg/mL CBD (CBD high), 1µg/mL THC (THC low), 2.5µg/mL THC (THC high), 1µg/mL CBD and 1 µg/mL THC (CBD low + THC low) or 2.5µg/ml methanol (MeOH). MDMs were defined as the single, live, larger FSC population of cells. Statistical analysis was done between the MDM and each condition calculated using Wilcoxon signed rank non-parametric test. p-values < 0.1 noted on graph, * indicates a p-value <0.05. Bars are plotted as mean with standard deviation.

In terms of classical markers used to characterize myeloid cells, we looked at CD14, CD16 and CD11b. CD14 appeared to be highly sensitive to the treatments used (Figure 21A). CD14 intensity

was decreased following low and high doses of both CBD and THC (p= 0.0312 for all). Interestingly, the fluorescent intensity of CD14 increased, though not significantly, with methanol alone (p = 0.0938). CD16 was significantly less sensitivity to cannabinoid treatment. No significant changes in fluorescent intensity were observed, but a non-significant decrease following high THC treatment is reported (p = 0.0625) (Figure 21B). No changes were seen in expression of pan-myeloid marker CD11b (Figure 21C).

Next, we assessed the expression of anti-inflammatory markers CD163, CD206, and CD71. CD163, is a scavenger receptor for hemoglobin-haptoglobin, which aids in the clearance of debris, pathogens, and apoptotic cells by macrophages. Although there were no significant changes in CD163 expression, non-significant decreases were observed in macrophages that underwent differentiation in the presence of high levels of CBD and THC (p = 0.0625 and p = 0.0938, respectively) (Figure 22A). High doses of CBD and THC appear to reduce the heterogeneity of CD163 expression. It is unclear if this is an artifact of the small sample size of our study, thus a larger sample size is necessary. The mannose receptor, CD206, which is important in antigen uptake, also experienced no significant changes, however, differentiation in the presence of a low dose of CBD led to increased CD206 fluorescent intensity (p = 0.0625) (Figure 22B). A similar effect was not noted in the CBD high treatment group. No changes were observed in the fluorescent intensity of transferrin receptor CD71 (Figure 22C).

Pro-inflammatory markers, TLR4 and CD86, were also assessed. Expression of TLR4, a pattern recognition receptor specifically targeted at LPS, decreased in the CBD high-dose treatment group (p = 0.0312). While expression of TLR4 also decreased in the THC high-dose treatment group, this decrease was not statistically significant (p = 0.0625) (Figure 23A). CD86,

which plays a critical role in T cell stimulation, was unaffected by cannabinoid treatment during macrophage differentiation (Figure 23B).

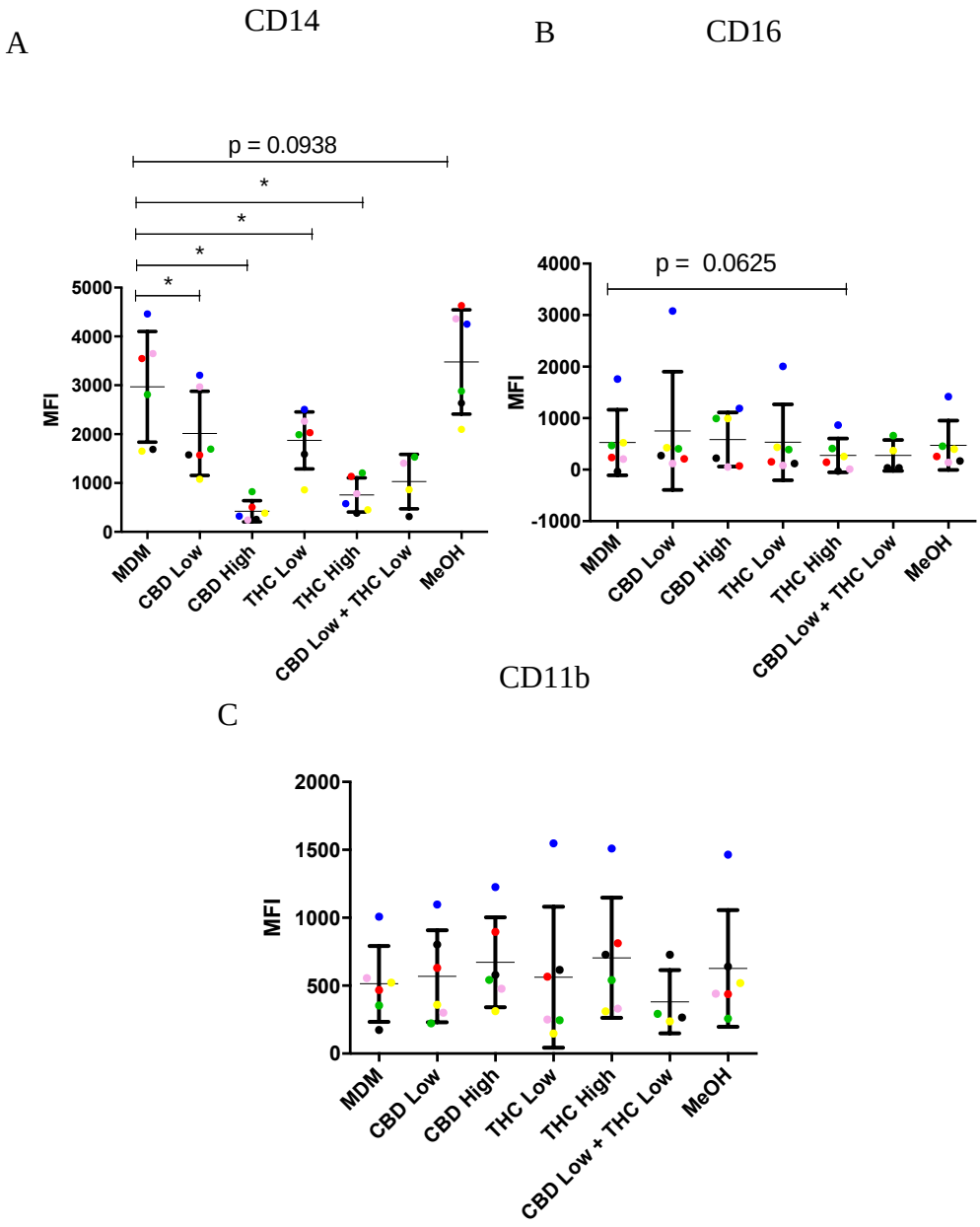


Figure 21. Classical myeloid markers on MDMs differentiated in the presence of cannabinoids. MFI of CD14 (A), CD16 (B), CD11b (C) on macrophages that underwent differentiation into MDMs using 10ng/mL of MCSF while being treated with 1µg/mL CBD (CBD low), 2.5µg/mL CBD (CBD high), 1µg/mL THC (THC low), 2.5µg/mL THC (THC high), 1µg/mL CBD and 1 µg/mL THC (CBD low + THC low) or 2.5µg/ml methanol (MeOH). MDMs were defined as the single, live, larger FSC population of cells. Statistical analysis was done between

the MDM and each condition calculated using Wilcoxon signed rank non-parametric test. p-values < 0.1 noted on graph, * indicates a p-value <0.05. Bars are plotted as mean with standard deviation.

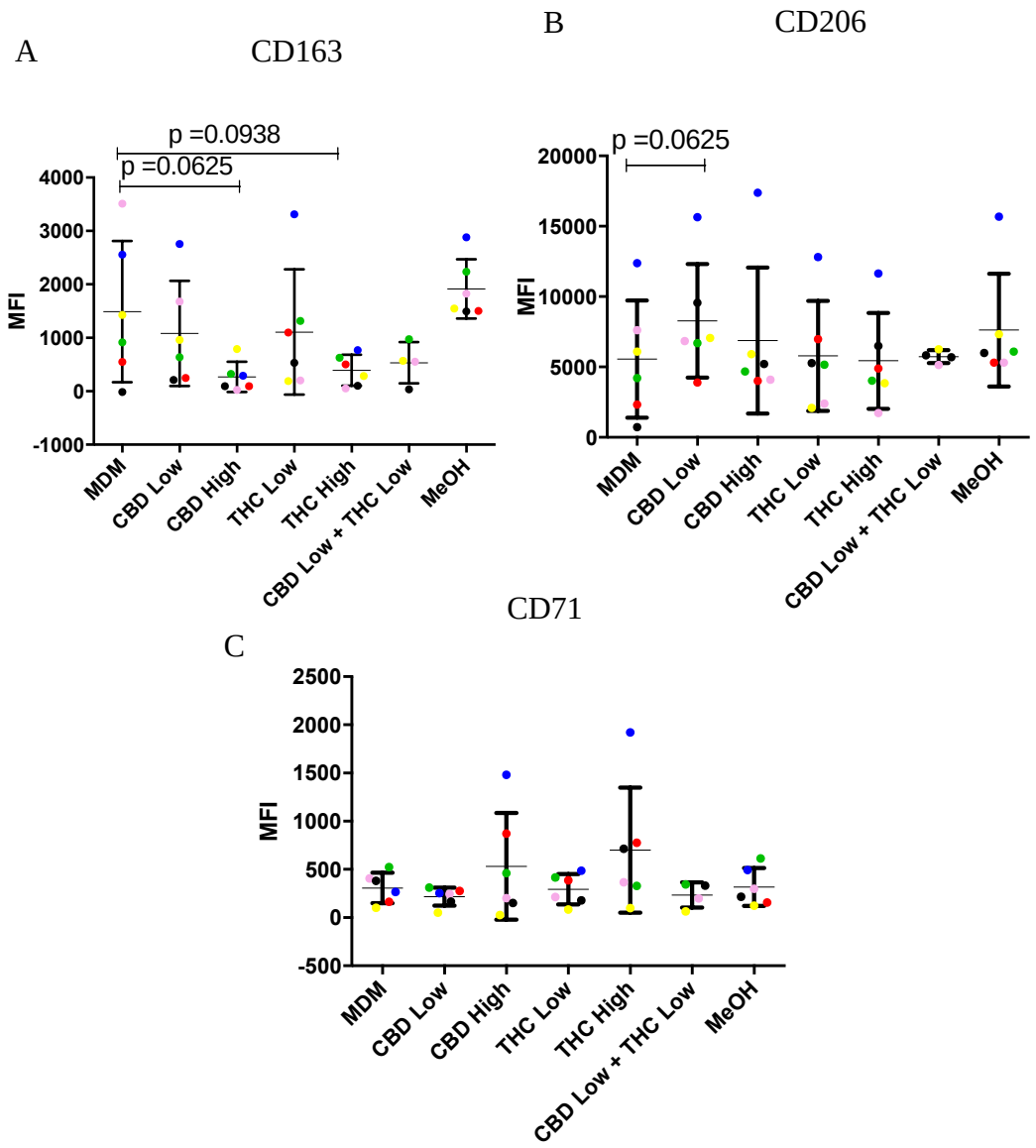


Figure 22. Anti-inflammatory marker fluorescence on MDMs differentiated in the presence of cannabinoids. MFI of CD163 (A), CD206 (B), and CD71 (C) on macrophages that underwent differentiation into MDMs using 10ng/mL of MCSF while being treated with 1µg/mL CBD (CBD low), 2.5µg/mL CBD (CBD high), 1µg/mL THC (THC low), 2.5µg/mL THC (THC high), 1µg/mL CBD and 1 µg/mL THC (CBD low + THC low) or 2.5µg/ml methanol (MeOH). MDMs were defined as the single, live, larger FSC population of cells. Statistical analysis was done between the MDM and each condition calculated using Wilcoxon signed rank non-parametric test. p-values < 0.1 noted on graph, * indicates a p-value <0.05. Bars are plotted as mean with standard deviation.

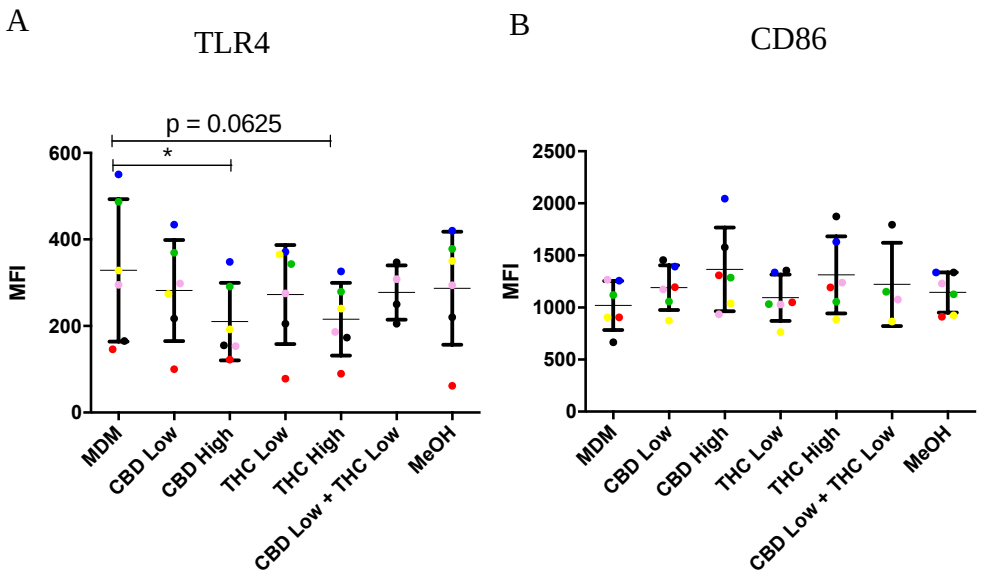


Figure 23. Pro-inflammatory marker fluorescence on MDMs differentiated in the presence of cannabinoids. MFI of TLR4 (A), CD86 (B) on macrophages that underwent differentiation into MDMs using 10ng/mL of MCSF while being treated with 1µg/mL CBD (CBD low), 2.5µg/mL CBD (CBD high), 1µg/mL THC (THC low), 2.5µg/mL THC (THC high), 1µg/mL CBD and 1 µg/mL THC (CBD low + THC low) or 2.5µg/ml methanol (MeOH). MDMs were defined as the single, live, larger FSC population of cells. Statistical analysis was done between the MDM and each condition calculated using Wilcoxon signed rank non-parametric test. p-values < 0.1 noted on graph, * indicates a p-value <0.05. Bars are plotted as mean with standard deviation.

3.3.2 *Aim 2*

To determine the effect of cannabinoid treatment on *in vitro* polarization of monocyte derived macrophages into a pro- or anti-inflammatory macrophages subset we analyzed the same cell surface markers of myeloid lineage, inflammation, as well as cannabinoid and HIV receptors as in Aim 1, using the same gating strategy outlined in Figure 14. Five unique samples taken on various days were used for the following experiments, four of which include a methanol control. M2a CBD treatment for one of the samples is not included in the results due to contamination. Each coloured dot represents a different participant. This colour coding is consistent across plots.

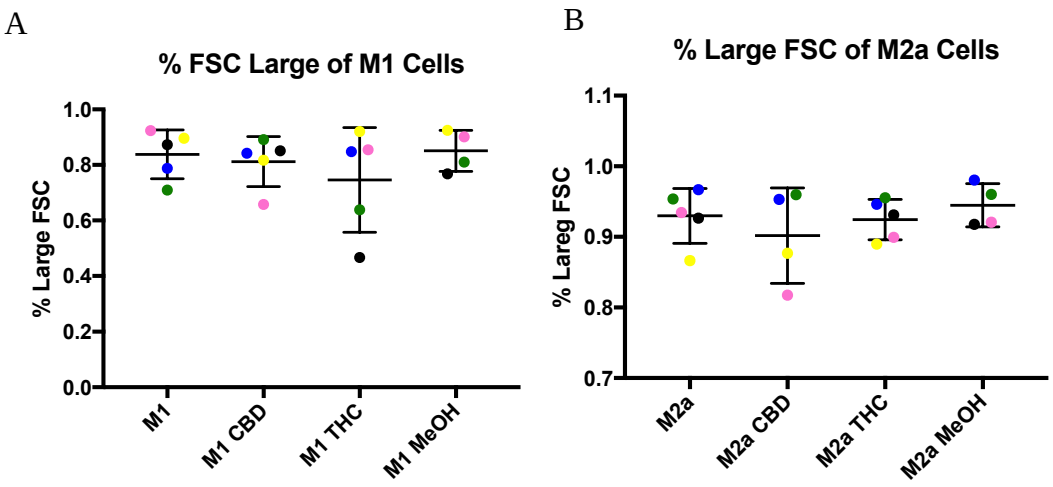


Figure 24. Large FSC MDMs following polarization in the presence of cannabinoids. Frequency of large FSC cells, of cells polarized to the M1 phenotype (A) and M2a phenotype (B) upon which further analysis is completed for each group. Cells were polarized from MDM using 20ng/mL IFN- γ and 1 μg/mL LPS for M1 and 20ng/mL IL-4 for M2a while being treated with 2.5μg/mL CBD, 2.5μg/mL THC or 2.5μg/ml methanol (MeOH). Statistical analysis was done between the M1 or M2a and each condition calculated using Wilcoxon signed rank non-parametric test. p-values < 0.1 noted on graph, * indicates a p-value <0.05. Bars are plotted as mean with standard deviation.

First, we looked at the percentage of total cells that were large, differentiated macrophages. There was no significant difference in the percentage of large, differentiated macrophages between treatment groups for both M1 and M2a cells (Figure 24). Next, we looked at expression of receptors crucial to the focus of our research, the cannabinoid receptor (CB_2) and the HIV co-receptor (CCR5). No statistically-significant differences were observed in the change of fluorescent intensity of either receptor following cannabinoid treatment (Figure 25). The expression of CD14, the classical macrophage marker that binds LPS, increased following THC treatment in M1 cells, though this increase was not statistically significant ($p = 0.0625$) (Figure 26A). This observation is opposite of that observed following THC treatment during macrophage differentiation, in which CD14 fluorescence significantly decreased. No other changes in CD14 expression were observed (Figure 26A&B).

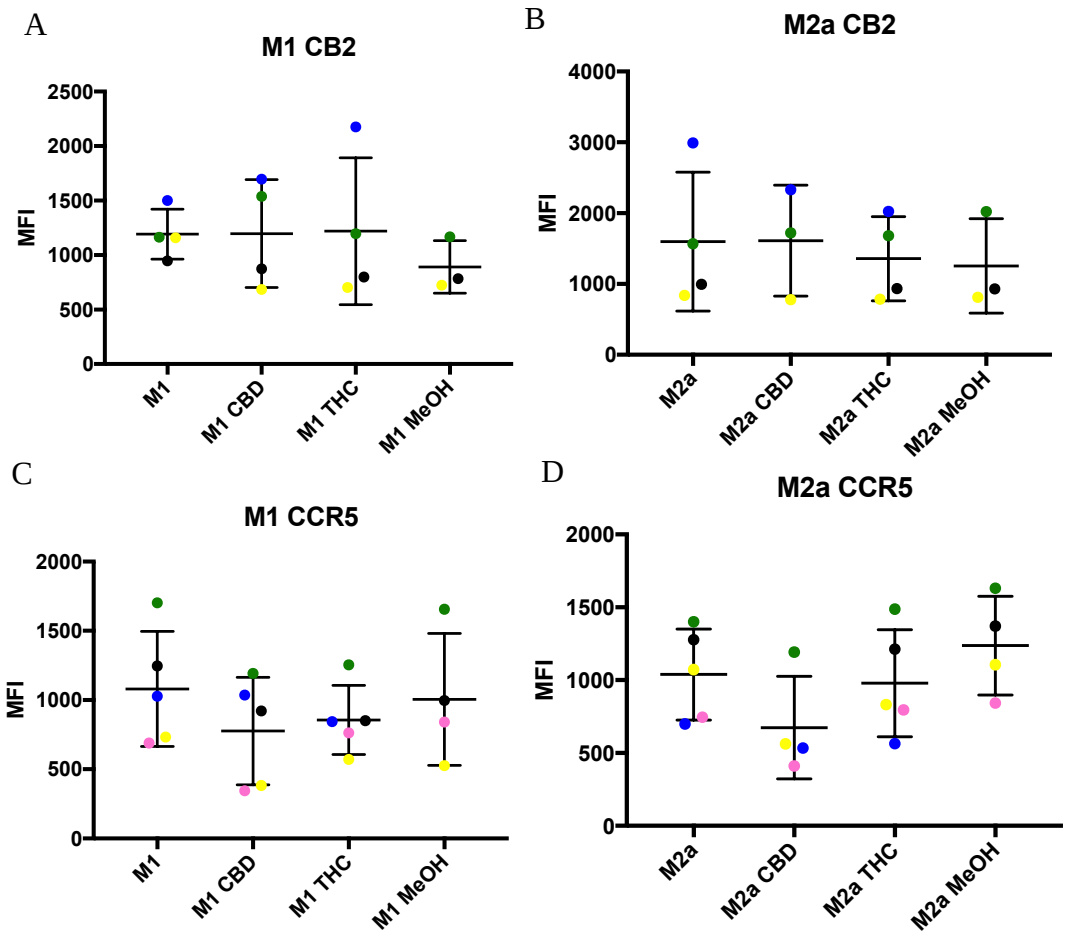


Figure 25. Cell surface receptor expression following MDM polarization in the presence of cannabinoids. Median fluorescent intensity of surface receptors for cannabinoids, CB_2, for M1 (A) and M2a (B) cells and the HIV co-receptor CCR5 for M1 (C) and M2a (D) cells. Cells were polarized from MDM using 20ng/mL IFN- γ and 1 μg/mL LPS for M1 and 20ng/mL IL-4 for M2a while being treated with 2.5μg/mL CBD, 2.5μg/mL THC or 2.5μg/ml methanol (MeOH). Statistical analysis was done between the M1 or M2a and each condition calculated using Wilcoxon signed rank non-parametric test. p-values < 0.1 noted on graph, * indicates a p-value <0.05. Bars are plotted as mean with standard deviation. Missing data points are due to supply issues in regards to antibodies used.

The expression of FC receptor CD16, was then assessed. In M2a cells, THC was found to non-significantly decrease CD16 fluorescence ($p = 0.0625$) (Figure 26D). A similar effect had been observed during macrophage differentiation. No other changes in CD14 expression were

observed (Figure 26C&D). Next, we assessed pan-myeloid marker CD11b. A non-significant increase was seen in M1 cells treated with THC ($p = 0.0625$) (Figure 26E). No other changes in CD14 expression were observed (Figure 26E&F).

The fluorescence of anti-inflammatory markers was then explored. No changes were observed in the expression of CD206 or CD71was seen in M1 or M2a cells (Figure 27 A, B, E, F). CD163 fluorescence was decreased following CBD and THC treatment in M1 cells (Figure 27C). This result was similar to what was seen following CBD and THC treatment during macrophage differentiation. No changes were observed in expression of CD163 on M2a cells following cannabinoid treatment (Figure 27D).

In terms of pro-inflammatory markers, we investigated the expression of TLR4 and CD86. No changes were observed in the expression of either marker on M1 or M2a cells following cannabinoid treatment (Figure 28).

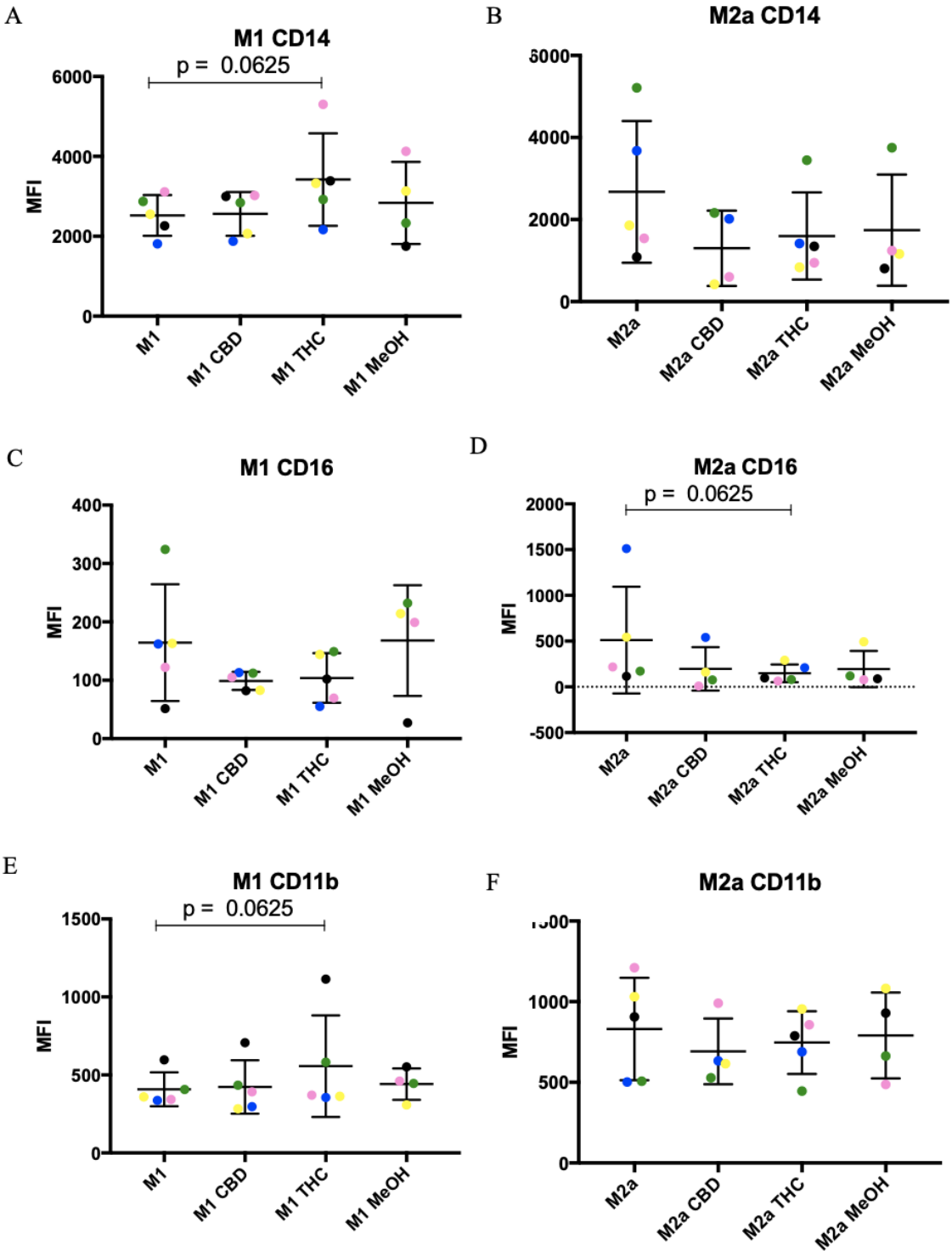
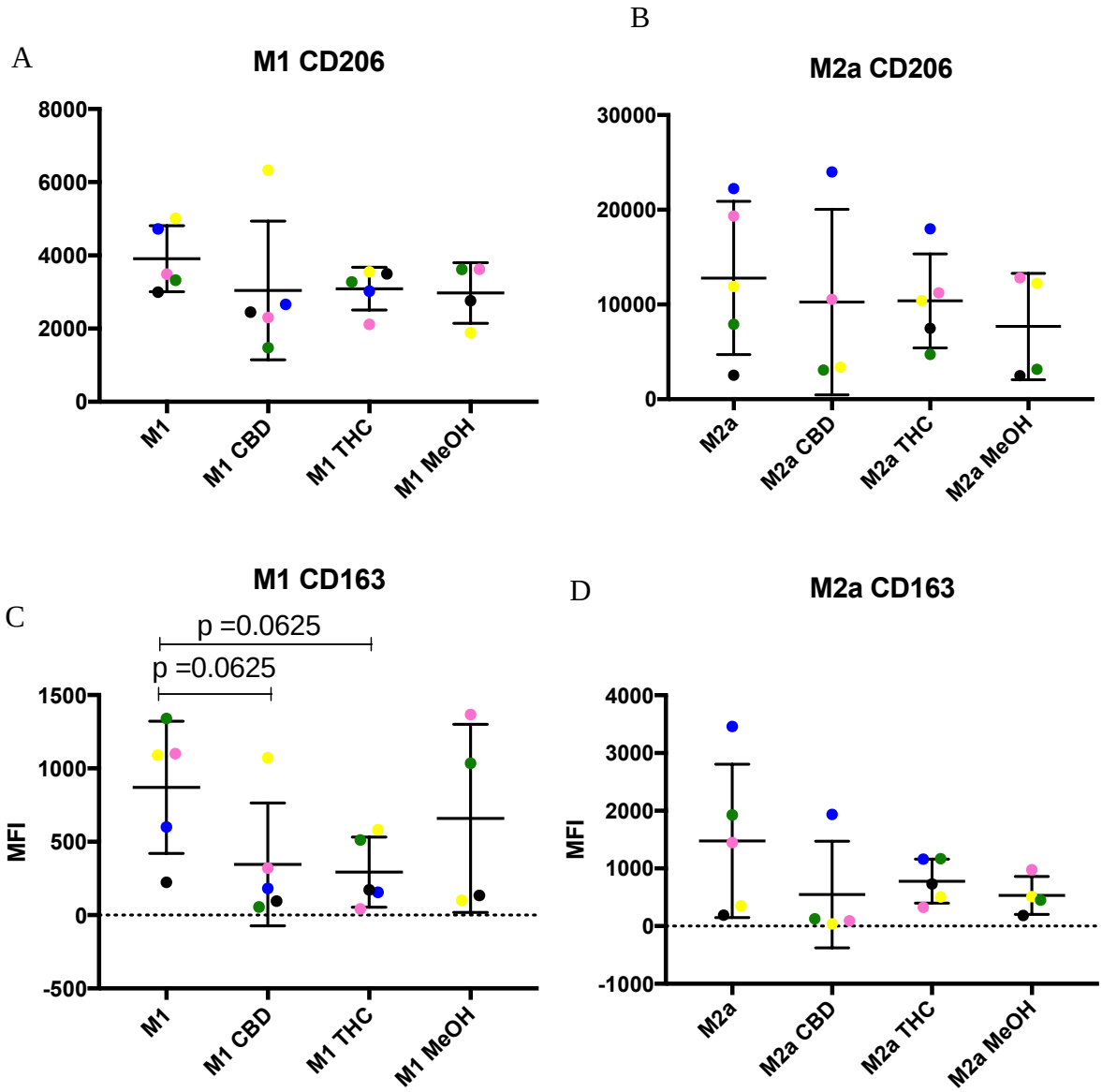

A
M1 CD14
p = 0.0625
MFI
6000
4000
2000
0
M1
M1 CBD
M1 THC
M1 MeOH
B
M2a CD14
6000
4000
2000
0
M2a
M2a CBD
M2a THC
M2a MeOH
C
M1 CD16
MFI
400
300
200
100
0
M1
M1 CBD
M1 THC
M1 MeOH
D
M2a CD16
p = 0.0625
MFI
2000
1500
1000
500
0
-500
M2a
M2a CBD
M2a THC
M2a MeOH
E
M1 CD11b
p = 0.0625
MFI
1500
1000
500
0
M1
M1 CBD
M1 THC
M1 MeOH
F
M2a CD11b
MFI
1000
500
0
M2a
M2a CBD
M2a THC
M2a MeOH

Figure 26. Classical myeloid markers on MDMs following polarization in the presence of cannabinoids. Median fluorescent intensity of CD14 for M1 (A) and M2a (B) cells, CD16 for M1 (C) and M2a (D) cells, and CD11b for M1 (E) and M2a (F) cells. Cells were polarized from MDM using 20ng/mL IFN- γ and 1 μg/mL LPS for M1 and 20ng/mL IL-4 for M2a while being treated with 2.5μg/mL CBD, 2.5μg/mL THC or 2.5μg/ml methanol (MeOH). Statistical analysis was done between the M1 or M2a and each condition calculated using Wilcoxon signed rank non-parametric test. p-values < 0.1 noted on graph, * indicates a p-value <0.05. Bars are plotted as mean with standard deviation. Missing data points are due to supply issues in regards to antibodies used.



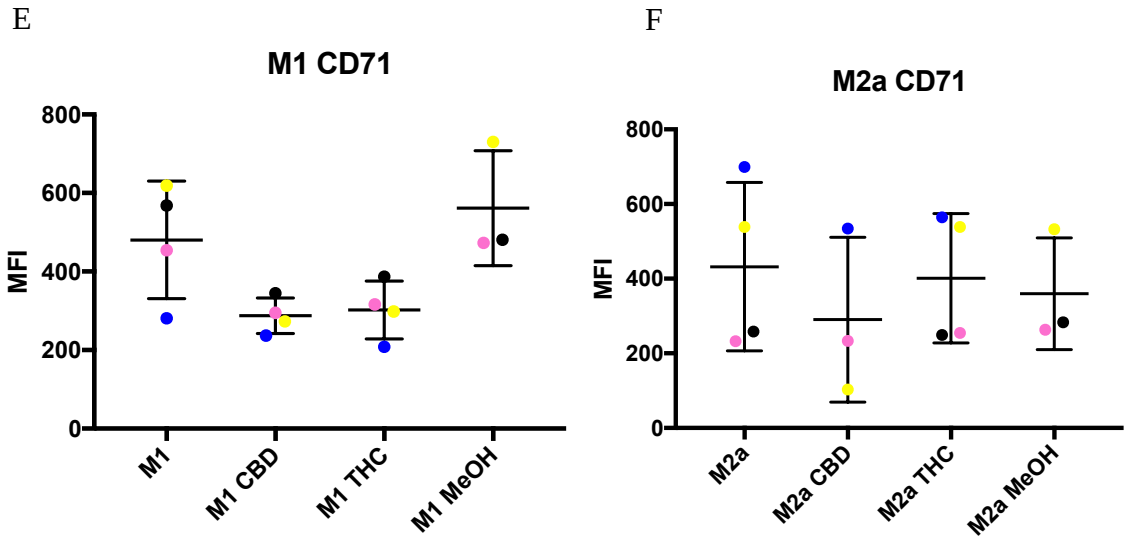
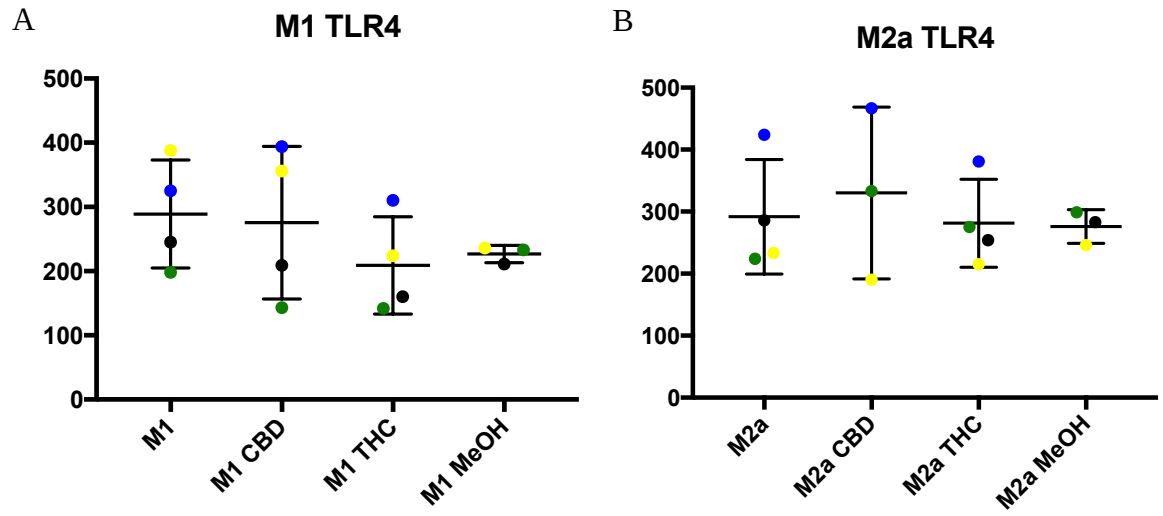


Figure 27. Anti-inflammatory marker fluorescence following MDM polarization in the presence of cannabinoids. Median fluorescent intensity of CD206 for M1 (A) and M2a (B) cells, CD163 for M1 (C) and M2a (D) cells, and CD71 for M1 (E) and M2a (F) cells. Cells were polarized from MDM using 20ng/mL IFN- γ and 1 μg/mL LPS for M1 and 20ng/mL IL-4 for M2a while being treated with 2.5μg/mL CBD, 2.5μg/mL THC or 2.5μg/ml methanol (MeOH). Statistical analysis was done between the M1 or M2a and each condition calculated using Wilcoxon signed rank non-parametric test. p-values < 0.1 noted on graph, * indicates a p-value <0.05. Bars are plotted as mean with standard deviation. Missing data points are due to supply issues in regards to antibodies used.



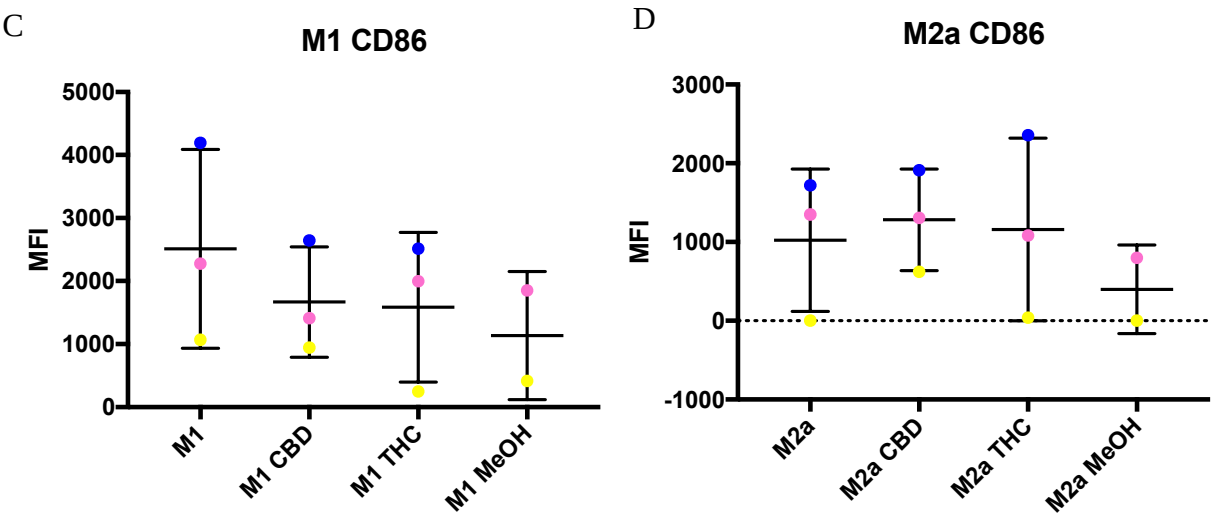


Figure 28. Pro-inflammatory marker fluorescence following MDM polarization in the presence of cannabinoids. Median fluorescent intensity of TLR4 for M1 (A) and M2a (B) cells and CD86 for M1 (C) and M2a (D) cells. Cells were polarized from MDM using 20ng/mL IFN-γ and 1 μg/mL LPS for M1 and 20ng/mL IL-4 for M2a while being treated with 2.5μg/mL CBD, 2.5μg/mL THC or 2.5μg/ml methanol (MeOH). Statistical analysis was done between the M1 or M2a and each condition calculated using Wilcoxon signed rank non-parametric test. p-values < 0.1 noted on graph, * indicates a p-value <0.05. Bars are plotted as mean with standard deviation. Missing data points are due to supply issues in regards to antibodies used.

3.3.3 *Aim 3*

To understand the effect of cannabinoids on macrophages in the context of HIV, we used the U1 monocyte cell line. Following differentiation into macrophages and subsequent treatment with cannabinoids, we characterized the resulting cells using the gating strategy outlined in Figure 29. It is unclear if the differentiation was successful, as CD206 expression is expected to be higher in macrophages[169].

First, we set out to determine if the cannabinoid receptor CB_2 was expressed on this cell line. In terms of absolute expression, there was a very small, but consistently expressed population of CB_2+ cells (Figure 30B). There were no changes for each repeat in absolute expression of CB_2, however, there appears to be a decrease in CB_2 MFI with both CBD and THC treatment (Figure 30A). Due to the small sample size, the presence of any significant differences could not be

ascertained. Additionally, fluorescence of CD14, CD16, and CD206 appears consistent across treatment groups (Figure 30C-E).

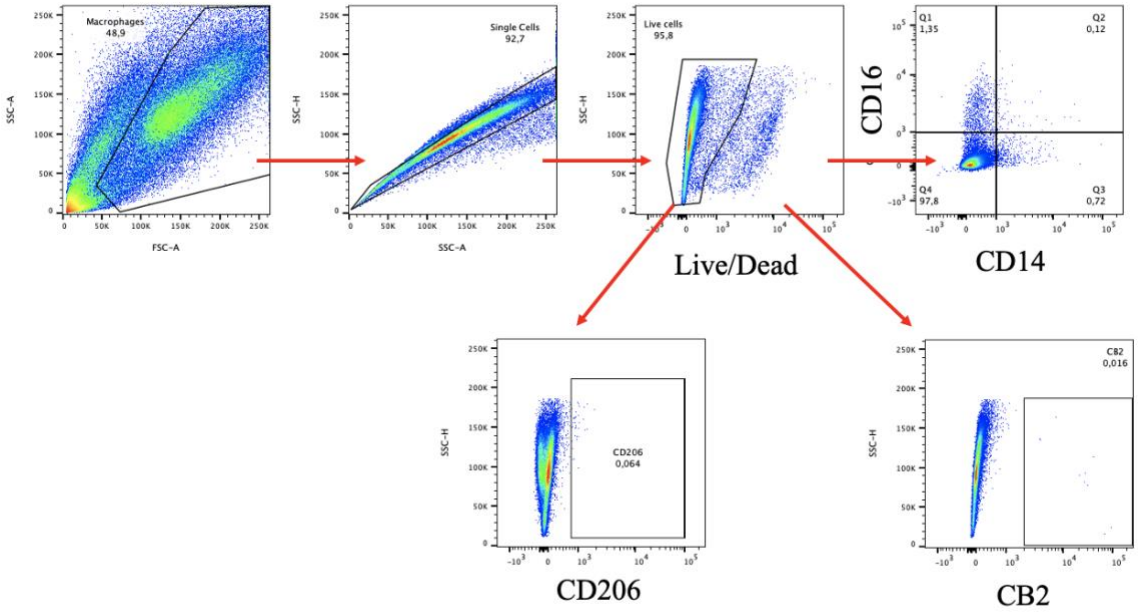
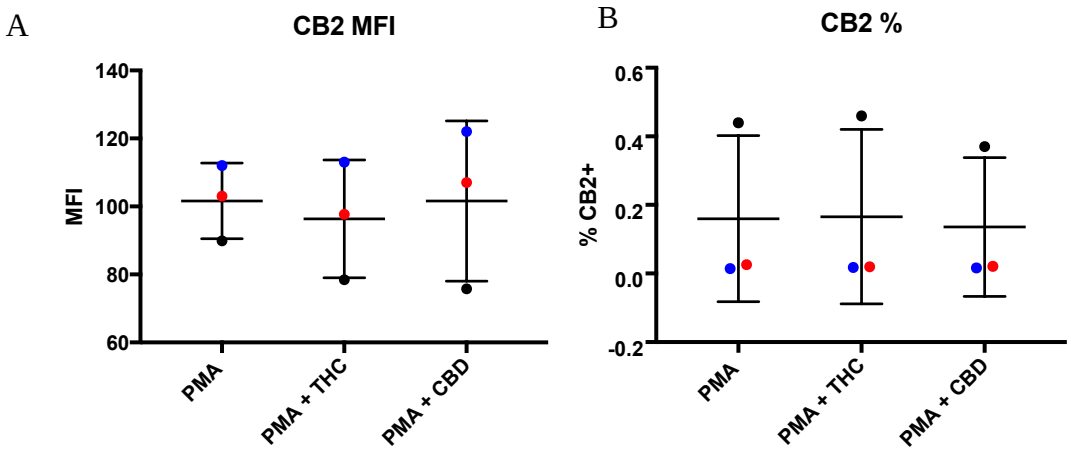


Figure 29. Gating strategy for U1 cells. Debris was first gated out, then U1 macrophages were defined as single, live cells. Within this population CD206, CB_2, CD16, and CD14 were analyzed.



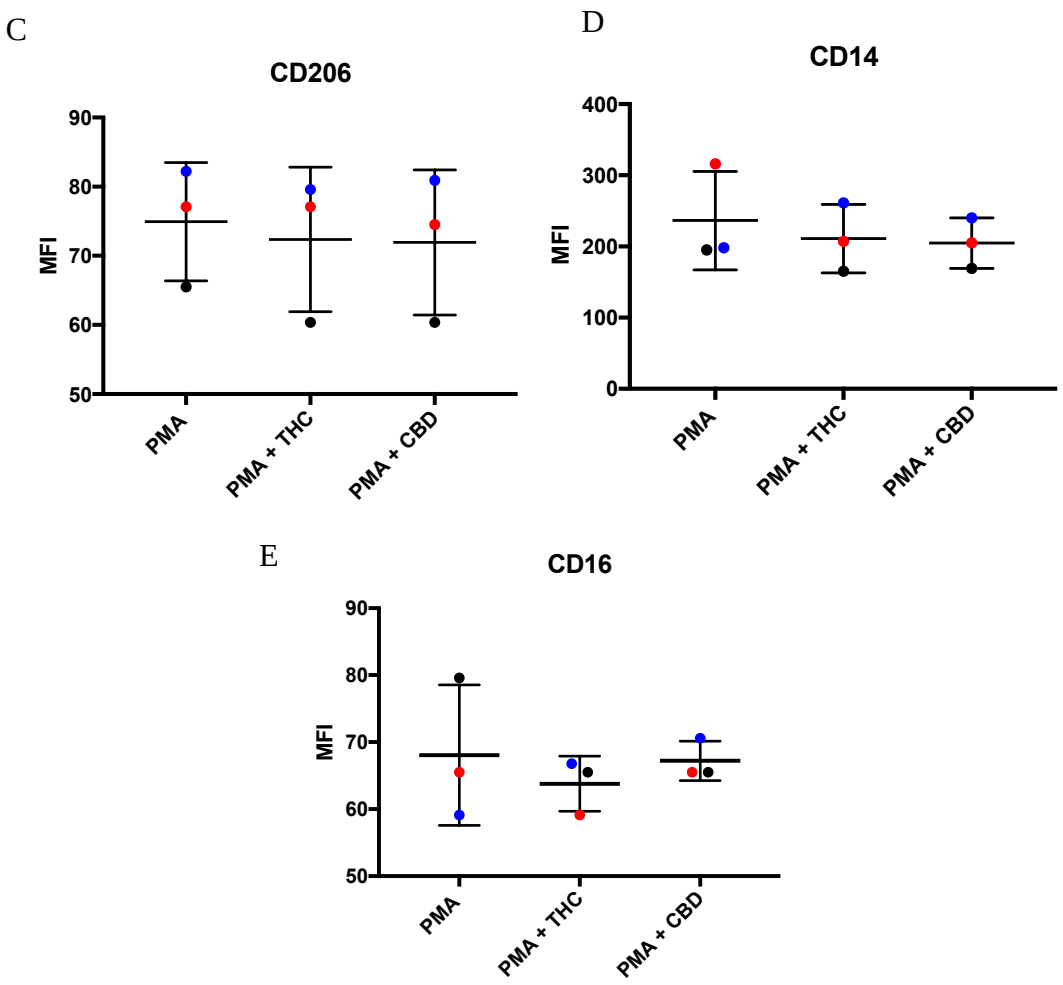


Figure 30. Characterization of U1 cells. The MFI (A) and frequency (B) of CB_2, as well as the MFI of CD206 (C), CD14 (D), and CD16 (E). U1 macrophages were defined as single, live cells.

3.4 Quantification of HIV-1

In parallel with the immunophenotyping of U1 cells following activation and cannabinoid treatment, the HIV-1 viral production by these cells was also quantified. From the supernatant of these cells, we identified a decrease in p24 concentration following both CBD and THC treatment (Figure 31A). The experiments were run in triplicate, with cells for each replicate having been split prior to treatment. Figure 31B was used to highlight that, while the absolute quantification of HIV-1 in the third replicate was much lower than the other two replicates, there remained a marked reduction in production of HIV-1 with CBD and THC treatments.

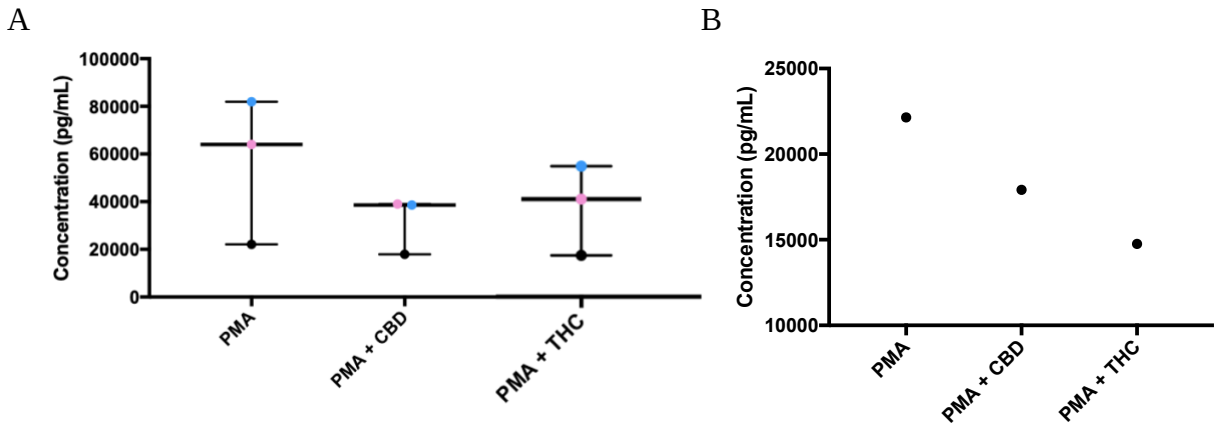


Figure 31. Quantification of HIV-1 on stimulated U1 cells following cannabinoid treatment. (A) U1 cells were activated with PMA for 48 hours, allowed to rest for 24 hours, and treated with 2.5µg/mL of CBD or THC for 24 hours. (B) The third replicate was plotted on its own graph. HIV-1 quantification was done using a p24 ELISA and measured in pg/mL immediately after the cannabinoid treatment. Triplicates were performed using U1 cells that had been cultured in different flasks.

Chapter 4: Discussion

To understand the impact of cannabinoids CBD and THC on macrophages and the application of such in the context of HIV infection, we assessed the impact on macrophage differentiation and polarization *in vitro*. We began by characterizing MDMs following differentiation in the presence of cannabinoids. We then studied the same characteristics in M1 and M2a cells that had been polarized while being treated with cannabinoids. Finally, we used U1 cells as a model of HIV infection to understand if and how the changes observed in healthy primary cells may materialize in the context of HIV infection.

Macrophages are an engaging cell type to study as they are tissue resident. Unlike other immune cells that can be isolated from blood, invasive procedures such as biopsies or bronchoalveolar lavage are often necessary to isolate macrophages. Thus, we used two models of macrophages. Firstly, we used U1 monocyte cells that have been differentiated into macrophages using PMA. Cell lines, such as the U1 cell line, are beneficial in that they are easily accessible and provide the opportunity to study reproducible phenomena in a controlled manner. In the context of activation of U1 cells with PMA, TNF-α and IL-1β are secreted, which as seen in MDMs, results in increased HIV production in an autocrine manner[179]. This is important for our experiments into HIV-1 production following PMA stimulation. U1 cells are a good model of HIV latency, as they have minimal constitutive expression of HIV-1[175]. This makes U1 cells a relevant model for our experiments as we are aiming to understand the effect of cannabis on chronic inflammation in PLWH on ART. Much of the virus in this population will be in the latent phase. These cells are derived from parent U937 cells. Future experiments can be aimed at comparing the effect of cannabinoids on the virally-infected and uninfected parent cells. U1 cells are often used in HIV research as a model of latent HIV infection. Recent work from DeMarino *et al.* involving U1 monocytes and primary macrophages demonstrated a decrease in extracellular vesicle (EVs)

release from CBD-treated cells[180]. However, the U1 cell line, like other cell lines, is derived from cancer cells. This makes the cells inherently different from the primary cells of which they are intended to replace.

Our second macrophage model was macrophages differentiated from peripheral monocytes *in vitro*, known as MDM. This model, while very common, is limited when considering the replenishment of tissue resident macrophages by circulating monocytes[59]. In this *in vitro* context the macrophages are missing tissue-specific signals, which can result in differential phenotypes[181]. Additionally, there is evidence suggesting that certain monocyte subsets, namely classical monocytes, are preferentially recruited to sites of infection, whereas nonclassical monocytes operate as patrollers[181-183]. Hence, when deriving macrophages from all monocytes present in the whole blood, this can result in phenotypic differences due to the composition of the starting population. Primary cells, by nature, are more complicated to manipulate due to inherent donor-to-donor differences. While these differences more accurately reflect reality, many factors cannot be controlled for, and can result in outliers or make data interpretation difficult, especially when using small samples sizes, such as those that we are working with.

Another challenge in the use of primary cells for the study of cannabinoid-based research is a result of the effect of individuals' endocannabinoid systems. The endocannabinoid system is greatly affected for various donor characteristics, many of which we cannot control for, including biological sex hormones, time of day, cannabis smoking status, stress, exercise, weight, sleep, food consumption, and others[110,184,185]. The impact of the presence of varying levels of endocannabinoids on monocytes prior to monocyte isolation is unknown. In addition, many PLWH are at a higher risk for other comorbidities, including various cancers, and the effects of cannabis in these conditions must also be evaluated prior to the decision to self-medicate or do so under the

supervision of a doctor. For example, a recent paper showed that in HIV-uninfected people undergoing nivolumab immunotherapy treatment for non-small cell lung cancer, renal cell carcinoma and melanoma, the response rate to treatment was decreased with cannabis usage[186]. Although this study was observational in nature, this paper has raised safety concerns regarding cannabis usage in this population. However, much additional research is nonetheless required before conclusions can be drawn.

Prior to any experiments on the effect of cannabinoids on myeloid cells, we first set out to determine concentrations of CBD and THC in methanol that were non-toxic. Using flow cytometry analysis of annexin V and live/dead dyes, we noticed marked increases in toxicity in monocytes at 5 μg/mL and at 10 μg/mL for lymphocytes. Overall, monocytes had a much higher mortality in comparison to lymphocytes following the overnight incubation, even in the case of the untreated controls. This is likely due to their short life span[177]. An alternative explanation of this increased mortality is that the monocytes are more sensitive to the cryopreservation technique used. A study by Pardali *et al.* found no effect of cryopreservation on monocyte survival, although we did not test this in our lab[187]. Thus, we selected 1 μg/mL as our "low" dose treatment and 2.5 μg/mL as our "high" dose treatment for further experiments on monocytes. Measured in molar, our "low" dose treatment is approximately equivalent to 3.18 μM and our high dose treatment is approximately equivalent to 7.95 μM for both THC and CBD.

While the concentrations of CBD and THC used in these experiments were found to be non-toxic into immune cells and are within the range or below the concentrations commonly used within *in vitro* research onto the effect of cannabinoids, the levels are well above what is observed in the plasma of cannabis users[127,140,178]. A randomized control trial reported peak plasma concentrations following consumption of 10mg THC and 5.4 mg of THC[158]. Peak plasma

concentrations were reported at 1.2 to 10.3 ng/mL, with a mean of 4.05 ng/mL for THC and between 0.2 and 2.6 ng/mL, with a mean of 0.95ng/mL for THC[188]. Other studies record peak plasma levels of THC between 50-262ng/mL; however, these peaks are short lived, occurring in around 6 to 10 minutes following smoking, and decrease rapidly within the first hour of smoking[163,164,166]. Inhalation of cannabinoids results in a higher bioavailability of cannabinoids than ingestion[166]. The bioavailability of CBD is an average of 31% of cannabis consumed through inhalation and a mere 6% of orally ingested cannabis[166]. For THC, the bioavailability through inhalation ranges from 10-35% and is a mere 4-12% of that when ingested orally[166]. This differs from our *in vitro* conditions in both the higher cannabinoid exposure concentrations as well as the sustained time in which the cells are exposed to these high levels. This is especially important to consider in the context of the two doses we used for the experiments on the differentiation of cannabis in the presence of cannabinoids. In these studies, we often observed a significant effect of the cannabinoids in our higher dose condition but could not reach significance with our lower dose. It is possible that with more power these lower doses would also reach significance. Importantly, one must also consider the fact that THC and CBD undergo metabolism upon consumption, and that their active metabolites may contribute to the biological effects observed *in vivo*[166]. Additionally, the cannabis plant has hundreds of other compounds, such as flavonoids, terpenes, and other cannabinoids, that are not included in the THC and CBD solutions we used in these experiments. For instance, cannabis includes more than 60 structurally related cannabinoids[137]. These compounds may contribute to an "entourage effect", or exert their own immune modulatory effects[104,112,127]. Furthermore, both CBD and THC are highly lipophilic meaning that they will bind lipids in the serum in addition to acting on cells of interest[189,190].

It is important to note that in our experiments we are using pure THC and CBD in a methanol suspension. In practice, smoking is the most common method by which individuals consume cannabis, and as discussed previously, terpenes and flavonoids contribute to the "entourage" effect which modifies responses. Thus, the results of our *in vitro* work with pure CBD and THC may be very different to the *in vivo* response to the smoked cannabis plant. However, pure CBD and THC are convenient for laboratory studies, as well as for clinical applications. Additionally, much of our research was done on CBD and THC independently. In many formulations of cannabinoids, both CBD and THC may be used. The co-formulation of these cannabinoids may be done to yield the beneficial medicinal effects of both compounds while increasing tolerability. For example, adding CBD to a THC-containing product reduces the adverse central nervous system effects characteristic of THC[191,192]. For the purposes of the current studies, the separation of the two chemicals was done to understand the direct contribution of each component although research into their combined impact is also warranted.

The mode of administration is also important to consider when evaluating the effects of cannabinoids on immune cells. In PLWH, the prevalence of tobacco smoking is three times higher than the general population[193]. When divided by smoking status, less than 10% of PLWH who cigarette smokers report not currently using cannabis whereas 40% of current smokers also report current cannabis use[193]. The interaction of these two drugs, especially on alveolar macrophage subsets, is important for future research given the critical role of alveolar macrophages which are at the forefront of pulmonary immune defense.

We subsequently aimed at characterizing the various MDM subsets; M1, M2a, M2b, and M2c. While CB_1 is not typically described on immune cells, CB_1 has been reported on human lung macrophages, as well as various murine macrophage[173,194]. Hence, we decided to explore the

presence of CB_1 on human MDMs. We did not observe the CB_1 expression on any of the MDM subsets and subsequently removed this marker from our panel for further experiments. CB_2, which is known to be expressed on immune cells, was expressed on all MDM subtypes, as was retained for further experiments.

Based on the differential expression of various markers, which is largely in accordance with expected MDM phenotypes, we determined that the polarization of macrophages was effective. For example, based on the literature, M1 was expected to be $CD163^{low}$, M2a was expected to be $CD14^{low}$, $CD163^{low}$, $CD206^{high}$ and $TLR4^{low}$, M2b was expected to be $CD14^{high}$, $CD206^{low}$, and M2c was expected to be $CD163^{high}$, which were in accordance with our observations[155,170,195]. Despite the effectiveness of our polarization of different MDM subsets, we decided that for the purposes of our aim 2 research question, to determine the effect of cannabinoid treatment on *in vitro* polarization of monocyte derived macrophages into a pro- or anti-inflammatory macrophage subset, it was best to simplify the experiments. We elected to polarize to M1 and M2a cells exclusively. This is important as M1 macrophages are a pro-inflammatory mediator of the Th1 response and M2a macrophages are an anti-inflammatory mediator of the Th2 response and will be used as a model of such in our experiments. This simplification is common within the literature[74,196]. In addition, we switched the stimulants used for M1 polarization from IFN- γ alone to IFN- γ and LPS, which is again in line with what is common in the literature[59,169].

To assess the effect of cannabinoid treatment on *in vitro* differentiation of monocytes to macrophages in aim 1, we differentiated monocytes with MCSF in the presence of cannabinoids. Following this differentiation, the CBD-treated cells appear to increase in density, potentially a result of cell division, but this was unstudied. While monocytes are typically considered non-proliferative, macrophages are known to proliferate at low-levels constitutively and with

proliferation rates increasing upon certain conditions such as macrophage depletion or an inflammatory challenge[197]. Danchine *et al.* have showed that CBD decreases proliferation of a macrophage cell line. The mechanism behind this observation is unclear at this time[198]. Using flow cytometry, we characterized these differentiated macrophages to understand the effects CBD and THC had on the cells during the differentiation process. When we first observe these cells on the FSC-A/SSC-A graph, we notice that the cells are differentiated into two distinct populations, which we call FSC-A small and FSC-A large. We gated on the cells of the large FSC-A population, as this is where we expect living, differentiated macrophages to be positioned, whereas the small FSC-A group is expected to be undifferentiated monocytes[199,200]. We then wanted to understand if there was variation in the percentage of cells within this FSC-large group. We noted no significant differences or trends indicating a change in the proportion of these FSC-large cells, suggesting that the cannabinoid treatment did not impact the ability of cells to differentiate from monocytes to macrophages.

We then investigated the expression of CB_2, an important cannabinoid receptor on immune cells. While we expect that the expression of CB_2 is associated with the function of cannabinoids on macrophages, it is important to note that cannabinoids have been shown to act in pathways independent of both CB_1 and CB_2[129]. We observed significant donor-to-donor variation in the percentage of cells expressing CB_2. This inter-donor variation has been previously described in monocytes[201,202]. We noted a significant decrease in CB_2 MFI following CBD high and THC high treatment. A similar effect has been shown with other endocannabinoids, including cannabichromene and noladin ether[203,204]. Prolonged application of these CB_2 agonists lead to decreased CB_2 expression on the cell surface and desensitization[203,204]. Acute receptor desensitization is a known mechanism to continuous drug exposure and is in part driven by the

decoupling of the receptor from the G-protein and subsequent internalization[205]. This is hypothesized to be a protective adaptation to prevent physiological drug tolerance, as drugs such as morphine that do not induce receptor internalization may be predisposed to tolerance[205]. CB_2 internalization has been reported following exposure of some agonists, but not others[206]. Interestingly, in a paper by Atwood *et al.,* THC did not produce CB_2 internalization in human embryonic kidney cells (HEK)[206]. This lack of effect could be due to differences in the nature of the cells. Additionally, internalization may explain why no changes in the mRNA levels of CB_2 were seen in THC dependent individuals in comparison to non-to dependent individuals, as the mRNA could still be present in the cell following internalization[207]. This internalization is easily reversible, however, with low CB_1 levels observed in cannabis dependent men returning to normal levels after just 2 days of abstinence[208]. In clinical applications, it may be beneficial to consider the incorporation of "abstinence days" to refresh the surface expression of cannabinoid receptors to reduce tolerance and increase drug effectiveness in patients. This practice is already performed amongst recreational cannabis users, colloquially referred to as "T-Breaks"[209]. Additionally, CB_2 levels have been shown to be increased during periods of inflammation, so a reduction of CB_2 may indicate a reduction of inflammation[116,117].

As we are interested in the effect of cannabinoids in reducing chronic inflammation and co-morbidities in PLWH, CCR5, an HIV co-receptor is of particular interest to us. We showed that low and high levels of CBD, and high levels of THC during macrophage differentiation led to a reduced expression of CCR5 on the cell surface. A dose dependent effect was observed between the CBD low and CBD high conditions. A similar decrease in CCR5 expression, measured in MFI, has been reported during macrophage differentiation by Williams *et al.* using 30 μM of THC and was associated with reduced HIV infection of macrophages[136]. Thus, we predict that CBD will

also have a similar effect in reducing macrophage infection. This is significant as it could contribute to reducing the spread of HIV to macrophages during periods of viral rebound.

We next looked at CD14, CD16, and CD11b, all surface receptors typically used to characterize macrophages. CD14 is critical to the response of monocytes and macrophages to LPS, resulting in an inflammatory response[42,43]. We show that there is a decrease in CD14 expression in cells differentiated in both low and high doses of CBD and THC in a dose dependent manner. Unexpectedly, we also observed a non-significant increase in CD14 expression in our vehicle control group, potentially indicating CD14 is sensitive to methanol treatment. Significantly, this effect is in opposition to the effect of the cannabinoid treatments, potentially indicating an even greater effect of the cannabinoids. In microglia, like many macrophages, CD14 is constitutively expressed, however, differing levels of expression are used to distinguish levels of macrophage activation[210]. Thus, the observed decrease we observed may be due to lower levels of macrophage activation. Following differentiation in the presence of THC Williams *et al.* observed a decrease in CD14, in addition to CD16 expression on macrophages[136]. CD14 is shed readily from human monocytes upon activation[211]. sCD14 levels in HIV infection is a predictor of mortality and is associated with increased inflammatory markers[29]. Further analysis of the supernatant of our differentiated MDMs would be beneficial to understand the role sCD14 may be playing in our experimental design. CD16, a marker of macrophage activation. It has been associated with inflammation, such as its role in promoting intestinal fibrosis[212]. Furthermore, CD16+, and not CD16- monocytes promote HIV replication in macrophages, CD4+T cells, and conjugates of macrophages and T cells following macrophage differentiation[213]. We observed a non-significant decrease in CD16 expression following THC treatment during macrophage differentiation,

potentially mitigating the inflammatory role of this receptor. There were no changes in the pan myeloid, CD11b receptor.

We then assessed anti-inflammatory macrophage markers CD163, CD206, and CD71. CD163 is a scavenger receptor hemoglobin (Hb) -haptoglobin (Hp) and is associated with anti-inflammatory effects of myeloid cells. It is thought that CD163 plays a role in resolving inflammation through the scavenging of pro-inflammatory Hb, thus, preventing free Hb associated damage to tissue and releasing anti-inflammatory cytokines including IL-10[214,215]. Unexpectedly, we observed a non-significant decrease in CD163 following macrophage differentiation in the presence of a high dose of CBD and THC. Despite the typical association of CD163 as being anti-inflammatory, Williams *et al.* also observed a decrease in CD163 in the presence of THC[136]. Recent work also shows that in the context of atherosclerosis, CD163+ macrophages were associated with vascular permeability, intraplaque angiogenesis and inflammation[216]. In rats, CD163 has been shown to enhance the production of pro-inflammatory mediators[217]. Additionally, Tuluc *et al.* HIV production was shown to be greater in $CD163^{high}$ macrophages[218]. This paper also showed that Hb-Hp attenuated HIV infection of macrophages in a dose-dependent manner[218]. Accordingly, the role of CD163 may be more complicated than once anticipated. CD206 is a mannose receptor, which also plays a role in antigen uptake. In colonic macrophages, CD206+ macrophages produced higher levels of IL-10 transcripts than CD206- macrophages[219]. In murine lungs, CD206+ macrophage depletion was associated with worsened lung injury and neutrophil infiltration, further supporting the role of these cells in the resolution of inflammation[220]. We observed a non-significant increase in CD206 in the low dose of CBD, but no effect of THC. Furthermore, we found no changes associated with CD71, an anti-inflammatory marker found in high levels in M2a cells over M1 cells and critical to cellular uptake of iron[169].

In regard to pro-inflammatory markers, we assessed the expression of TLR4 and CD86. TLR4 recognizes gram-negative bacteria through binding LPS and initiates pro-inflammatory signaling cascades[221]. One of these cascades involves TRAM and TRIF and only occurs following endocytosis of TLR4[221]. Significantly, the internalization of TLR4 is regulated by CD14[221]. Hence, the decreased expression of TLR4 we observed in macrophages differentiated with high doses of CBD and the non-significant, yet visible effect observed on macrophages treated with THC, makes sense in combination with the decreases in CD14 expression we also observed as they both work on the same pathway. CD86 is a co-stimulatory molecule important for T cell activation and survival, thus expanding the immune response[222]. We observed no changes in the expression of CD86. Taken together, these results suggest that both CBD and THC may be associated with reduced responsiveness to LPS. While some observations were unexpected, such as a non-significant decrease of CD163 expression, this result is better understood within the framework of the literature, which calls the entirely anti-inflammatory role of CD163 into question.

We determined the effect of cannabinoid treatment on *in vitro* polarization of monocyte derived macrophages into a pro- or anti-inflammatory macrophage subset to address aim 2. We analyzed the effect of cannabinoids on M1 and M2a subsets independently to determine any effect within a subset. For aim 2, we had one less sample than we used in aim 1. Subsequently, we have not reached significance in any of the parameters assessed. This is likely due to the smaller sample size, and thus, lower power. I will report and discuss all non-significant p-values below 0.1. Further work should be done to increase the sample size. Following polarization, morphologically, M2a treated cells were highly elongated extensions, that may potentially be nanotubules, which have recently been suggested to be widespread and versatile in the context of macrophages[223]. We first looked at the percentage of differentiated, large FSC MDMs, and noted no difference in either

group. Thus, we believe the cannabinoid treatment did not affect the cell's ability to polarize. We then looked at the expression of receptors CB_2 and CCR5. For the cannabinoid receptor CB_2, we observed no changes in expression for either M1 or M2a. This could be due to the time the cells in aim 2 spent in the presence of cannabinoids in comparison to those in aim 1. The cells treated with cannabinoids during polarization were only treated for the length of polarization, which is 2 days, whereas macrophage differentiation takes 6 days. Thus, the cells may not have been desensitized in this shorter timeframe. Additionally, there were no changes to CCR5 expression. This may be in part a result of the increased expression of CCR5 that occurs during the differentiation of monocytes to MDMs[171]. There may be some mechanism inhibiting the production of CCR5 during this transition but does not impact the already expressed CCR5 observed on macrophages undergoing polarization.

Next, we looked at CD14, CD16, and CD11b, our traditional macrophage markers. For M1 cells, we observed a non-significant increase in CD14 and CD11b expression on cells that underwent polarization with THC. This differential response of CD14 when compared to the decrease observed in our MDMs may be due to the pro-inflammatory nature of M1 cells. Changes in CD11b expression were not observed during macrophage differentiation. CD11b on macrophages could inhibit the inflammatory response via the TLR pathway[224]. CD11b-deficient microglia cells in culture have been shown to produce more IL-6 and TNF-a[224]. CD11b+ alveolar macrophages have also been shown to decrease inflammation in acute liver injury in mice[225]. Thus, increased CD11b may contribute to the resolution of inflammation. However, the role of CD11b in myeloid inflammation, especially within the lungs, is unclear as high CD11b expression on alveolar macrophages has been associated with acute lung irritation in a mouse model of COPD and in patients with pneumonia and acute respiratory distress syndrome[226-228]. This pro-

inflammatory role in lung macrophages may be tissue specific, as each macrophage tissue subset is distinct in activation and functionality. In M2a macrophages, CD16 fluorescent expression was non-significantly decreased in cells that underwent polarization in the presence of THC. This is likely beneficial to chronic inflammation, due to the association of CD16 with inflammation, fibrosis, and HIV replication[212,213].

In regard to the traditional anti-inflammatory markers, no changes were observed in expression of CD206 or CD71. Non-significant decreases in CD163 expression were observed in M1 cells polarized with both CBD and THC treatment. This change corresponds with the decrease in CD163 observed in macrophages that underwent differentiation in the presence of a high dose of both CBD and THC. While initially thought to be anti-inflammatory, the role of CD163 in inflammation is likely more nuanced, potentially playing a role in contributing to inflammation, including during HIV infection. We finally looked at the expression of pro-inflammatory markers TLR4 and CD86 and found no changes in expression associated with cannabinoid treatment.

Overall, the anti-inflammatory role of CBD and THC on the polarization of already differentiated macrophages into a more pro- or anti-inflammatory phenotype is less clear than the anti-inflammatory role observed during the differentiation of macrophages. For M1 cells, some changes including decreased levels of CD14, CD11b, and CD163 could contribute to an anti-inflammatory role. Most of these anti-inflammatory changes were observed following THC treatment, with only the decrease in CD163 also a result of CBD treatment. In M2a cells only THC reduced a single marker of activation, CD16. Much research into anti-inflammatory roles of cannabinoids, especially in the context of HIV infection have focused on the role of THC, and our results support the finding that THC may be critical to the anti-inflammatory role of cannabis in PLWH.

To explore the anti-inflammatory role of cannabinoids observed in fresh monocytes, we extended our studies to include a monocytic cell line with latent HIV infection in aim 3. We first quantified HIV production on MDMs following 24 hours of cannabinoid treatment. We repeated this experiment three times, and due to the low sample size, we could not achieve the power to run meaningful statistical tests. However, there is a clear decrease in concentration of HIV p24 following both treatment with CBD and THC. Additionally, one of the replicates, while showing a clear reduction in HIV-1 production following CBD and THC treatments, was found to produce much less p24 than the other replicates, supporting the need for a larger sample size. As reviewed in Chapter 1, cannabinoids including CBD and THC have been shown in *in vitro*, animal, and human studies to decrease HIV infection macrophages and subsequent viral production.

We next aimed to characterize phenotypic changes to the U1 cells associated with decreased viral production. We analyzed markers found to be important in our *in vitro* differentiation and polarization experiments, and that were known to be expressed by U1 cells or their parent, uninfected U937 cells[229-231]. Critically CD14 expression on U1 cells is necessary for LPS induced stimulation of HIV production from U1 cells[229]. As we stimulate the cells with PMA instead of LPS, we aimed to understand the role of CD14 in HIV production in this manner. We additionally added CB_2 as an exploratory marker, as to our knowledge CB_2 has not been described on U1 cells or U937 cells. Overall, we observed no changes in the expression of any of the markers we assessed. Significantly, CD206 expression was very low in all samples. Taniguchi *et al.* found that only U937 cells activated with IL-4 expressed significant levels of CD206. It is possible that PMA does not activate the U1 cells in the same way. Additionally, we observed a clearly positive, yet very small population of CB_2+ cells, at less that 1% in all samples and conditions. Future

experiments should be done to characterize CB_2 mRNA before and after differentiation with PMA to gain a greater understanding of CB_2 in U1 cells.

Conclusions and Future Directions

Overall, it appears that cannabinoids CBD and THC promote a shift towards an anti-inflammatory macrophage phenotype during differentiation of monocytes into macrophages, and to a lesser extent, during the polarization of macrophages into M1 and M2a phenotypes. This is likely due to the strong effect of the cytokines used to promote the various subtypes overpowering the potential role the cannabinoids may have. This is significant as our understanding that cannabinoids may be critical to limiting the inflammation caused by initial monocyte infiltration but may be less important in tissue resident macrophages. We further showed that in a monocytic cell line differentiated into macrophages, cannabinoids inhibited viral production. This research will contribute to the general understanding of cannabinoids in the context of persistent immune activation and potentially support the development of their use as an interventional strategy for HIV-associated chronic inflammation in conjunction with ART.

While we have shown that there is a shift towards a more anti-inflammatory phenotype of macrophages that underwent differentiation in the presence of cannabinoids, we have additionally preserved supernatants of these samples to understand the pro- and anti-inflammatory cytokines and other products produced by these differentiating cells. This will allow us to better understand the effect that the cannabinoid have exerted on cell function. Cytokines themselves may also be classified as soluble or EV-associated cytokines[180,132]. EVs are released by almost all cells and during viral infection they incorporate viral-encoded molecules[180,232]. HIV infection has been previously shown to alter the distribution of cytokines between soluble and EV-associated forms and this effect is not completely reversed by ART[233]. A recent study showed that CBD treatment reduced the release of EVs from U1 cells and primary macrophages in the context of HIV infection, thus, we are particularly interested in understanding the ratio of soluble to EV associated

cytokines[180]. Luminex technology will be used to quantify EV- associated and soluble substances in the supernatant from the MDMs treated with cannabinoids. Targets include sCD14, sCD163, IL-1β, IL-7, IL-18, IFN-γ, MIP-1α, MIP-1β, and RANTES. Additionally, Cassol *et al. 2009* has shown that both M1 and M2a cells have distinct mechanisms in which they both decrease productive HIV-1 infection in comparison to non-polarized MDMs[66]. The next logical step would perform an infection assay of MDM, M1, and M2a cells in the presence of cannabinoids to elucidate the role of cannabinoids in HIV viral production in all three cell types. Additionally, future directions may include repeating these cytotoxicity experiments on isolated monocytes to ensure that the experimental concentrations of cannabinoids used are non-toxic.

The mechanisms underpinning the downregulation of HIV production following cannabinoid treatment of U1 cells should be further investigated. Firstly, the expression of CB_2 mRNA can be analyzed through quantitative polymerase chain reaction (qPCR) to understand the expression of CB_2 in U1 cells before and after stimulation with PMA, and additionally following cannabinoid treatment. This will help our understanding of the effects of cannabinoids which are dependent on the CB_2 receptor. I have established a working protocol and primer set to perform this qPCR at our facilities. Cytokine analysis could additionally be done on the supernatant of the U1 cells to help decipher the signals cells are receiving, and potential pathways that are being activated to inhibit viral replication, as previously described for the primary PBMCs.

In addition to expanding on the experiments described in this thesis, future experiments should be aimed at understanding the physiological effects of some of the anti-inflammatory effects reported in this thesis. Our lab is currently undertaking a randomized clinical trial into the safety, tolerability, and immune impact of oral cannabinoids on PLWH, as described in the study protocol published in British Medical Journal[191]. Upon enrolment at McGill this project was

originally to be a major component of my thesis but has since been removed due to delays caused by COVID-19. This research will assess changes in markers of microbial translocation such as LPS and sCD14, cytokines including IL-6, IL-10, and IP-10, and various T cell subsets following 12 weeks of daily cannabinoid treatment[191]. This clinical trial addresses many issues of importance for PLWH, including quantification of the size of the HIV viral reservoir pre and post cannabinoid treatment.

Due to the high rates of co-morbidities within PLWH, it is also important to study the effect of cannabinoids within these populations. Work in our lab also sets out to determine the effects of Direct Acting antivirals (DAA) treatment, resulting in sustained virologic response (SVR) or Hepatitis C (HCV) cure, on markers of chronic inflammation and immune activation in individuals with HIV-Hepatitis C co-infection, and whether these changes correlate with changes in endocannabinoid system. Banked plasma and PBMCs from individuals with HIV-HCV co-infection (half males and half females) enrolled in the Canadian Co-infection Cohort (CCC) study, before and after HCV cure, will be used. I have assisted in developing a flow cytometry panel to assess various markers of cellular activation, senescence and exhaustion in addition to CB_2 expression. Levels of important endocannabinoid mediators will be quantified in plasma. Through this research, we aim to determine whether any changes in endocannabinoid levels following SVR correlate with changes in monocyte or lymphocyte activation profiles.

Overall, this work shows the potential anti-inflammatory role of CBD and THC on macrophage differentiation and polarization. We further explored this effect in the context of HIV infected cell lines and were able to show decreased viral production. This work will help contribute to the growing body of work on the impact of cannabinoid usage, especially in the context of chronic viral infections.

References

1. Centers for Disease Control. A cluster of Kaposi's sarcoma and Pneumocystis carinii pneumonia among homosexual male residents of Los Angeles and Orange Counties, California. *MMWR Morb Mortal Wkly Rep.***31**(23):305-307 (1982).
2. F. Barré-Sinoussi, Chermann, J. C. *et al.* Isolation of a T-Lymphotropic Retrovirus from a Patient at Risk for Acquired Immune Deficiency Syndrome (AIDS) Isolation of a T-Lymphotropic Retrovirus from a Patient at Risk for Acquired Immune Deficiency Syndrome (AIDS). *Science.* **220**, 868–871 (1983).
3. Gallo, R. C. *et al.* Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science.* **224**, 500–503 (1984).
4. Korber, B. *et al.* Timing the ancestor of the HIV-1 pandemic strains. *Science.* **288**, 1789–1796 (2000).
5. Zhu, T. *et al.* An African HIV-1 sequence from 1959 and implications for the of the epidemic. *Nature* **391**, 594–597 (1998).
6. Désiré, N. *et al.* Characterization update of HIV-1 M subtypes diversity and proposal for subtypes A and D sub-subtypes reclassification. *Retrovirology* **15**, 1–7 (2018).
7. Chapter 26 - Acquired Immune Deficiency Syndrome. In: Flaherty DK, ed. *Immunology for Pharmacy.* Saint Louis: Mosby, 214-223 (2012).
8. Hirsch, V. M., Olmsted, R. A., Murphey-Corb, M., Purcell, R. H. & Johnson, P. R. An African primate lentivirus (SIVsm closely related to HIV-2. *Nature* **339**, 389–392 (1989).
9. Sharp, P. M. & Hahn, B. H. Origins of HIV and the AIDS pandemic. *Cold Spring Harb. Perspect. Med.* **1**, 1–22 (2011).
10. Gottlieb, G. S. *et al.* A call for randomized controlled trials of antiretroviral therapy for HIV-2 infection in West Africa. *AIDS* **22**, 2069–2072 (2008).
11. UNAIDS Data 2020. *UNAIDS* (2020).
12. Summary: Estimates of HIV incidence, prevalence, and Canada's progress on meeting the 90-90-90 HIV targets, 2016. *Public Health Agency of Canada* (2018).
13. Kordy, K., Tobin, N. H. & Aldrovandi, G. M. HIV and SIV in Body Fluids: From Breast Milk to the Genitourinary Tract. *Curr. Immunol. Rev.* **15**, 139–152 (2018).
14. Patel, P. *et al.* Estimating per-act HIV transmission risk: A systematic review. *AIDS* **28**, 1509–1519 (2014).
15. Trickey, A. *et al.* Survival of HIV-positive patients starting antiretroviral therapy between 1996 and 2013: a collaborative analysis of cohort studies. *Lancet HIV* **4**, e349–e356 (2017). Figures used in accordance with CC BY.
16. Marcus, J. L. *et al.* Comparison of overall and comorbidity-free life expectancy between insured adults with and without HIV Infection, 2000-2016. *JAMA Netw. open* **3**, e207954 (2020).
17. UNAIDS Fact Sheet – World AIDS Day 2021. *UNAIDS* (2021).
18. Atta, M. G., De Seigneux, S. & Lucas, G. M. Clinical pharmacology in HIV therapy. *Clin. J. Am. Soc. Nephrol.* **14**, 435–444 (2019). CCC License 1206109-1
19. HIV.gov CI. What to Start: Initial Combination Regimens for the Antiretroviral-Naive Patient. *Guidelines for the Use of Antiretroviral Agents in Adults and Adolescents Living with HIV* 2019; https://clinicalinfo.hiv.gov/en/guidelines/adult-and-adolescent-arv/what-start-initial-combination-regimens-antiretroviral-naive?view=full, 2021.

20. Bavinton, B. R. *et al.* Viral suppression and HIV transmission in serodiscordant male couples: an international, prospective, observational, cohort study. *Lancet HIV* **5**, e438–e447 (2018).
21. Rodger, A. J. *et al.* Sexual activity without condoms and risk of HIV transmission in serodifferent couples when the HIV-positive partner is using suppressive antiretroviral therapy. *JAMA - J. Am. Med. Assoc.* **316**, 171–181 (2016).
22. Rodger, A. J. *et al.* Risk of HIV transmission through condomless sex in serodifferent gay couples with the HIV-positive partner taking suppressive antiretroviral therapy (PARTNER): final results of a multicentre, prospective, observational study. *Lancet* **393**, 2428–2438 (2019).
23. Challacombe L. The Epidemiology of HIV in Canada. (2020). https://www.catie.ca/en/fact-sheets/epidemiology/epidemiology-hiv-canada.
24. Delgado-Vélez, M. & Lasalde-Dominicci, J. A. The cholinergic anti-inflammatory response and the role of macrophages in HIV-induced inflammation. *Int. J. Mol. Sci.* **19**, (2018).
25. Frank, T. D. *et al.* Global, regional, and national incidence, prevalence, and mortality of HIV, 1980-2017, and forecasts to 2030, for 195 countries and territories: A systematic analysis for the Global Burden of Diseases, Injuries, and Risk Factors Study 2017. *Lancet HIV* **6**, e831–e859 (2019). CCC Licence 1187319-1.
26. Deeks, S. G., Russell, T. & Douek, D. C. Systemic effects of inflammation on health during chronic HIV infection. *Immunity* **39**, 633-645 (2013).
27. French, M. A., King, M. S., Tschampa, J. M., Da Silva, B. A. & Landay, A. L. Serum immune activation markers are persistently increased in patients with HIV infection after 6 years of antiretroviral therapy despite suppression of viral replication and reconstitution of CD4+ T Cells. *J. Infect. Dis.* **200**, 1212–1215 (2009).
28. Neuhaus, J. *et al.* Markers of inflammation, coagulation, and renal function are elevated in adults with HIV infection. *J. Infect. Dis.* **201**, 1788–1795 (2010).
29. Sandler, N. G. *et al.* Plasma levels of soluble CD14 independently predict mortality in HIV infection. *J. Infect. Dis.* **203**, 780–790 (2011).
30. Costiniuk, C. T. & Angel, J. B. Human immunodeficiency virus and the gastrointestinal immune system: Does highly active antiretroviral therapy restore gut immunity. *Mucosal Immunol.* **5**, 596–604 (2012).
31. Hileman, C. O. *et al.* Differential Reduction in Monocyte Activation and Vascular Inflammation with Integrase Inhibitor-Based Initial Antiretroviral Therapy among HIV-Infected Individuals. *J. Infect. Dis.* **212**, 345–354 (2015).
32. McCausland, M. R. *et al.* Altered monocyte phenotype in HIV-1 infection tends to normalize with integrase- inhibitor-based antiretroviral therapy. *PLoS One* **10**, 1–19 (2015).
33. Papagno, L. *et al.* Immune activation and CD8+ T-cell differentiation towards senescence in HIV-1 infection. *PLoS Biol.* **2**, (2004).
34. Martin, M. D., Shan, Q., Xue, H. H. & Badovinac, V. P. Time and antigen-stimulation history influence memory CD8 T cell bystander responses. *Front. Immunol.* **8**, (2017).
35. Smith, M. Z., Bastidas, S., Karrer, U. & Oxenius, A. Impact of antigen specificity on CD4+ T cell activation in chronic HIV-1 infection. *BMC Infect. Dis.* **13**, 1 (2013).
36. Wittkop, L. *et al.* Effect of cytomegalovirus-induced immune response, self antigen-induced immune response, and microbial translocation on chronic immune activation in

successfully treated HIV type 1-infected patients: the ANRS CO3 Aquitaine Cohort. *J. Infect. Dis.* **207**, 622–627 (2013).
37. Naeger, D. M. *et al.* Cytomegalovirus-specific T cells persist at very high levels during long-term antiretroviral treatment of HIV disease. *PLoS One* **5**, 1–9 (2010).
38. Mannioui, A. *et al.* Dynamics of viral replication in blood and lymphoid tissues during SIVmac251 infection of macaques. *Retrovirology* **6**, 1–15 (2009).
39. Guadalupe, M. *et al.* Severe CD4+ T -cell depletion in gut lymphoid tissue during primary human immunodeficiency virus type 1 infection and substantial delay in restoration following highly active antiretroviral therapy. *J. Virol* **77**, 11708-11717 (2003).
40. Sharpstone, D. *et al.* Small intestinal transit, absorption, and permeability in patients with AIDS with and without diarrhoea. *Gut* **45**, 70–76 (1999).
41. Brenchley, J. M. *et al.* Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat. Med.* **12**, 1365–1371 (2006). CCC License 1187324-1.
42. Poltorak, A. *et al.* Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in TLR4 gene. *Science.* **282**, 2085–2088 (1998).
43. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J. & Mathison, J. C. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science.* **249**, 1431–1433 (1990).
44. Kumar, V. *et al.* Cannabinoid attenuation of intestinal inflammation in chronic SIV-infected rhesus macaques involves T cell modulation and differential expression of micro-RNAs and pro-inflammatory genes. *Front. Immunol.* **10**, 1–23 (2019). Figures used in accordance with CC BY.
45. Hsue, P. Y. Mechanisms of Cardiovascular Disease in the Setting of HIV Infection. *Can. J. Cardiol.* **35**, 238–248 (2019).
46. Dein Terra Mota Ribeiro, A. B., Heimesaat, M. M. & Bereswill, S. Changes of the intestinal microbiome—host homeostasis in HIV-infected individuals — a focus on the bacterial gut microbiome. *Eur. J. Microbiol. Immunol.* **7**, 158–167 (2017).
47. Zhou, J. *et al.* Gut Microbiome Changes Associated With HIV Infection and Sexual Orientation. *Front. Cell. Infect. Microbiol.* **10**, (2020).
48. Vázquez-Castellanos, J. F. *et al.* Altered metabolism of gut microbiota contributes to chronic immune activation in HIV-infected individuals. *Mucosal Immunol.* **8**, 760–772 (2015).
49. Routy J.P., Dupuy F.P., Lin J. & Isnard S. More than a Gender Issue: Testis as a Distinctive HIV Reservoir and Its Implication for Viral Eradication. *Methods Mol Biol.* **2407**, 173-186 (2022).
50. Van Den Hof, M. *et al.* CNS penetration of ART in HIV-infected children. *J. Antimicrob. Chemother.* **73**, 484–489 (2018).
51. Greener, B. N. *et al.* Dolutegravir pharmacokinetics in the genital tract and colorectum of HIV negative men after single and multiple dosing. *J. Acquir. Immune Defic. Syndr.* **64**, 39-44 (2013).
52. Thompson, C. G., Gay, C. L. & Kashuba, A. D. M. HIV Persistence in Gut-Associated Lymphoid Tissues: Pharmacological Challenges and Opportunities. *AIDS Res. Hum. Retroviruses* **33**, 513–523 (2017).
53. Choi, Y. *et al.* Low-Level Anorectal HIV Shedding despite Effective Antiretroviral Therapy Is Not Driven by Mucosal Inflammation. *J. Immunol.* **207**, 685–695 (2021).

54. Varol, C., Mildner, A. & Jung, S. *Macrophages: Development and tissue specialization*. *Annual Review of Immunology* vol. 33 (2015). CCC License 1187325-1.
55. Palis, J., Robertson, S., Kennedy, M., Wall, C. & Keller, G. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* **126**, 5073–5084 (1999).
56. Lang, R. A. & Bishop, J. M. Macrophages are required for cell death and tissue remodeling in the developing mouse eye. *Cell* **74**, 453–462 (1993).
57. Fantin, A. *et al.* Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood* **116**, 829–840 (2010).
58. Yang, D. *et al.* A sweet spot for macrophages: Focusing on polarization. *Pharmacol. Res.* **167**, (2021).
59. Hashimoto, D. *et al.* Tissue resident macrophages self-maintain locally. *Immunity* **38**, 792–804 (2013).
60. Yao, Y., Xu, X. H. & Jin, L. Macrophage polarization in physiological and pathological pregnancy. *Front. Immunol.* **10**, 1–13 (2019). Figures used in accordance with CC BY.
61. Abdelaziz, M. H. *et al.* Alternatively activated macrophages; A double-edged sword in allergic asthma. *J. Transl. Med.* **18**, 1–12 (2020).
62. Murray, P. J. *et al.* Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity* **41**, 14–20 (2014).
63. Wang, L., Zhang, S., Wu, H., Rong, X. & Guo, J. M2b macrophage polarization and its roles in diseases. *J. Leukoc. Biol.* **106**, 345–358 (2019).
64. Zizzo G., Hilliard B.A., Monestier M. & Cohen P.L. Efficient clearance of early apoptotic cells by human macrophages requires M2c polarization and MerTK induction. *J Immunol.***189**, 3508–3520 (2012).
65. Ley, K. M1 Means Kill; M2 Means Heal. *J. Immunol.* **199**, 2191–2193 (2017).
66. Cassol, E., Cassetta, L., Rizzi, C., Alfano, M. & Poli, G. M1 and M2a Polarization of Human Monocyte-Derived Macrophages Inhibits HIV-1 Replication by Distinct Mechanisms. *J. Immunol.* **182**, 6237–6246 (2009).
67. Clerici, M. & Shearer, G. M. viewpoint A THI- T.2 switch is a critical step in the etiology of HIV infection. *Immunol. Today* (1993).
68. Hocini, H. *et al.* HIV Controllers Have Low Inflammation Associated with a Strong HIV-Specific Immune Response in Blood. *J. Virol.* **93**, 1–14 (2019).
69. Na, Y. R., Stakenborg, M., Seok, S. H. & Matteoli, G. Macrophages in intestinal inflammation and resolution: a potential therapeutic target in IBD. *Nat. Rev. Gastroenterol. Hepatol.* **16**, 531–543 (2019). CCC License 5244890683450.
70. Lu, H., Huang, D., Ransohoff, R. M. & Zhou, L. Acute skeletal muscle injury: CCL2 expression by both monocytes and injured muscle is required for repair. *FASEB J.* **25**, 3344–3355 (2011).
71. Barker, R. N. *et al.* Antigen presentation by macrophages is enhanced by the uptake of necrotic, but not apoptotic, cells. *Clin. Exp. Immunol.* **127**, 220–225 (2002).
72. Fox, S., Leitch, A. E., Duffin, R., Haslett, C. & Rossi, A. G. Neutrophil apoptosis: Relevance to the innate immune response and inflammatory disease. *J. Innate Immun.* **2**, 216–227 (2010).

73. Medina, C. B. & Ravichandran, K. S. Do not let death do us part: 'Find-me' signals in communication between dying cells and the phagocytes. *Cell Death Differ.* **23**, 979–989 (2016).
74. Gude, D. R. *et al.* Apoptosis induces expression of sphingosine kinase 1 to release sphingosine-1-phosphate as a "come-and-get-me" signal. *FASEB J.* **22**, 2629–2638 (2008).
75. Truman, L. A. *et al.* CX3CL 1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. *Blood* **112**, 5026–5036 (2008).
76. Elliott, M. R. *et al.* Nucleotides released by apoptotic cells act as a find-me signal for phagocytic clearance. *Nature* **461**, 282–286 (2009).
77. Schett, G. & Neurath, M. F. Resolution of chronic inflammatory disease: universal and tissue-specific concepts. *Nat. Commun.* **9**, (2018).
78. Arnold, L. *et al.* Inflammatory monocytes recruited after skeletal muscle injury switch into anti-inflammatory macrophages to support myogenesis. *J. Exp. Med.* **204**, 1057–1069 (2007).
79. Zhu, Y. *et al.* The pentacyclic triterpene Lupeol switches M1 macrophages to M2 and ameliorates experimental inflammatory bowel disease. *Int. Immunopharmacol.* **30**, 74–84 (2016).
80. Papayianni, A., Serhan, C. N., Phillips, M. L., Rennke, H. G. & Brady, H. R. Transcellular biosynthesis of lipoxin A4 during adhesion of platelets and neutrophils in experimental immune complex glomerulonephritis. *Kidney Int.* **47**, 1295–1302 (1995).
81. Kelesidis, T., Kendall, M. A., Yang, O. O., Hodis, H. N. & Currier, J. S. Biomarkers of microbial translocation and macrophage activation: Association with progression of subclinical atherosclerosis in HIV-1 infection. *J. Infect. Dis.* **206**, 1558–1567 (2012).
82. McKibben, R. A. *et al.* Elevated levels of monocyte activation markers are associated with subclinical atherosclerosis in men with and those without HIV infection. *J. Infect. Dis.* **211**, 1219–1228 (2015).
83. Anderson, A. M. *et al.* Cognitive and Neuronal Link With Inflammation: A Longitudinal Study in People With and Without HIV Infection. *J. Acquir. Immune Defic. Syndr.* **85**, 617–625 (2020).
84. Lyons, J. L. *et al.* Plasma sCD14 is a biomarker associated with impaired neurocognitive test performance in attention and learning domains in HIV infection. *J. Acquir. Immune Defic. Syndr.* **57**, 371-379 (2011).
85. Navia, B.A., Cho, E., Petito, C. K., Price, R. W. The AIDS dementia complex: II neuropathology. *Ann Neurol* **19**, 525-535 (1986).
86. Williams, D. *et al.* Monocytes Mediate HIV Neuropathogenesis: Mechanisms that Contribute to HIV Associated Neurocognitive Disorders. *Curr. HIV Res.* **12**, 85–96 (2014).
87. Saylor, D. *et al.* Corrigendum: HIV-associated neurocognitive disorder - Pathogenesis and prospects for treatment. *Nat. Rev. Neurol.* **12**, 309 (2016).
88. Hillig, K. W. & Mahlberg, P. G. A chemotaxonomic analysis of cannabinoid variation in Cannabis (Cannabaceae). *Am. J. Bot.* **91**, 966–975 (2004).
89. Kalant, H. Medicinal use of cannabis: History and current status. *Pain Res. Manag.* **6**, 80–91 (2001).
90. Russo, E. B. History of cannabis and its preparations in saga, science, and sobriquet. *Chem. Biodivers.* **4**, 1614–1648 (2007).

91. Wallace, M. S., Marcotte, T. D., Umlauf, A., Gouaux, B. & Atkinson, J. H. Efficacy of Inhaled Cannabis on Painful Diabetic Neuropathy. *J. Pain* **16**, 616–627 (2015).
92. Chaves, C., Bittencourt, P. C. T. & Pelegrini, A. Ingestion of a THC-Rich Cannabis Oil in People with Fibromyalgia: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial. *Pain Med. (United States)* **21**, 2212–2218 (2020).
93. Serpell, M. *et al.* A double-blind, randomized, placebo-controlled, parallel group study of THC/CBD spray in peripheral neuropathic pain treatment. *Eur. J. Pain (United Kingdom)* **18**, 999–1012 (2014).
94. Peyravian, N., Deo, S., Daunert, S. & Jimenez, J. J. Cannabidiol as a Novel Therapeutic for Immune Modulation. *ImmunoTargets Ther.* **Volume 9**, 131–140 (2020).
95. Arzimanoglou, A. *et al.* Epilepsy and cannabidiol: a guide to treatment. *Epileptic Disord.* **22**, 1–14 (2020).
96. Costiniuk, C. T. & Jenabian, M. A. Cannabinoids and inflammation: Implications for people living with HIV. *Aids* **33**, 2273–2288 (2019).
97. Harris, G. E. *et al.* Patterns and correlates of cannabis use among individuals with HIV/AIDS in maritime Canada. *Can. J. Infect. Dis. Med. Microbiol.* **25**, 1–7 (2014).
98. Shiau, S., Arpadi, S. M., Yin, M. T. & Martins, S. S. Patterns of drug use and HIV infection among adults in a nationally representative sample. *Addict. Behav.* **68**, 39–44 (2017).
99. Klumpers, L. E. & Thacker, D. L. A brief background on cannabis: From plant to medical indications. *J. AOAC Int.* **102**, 412–420 (2019).
100. Lorenzetti, B. B., Souza, G. E. P., Sarti, S. J., Santos Filho, D. & Ferreira, S. H. Myrcene mimics the peripheral analgesic activity of lemongrass tea. *J. Ethnopharmacol.* **34**, 43–48 (1991).
101. Do Vale, T. G., Furtado, E. C., Santos, J. G. & Viana, G. S. B. Central effects of citral, myrcene and limonene, constituents of essential oil chemotypes from Lippia alba (mill.) N.E. Brown. *Phytomedicine* **9**, 709–714 (2002).
102. Carvalho-Freitas MI, Costa M. Anxiolytic and sedative effects of extracts and essential oil from Citrus aurantium L. *Biol Pharm Bull* **25**: 1629–1633 (2002).
103. Siani, A. C., Souza, M. C., Henriques, M. G. M. O. & Ramos, M. F. S. Anti-inflammatory activity of essential oils from Syzygium cumini and Psidium guajava. *Pharm. Biol.* **51**, 881–887 (2013).
104. Russo, E. B. Taming THC: Potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *Br. J. Pharmacol.* **163**, 1344–1364 (2011).
105. Morales, P., Hurst, D. P. & Reggio, P. H. Molecular targets of the phytocannabinoids: A complex picture. *Phytocannabinoids.* **103** (2017).
106. Mechoulam, R. & Ben-Shabat, S. From gan-zi-gun-nu to anandamide and 2-arachidonoylglycerol: The ongoing story of cannabis. *Nat. Prod. Rep.* **16**, 131–143 (1999).
107. Pacher, P., Kogan, N. M. & Mechoulam, R. Beyond THC and endocannabinoids. *Annu. Rev. Pharmacol. Toxicol.* **60**, 637–659 (2020). CCC Licence 1187317-1.
108. de Fonseca, F. R. *et al.* The endocannabinoid system: Physiology and pharmacology. *Alcohol Alcohol.* **40**, 2–14 (2005).
109. Salzet, M. & Stefano, G. B. The endocannabinoid system in invertebrates. *Prostaglandins Leukot. Essent. Fat. Acids* **66**, 353–361 (2002).

110. Hillard, C. J. Circulating Endocannabinoids: From Whence Do They Come and Where are They Going? *Neuropsychopharmacology* **43**, 155–172 (2018).
111. Lee, Y., Jo, J., Chung, H. Y., Pothoulakis, C. & Im, E. Endocannabinoids in the gastrointestinal tract. *Am. J. Physiol. - Gastrointest. Liver Physiol.* **311**, G655–G666 (2016).
112. Russo, E. B. The case for the entourage effect and conventional breeding of clinical cannabis: No "Strain," no gain. *Front. Plant Sci.* **9**, 1–8 (2019).
113. Ben-Shabat, S. *et al.* An entourage effect: inactive endogenous fatty acid glycerol esters enhance 2-arachidonoyl-glycerol cannabinoid activity. *Eur. J. Pharmacol.* **353**, 23–31 (1998).
114. Chanda, D., Neumann, D. & Glatz, J. F. C. The endocannabinoid system: Overview of an emerging multi-faceted therapeutic target. *Prostaglandins Leukot. Essent. Fat. Acids* **140**, 51–56 (2019).
115. Galiègue, S. *et al.* Expression of Central and Peripheral Cannabinoid Receptors in Human Immune Tissues and Leukocyte Subpopulations. *Eur. J. Biochem.* **232**, 54–61 (1995).
116. Wright, K. *et al.* Differential Expression of Cannabinoid Receptors in the Human Colon: Cannabinoids Promote Epithelial Wound Healing. *Gastroenterology* **129**, 437–453 (2005).
117. Carlisle, S. J., Marciano-Cabral, F., Staab, A., Ludwick, C. & Cabral, G. A. Differential expression of the CB2 cannabinoid receptor by rodent macrophages and macrophage-like cells in relation to cell activation. *Int. Immunopharmacol.* **2**, 69–82 (2002).
118. Reddy, P.M., Maurya, N. & Velmurugan, B. K. Medicinal Use of Synthetic Cannabinoids—a Mini Review. *Curr. Pharmacol. Reports* **5**, 1–13 (2019). CCC Licence 1187339-1.
119. Muthumalage, T. & Rahman, I. Cannabidiol differentially regulates basal and LPS-induced inflammatory responses in macrophages, lung epithelial cells, and fibroblasts. *Toxicol. Appl. Pharmacol.* **382**, 114713 (2019).
120. Yeisley, D. J., Arabiyat, A. S. & Hahn, M. S. Cannabidiol-Driven Alterations to Inflammatory Protein Landscape of Lipopolysaccharide-Activated Macrophages in Vitro May Be Mediated by Autophagy and Oxidative Stress. *Cannabis Cannabinoid Res.* **6**, 253–263 (2021).
121. Liu, C., Ma, H., Slitt, A. L. & Seeram, N. P. Inhibitory Effect of Cannabidiol on the Activation of NLRP3 Inflammasome Is Associated with Its Modulation of the P2X7 Receptor in Human Monocytes. *J. Nat. Prod.* **83**, 2025–2029 (2020).
122. Wu, H. Y. *et al.* Cannabidiol induced a contrasting pro-apoptotic effect between freshly isolated and precultured human monocytes. *Toxicol. Appl. Pharmacol.* **246**, 141–147 (2010).
123. Specter, S., Lancz, G. & Goodfellow, D. Suppression of human macrophage function in vitro by Δ9-tetrahydrocannabinol. *J. Leukoc. Biol.* **50**, 423–426 (1991).
124. Shivers, S.C., Newton, C., Freidman, H. & Klein, T. W. Δ^9-tetrahydrocannabidiol (THC) modulates Il-1 bioactivity in human monocyte/macrophage cell lines. *Life Sci.* **54**, 1281-1289 (1994).

125. Roth, M. D, Castaneda, J. T. & Kiertscher, S. M. Exposure to 9-tetrahydocannabidiol impairs the differentiation of human monocyte-derived dendritic cells and their capacity for T cell activation. *J. Neuroimmune Pharmacol.* **10**, 333-343 (2015).
126. Börner, C., Smida, M., Höllt, V., Schraven, B. & Kraus, J. Cannabinoid receptor type 1- and 2-mediated increase in cyclic AMP inhibits T cell receptor-triggered signaling. *J. Biol. Chem.* **284**, 35450–35460 (2009).
127. Ngaotepprutaram, T., Kaplan, B.L.F. & Kaminski, N. E. Impaired NAFT and NFκB activation are involved in suppression of CD40 expresssion by Δ^9-tetrahydrocannabidiol in human CD4+ T cells. *Toxicol. Appl. Pharmacol.* **273**, 209-218 (2013).
128. Henriquez, J. E., Bach, A. P., Matos-Fernandez, K. M., Crawford, R. B. & Kaminski, N. E. Δ9-Tetrahydrocannabinol (THC) Impairs CD8+ T Cell-Mediated Activation of Astrocytes. *J. Neuroimmune Pharmacol.* **15**, 863–874 (2020).
129. Karmaus, P. W. F., Chen, W., Kaplan, B. L. F. & Kaminski, N. E. Δ9-tetrahydrocannabinol suppresses cytotoxic T lymphocyte function independent of CB1 and CB2, disrupting early activation events. *J. Neuroimmune Pharmacol.* **7**, 843–855 (2012).
130. Costa, B., Giagnoni, G., Franke, C., Trovato, A. E. & Colleoni, M. Vanilloid TRPV1 receptor mediates the antihyperalgesic effect of the nonpsychoactive cannabinoid, cannabidiol, in a rat model of acute inflammation. *Br. J. Pharmacol.* **143**, 247–250 (2004).
131. Patwardhan, A. M. *et al.* The cannabinoid WIN 55,212-2 inhibits transient receptor potential vanilloid 1 (TRPV1) and evokes peripheral antihyperalgesia via calcineurin. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 11393–11398 (2006).
132. Anand, U. *et al.* CBD effects on TRPV1 signaling pathways in cultured drg neurons. *J. Pain Res.* **13**, 2269–2278 (2020).
133. Yuan, M. *et al.* Δ9-Tetrahydrocannabinol regulates Th1/Th2 cytokine balance in activated human T cells. *J. Neuroimmunol.* **133**, 124–131 (2002).
134. Srivastava, M. D., Srivastava, B. I. S. & Brouhard, B. Δ9 Tetrahydrocannabinol and cannabidiol alter cytokine production by human immune cells. *Immunopharmacology* **40**, 179–185 (1998).
135. Derocq, J. M., Ségui, M., Marchand, J., Le Fur, G. & Casellas, P. Cannabinoids enhance human B-cell growth at low nanomolar concentrations. *FEBS Lett.* **369**, 177-182 (1995).
136. Williams, J. C. *et al.* Δ9-tetrahydrocannabinol treatment during human monocyte differentiation reduces macrophage susceptibility to HIV-1 infection. *J. Neuroimmune Pharmacol.* **9**, 369–379 (2014).
137. Costantino, C. M. *et al.* Cannabinoid receptor 2-mediated attenuation of CXCR4-tropic HIV infection in primary CD4+ T cells. *PLoS One* **7**, (2012).
138. Ramirez, S. H. *et al.* Attenuation of HIV-1 replication in macrophages by cannabinoid receptor 2 agonists. *J. Leukoc. Biol.* **93**, 801–810 (2013).
139. Cocchi, F. *et al.* Identification of RANTES, MIP-1a and MIP-1b as the major HIV-suppressive factors produced by CD8+ T cells. *Science.* **270**, 1811-1815 (1995).
140. Henriquez, J. E., Rizzo, M. D., Crawford, R. B., Gulick, P. & Kaminski, N. E. Interferon-A–mediated activation of T cells from healthy and HIV-infected individuals is suppressed by Δ^9-tetrahydrocannabinol. *J. Pharmacol. Exp. Ther.* **367**, 49–58 (2018).
141. Chen, W. *et al.* Magnitude of stimulation dictates the cannabinoid-mediated differential T cell response to HIV gp120. *J. Leukoc. Biol.* **92**, 1093–1102 (2012).

142. Whitney, J. B. & Jones, R. B. Chapter 10 – In vitro and in vivo models of HIV Latency. In: L. Zhang, S. R. Lewin (eds.), *HIV Vaccines and Cure: Advances in Experimental Medicine and Biology*. **1075**, 241-263 (2018).
143. Lockridge, J. L. *et al.* Mice engrafted with human fetal thymic tissue and hematopoietic stem cells develop pathology resembling chronic graft-versus-host disease. *Biol. Blood Marrow Transplant.* **19**, 1310–1322 (2013).
144. Greenblatt, M. B. *et al.* Graft versus Host Disease in the Bone Marrow, Liver and Thymus Humanized Mouse Model. *PLoS One* **7**, (2012).
145. Yasuda, M. *et al.* Incidence of spontaneous lymphomas in non-experimental NOD/Shi-scid, IL-2Rγnull (NOG) mice. *Exp. Anim.* **66**, 425–435 (2017).
146. Molina, P. E. *et al.* Modulation of Gut-specific mechanisms by chronic Δ9-Tetrahydrocannabinol administration in male rhesus macaques infected with simian immunodeficiency virus: A systems biology analysis. *AIDS Res. Hum. Retroviruses* **30**, 567–578 (2014).
147. Chandra, L. C. *et al.* Chronic Administration of Δ 9 -Tetrahydrocannabinol Induces Intestinal Anti-Inflammatory MicroRNA Expression during Acute Simian Immunodeficiency Virus Infection of Rhesus Macaques. *J. Virol.* **89**, 1168–1181 (2015).
148. O'Brien, J., Hayder, H., Zayed, Y. & Peng, C. Overview of microRNA biogenesis, mechanisms of actions, and circulation. *Front. Endocrinol.* **9**, 1–12 (2018).
149. Molina, P. E. *et al.* Cannabinoid administration attenuates the progression of simian immunodeficiency virus. *AIDS Res. Hum. Retroviruses* **27**, 585–592 (2011).
150. Amedee, A. M. *et al.* Chronic Δ9-tetrahydrocannabinol administration may not attenuate simian immunodeficiency virus disease progression in female rhesus macaques. *AIDS Res. Hum. Retroviruses* **30**, 1216–1225 (2014).
151. Schauer, G. L., Njai, R. & Grant-Lenzy, A. M. Modes of marijuana use – smoking, vaping, eating, and dabbing: Results from the 2016 BRFSS in 12 States. *Drug Alcohol Depend.* **209**, 107900 (2020).
152. Keen L. *et al.* Confirmed marijuana use and lymphocyte count in black people living with HIV. *Drug Alcohol Depend*. **198**, 112-115 (2019).
153. Bonn-Miller, M. O., Oser, M. L., Bucossi, M. M. & Trafton, J. A. Cannabis use and HIV antiretroviral therapy adherence and HIV-related symptoms. *J. Behav. Med.* **37**, 1–10 (2014).
154. Rizzo *et al.* HIV-infected cannabis have low circulating CD16+monocytes and IP-10 levels compared to non-using HIV-patients. *AIDS.* **32**, 419-429 (2018).
155. de Oliveira Feitosa de Castro, F. *et al.* Distinct inflammatory profiles in HIV-infected individuals under antiretroviral therapy using cannabis, cocaine or cannabis plus cocaine. *AIDS* **33**, 1831–1842 (2019).
156. Adams, J.W. *et al.* Association of cannabis, stimulant, and alcohol use with mortality prognosis among HIV-infected men. *AIDS Behav.* **22**,1341–1351 (2018).
157. Manuzak, J. A. *et al.* Heavy Cannabis Use Associated with Reduction in Activated and Inflammatory Immune Cell Frequencies in Antiretroviral Therapy-Treated Human Immunodeficiency Virus-Infected Individuals. *Clin. Infect. Dis.* **66**, 1872–1882 (2018).
158. Vidot, D.C. *et al.* Hazardous cannabis use and monocyte activation among methamphetamine users with treated HIV infection. *J. Acquir. Immune Defic. Syndr.* **81**, 361–364 (2019).

159. Castillo-Mancilla, J. R. *et al. J.* Higher ART adherence is associated with lower systemic inflammation in treatment-naïve Ugandans who achieve virological suppression. *Acquir. Immune Defic. Syndr.* **77**, 507–513 (2019).
160. Castillo-Mancilla, J. R. *et al.* Incomplete ART adherence is associated with higher inflammation in individuals who achieved virologic suppression in the START study. *J. Int. AIDS Soc.* **22**, (2019).
161. Bredt, B. M. *et al.* Short-term effects of cannabinoids on immune phenotype and function in HIV-1-infected patients. *J. Clin. Pharmacol.* **42**, 82–89 (2002).
162. Abrams, D. I. *et al.* Short-Term Effects of Cannabinoids in Patients with HIV-1 Infection: A Randomized, Placebo-Controlled Clinical Trial. *Ann. Intern. Med.* **139** (2003).
163. Huestis, M. A. & Cone, E. J. Relationship of Δ9-tetrahydrocannabinol concentrations in oral fluid and plasma after controlled administration of smoked cannabis. *J. Anal. Toxicol.* **28**, 394–399 (2004).
164. Huestis, M. A., Henningfield, J. E. & Cone, E. J. Blood cannabinoids absorption of THC and formation of 11-oh-thc and THC-COOOH during and after smoking marijuana. *J. Anal. Toxicol.* **16**, 276–282 (1992).
165. Millar, S. A., Stone, N. L., Yates, A. S. & O'Sullivan, S. E. A systematic review on the pharmacokinetics of cannabidiol in humans. *Front. Pharmacol.* **9**, (2018).
166. Chayasirisobhon, S. Mechanisms of Action and Pharmacokinetics of Cannabis. *Perm J* **25**, 1-3 (2020).
167. Ujváry, I. & Hanuš, L. Human Metabolites of Cannabidiol: A Review on Their Formation, Biological Activity, and Relevance in Therapy. *Cannabis Cannabinoid Res.* **1**, 90–101 (2016).
168. Devinsky, O. *et al.* Cannabidiol: Pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. *Epilepsia* **55**, 791-802 (2014).
169. Rey-Giraud, F., Hafner, M. & Ries, C. H. In vitro generation of monocyte-derived macrophages under serum-free conditions improves their tumor promoting functions. *PLoS One* **7**, (2012).
170. Iqbal, S. & Kumar, A. Characterization of In vitro Generated Human Polarized Macrophages. *J. Clin. Cell. Immunol.* **06**, (2015).
171. Tuttle, D. L., Harrison, J. K., Anders, C., Sleasman, J. W. & Goodenow, M. M. Expression of CCR5 Increases during Monocyte Differentiation and Directly Mediates Macrophage Susceptibility to Infection by Human Immunodeficiency Virus Type 1. *J. Virol.* **72**, 4962–4969 (1998).
172. Williams, J. C. *et al.* Δ9-tetrahydrocannabinol treatment during human monocyte differentiation reduces macrophage susceptibility to HIV-1 infection. *J. Neuroimmune Pharmacol.* **9**, 369–379 (2014).
173. Han, K. H. *et al.* CB1 and CB2 cannabinoid receptors differentially regulate the production of reactive oxygen species by macrophages. *Cardiovasc. Res.* **84**, 378–386 (2009).
174. NIH HIV Reagent Program. ARP-165 Human Immunodeficiency Virus- 1 (HIV-1) infected U937 Cells (U1). (2022). https://www.hivreagentprogram.org/Catalog/HRPCellLinesInfected/ARP-165.aspx

175. Emiliani, S. *et al.* Mutations in the tat Gene Are Responsible for Human Immunodeficiency Virus Type 1 Postintegration Latency in the U1 Cell Line. *J. Virol.***72**, 1666–1670 (1998).
176. Song, M. G. *et al.* NRF2 signaling negatively regulates phorbol-12-myristate-13-acetate (PMA)-induced differentiation of human monocytic U937 cells into pro-inflammatory macrophages. *PLoS One* **10**, 1–18 (2015).
177. Patel, A. A. *et al.* The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. *J. Exp. Med.* **214**, 1913–1923 (2017).
178. Condie, R., Herring, A., Koh, W. S., Lee, M. & Kaminski, N. E. Cannabinoid inhibition of adenylate cyclase-mediated signal transduction and interlukin-2 (IL-2) expression in the murine T-cell line, EL4.IL-2*. *J. Biol. Chem.* **271**, 13175-13183 (1996).
179. Cassol, E., Alfano, M., Biswas, P. & Poli, G. Monocyte-derived macrophages and myeloid cell lines as targets of HIV-1 replication and persistence. *J. Leukoc. Biol.* **80**, 1018–1030 (2006).
180. DeMarino, C. *et al.* Antiretroviral Drugs Alter the Content of Extracellular Vesicles from HIV-1-Infected Cells. *Sci. Rep.* **8**, 1–20 (2018).
181. Luque-Martin, R., Mander, P. K., Leenen, P. J. M. & Winther, M. P. J. Classic and new mediators for in vitro modelling of human macrophages. *J. Leukoc. Biol.* **109**, 549–560 (2021).
182. Audoy-Rémus, J. *et al.* Rod-shaped monocytes patrol the brain vasculature and give rise to perivascular macrophages under the influence of proinflammatory cytokines and angiopoietin-2. *J. Neurosci.* **28**, 10187–10199 (2008).
183. Auffray *et al.* Monitoring of blood vessels and tissue by population of monocytes with patrolling behaviour. *Science.* **317**, 666-670 (2007).
184. Rubino, T. & Parolaro, D. Sexually dimorphic effects of cannabinoid compounds on emotion and cognition. *Front. Behav. Neurosci.* **5**, 1–5 (2011).
185. Wagner, E. J. Sex differences in cannabinoid-regulated biology: A focus on energyhomeostasis. *Front Neuroendocrinol.* **40**, 101-109 (2016).
186. Taha, T. *et al.* Cannabis Impacts Tumor Response Rate to Nivolumab in Patients with Advanced Malignancies. *Oncologist* **24**, 549–554 (2019).
187. Pardali, E. *et al.* Cryopreservation of primary human monocytes does not negatively affect their functionality or their ability to be labelled with radionuclides: basis for molecular imaging and cell therapy. *EJNMMI Res.* **6**, (2016).
188. Nadulski, T. *et al.* Simultaneous and sensitive analysis of THC, 11-OH-THC, THC-COOH, CBD, and CBN by GC-MS in plasma after oral application of small doses of THC and cannabis extract. *J. Anal. Toxicol.* **29**, 782–789 (2005).
189. Springs, A. E. B., Karmaus, P. W. F., Crawford, R. B., Kaplan, B. L. F. & Kaminski, N. E. Effects of targeted deletion of cannabinoid receptors CB 1 and CB 2 on immune competence and sensitivity to immune modulation by Δ 9 -tetrahydrocannabinol . *J. Leukoc. Biol.* **84**, 1574–1584 (2008).
190. Christophersen, A. S. Tetrahydrocannabinol stability in whole blood: Plastic versus glass containers. *J. Anal. Toxicol.* **10**, 129–131 (1986).
191. Costiniuk, C. T. *et al.* Oral cannabinoids in people living with HIV on effective antiretroviral therapy: CTN PT028-study protocol for a pilot randomised trial to assess safety, tolerability and effect on immune activation. *BMJ Open* **9**, 1–14 (2019).

192. Zuardi, A. W. *et al.* Cannabidiol for the treatment of psychosis in Parkinsons disease. *J. Psychopharmacol.* **23**, 979–983 (2009).
193. Cui, Q. *et al.* Effect of smoking on lung function, respiratory symptoms and respiratory diseases amongst HIV-positive subjects: A cross-sectional study. *AIDS Res. Ther.* **7**, 1–10 (2010).
194. Mai, P. *et al.* Endocannabinoid System Contributes to Liver Injury and Inflammation by Activation of Bone Marrow–Derived Monocytes/Macrophages in a CB1-Dependent Manner. *J. Immunol.* **195**, 3390–3401 (2015).
195. Chimal-Ramírez, G. K., Espinoza-Sánchez, N. A., Chávez-Sánchez, L., Arriaga-Pizano, L. & Fuentes-Pananá, E. M. Monocyte Differentiation towards Protumor Activity Does Not Correlate with M1 or M2 Phenotypes. *J. Immunol. Res.* **2016**, (2016).
196. Gobeil, L.-A., Lodge, R. & Tremblay, M. J. Differential HIV-1 Endocytosis and Susceptibility to Virus Infection in Human Macrophages Correlate with Cell Activation Status. *J. Virol.* **86**, 10399–10407 (2012).
197. Italiani, P. & Boraschi, D. From monocytes to M1/M2 macrophages: Phenotypical vs. functional differentiation. *Front. Immunol.* **5**, 1–22 (2014).
198. Danchine, V. D. & Caldari, C. Effects of Cannabidiol on RAW 264.7 Macrophage Viability and Inflammatory Markers. *FASEB J.* **34**, 1–1 (2020).
199. Liu, Z., Gu, Y., Shin, A., Zhang, S. & Ginhoux, F. Analysis of Myeloid Cells in Mouse Tissues with Flow Cytometry. *STAR Protoc.* **1**, 100029 (2020).
200. Patel, V. I. *et al.* Transcriptional Classification and Functional Characterization of Human Airway Macrophage and Dendritic Cell Subsets. *J. Immunol.* **198**, 1183–1201 (2017).
201. Greco, R. *et al.* Characterization of CB2 Receptor Expression in Peripheral Blood Monocytes of Acute Ischemic Stroke Patients. *Transl. Stroke Res.* **12**, 550–558 (2021).
202. Graham, E. S., Angel, C. E., Schwarcz, L. E., Dunbar, P. R. & Glass, M. Detailed characterisation of CB2 receptor protein expression in peripheral blood immune cells from healthy human volunteers using flow cytometry. *Int. J. Immunopathol. Pharmacol.* **23**, 25–34 (2010).
203. Shoemaker, J. L., Joseph, B. K., Ruckle, M. B., Mayeux, P. R. & Prather, P. L. The endocannabinoid noladin ether acts as a full agonist at human CB2 cannabinoid receptors. *J. Pharmacol. Exp. Ther.* **314**, 868–875 (2005).
204. Udoh, M., Santiago, M., Devenish, S., McGregor, I. S. & Connor, M. Cannabichromene is a cannabinoid CB2 receptor agonist. *Br. J. Pharmacol.* **176**, 4537–4547 (2019).
205. Borgland, S. L. Acute opioid receptor desensitization and tolerance: Is there a link? *Clin. Exp. Pharmacol. Physiol.* **28**, 147–154 (2001).
206. Atwood, B. K., Wager-Miller, J., Haskins, C., Straiker, A. & Mackie, K. Functional selectivity in CB 2 cannabinoid receptor signaling and regulation: Implications for the therapeutic potential of CB 2 ligands. *Mol. Pharmacol.* **81**, 250–263 (2012).
207. Rotter, A. *et al.* CB1 and cb2 receptor expression and promoter methylation in patients with cannabis dependence. *Eur. Addict. Res.* **19**, 13–20 (2013).
208. D'Souza *et al.* Rapid changes in CB1 receptor availability in cannabis dependant males after abstinence from cannabis. *Biol. Psychiatry. Cogn. Neurosci. Neuroimaging.* **1**, 60-67 (2016).
209. T-Break Guide. *The University of Vermont.* Volume 4, (2022).

210. Walker, D. G. & Lue, L. F. Immune phenotypes of microglia in human neurodegenerative disease: Challenges to detecting microglial polarization in human brains. *Alzheimer's Res. Ther.* **7**, 1–9 (2015).
211. Bazil, V. & Strominger, J. L. Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. *J Immunol* **147**, 1567-1574 (1991).
212. Salvador, P. *et al.* CD16+ macrophages mediate fibrosis in inflammatory bowel disease. *J. Crohn's Colitis* **12**, 589–599 (2018).
213. Ancuta, P. *et al.* CD16+ monocytes exposed to HIV promote highly efficient viral replication upon differentiation into macrophages and interaction with T cells. *Virology* **344**, 267–276 (2006).
214. Tippett, E. *et al.* Differential Expression of CD163 on Monocyte Subsets in Healthy and HIV-1 Infected Individuals. *PLoS One* **6**, (2011).
215. Philippidis, P. *et al.* Hemoglobin Scavenger Receptor CD163 Mediates Interleukin-10 Release and Heme Oxygenase-1 Synthesis: Anti-inflammatory Monocyte-Macrophage Responses In Vitro, in Resolving Skin Blisters In Vivo, and after Cardiopulmonary Bypass Surgery. *Circ. Res.* **94**, 119–126 (2004).
216. Guo, L. *et al.* CD163+ macrophages promote angiogenesis and vascular permeability accompanied by inflammation in atherosclerosis. *J. Clin. Invest.* **128**, 1106–1124 (2018).
217. Polfliet, M. M. J., Fabriek, B. O., Daniëls, W. P., Dijkstra, C. D. & van den Berg, T. K. The rat macrophage scavenger receptor CD163: Expression, regulation and role in inflammatory mediator production. *Immunobiology* **211**, 419–425 (2006).
218. Tuluc, F., Meshki, J., Spitsin, S. & Douglas, S. D. HIV infection of macrophages is enhanced in the presence of increased expression of CD163 induced by substance P. *J. Leukoc. Biol.* **96**, 143–150 (2014).
219. Wright, P. B. *et al.* The mannose receptor (CD206) identifies a population of colonic macrophages in health and inflammatory bowel disease. *Sci. Rep.* **11**, 1–12 (2021).
220. Kambara, K. *et al.* In vivo depletion of CD206+ M2 macrophages exaggerates lung injury in endotoxemic mice. *Am. J. Pathol.* **185**, 162–171 (2015).
221. Ciesielska, A., Matyjek, M. & Kwiatkowska, K. TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. *Cell. Mol. Life Sci.* **78**, 1233–1261 (2021).
222. Wang, L.X., Zhang, S.X., Wu, H.J., Rong, X.L. & Guo, J. M2b macrophage polarization and its roles in diseases. *J. Leukoc. Biol.* **106**, 345–358 (2019).
223. Goodman, S., Naphade, S., Khan, M., Sharma, J. & Cherqui, S. Macrophage polarization impacts tunneling nanotube formation and intercellular organelle trafficking. *Sci. Rep.* **9**, 1–15 (2019).
224. Yang, M. *et al.* CD11b-activated Src signal attenuates neuroinflammatory pain by orchestrating inflammatory and anti-inflammatory cytokines in microglia. *Mol. Pain* **14**, (2018).
225. Joshi, J. C. *et al.* SPHK2- generated S1P in CD11b+ macrophage blocks STING to suppress the anti-inflammatory function of alveolar macrophages. *Cell Rep.* **30**, 4096-4109 (2020).
226. Rosseau, S. *et al.* Phenotypic characterization of alveolar monocyte recruitment in acute respiratory distress syndrome. *Am. J. Physiol. - Lung Cell. Mol. Physiol.* **279**, 25–35 (2000).

227. Bühling, F *et al.* MRP8 / MRP14, CD11b and HLA-DR expression of alveolar macrophages in pneumonia. *Immunol. Lett.* **71**, 185–190 (2000).
228. Duan, M. *et al.* CD11b immunophenotyping identifies inflammatory profiles in the mouse and human lungs. *Mucosal Immunol.* **9**, 550–563 (2016).
229. Bagasra, O., Wright, S. D., Seshamma, T., Oakes, J. W. & Pomerantz, R. J. CD14 is involved in control of human immunodeficiency virus type 1 expression in latently infected cells by lipopolysaccharide. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 6285–6289 (1992).
230. Taniguchi, K. *et al.* Essential Role of Lysophosphatidylcholine Acyltransferase 3 in the Induction of Macrophage Polarization in PMA-Treated U937 Cells. *J. Cell. Biochem.* **116**, 2840–2848 (2015).
231. Ikewaki, N., Tamauchi, H. & Inoko, H. Modulation of cell surface antigens and regulation of phagocytic activity mediated by CD11b in the monocyte-like cell line U937 in response to lipopolysaccharide. *Tissue Antigens* **42**, 125–132 (1993).
232. Hoen, E. N., Cremer, T., Gallo, R. C. & Margolis, L. B. Extracellular vesicles and viruses: Are they close relatives? *Proc. Natl. Acad. Sci. U. S. A.* **113**, 9155–9161 (2016).
233. Mercurio, V., Fitzgerald, W., Molodtsov, I. & Margolis, L. Persistent Immune Activation in HIV-1-Infected Ex Vivo Model Tissues Subjected to Antiretroviral Therapy: Soluble and Extracellular Vesicle-Associated Cytokines. *J. Acquir. Immune Defic. Syndr.* **84**, 45–53 (2020).