EFFECTS OF MARIJUANA ON PERIPHERAL AND CENTRAL NERVOUS IMMUNE MARKERS IN MARIJUANA SMOKERS AND HIV-INFECTED SUBJECTS

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Sody Mweetwa Munsaka

Dissertation Committee:

Linda Chang, Chairperson
Sandra Chang
Vivek Nerurkar
Christine Cloak
George King

Key words: HIV, Immune markers, Marijuana
DEDICATION

I dedicate this Doctor of Philosophy dissertation to my two lovely and beautiful daughters Lillian Leilani and Lisa Luyando Lehua who have brought great Joy to my life and have motivated me to pursue this work. This dissertation would not have been completed without the loving care and support that I have received from my darling wife, Fungai Harriet Lungu. Finally to my parents, Mr. Solomon and Mrs. Esther Munsaka who gave me the gift of life and the best gift you can ever give a child; education, I say Leza amuleleke alimwi ndalumba kapati.
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ABSTRACT

Marijuana can lower cellular immune responses and inhibit inflammation. The goal of this dissertation was to examine the effects of marijuana on peripheral and central nervous system (CNS) immune markers in current marijuana users (MJ) and HIV-infected marijuana users (HIV+MJ). We hypothesized that marijuana would lower cell activation and reduce inflammatory cytokine secretion in the CNS.

An in vitro monocyte activation model using lipopolysaccharide was developed to assess baseline and activation immune markers in healthy individuals using flow cytometry and the kinetics of HIV infection between activated and non-activated monocytes was studied. Peripheral blood mononuclear cells were isolated from HIV+MJ, HIV+ subjects (HIV+), MJ and sero-negative controls (SN). Cells were stained for cannabinoid receptors (CB) and immune markers and were analyzed using flow cytometry. Cytokines from age-matched subjects who provided cerebral spinal fluid (CSF) were measured by Luminex.

After lipopolysaccharide stimulation, monocytes increased CD16 and CD69 surface expression (p<0.05). The activated monocyte phenotype was supported by an increase in TNF-α production (p<0.05). The activated monocytes also had increased CCR5 expression (p=0.05) and bound more R5-tropic HIV than non-activated monocytes (p<0.05). CB1 and CB2 receptors were found on monocytes and significant group differences were observed in the expression of inflammatory CD16 (p<0.0001) and CCR5 (p<0.003) on monocytes. CD14+CD16+ monocytes were higher in HIV+MJ than in HIV+ subjects (p=0.0054), and in MJ users than in SN subjects (p<0.0001). CCR5 expression was significantly higher in MJ users than SN subjects (p<0.0001); however, no difference in CCR5 expression was found between HIV+MJ users and HIV subjects (p=0.4). The duration of marijuana use correlated with the levels of CD14+CD16+ (r=0.53, p<0.001) and CD14+CCR5+ expression (r=0.24, p=0.049). Inflammatory cytokines (fractalkine, INF-α2 and IL-1α) and
chemoattractants (MCP-1, IP-10 and IL-8) were all significantly higher in the HIV+MJ and MJ than in respective controls.

The higher levels of inflammatory cytokines and activated monocytes in HIV+MJ and MJ groups, as well as their correlation with lifetime MJ use, suggest marijuana induced inflammation in the periphery and CNS. Moreover, higher CCR5 expression on the activated monocytes in MJ suggests that marijuana use may render susceptibility to HIV infection.
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<td>°C</td>
<td>Degree Celsius</td>
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<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<td>ANI</td>
<td>Asymptomatic neurocognitive impairment</td>
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<tr>
<td>APC</td>
<td>Allophycocyanin</td>
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<td>APOE</td>
<td>Apolipoprotein E</td>
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<tr>
<td>cART</td>
<td>combinational antiretroviral therapy</td>
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<tr>
<td>CB</td>
<td>Cannabinoid</td>
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<tr>
<td>CCR5</td>
<td>C-C motif Chemokine receptor -5</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebral spinal fluid</td>
</tr>
<tr>
<td>CXCR4</td>
<td>C-X-C motif Chemokine receptor-4</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>FACS</td>
<td>Flow activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>Fc</td>
<td>Crystallizable fragment</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>G-CSF</td>
<td>granulocyte colony stimulating factor</td>
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<tr>
<td>GRO</td>
<td>growth regulated oncogene</td>
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<tr>
<td>HAART</td>
<td>Highly active antiretroviral therapy</td>
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<td>HAD</td>
<td>HIV-associated dementia</td>
</tr>
<tr>
<td>HAND</td>
<td>HIV-associated neurocognitive disorders</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>INF</td>
<td>Interferon</td>
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<tr>
<td>IP</td>
<td>γ-Interferon induced protein</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
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<td>MDC</td>
<td>macrophage derived chemokine</td>
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<td>MIP</td>
<td>Macrophage inflammatory protein</td>
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<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
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<tr>
<td>NF</td>
<td>nuclear factor</td>
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<tr>
<td>NK</td>
<td>Natural killer cells</td>
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<tr>
<td>p</td>
<td>Probability</td>
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<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>R5</td>
<td>Macrophage/CCR5-tropic HIV strain</td>
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<tr>
<td>R5/X4</td>
<td>Dual tropic HIV strain</td>
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<td>RANTES</td>
<td>regulated upon activation normal T-cell expressed</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RT-PCR</td>
<td>Real-time polymerase chain reaction</td>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<td>TC</td>
<td>Cytotoxic T-cells</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>TH</td>
<td>helper T-cell</td>
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<td>THC</td>
<td>Tetrahydrocannabinol</td>
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<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>X4</td>
<td>T-cell tropic HIV strain</td>
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<td>α</td>
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<td>β</td>
<td>beta</td>
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CHAPTER 1
INTRODUCTION

1.1 Background

In the last 20 years, the global prevalence of people living with human immunodeficiency virus (HIV) has increased from an estimated 8 million to 33.3 million cases (UN AIDS, 2010). However, the incident rate has declined from 3.1 million cases/year to 2.6 million cases/year (UN AIDS, 2010). Sub-Saharan Africa accounts for about two-thirds (67.7%) of all HIV cases in the world and is followed by Southeast Asia at 14.4% (UN AIDS, 2010). Zambia, a sub-Saharan country where I come from, has been ravaged by HIV. The current HIV prevalence in Zambia is estimated to be about 16% (ranked 7th in the world) and even though the incidence appears to be decreasing, the socio-economic impact continues to worsen. The major socio-economic issues include the high burden of caring for the ill, loss of work time (for funerals and attending to the sick), the increased number of orphaned children and the loss due to death and sickness of the productive worker force. The HIV scourge has exerted an enormous strain on public health services, destroyed the economic fibers of society and has broken down cultural and family structures in the region. Despite advances in potent combination antiretroviral treatment (cART) in the western countries, the availability and access to such drugs remains elusive in sub-Saharan Africa.

1.2 HIV/AIDS in Hawai‘i and United States of America

In Hawai‘i, a cumulative number of 4,209 acquired immune deficiency syndrome (AIDS) cases have been reported as of December 2010 (Hawai‘i State Department of Health, 2010). Currently there are 3,592 people living with HIV in Hawai‘i (Hawai‘i State Department of Health, 2010). The gender distribution of HIV cases in Hawai‘i is about 70-80% males and 20-30% females, suggesting homosexual transmission and injection drug use as major modes of transmission; whereas in sub-Saharan Africa, the ratio is almost 50:50 (male: female) which would be consistent with heterosexual transmission. Two-thirds of HIV cases in the continental United States occur amongst ethnic minorities
(African-Americans and Hispanics), but in Hawai‘i, where the ethnic distribution is different from the mainland United States, almost half of HIV cases are Caucasiанс and the remaining half are Asians and Pacific Islanders with a small number of African-Americans and Hispanics. cART has significantly reduced the death rate, increased survival and has improved the quality of life of HIV patients in western countries (Sterne et al 2005). However, up to 50% of these individuals may present with cognitive problems (Heaton et al 2011). Apart from age, other co-morbid conditions including co-infections (e.g. Hepatitis C), and psychotropic substance abuse may further increase the risk of development of neurocognitive problems in HIV-infected individuals.

1.3 Main aim and central hypothesis

Marijuana use is common amongst HIV-infected individuals with reported benefits including relief of anxiety and depression, improved appetite and reduced neuropathic pain (Prentiss et al 2004). However, the other biological effects of marijuana use in HIV-infected patients are not well studied. Less is known about the effects of marijuana use on immune cells and cytokine secretion in HIV patients. This dissertation aims to address and fill this void. Studies have shown that acute exposure to Tetrahydrocannabinol (THC), the main psychoactive ingredient in marijuana, can suppress immune cell function, increase HIV co-receptor expression, and act as a cofactor to significantly enhance HIV replication in vivo (Roth et al 2005). Cannabinoids exhibit immunosuppressive properties by interfering with humoral immunity, cell-mediated immunity and cellular defenses against infectious agents (Sanders et al 1991). Other studies have shown that at higher doses (>3nM), THC may promote inflammation and secretion of proinflammatory cytokines (Berdyshev et al 1997). However, these studies were conducted in-vitro with no in-vivo data is available. Because cannabinoids suppress immune function in vitro, we hypothesized that marijuana users (HIV-infected marijuana users and HIV-negative marijuana users) would have lower levels of activated immune cells and would have lower secretion of proinflammatory cytokines than the respective
non-marijuana using individuals (HIV-infected subjects and seronegative control). Immune suppression in HIV infection may lead to high HIV replication, which may increase the pathology leading to cognitive dysfunction. This project is innovative in its use of a well characterized cohort of subjects enrolled in several HIV and drug abuse clinical research projects led by Dr. Linda Chang (Dissertation Committee Chair). Due to the high prevalence of marijuana use amongst HIV-infected individuals (~50%), the available cohort from these clinical research studies provided a unique opportunity for the current dissertation project. All the subjects were co-enrolled from this cohort.

The relevance of this project is that because marijuana use may affect cognition, attention and memory, it could pose a problem to public health and safety. Also because marijuana may suppress immune function it could further impair the immune system in HIV-infected marijuana users. Marijuana is widely used and in the United States there is also immense pressure on state governments to legalize marijuana; 16 States and the District of Columbia in the (Hawai’i included) currently have legislation for prescription marijuana and THC (dronabinol or Marinol®) for medical uses, including symptoms associated with HIV infection like wasting syndrome and peripheral neuropathic pain. HIV causes cognitive and behavioral dysfunction and the use of marijuana may exacerbate these problems in patients with HIV-associated neurological disorders (HAND).

Lastly, the effect of marijuana on inflammation in HIV disease is not well characterized. This study aims to characterize how marijuana may affect inflammatory responses in HIV disease. This study will help elucidate the impact of marijuana use and the combined effect of marijuana and HIV on the peripheral and CNS immune system.
1.4 The immune system and the central nervous system

Historically, the brain has been regarded as an anatomically immune privileged organ with the blood-brain barrier preventing entry of toxins, immune cells and pathogens from systemic circulation into the CNS (Streilein 1993). In the last decade, research findings clearly indicate that resident cells in the brain may produce immune responses and inflammatory processes that are similar to those in the peripheral immune system. It is now widely accepted that microglia and perivascular macrophages are involved in inflammatory processes in the brain and are implicated in the development of inflammatory neurodegenerative diseases such as multiple sclerosis and Alzheimer’s disease (Gendelman 2002).

As part of the normal homeostasis (immune surveillance theory), leukocytes (lymphocytes and mononuclear phagocytes) traffic back and forth between the central nervous system and systemic circulation, and neuroimmunological processes in the brain during disease and inflammation can recruit immune cells from the periphery which can affect disease outcomes (Schwartz & Kipnis 2004). The concept of immune surveillance suggests that the immune system, via immune cells and mediators, patrols the body not only to recognize and destroy invading pathogens but also host cells that are altered or become cancerous. Neuroimmune processes in the brain play important roles in health and disease. They regulate development, maintenance, and support brain cells and their networks (Crutcher et al 2006).

The human immune system is very complex and its key role is to defend the body against pathogens, including viruses, bacteria, fungi and parasites, and abnormal cells and proteins. When the immune system is compromised, diseases can occur ranging from autoimmune disorders, where the immune system attacks the host self components (e.g. systemic lupus erythematosus, myasthenia gravis, etc.), to immune deficiency disorders where the immune system does not respond appropriately to eliminate a pathogen (e.g. HIV/AIDS). The immune system functions using two main pathways, the innate and adaptive immunity. Innate immune mechanisms include barriers such as the skin and mucous membranes which prevent microbes and pathogens from invasion, and
soluble molecules such as interferons and complement, and cell populations such as natural killer cells, mononuclear phagocytes and dendritic cells. Adaptive immune responses involve cell mediated immunity produced by mononuclear phagocytes, T cells and dendritic cells, and humoral immunity mediated by antibodies produced by B cells. The main features of the adaptive immune system are recognition, memory and adaptability. Immune memory is the basis for vaccination. The immune system must discriminate whether an invading microbe is a pathogen or normal flora to mount an appropriate response. After the pathogen is cleared by the immune response, the immune system must shut down (except for the persistence of memory responses) to prevent further tissue damage and unwanted immune effects. Memory responses perpetuate so that when the same threat recurs, there is a robust response against the threat. There is a fine balance between stimulation of immune mechanisms needed to clear a pathogen and prevention of an excessive response which could lead to immunopathology. Most immune diseases ranging from immune deficiency to chronic inflammatory conditions involve the loss of this balance or dysregulation of immune responses. To maintain this balance, the immune system is in constant communication among major components (leukocytes and immune organs e.g. lymph nodes, spleen, thymus etc) via cell-to-cell contact, cytokines and chemokines.

Communications and interactions occur between the peripheral immune system and the central nervous system via multiple mechanisms. Leukocytes are the main participants in the peripheral immune system, but also have receptors for neuropeptides, neurotransmitters, and neuromodulators like endocannabinoids (Andres et al 2010). Leukocytes, whose primary function is to fight off microbes and foreign molecules, can also produce neuropeptides (Smith 2008). On the other hand, neurons which are the main effector cells in the brain express toll-like receptors (TLR-7) (Liu et al 2010) and chemokine receptors, produce immune signaling molecules including chemokines (Lavi et al 1998), and are involved in peripheral immune responses [e.g. via the vagus nerve (Borovikova et al 2000)]. Toll-like receptors recognize molecular patterns on
potential pathogens and trigger the development of immune reactions, whereas chemokines signal other immune cells to traffic to sites of infection or to produce immune mediators like interferons or antibodies to neutralize threats. Therefore, a bidirectional regulatory circuit exists between the nervous and immune systems. Commonly abused drugs, such as marijuana, could modulate both peripheral and CNS immune responses since the receptors that these drugs act upon in the brain are also expressed on peripheral immune cells. For instance, THC acts via cannabinoid receptors expressed on neurons in the CNS; since leukocytes also express cannabinoid receptors, THC can also modulate their function. Furthermore, cannabinoid receptors respond to chemical stimuli from brain cells, mediated by lipid-like compounds known as endocannabinoids. The immune system and its components communicate through cell-to-cell contact and cytokines and many of these signals are sent or produced in the brain.

Pathways which transmit information from the immune system to the brain include cytokines, such as interleukins (IL) and tumor necrosis factor (TNF) (e.g. IL-1α, IL-6, TNF), which circulate in blood stream and influence brain activity via circumventricular organs or via interaction with brain endothelial cells (Mravec et al 2006). In the periphery, binding of cytokines (e.g. IL-1α) to receptors on vagal paraganglion dendritic cells, or directly to receptors of the vagus nerve, also may activate vagus nerve afferents (Figure 1.1). Inflammatory cytokines like IL-1α activate sensory afferents of the vagus nerve and this transmits information to the brain about inflammatory processes or tumorigenesis. Other brain derived neurochemicals, such as endorphins, also might bind to somatic afferents and produce an analgesic effect (Mravec et al 2006) (Figure 1.1).
Figure 1.1 Pathways which transmit information from the immune system to the brain. During inflammation or tumorigenesis the peripheral immune system sends messages to the brain via cytokines and chemokines. The brain and resident immune cells in the brain also have cytokine and chemokine receptors. Adapted from (Mravec et al 2006)

During normal physiological processes, the brain sends messages to the immune system via hormones released from the pituitary gland (e.g. Adrenocorticotropic hormone (ACTH), prolactin, growth hormone (GH)) that has the potential to modulate immune function (Figure 1.2). Acetylcholine released from postganglionic vagal neurons binds to nicotine receptors of immune cells and produces an anti-inflammatory effect. Norepinephrine released from postganglionic sympathetic neurons (SNpo) and epinephrine/norepinephrine released from adrenal medulla might influence immune functions after binding to adrenergic receptors on the immune cells and glucocorticoids released from adrenal cortex also have complex effects on the immune system (Mravec et al
Neurons also synthesize and secrete endocannabinoids to modulate physiological processes including appetite, pain-sensation, mood and memory. Endocannabinoids signal through G protein-coupled receptors modulating various signal transduction pathways involved in controlling cell proliferation, differentiation and survival (Howlett et al 2002). Endocannabinoids and cannabinoids may modulate immune function via specific receptors on immune cells.

**Figure 1.2 Pathways which transmit information from the brain to the immune system.** Under conditions of inflammation or tumorigenesis the brain and immune cells in the brain send signals to the peripheral immune system through hormones, cytokines, chemokines, neuropeptides and endocannabinoids. Peripheral immune cells and organs have receptors for hormones, cytokines, chemokines, neuropeptides and endocannabinoids. Adapted from (Mravec et al 2006)
1.5 HIV and Marijuana

About half of HIV-infected subjects that are screened for our studies self-report chronic use of marijuana to reduce nausea and/or neuropathic pain. The use of cannabis as a therapeutic agent dates back about 5,000 years (Croxford 2003). Cannabinoids are best known for their effects on the CNS. They produce euphoria, alterations in cognition and analgesia, have anticonvulsant properties, and affect temperature regulation, sleep and appetite (Correa et al 2005). Medicinal properties include: analgesia, muscle relaxation, immunosuppression (immunomodulation), anti-emesis, lowering of intraocular pressure, bronchodilation, neuroprotection and induction of apoptosis in cancer cells.

Acute marijuana intoxication impairs learning and memory (Hooker & Jones 1987) and adversely affects psychomotor and cognitive performance (Heishman et al 1990). Neuropsychological studies have shown that chronic marijuana users perform poorer on attention and cognition tasks (Solowij et al 2002); thus, marijuana use may contribute to HIV-associated neurocognitive dysfunction. THC can also affect a number of other systems including circulation, hormonal, digestive and immune systems. THC exerts its pharmaceutical and immunological effects through cannabinoid receptors distributed in the CNS, immune organs and on circulating immune cells.

1.6 The Endocannabinoids and Cannabinoid Receptors

Two cannabinoid receptors have been identified; cannabinoid receptor (CB) 1 receptors (Matsuda et al 1990) are mainly expressed in the CNS (neurons and microglia) while CB2 receptors are mainly expressed in the periphery, on immune cells (Munro, Thomas et al. 1993) and other organs. Endogenous cannabinoid ligands include anandamide (CB1 agonist) and 2-arachydonoylglycerol (2-AG) (CB2 agonist) (Devane et al 1992; Mechoulam et al 1995). Leukocytes have been shown to possess both CB1 and CB2 receptors, but CB2 receptors are associated primarily with the immunomodulatory activities of cannabinoids (Kaminski et al 1992). As early as 1973, THC was shown to modulate T cell responses in animals (Nahas et al 1973). Cannabinoids and
endogenous cannabinoid ligands, signal through G protein-coupled receptors that modulate various signal transduction pathways involved in controlling cell proliferation, differentiation and survival (Howlett et al 2002). The G-protein-coupled CB2 receptor modulates several different cellular pathways including inhibition of adenylyl cyclase, activation of MAP kinase cascades and activation of the PI3K-AKT pathway. 2AG is the endogenous ligand for CB2 receptors (Sugiura et al 2006a) while other selective synthetic ligands for CB2 are HU-308, JWH-133, AM1241, and non-selective ligands that stimulate both CB1 and CB2 are THC, cannabidiol, WIN55 212-2, and HU-210 (Bouaboula et al 1996; Caldwell & Evans 2008; Howlett et al 2002). THC and cannabidiol have the same affinity for CB2 receptors. In contrast, THC binds to CB1 with a tenfold higher affinity than cannabidiol (Fernandez-Ruiz et al 2007). CB2 receptor antagonists include synthetic SR144528 and AM630. Endogenous ligand anandamide is more active for CB1 receptors than CB2 receptors.

Cannabinoids exhibit immunosuppressive properties by interfering with humoral immunity, cell-mediated immunity and cellular defenses against infectious agents (Sanders et al 1991). Acute exposure to THC in vivo can suppress immune cell function, increase HIV co-receptor expression, and act as a cofactor to significantly enhance HIV replication in the huPBL-SCID mouse model (Roth et al 2005). However, higher doses of THC (>3nM) may promote cellular activation and cause secretion of proinflammatory cytokines (Berdyshev et al 1997). Expression of CB2 receptors in cells of monocyte/macrophage lineage is modulated by cell activation and has been shown to increase under inflammatory conditions (Davis et al 1995; Di Marzo et al 1994). Elevated CB receptor expression was found on peripheral blood mononuclear leukocytes of marijuana smokers (Nong et al 2002). THC and cannabidiol may decrease production of inflammatory cytokines by human immune cells (Srivastava et al 1998). Dendritic cells, as well as macrophages, generate anandamide and 2-AG in response to inflammatory conditions and express CB receptors and the enzyme responsible for endocannabinoid hydrolysis (Horswill et al 1994; Varga et al 1998), suggesting a physiological role of the endocannabinoid system in
immune function. This may have important implications in pathological inflammatory conditions, including immune related disorders of the brain such as HIV encephalitis. The rank order of cannabinoid CB2 mRNA expression on human blood leukocytes is B cells >NK cells>monocytes>neutrophils> CD8 cells >CD4 cells (Bouaboula et al 1993). Leukocytes also express both CB1 and CB2 receptors but primarily CB2 receptors have been are associated with the immunosuppressive properties of cannabinoids (Kaminski et al 1992).

1.7 Pharmacokinetics of THC

THC and its metabolites have monoterpenoid or dibenzopyran ring-structures that make them highly lipophilic and essentially water-insoluble. THC can be administered by several routes: by inhalation, orally, ophthalmically, sublingually and transdermally. The pharmacokinetics of THC varies depending on the route of administration. Inhalation of THC results in maximum plasma concentrations within minutes (3-10 minutes on average), while psychoactivity starts within seconds to a few minutes, reaches a maximum after 15 to 30 minutes, and tapers off after 2-3 hours (Grotenhermen 2003). Systemic bioavailability ranges between 10-35% and regular users are more efficient in metabolizing THC than first time users (Lindgren et al 1981). Bioavailability also varies according to the depth of inhalation, puff duration and the time the breath is held.

Following oral ingestion of THC, psychoactivity is delayed for up to 30-90 minutes, reaches a maximum after 2-3 hours and lasts between 4-12 hours depending on the dose (Grotenhermen 2003). Oral administration results in low or erratic absorption with maximum plasma concentrations after 60-120 minutes and as late as 4 hrs or 6 hours. THC is degraded by the acidic environment in the stomach or gut. Bioavailability is increased if an oil formulation is ingested. Most of the THC is metabolized in the liver before it reaches the site of action. Ingestion of 20mg THC in a chocolate cookie and oral administration of 10mg dronabinol (THC) resulted in a very low systemic bioavailability of 6±3% (Sporkert F 2001). Systemic bioavailability in ophthalmic administration varies
and a rabbit study using oil formulated THC showed a 4-6% bioavailability. Peak plasma concentrations were detected after 1 hour and remained high for several hours (Chiang CW 1983). Bioavailability varies too in sublingual and dermal administration of THC, with plasma concentration peaking fast in sublingual administration and oil formulated THC is increasingly taken up via the skin. Rectal administration also has varied bioavailability and the bioavailability depends on formulation; some people use this route to by-pass the liver for sustained and longer effects (Sporkert F 2001).

1.8 Metabolism of THC

The metabolism of THC mainly occurs in the liver by microsomal hydroxylation and oxidation catalyzed by enzymes of the cytochrome P450 (CyP) complex. Nearly 100 THC metabolites have been identified and the major active metabolites are the monohydroxylated compounds of THC (11-OH-THC, THC-COOH and 11-nor-9-Carboxyl-THC glucuronide). The average clearance rate is reported as 11.8±3L/h and is higher in men (Grotenhermen 2003). The elimination half-life of THC from plasma is difficult to calculate as the plasma/fatty tissue equilibrium concentration is reached slowly. THC is also eliminated slowly from plasma because the diffusion of THC from body fat and other tissues is slow. The half-life of THC ranges from 25-36 hours, 12-36 hours for 11-OH-THC and from 25-55 hours for THC-COOH after oral or intravenous administration (Grotenhermen 2003).

THC is excreted as metabolites in urine and feces within days and weeks (20-35% in urine and 65-80% in feces) and as THC in feces (less than 5%). A single oral dose can result in detectable amounts of THC or its metabolites up to 12 days in urine (usually 3-5 days). The average time until last urine positive is 31 days for heavy users and 12 days for light users. Urine drug screening for THC uses chromatographic immunoassays with antibody-dye conjugates specific for THC and or its major metabolites. These tests give a preliminary test result while more specific chemical methods such as chromatography/ mass spectrometry are required for confirmation. Several methods and models are
used to predict time of last use. Most are based on the THC plasma concentrations or the ratio of THC and its metabolites (THC-COOH and 11-OH-THC) in plasma with the higher the THC-COOH/THC ratio, the longer the time since last consumption (Huestis et al 1992).

1.9 HIV and chronic immune activation

HIV disease progression and neuropathogenesis is believed to be promoted by chronic immune activation and inflammation. During acute HIV infection, there is massive loss of CD4+ cells especially in the gut resulting in impairment of mucosal homeostasis (‘leaky gut’), which leads to translocation of microbes and microbial products like lipopolysaccharide (LPS) from the gut into systemic circulation (Brenchley et al 2006). There are persistently elevated plasma LPS levels in HIV-infected patients (Ancuta et al 2008). The elevated LPS levels lead to chronic immune activation. Activated CD4+ T cells and monocytes (CD14+ CD16+ cells) are more susceptible to HIV infection than resting cells and these activated cells are thought to play an important role in HIV disease pathogenesis (Kusdra et al 2002; Williams et al 2001). An expanded CD14+ CD16+ monocyte subpopulation producing IL-1α and TNF-α has been identified in HIV-infected individuals (Thieblemont et al 1995). Individuals with HIV-associated dementia have higher percentages of circulating activated monocytes/macrophages (Fischer-Smith et al 2001; Pulliam et al 2004) and these activated monocytes have been found to infiltrate perivascular brain regions (Fischer-Smith et al 2001).

1.10 HIV Biology

HIV is a spherical, enveloped, single stranded positive sense RNA lentivirus with two copies of its genome. It attaches to CD4 cell surface receptor and chemokine co-receptor CXCR4 and/or CCR5 via two envelope glycoproteins, gp120 and gp41. T-lymphocytes, monocytes/macrophages, dendritic cells, perivascular macrophages, microglia, astrocytes, oligodendrocytes, brain microvascular endothelial cells and neurons express one
or more of the HIV receptors but productive infection only occurs in CD4+ cells (Martin-Garcia et al 2002). The HIV virus has a 9 kilobase genome with 9 genes encoding 15 proteins. The three main structural genes are \textit{gag} (encoding the core proteins), \textit{pol} (encoding the polymerase) and \textit{env} (encoding the envelope proteins, gp120 and gp41). \textit{Tat, rev, vpu, nef, vpr,} and \textit{vif} genes code for regulatory and accessory proteins needed for infection and replication. The high error rate of the HIV polymerase makes the virus mutate at very high rates and evade immune mechanisms. The virus also is able to integrate into the host genome making it impossible to eliminate, especially in resting memory cells and long lived myeloid cells (monocytes, macrophages, perivascular macrophages and microglia). The chronically infected cells serve as reservoirs of HIV infection (Alexaki et al 2008; Haggerty et al 2006).

1.11 HIV Neuropathogenesis

Animal studies and postmortem studies of humans have shown that HIV enters the CNS very early during acute infection (Davis et al 1992; Zink & Clements 2002). The neuropathology of HIV infection shows monocyte/macrophage infiltration, multi-nucleated giant cells (Koenig et al 1986), inflammation, astrocytosis and, ultimately, neuronal death manifesting as neurological complications (Gonzalez et al 2002). One of the neurological complications of HIV is HIV-associated neurocognitive disorder (HAND), which the rates continue to rise despite the use of cART. Although the spectrum of the disorder has changed with cART, milder forms still occur (Antinori et al 2007). Increasing evidence also supports the theory that activated monocytes/macrophages harbor the virus and serve as potential reservoirs of infection (Crowe 2007; Eilbott et al 1989; Gendelman et al 1988; Koenig et al 1986). Therefore, monocytes/macrophages are believed to play a role in the pathogenesis of HAND as they may carry HIV-1 into the CNS by a 'Trojan horse' mechanism (Nottet & Gendelman 1995) first described for the dissemination of visna virus by infected monocytes (Peluso et al 1985). A cascade of events in the CNS as a result of proinflammatory cytokines /chemokines and viral proteins
released from HIV-infected cells in the CNS, may induce neuronal death, leading to cognitive and motor dysfunction (Martin-Garcia et al 2002). Both direct and indirect neurotoxic mechanisms are believed to occur (Kaul et al 2001).

**Figure 1.3 Mechanisms of HIV-1 neuropathogenesis.** HIV enters the brain through monocytes and lymphocytes during acute infection. HIV infection of microglia causes activation and release of neurotoxic viral proteins, cytokines, chemokines and excitotoxic mediators causing astrocyte dysfunction and neuronal death leading to neuropathology. Adapted from (Martin-Garcia, et al 2002).

Direct neurotoxic mechanisms involve release of viral molecules (gp120, Tat, Nef, Vpr) shed by HIV from infected monocytes/macrophages and microglia that directly injure neurons (Nath et al 1996). Indirect neuropathogenic mechanisms involve influx of activated monocytes/macrophages through the brain vasculature that activate perivascular microglia to release proinflammatory cytokines and chemokines (interleukine-1α, interleukin-1β and tumor necrosis factor-α) and other excitatory mediators (TRAIL, glutamate, quinolinic acid) resulting in neuronal apoptosis (Anderson et al 2002; Huang et al 2005; Kaul et al 2001; Merrill & Chen 1991) (Figure 1.3). Proinflammatory cytokines increase
expression of adhesion factors on brain endothelial cells enabling further transmigration of infected and activated monocytes/macrophages into the brain (Brabers & Nottet 2006; Gonzalez-Scarano & Martin-Garcia 2005). Furthermore, activation and excitation of astrocytes modifies the permeability of the blood-brain barrier and promotes the migration of more monocytes into the brain (Gonzalez-Scarano & Martin-Garcia 2005) (Figure 1.3). Microglia and glial (astrocyte) activation is another feature in HIV disease that leads to release of neurotoxic substances including monocyte chemoattractant protein-1 (MCP-1) that may contribute to neuronal apoptosis or neuronal dysfunction (Chang et al 2004a). Activated astrocytes have reduced glutamate uptake and thus cause increased extracellular glutamate which can lead to excitotoxicity and neuronal damage. Furthermore, animal models have shown that HIV/SIV induce production of matrix metalloproteinases (MMPs) (Louboutin et al 2010), endothelial adhesion molecules (Sasseville et al 1992) and dampen expression of tight junction proteins (Kanmogne et al 2005), all of which would further compromise the blood-brain barrier.

Because the pathogenesis of HIV is promoted by chronic systemic immune activation and inflammation, it is important to study other modulators of immune activation beyond viral and microbial products. One possible cofactor in immune activation is drug abuse. For example, cocaine has been shown to potentiate HIV-1 replication and thus may promote HIV diseases in HIV-infected cocaine abusers (Peterson et al 1991). Other drugs like cannabis are promoted because of their anti-inflammatory properties to control inflammatory neuropathic pain, nausea and to improved appetite in patients with multiple sclerosis, cancer and HIV-infection. According to the United Nations’ 2010 world drug report, marijuana is the most widely used illicit drug in the world. In Hawai‘i, marijuana is the most abused drug among youth and often is used as a “gateway drug” to other drugs. In-vitro studies showed that high doses of THC may promote inflammation and thus promote immune activation (Berdyshev et al 1997). However these studies were conducted in vitro using THC and not marijuana.
1.12 Significance

The significance of this study continues to build on the hypothesis that HIV pathology is driven by immune activation and secretion of proinflammatory mediators both in the periphery and subsequently in the CNS. We hypothesized that marijuana use would suppress immune function and lower cytokine expression in marijuana users. This hypothesis is based in other research studies that have shown that THC (the active ingredient in marijuana) suppresses immune function, and shifts immune responses from TH-1 to TH-2 (Mulders et al 1999; Roth et al 2005). The pathology of HIV is sustained by systemic immune activation and inflammation. This is supported by evidence that activated monocytes are more permissive to infection and preferentially harbor HIV-1 in-vivo and in-vitro (Crowe 2007) and in-vitro activated monocytes have elevated HIV chemokine receptor, CCR5, surface expression and support R5-tropic HIV (Munsaka et al 2009; Tuttle et al 1998). The pathogenesis of HAND involves HIV infection of peripheral monocytes, and preferentially activated monocytes then ingress into the CNS- trafficking the virus to the brain. Individuals with HIV-associated dementia (HAD) have higher percentages of circulating activated monocytes/macrophages (CD14+/CD16+) (Fischer-Smith et al 2001; Pulliam et al 2004; Shiramizu et al 2005) and activated monocytes have been found to infiltrate perivascular brain regions (Fischer-Smith et al 2001).

The results of this project will have clinical implications and may inform practice if marijuana use in HIV-infected individuals may be contributing to the pathology of HIV disease. The results may also guide future legislative decisions on whether marijuana use should be legalized for both recreational and medicinal purposes, especially in the HIV+ population. This project will further knowledge on how immune responses may be modulated by an agent that also targets the CNS and thus reduce the gap between the immune system and the CNS once thought as independent/compartmentalized systems.

The second chapter of this dissertation will focus on studying the mechanistic role of activated monocytes in HIV infection. An in-vitro monocyte
activation model was developed to assess expression of CD16 (marker for monocyte activation and differentiation) and CCR5 (chemokine co-receptor for macrophage tropic HIV-1). HIV-1 infection kinetics between activated and non-activated monocytes were examined. The third chapter will focus on characterization of activated monocytes (CD14+CD16+ cells) and HIV chemokine co-receptor-5 (CCR5) in HIV-infected marijuana users and marijuana users. The fourth chapter will examine cerebral spinal fluid (CSF) cytokine expression in the above four subject groups by Luminex multi-plex immune assay. Chapters 5 and 6 will explore the expression patterns of cannabinoid receptors in HIV subjects with and without marijuana use, and seronegative subjects with and without marijuana use.

While overwhelming evidence in the literature supports that THC is immunosuppressive and inhibits secretion of proinflammatory cytokines very few studies have examined effects of THC in humans. Additionally, smoked marijuana contains several other cannabinoids and other compounds which may affect the immune system differently from pure THC used by in vitro studies. Furthermore, HIV infection impairs the immune system and using marijuana could make it worse. Therefore, the goal of this study is to study the effects of marijuana on peripheral immune cells and cytokine secretion in the CNS.
1.13 SPECIFIC AIMS

Specific aim 1: To characterize baseline and activation immune markers in monocytes of healthy individuals. To further study the kinetics of HIV infection (binding) to CCR5 in activated versus non-activated monocytes from the same individuals.

Hypothesis: Activated monocytes will have a higher expression of activation markers (CD16, CD69 and TNF-α) and CCR5 compared to non-activated monocytes. Hence, activated monocytes will have higher amounts of HIV-1 binding compared to non-activated monocytes.

Rationale: Activated monocytes will have altered expression of activation markers and CCR5 compared to non-activated monocytes (Tuttle et al 1998). This may cause activated monocytes to bind more HIV and thus be more susceptible to viral infection (Fischer-Smith et al 2001; Pulliam et al 2004). Activated monocytes are more permissive to infection and preferentially harbor HIV-1 in-vivo and in-vitro (Crowe 2007). In-vitro activated monocytes have elevated HIV chemokine receptor, CCR5, surface expression and support R5-tropic HIV (Munsaka et al 2009; Tuttle et al 1998).

Specific aim 2: To characterize the amount of activated monocytes in HIV+MJ users and MJ users compared to HIV and SN controls. Because HIV chemokine co-receptor, CCR5, is elevated in activated monocytes (Tuttle et al 1998), CCR5 expression will be measured on monocytes and since immune modulation by cannabinoids is mediated through cannabinoid receptors, CB1 and CB2 on leukocytes from the subject groups will be measured.

Hypothesis: Marijuana users [both HIV-infected (HIV+MJ) and HIV-seronegative (MJ)] would have lower levels of activated immune cells than non-marijuana users [both HIV-infected (HIV) and seronegative (SN)]. In addition, lymphocytes and monocytes will primarily express CB2 receptors, while CB1 receptor levels will be low (or non-detectable) in PBMCs across all groups since CB1 is mainly expressed in the brain.
Rationale: Cannabinoids including marijuana have immunosuppressive properties. Cannabinoids interfere humoral immunity and cell-mediated immunity in vitro, and lower cellular defenses against infectious agents in vivo (Sanders et al 1991). Cannabinoids induce immunosuppression and modulate antigen presenting cells including monocytes (Klein & Cabral 2006). CB2 receptor expression in monocytes and macrophage is increased under inflammatory conditions (Davis et al 1995; Di Marzo et al 1994). Therefore, activated monocytes will have higher expression of CB2 receptors. In contrast, CB1 receptors are mainly expressed in the CNS and rarely on PBMCs.

Specific aim 3: To examine the effects of chronic active MJ use on CSF cytokine expression in both HIV-infected and HIV-seronegative subjects

Hypothesis: Marijuana use would suppress immune function and reduce secretion of proinflammatory cytokines in the CSF of MJ users and HIV-infected MJ users relative to non-MJ users.

CHAPTER 2
CHARACTERISTICS OF ACTIVATED MONOCYTE PHENOTYPE SUPPORT
R5-TROPIC HUMAN IMMUNODEFICIENCY VIRUS

2.1 Summary

Microbial translocation has been recognized as an important factor in monocyte activation and contributes to AIDS pathogenesis with elevated plasma lipopolysaccharide (LPS) levels, as a biomarker for microbial translocation, seen in advanced HIV disease. Therefore, the current study was undertaken to assess monocyte activation in vitro by LPS and to determine its impact on monocyte phenotype.

Monocytes from non-HIV-infected donors were analyzed for CD14, CD16, CD69, TNF-α, and CCR5 by flow cytometry pre- and post- stimulation with LPS. In-vitro cultures were then set up to expose non-activated and activated monocytes to R5-, X4-, and dual- tropic (R5/X4) HIV strains and the amount of HIV present on the cells was assayed.

Non-HIV-infected monocytes, after LPS stimulation, were confirmed to have an activated phenotype with increased CD16 and CD69 surface expression (p<0.05). The activated monocyte was supported by an increase in TNF-α production (p<0.05). The activated monocyte phenotype had increased CCR5 surface expression (from 21% to 98%; p=0.05) and were found to have more R5-tropic virus than non-activated monocytes (p<0.05).

Following activation by LPS, non-activated monocytes were found to have an increase in surface CCR5. These activated monocytes, when exposed to R5-tropic virus, were found to have more virus compared to non-activated monocytes. These findings may explain how microbial translocation plays a role in HIV progression by possibly promoting CCR5-directed strategies in treating HIV.
2.2 **Background**

Systemic immune activation in chronic HIV-infection is thought to be promoted by persistently elevated plasma LPS levels due to microbial translocation from the gut (Ancuta et al 2008). This suggests a role for elevated LPS in driving monocyte activation in HIV disease progression. The elevated LPS levels lead to monocyte activation and these activated monocytes are thought to play an important role in HIV disease pathogenesis (Kusdra et al 2002; Williams et al 2001). Because LPS can lead to monocyte activation, we evaluated if the activated monocyte phenotype supports this clinical observation by assessing its effects on CCR5, the co-receptor for macrophage-tropic HIV (R5-tropic HIV). Other studies have shown that CCR5 expression is upregulated on HIV-infected monocytes.

We hypothesized that the initial activation of monocytes by LPS is an important mechanism leading to increased CCR5 expression on the cells. While the variability in CCR5 expression on cells is dependent on multiple factors, the initial activation by LPS could promote increase in CCR5, thus leading to an increase in R5-tropic virus on monocytes (Gorry et al 2002; Kaufmann et al 2001). If, after activation with LPS, monocytes have increased CCR5 expression and more R5-tropic HIV can be recovered from activated cells, then the paradigm for CCR5-adapted treatment or prevention strategies for HIV-infection can be reexamined.
2.3 Specific aim

The first objective was to characterize baseline and activation immune markers in monocytes of healthy individuals. To further assess the impact of monocyte activation, we planned to study the kinetics of HIV infection (binding) to CCR5 in activated versus non-activated monocytes from the same individuals.

Hypothesis

We hypothesized that activated monocytes will have a higher expression of activation markers (CD69 and TNF-α), CD16 and CCR5 compared to non-activated monocytes. Hence, activated monocytes will have higher amounts of HIV-1 binding compared to non-activated monocytes.

Rationale

Activated monocytes will have altered expression of activation markers and CCR5 compared to non-activated monocytes. This may cause activated monocytes to bind more HIV and thus be more susceptible to viral infection. (Fischer-Smith et al 2001; Pulliam et al 2004). It has been shown that activated monocytes are more permissive to infection and preferentially harbor HIV-1 in-vivo and in-vitro (Crowe 2007). In-vitro activated monocytes have elevated HIV chemokine receptor, CCR5, surface expression and support R5-tropic HIV (Munsaka et al 2009; Tuttle et al 1998).
2. 4 **Experimental Design and Methods**

2. 4.1 **Experimental Design:**

To characterize baseline and activation immune markers in monocytes of healthy individuals and to further study the kinetics of HIV infection (binding to CCR5) in activated versus non-activated monocytes from the same healthy individuals, peripheral blood mononuclear cells (PBMC) were obtained from Ficoll-paque centrifugation of whole blood from 4 volunteers following approval of the study by the University of Hawai‘i Institutional Review Board, CHS# 14313 (Principal Investigator: Dr. Bruce Shiramizu). Monocytes were isolated by magnetic bead separation and analyzed for baseline immune markers by flow cytometry. Activated monocytes were obtained from LPS stimulated PBMCs from the same donors and analyzed for activation markers and CCR5. Activated and non-activated monocytes were exposed to X4 (LAI), R5 (BaL) and dual tropic (p89.6) HIV-1 strains and the amount of HIV recovered was measured by qRT-PCR and densitometry. Experiments were run in triplicate for each viral isolate (LAI; T-tropic, BaL; M-tropic and p89.6; dual tropic). Differences in amounts of HIV recovered from each of the two groups was considered significant by Student’s t-test if $p<0.05$. Statistics were performed using Sigma Stat 3.0 (SPSS Inc) of the triplicate experiments from 4 volunteers with the average ratio of HIV Gag/β-actin.

2.4.2. **Methods**

PBMCs were obtained from whole blood from healthy volunteers by Ficoll centrifugation (GE Heath Care, Piscataway, NJ), primed with IL-10 and stimulated with endomethacin and 2μg/mL *Escherichia coli* LPS O111:B4 (Sigma Aldrich, St. Louis, Missouri) in Teflon flasks for 6 and 20 hours. Monocytes were separated from stimulated and unstimulated cells using magnetic beads (Easysep monocytes isolation kit and monocytes isolation kit with CD16 depletion, Stem Cell Technologies, Vancouver, Canada). Isolated monocytes were stained for monocyte markers (CD14, CD33), activation markers (CD69, TNF-α), CD16 and CCR5 HIV Chemokine co-receptor. Intracellular TNF-α
staining was conducted after staining cells with all the surface markers and treatment with brefeldin A to block intracellular protein transport. The cells were fixed in 4% paraformaldehyde and analyzed using FACSARia (BD Biosciences, San Jose, CA). Aliquots of stimulated and unstimulated monocytes were set up in a 96 well plate at 5 x 10^5 cells/100μL media (RPMI media supplemented with 10% FBS, 100 units/mL Penicillin/Streptomycin, 4mM L-glutamine) per well and exposed to 2ng p24 equivalents of HIV (LAI; T-tropic, BaL; M-tropic and p89.6; dual tropic) for 1 hour in a humidified incubator at 37°C, 5% CO₂ to allow for viral binding. Cells were washed three times with 400μL PBS and then lysed with lysis buffer to extract RNA.

RNA was extracted using RNeasy kit (Qiagen, Valencia, CA). RNA purity and concentration was measured by using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). The A_{260/280} ratio was used to assess RNA quality. Good quality RNA of A_{260/280} ratio between 1.7 and 2.2 was used for RT-PCR and/or stored at -80ºC. cDNA was synthesized from RNA using a mixture of Oligo dT and random hexamer primers and reverse transcriptase from Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Viral binding was measured by quantitative real-time PCR (ΔΔCT method) using HIV Gag primers (for: ATCAAGCAGCCATGCAAATGTT, rev: CTGAAGGGTACTAGTAGTTCCCTGCTATATC) (Klein et al 2003) normalized with β-actin (for: CCTGTACGCCAACACAGTGC, rev: ATACTCCTGCTTGCTGATCC designed using Primer Express Software). The reactions were performed in 96 well plates and run on a StepOnePlus™ real-time-PCR thermocycler (ABI, Foster City, CA) using Sensi Mix dT (2x) polymerase mix and Sybr green (50x). PCR cycling conditions were set as follows: 95ºC hold for 10 minutes and 40 one step cycles of 95ºC for 10 seconds (melting), 60ºC for 30 seconds (annealing and extension). Final extension was done at 72ºC for 3 minutes followed by a melt curve cycling at 95ºC for 15 seconds (melting) and 80ºC for 10 seconds (annealing) and then the reaction was held at 4ºC. Melting curve analysis as well as gel electrophoresis was used to assess specific amplification.
Amplified fragments were resolved on 2.5% agarose gels to confirm specific amplification and scanned with a densitometer for quantification. The ratio of HIV \( Gag/\beta\text{-actin} \) was compared between the two cell populations. Differences in binding capacities between each of the two groups was considered significant by Student’s t-test if \( p<0.05 \). The statistics was performed using Sigma Stat 3.0 (SPSS Inc) of the triplicate experiments from 4 volunteers with the average ratio of HIV \( Gag/\beta\text{-actin} \).

### 2.4.3. Cells and Cell Cultures

Heparinized blood from four non-HIV-infected donors were processed with Dulbecco’s phosphate buffered saline (PBS) and Ficoll-paque (Amersham Biosciences Inc, Piscataway, NJ) to isolate PBMCs (Ruitenberg et al 2006). The cells were resuspended in PBS, 2% fetal bovine serum (FBS) (Mediatech Inc., Herndon, VA); washed and re-suspended in RPMI (Mediatech Inc., Herndon, VA) supplemented with antibiotics (100IU/mL Penicillin, 100ng/mL Streptomycin) and 0.2% normal human serum. Plasma IL-10 levels are known to be elevated in HIV-infected patients (Adib-Conquy et al 1999; Benveniste et al 2000; Caldwell & Evans 2008; Chen et al 2006), hence, IL-10 (10ng/mL rhIL-10) was used to prime the cells, which were then incubated at 37°C, 5% CO2 for 20 hours. This protocol was established from published work and in our laboratory provided consistent results with recovery of cells (Adib-Conquy et al 1999). The primed cells were resuspended in RPMI supplemented with antibiotics, 0.2% normal human serum and 1μg/mL endomethacin (Sigma Aldrich, St. Louis, Missouri), at \( 6 \times 10^6 \) cells/mL; and incubated in Teflon flasks at 37°C, 5% CO2 for 20 hours by stimulating with 20ng/mL \textit{Escherichia coli} LPS O111:B4 (Sigma Aldrich, St. Louis, Missouri)(Adib-Conquy et al 1999). Stimulated PBMC recovered from Ficoll centrifugation were suspended in 100μL PBS, 2% FBS, 1mM EDTA. Monocyte isolation was achieved using the Human Monocyte Enrichment Kit ‘without CD16 depletion’ (Stem Cell Technologies, Vancouver, Canada).

Briefly 10μL of \( \alpha\text{CD32} \) blocking antibody was added to prevent non-specific binding to monocytes by blocking monocyte Fc receptors. The cells were
kept at 4°C for 5 minutes; followed by the addition of 5μL of the antibody cocktail (CD2, CD3, CD19, CD20, CD56, CD66b, CD123, glycophorin A) and dextran; and kept at 4°C for 5 minutes. Magnetic beads were then added to the cells followed by PBS, 2% FBS, 1mM EDTA buffer; incubated at 4°C; and the cells were magnetically separated. The unbound monocytes were poured off; and the cells re-suspended in 2.5mL 2% FBS PBS, 1mM EDTA. The recovered activated monocytes (CD14+/CD16+ phenotype) were washed twice with 2% FBS, PBS. An aliquot of the activated monocytes was analyzed by flow cytometry. The remaining cells were used for the virus culture experiments described below.

Non-activated monocytes were obtained from PBMC isolated from fresh whole blood from the same donors by Ficoll centrifugation followed by Easysep Monocyte Enrichment Kit ‘with CD16 depletion’ as described above (Stem Cell Technologies, Vancouver, Canada). The antibody cocktail consisted of CD2, CD3, CD19, CD20, CD56, CD66b, CD123, glycophorin A; and anti-CD16 to deplete CD16+ monocytes. An aliquot of the non-activated monocytes were stained and analyzed by flow cytometry; with the remainder of the non-activated monocytes placed in culture for the virus experiments. As described by Adib-Conquy et al., 1999, similar experiments were set up by stimulating whole blood and monocytes to determine if differences in stimulating in the presence of other cells is noted (Adib-Conquy et al 1999).

2.4.4. Cell Staining and Phenotypic Characterization

Monocytes, both activated and non-activated, were washed twice and re-suspended in 100μL PBS, 2% FBS. Fluorochrome-conjugated antibodies (anti-CD14-FITC, anti-CD16-Alexa 674, anti-CCR5-APC Cys7, and anti-CD69-Cy7; BD Biosciences Pharmingen, San Diego, CA.) were added as per manufacturer’s specifications. The cells were incubated for 20 minutes at room temperature; washed with PBS 2% FBS twice and then fixed in 200μL 4% Paraformaldehyde, 2% FBS; and analyzed using FACSARia (BD Biosciences, San Jose, CA) and FACSDiva software (BD Biosciences, San Jose, CA). Intracellular staining for TNF-α was carried out following LPS stimulation by adding brefeldin A (Sigma
Aldrich, St. Louis, Missouri) to the cells three hours before harvesting. The cells were washed twice in PBS, 2% FBS and stained for cell surface markers as above. The cells were then permeabilized with 200μL fixation and permeabilization buffer (BD Biosciences, San Jose, CA); and incubated for 20 minutes. After permeabilization, the cells were washed with the wash buffer; centrifuged; re-suspended in 100μL PBS, 2% FBS with anti-TNF-α-PE; and incubated for 20 minutes at room temperature. The cells were washed with PBS, 2% FBS; fixed in PBS, 4% Paraformaldehyde, 2% FBS; and analyzed as noted above.

2. 4.5. HIV-1 Exposure on Activated Monocytes and Non-Activated Monocytes

Monocytes (activated and non-activated monocytes) were isolated and purified from fresh whole blood from the same donors noted above using Ficoll and magnetic bead selection as outlined above. To expose cells to virus, activated and non-activated monocytes were placed separately in 96-well plates (5 x10^5 cells/well) with the addition of 2ng p24 units of LAI (X4-tropic strain; NIH AIDS Research and Reference Reagent Program, Bethesda, MD), BaL (R5-tropic strain; NIH AIDS Research and Reference Reagent Program, Bethesda, MD); or p89.6 (dual-X4/R5-tropic strain) HIV-1 strains for 1 hour in triplicate. Following three washes, the cells and virus were lysed; and RNA isolated. The final supernatants from the third washes were recovered to verify that no virus was present in the last wash. The supernatants were also assessed for HIV p24 (XpressBio, Thurmont, MD) to verify that no active virus was present that could suggest that the cells were infected. The amount of virus that was recovered from the remaining cells was assayed from the isolated RNA by Real-time PCR and densitometry using HIV Gag and β-actin primers with appropriate positive and negative control RNA. Amplified fragments were resolved on 2.5% agarose gels and analyzed by densitometry. From the scanned gels, the ratio of HIV gag light units/β-actin light units was compared between the activated and non-activated monocytes and between the viruses.
2.4.6. **Statistical Analyses**

Results from the experiments carried out in triplicate are expressed as percentage of cells stained for the selected marker before and after stimulation; and the data were analyzed for statistical significance using the Student’s t test for paired values. Differences were considered significant at $p<0.05$. Similarly, experiments, carried out in triplicate for each viral strain, showing differences in the amount of virus detected between cell groups were considered significant by Student’s t-test if $p<0.05$. 
2.5 Results

Monocytes from non-HIV-infected donors were characterized by flow cytometry before and after activation to determine phenotypic changes due to LPS stimulation. Experiments were done using four volunteers and were run in triplicate. Monocytes before and after activation maintained their CD14-expression (87% versus 92% respectively, p>0.05) as shown in Figure 2.1C. To confirm activation, monocytes which were stained for CD16 expression showed significant increase from 9.5% to 89% (p<0.05; Figure 2.1C). Other markers of monocyte activation confirmed the phenotype after LPS stimulation and staining with CD69 and assessment of TNF-α production (Figure 2.1A).

Following LPS stimulation, expression of CD69 on monocytes increased from 18% to 91% (p<0.05; Figure 2.1B). Similarly, production of intracellular TNF-α increased from 0% to 33% post LPS stimulation (p<0.05; Figure 2.1B). Surface CCR5 expression was assessed on activated monocytes and shows increased from 21% to 98% (p=0.05; Figure 2.1C). Consistent to our findings, parallel experiments involving stimulating both whole blood and monocytes showed similar results in activation phenotype as previously reported (Adib-Conquy et al 1999). We assessed the effect of IL-10 itself on monocyte phenotype because plasma IL-10 is increased in HIV-infected individuals (Benveniste et al 2000). Others also have reported that when IL-10 was used to prime the PBMCs prior to LPS stimulation, more consistency in the amount of activation was observed (Adib-Conquy et al 1999).
Figure 2.1 CD14, CD16, CD69, TNF-α and CCR5 expression after monocyte activation with LPS. CD16, CD69 and CCR5 surface expression on monocytes was detected by flow cytometry using anti-CD14-FITC, anti-CD16-APC Alexa fluor, anti-CD69-PE Cy7, TNF-α-PE and anti-CCR5-APC Cy7. Panel A shows the expression profile of monocytes before stimulation and panel B shows the expression profile of monocytes after 6hrs LPS stimulation. Panel C shows the monocyte profile after 20hrs LPS stimulation.
To assess the impact of increased CCR5 expression on activated monocytes compared to non-activated monocytes, the experiments with the three different types of HIV-strains (LAI, BaL, and p89.6) confirmed that activated monocytes had more BaL (X5-tropic) compared to non-activated monocytes exposed to the same virus (p<0.05; Figure 2.2A). For both LAI (X4-tropic) and p89.6 (R5/X4-tropic), the amount of virus recovered from activated monocytes did not differ from non-activated monocytes (p>0.05; Figures 2.1C and 2.2B). The effect of IL-10, alone, on CCR5 expression, showed a slight increase in CCR5 expression on monocytes, as previously shown (Houle et al 1999). However, in contrast, IL-10 priming showed consistent level of activation and larger CCR5 increase than in monocytes treated with IL-10 alone. The supernatants recovered from the washing steps after an hour-exposure of the cells to the virus showed decreasing amounts of HIV with the last wash having no detectable HIV. This confirmed that the cells left in the culture wells had no free virus. The last wash was negative for p24, consistent with undetectable virus replication.
Figure 2.2 Relative Amounts of Virus Recovered from Activated versus Non-Activated Monocytes. A: Higher amount of BaL virus (R5-tropic) recovered from activated monocytes compared to non-activated monocytes, p<0.05, n=3; B & C: For both LAI (X4-tropic) and p89.6 (R5/X4-tropic), the amount of virus recovered from activated monocytes did not differ from non-activated monocytes (p>0.05, n=3) for both viruses.
2.6 Discussion and Conclusion

The current study focused on assessing the phenotype of non-HIV-infected monocytes before and after activation, and determined if activated monocytes had more R5-tropic virus. Following activation by LPS, monocytes in culture consistently had higher CD16 expression on the surface of monocytes, which was expected since CD16 is increased after monocyte activation (Carracedo et al 2006). Other markers of monocyte activation were also increased: TNF-α production and CD69 expression (Aguado et al 2006). While CCR5 is a known receptor on monocytes, these results demonstrate that an increase in CCR5 surface expression could be induced on monocytes upon stimulation with LPS, with some heterogeneity noted in the staining. While we also demonstrated that IL-10, by itself, up-regulates CCR5 gene expression on human monocytes, as previously shown; using IL-10 to prime the cells prior to LPS stimulation provided a more consistent model for activation, as shown by us and by others (Adib-Conquy et al 1999; Houle et al 1999).

These results are in contrast to a previous study showing decreased CCR5 expression after LPS-stimulation of monocyte derived macrophages (Franchin et al 2000). However, our data were obtained from primary monocytes, which is a different model than what was used by Franchin et al (Franchin et al 2000). Our data are limited by the number of donors who provided PBMC and monocytes for the study. Because the baseline phenotype of monocytes varies amongst individuals, and some volunteers had an activated baseline phenotype, we limited the current studies to donors with low baseline activated monocytes (de Man et al 1996). Larger donor pools are currently being planned as resources become available to assess the generality of the results. Additional limitations of the study include a limited number of replicates performed, which will need to be increased.

The importance of monocyte activation occurring in parallel with increase surface expression of CCR5 HIV co-receptor lies in the increased potential for CCR5-tropic viral strains to bind and infect the cells. In the setting of microbial translocation with increase plasma LPS levels, non-HIV-infected monocytes
could be exposed to this chronic inflammatory environment which could increase surface expression of CCR5. This may, in turn, provide a mechanism for these cells to bind more R5-tropic virus and infect the cells. These results are consistent with recent clinical findings where elevated plasma LPS was suggested to be a likely cause of systemic immune activation in chronic HIV infection, which induces monocyte activation (Ancuta et al 2008).

The implications are that peripheral immunological events may play a key role in the pathogenesis of HIV-1. Therefore, these activated cells could be targets for preventing newly-formed monocytes from becoming infected as they enter the circulation. New R5-inhibitor drugs could be identified as targets for future consideration in treatment or preventative strategies (Caldwell & Evans 2008). In conclusion, we believe that monocyte activation by LPS leads to a phenotype that supports R5-tropic virus. If confirmed through expanded studies, the information could shift the paradigm of how to treat or intervene to prevent monocytes from being infected chronically.
CHAPTER 3
INCREASED CD14+CD16+ INFLAMMATORY MONOCYTES AND CCR5 IN PBMCS OF MARIJUANA USERS AND HIV-INFECTED MARIJUANA USERS

3.1 Summary
Marijuana use can lower cellular immune responses and inhibit inflammatory processes. The goal of this study was to examine the effects of marijuana on monocyte activation and CCR5 expression in HIV-infected marijuana users and HIV seronegative marijuana users.

Peripheral blood mononuclear cells (PBMCs) were isolated from HIV+marijuana users (HIV+MJ, aged 41.1±2.8, n=22), HIV subjects (HIV, aged 47.2±2.3, n=18), HIV-seronegative marijuana users (MJ, aged 32.1±2.7, n=15) and seronegative controls (SN, aged 44.3±2.5, n=19). Cells were stained for CD14, CD16 and CCR5 and analyzed by flow cytometry.

There were significant differences in expression of inflammatory CD16+ (Two-way ANOVA p<0.0001) and CCR5 (Two-way ANOVA p<0.003) on CD14+ monocytes among the groups. CD14+CD16+ monocytes were higher in HIV+MJ than in HIV+ subjects (23.1% vs. 11.9%, p=0.0054), and in MJ users than in SN subjects (25.1% vs. 11.5%, p<0.0001). CCR5 expression was significantly higher in MJ users than SN subjects (27.3% vs. 8.0%, p<0.0001); however, no difference in CCR5 expression was found between HIV+MJ users and HIV subjects (14.0% vs. 18.0%, p=0.4). The duration of marijuana use correlated with the levels of CD14+CD16+ (r=0.53, p<0.001) and CD14+CCR5+ expression (r=0.24, p=0.049).

Higher levels of inflammatory monocytes in HIV+MJ and MJ groups, as well as their correlation with lifetime MJ use, suggest MJ smoking induced inflammation in the periphery. Moreover, higher CCR5 expression on the activated monocytes in MJ users than SN subjects suggests that MJ use may render SN subjects more susceptible to HIV.
3.2 Introduction

Marijuana use can lower cellular immune responses and inhibit production of inflammatory cytokines. Marijuana is derived from hemp, *Cannabis sativa*, and comprises numerous cannabinoids including cannabidiol, cannabinol, cannabigerol and tetrahydrocannabinol (THC). THC is the major psychoactive and immunosuppressive constituent (Mechoulam & Gaoni 1965; Pertwee 2005). Cannabinoids exhibit immunosuppressive properties by interfering with humoral immunity, cell-mediated immunity and cellular defenses against infectious agents (Sanders et al 1991). Mice treated with THC displayed reduced resistance to bacterial challenge and had suppressed INF-γ and IL-12 production (Klein et al 1994; Klein et al 2000; Newton et al 1994). Acute exposure to THC *in vivo* can suppress immune cell function, increase HIV co-receptor expression, and act as a cofactor to significantly enhance HIV replication (Roth et al 2005). Furthermore, alveolar macrophages from chronic marijuana users had altered cytokine production (Baldwin et al 1997; Cabral & Vasquez 1991) and THC exposed mice were less responsive to microbial challenge (Cabral et al 1986; Cabral & Marciano-Cabral 2004). However, other studies showed that THC has biphasic action on inflammatory cytokine secretion of stimulated human peripheral blood mononuclear cells by inhibiting TNF-α, IL-6 and IL-8 at 3nM and promoting INF-γ, TNF-α, IL-6 and IL-8 at 3µM concentrations (Berdyshev et al 1997). These conflicting reports need to be examined and validated by human subject studies.

Marijuana is the most commonly abused illicit drug in the world (2010 UN Drug Report). Marijuana smoking is also common amongst HIV-infected individuals with reported benefits including relief of anxiety and depression, improved appetite and reduced neuropathic pain (Prentiss et al 2004). Although marijuana has been promoted for its anti-inflammatory properties, its effects on the immune system of HIV-infected patients are not well studied. Most of the studies that evaluated the effects of marijuana or THC on immune function were conducted *in vitro* or in animal studies. Based on the aforementioned literature that cannabinoids would suppress immune function both *in vivo* and *in vitro*, we hypothesized that marijuana users [both HIV-infected (HIV+MJ) and HIV-
seronegative (MJ)] would have lower levels of activated immune cells than non-marijuana users [both HIV-infected (HIV) and seronegative (SN)]. Therefore, the aim of this study was to measure the amount of activated monocytes in these four subject groups. In addition, since CCR5, the chemokine co-receptor for macrophage-tropic or R5-tropic HIV variants (Deng et al 1996), was found to be elevated in activated monocytes (Munsaka et al 2009; Tuttle et al 1998), we also measured CCR5 surface expression on the monocytes from these subjects.
3.3 Methods

3.3.1 Participants

Seventy-three subjects (84% males) with ages ranging from 19 to 72 years old were recruited from local communities in Honolulu, Hawai'i (Table 3.1). Each subject provided written informed consent in a format approved by the Institutional Review Board at our institution prior to the study and was evaluated to ensure fulfillment of eligibility criteria. Seronegative controls were included in the study if they were \( \geq 18 \) years of age, negative for HIV by blood test (ELISA), had negative urine toxicology results and had no prior history of drug dependency except for nicotine. HIV+ subjects (HIV) were enrolled if they were \( \geq 18 \) years of age, were documented to be HIV-seropositive, had nadir CD4 count \( \leq 500 \) cells/ml, were stable on combinational antiretroviral therapy (cART) for at least six months or cART naïve, had no drug dependency except for nicotine, and did not fulfill other exclusion criteria (Chang et al 2008b).

Marijuana users (MJ) were recruited if they were \( \geq 18 \) years of age, had been actively using marijuana for at least 3 times per week for the past six months, were negative for HIV blood test, had negative urine toxicology results and had no previous drug dependency except for marijuana and nicotine. HIV-positive marijuana users (HIV+MJ) were included in the study if they were \( \geq 18 \) years of age, tested positive for HIV, used marijuana for at least 3 times per week in the past six months, had negative urine toxicology results and had no prior drug dependency, except for marijuana and nicotine, had nadir CD4 count \( < 500 \) cells/ml, were stable on cART for at least six months or were cART naïve and fulfilled other inclusion criteria (Chang et al 2008b).
Table 3.1: Participants Demographics and Clinical Variables (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>HIV+MJ</th>
<th>HIV</th>
<th>MJ</th>
<th>SN</th>
<th>p-value</th>
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<tr>
<td>n= 73 (males/females)</td>
<td>20/2</td>
<td>14/3</td>
<td>11/4</td>
<td>16/3</td>
<td>0.57</td>
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<tr>
<td>Age (years)</td>
<td>41.1 ± 2.3</td>
<td>47.2 ± 3.2</td>
<td>32.1 ± 1.6</td>
<td>44.3 ± 3.2</td>
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<td>Estimated Verbal IQ (WTAR)</td>
<td>100.8 ± 2.8</td>
<td>106.9 ± 2.3</td>
<td>107.4 ± 2.5</td>
<td>100 ± 4.0</td>
<td>0.13</td>
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<td>CES-Depression Scale (0-30)</td>
<td>19.8 ± 3.1</td>
<td>11.9 ± 2.5</td>
<td>10.6 ± 3.0</td>
<td>5.2 ± 0.92</td>
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<tr>
<td>Taking cART (+/-)</td>
<td>17/5</td>
<td>12/5</td>
<td></td>
<td></td>
<td>0.72</td>
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<tr>
<td>Duration of HIV (months)</td>
<td>177.1 ± 60.6</td>
<td>258.1 ± 82.6</td>
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<td>CD4 Count (#/mm³)</td>
<td>561.9 ± 49.9</td>
<td>575.5 ± 51.0</td>
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<td>Nadir CD4 Count (#/mm³)</td>
<td>227.8 ± 39.6</td>
<td>209.5 ± 45.9</td>
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<td>Karnofsky Scale (0-100)</td>
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<td>88.3 ± 2.5</td>
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<td>HIV Dementia Scale (0-16)</td>
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<td>14 ± 0.7</td>
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<td>Log Viral Load (copies/mL)</td>
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<td>2.2 ± 0.36</td>
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<td>Nicotine/Cigarette Smokers</td>
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<td>8</td>
<td>11</td>
<td>6</td>
<td>0.88</td>
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<td>Total lifetime nicotine use (g)</td>
<td>321.8 ± 181.3</td>
<td>103.5 ± 43.7</td>
<td>219.6 ± 67.5</td>
<td>12.9 ± 8.0</td>
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<td>Duration of MJ use (months)</td>
<td>199.5 ± 32.8</td>
<td>16 ± 4.4</td>
<td>38.2 ± 23.6</td>
<td>1.3 ± 0.99</td>
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<td>Average Daily MJ use (g)</td>
<td>1.66 ± 0.37</td>
<td>0.18 ± 0.10</td>
<td>1.57 ± 0.48</td>
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<td>Total Lifetime MJ use (Kg)</td>
<td>9.75 ± 2.8</td>
<td>0.99 ± 0.7</td>
<td>5.91 ± 2.5</td>
<td>0.006 ± 0.005</td>
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</table>
3.3.2 PBMC isolation and FACS Staining

Following informed consent, whole blood was obtained from research subjects by venipuncture in 2x 6mL Potassium Ethylene-diamine-tetraacetic acid (EDTA) tubes (BD Vacutainer, BD Franklin, Lakes, NJ). Whole blood was diluted 1:1 with 1x phosphate buffered saline (PBS) (Mediatech Inc, Manassas, VA) and mixed. The blood PBS mixture was layered on a Ficoll-paque (GE Healthcare, Uppsala, Sweden) gradient and centrifuged at 700g for 25 minutes at 4°C with the brake off to isolate PBMCs. Isolated buffy coat PBMCs were washed (300g for 25 minutes at 4°C) twice in 20mL 1x PBS supplemented with 2% fetal bovine serum (FBS) (Mediatech Inc, Manassas, VA). PBMCs were then resuspended in 100µL PBS in 15ml BD FACS tubes and stained with CD14-FITC (eBioscience, clone 61D3), CD16-PerCP (Biolegend, clone 3G8) and CCR5-PE (eBioscience, clone eBio T21/8) according to manufacturer specifications. The cells were incubated at 4°C in the dark for 30 minutes. Cells were then washed twice with up to 4mL PBS, 2% FBS, to remove excess antibody. The cells were stored at 4°C in the dark and run on BD FACSCalibur (BD Biosciences, San Diego, CA) on the same day, collecting 50,000 events. For comparison, unstained cells or cells stained with mouse IgG2A control antibody were used. FACS analysis was done using Summit V4.2 software subtracting the mean fluorescent intensity (MFI) of stained cells from background MFI of unstained or isotype stained cells.

3.3.3 Statistical Analysis

Statistical analysis was performed using SAS version 9.1 (SAS institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) and post-hoc test was used for group comparison. Multiple linear regression was used to examine interactions between immune markers (CD16 and CCR5), amount of marijuana smoked, duration of marijuana use, HIV and marijuana status of the subjects. Data are represented as mean ± standard error of the mean (SEM). p values < 0.05 were considered statistically significant. Because subjects’ ages were different and age could affect CD16 and CCR5 expression, age was used as a covariate in all analyses. The duration of marijuana use was log transformed to
get an even distribution (normal distribution) between the marijuana smoking groups. Partial correlation analysis was used to control for cofounding variables that were significantly different among the groups (e.g. nicotine).
3.4 Results

The study population of 73 mostly male subjects (84%) is reflective of the sex-proportion of HIV infected individuals in Hawai'i. The ethnic distribution comprised 45% Caucasian whites, 23% Asians, 5% native Hawai'ians/Pacific islanders, 9% African Americans and 18% mixed races which is consistent with the local population. The HIV groups (HIV+MJ and HIV) were well matched for the duration of HIV infections, CD4 counts, nadir CD4, HIV dementia scale and HIV viral load (Table 3.1). However, HIV subjects had a lower Karnofsky scale than the HIV+MJ (p=0.01). The seronegative MJ users were the youngest subjects compared to the other three groups (p=0.005). Among the marijuana using groups (HIV+MJ and MJ), the MJ group had a longer duration of marijuana use, higher average daily marijuana use and higher total lifetime marijuana exposure (p=0.0001, Table 3.1). The number of cigarette (nicotine) smokers was not different among the groups (chi² p=0.88) but the amount of lifetime nicotine smoked was significantly different (ANOVA p=0.03) with MJ users (HIV+MJ and MJ) using more nicotine. Using nicotine as a covariate (partial correlation) in the analysis did not change the significant differences of observations made.

Forward and side scatter was used to draw gates and discriminate between monocytes and lymphocytes (Figure 3.1A). The percentage of CD14+CD16+ and CD14+CCR5 expressing monocytes was measured from the CD14+ gate with the top right quadrants showing double positively stained cells (Figure 3.1B, C and D). The gates were drawn using unstained or isotype stained cells as control (dot plots not shown).
Figure 3.1 Scatter plots showing gating strategies used to quantify CD16 and CCR5 staining of CD14+ monocytes. 1A. Forward and side scatter was used to gate on the monocytes. 1B. Monocyte population was selected from the CD14+ gate. 1C & 1D. Amounts of CD16+ and CCR5+ monocytes were measured, using unstained sample as control (not shown).
Adjusting for age, inflammatory CD14+CD16+ monocytes were significantly higher in HIV+MJ users than HIV+ subjects (26.6% vs. 14.4%, p=0.0054), and in MJ users compared to SN subjects (25.1% vs. 11.5%, p<0.0001) (Figure 3.2A). 2-way ANOVA showed a significant marijuana effect (p<0.0001) in the expression of the CD14+CD16+ phenotype on monocytes. MJ users had higher CCR5 expression than SN subjects (27.3% vs. 8.0%, p<0.0001). HIV+MJ users and HIV subjects had no difference in CCR5 expression (14.0% vs. 18.0%, p=0.4) (Figure 3.2B). There was an interaction of marijuana and HIV (p=0.003) and a marijuana effect (p=0.0038) in the expression of CCR5 on monocytes by 2-way ANOVA.

The CD14+CD16+ phenotype also correlated positively with the log duration of marijuana use (r=0.24, p=0.043) where as the CD14+CCR5 phenotype did not (Figure 3. 2C and D). The partial correlation of CD14+CD16+ with duration of marijuana use after controlling for nicotine use was r=0.18, p=0.50. However, the CD14+CCR5+ phenotype was correlated with the total lifetime amounts of marijuana smoked (r=0.36, p=0.002) and the average daily marijuana use (r=0.39, p=0.001). The inflammatory monocyte phenotype was also correlated with the total lifetime amounts of marijuana smoked (r=0.49, p<0.001) and the average daily marijuana use (r=0.42, p=0.003) by Spearman Rank Correlation test. There was no correlation of immune phenotypes CD16 and CCR5 expression with age (data not shown).
Figure 3.2 Expression of CD16 and CCR5 on monocytes and correlations with duration of marijuana use. PBMCs from HIV+MJ, HIV+, MJ and SN were isolated by Ficoll centrifugation and stained for CD14, CD16 and CCR5. 2A. Percentage of monocytes expressing CD14 and CD16. 2B. Percentage of cells expressing CD14 and CCR5. 2C. Expression of CD16 and the log duration of marijuana use in HIV+MJ and MJ users. 2D. Expression of CCR5 and the log duration of marijuana use in HIV+MJ and MJ users.
3.5 Discussion and Conclusion

To study the effects of HIV and marijuana on inflammatory processes in HIV infection, we evaluated and measured the amount of inflammatory CD14+CD16+ cells in the HIV-infected subjects, with and without MJ use, as well as in HIV-negative subjects, with and without marijuana use. We also measured the amount of CCR5 expression on monocytes from these four subject groups by flow cytometry. We observed higher CD16+ monocytes in marijuana using groups suggesting that marijuana use may promote monocyte activation. The activated monocyte phenotype (CD14+CD16+) was higher in our marijuana smoking groups, both in the HIV+ and HIV- populations. This is contrary to prior studies that showed THC may suppress immune function. However, previous studies were conducted in vitro or using animal model treated with pure THC (Roth et al 2005), but not marijuana smoke which contains numerous compounds in addition to THC, the major psychoactive component (Mechoulam & Gaoni 1965). Marijuana also contains other cannabinoids including cannabidiol, cannabinoi and tetrahydrocannabinvarin which are less psychoactive but have sensory effects (Fusar-Poli et al 2009; Pertwee & Ross 2002) and may affect the immune system. For instance, Cannabidiol was found to inhibit IL-10 production in HUT-78 T-cells (Srivastava et al 1998) suggesting that the effects of marijuana on the immune system are not only influenced by THC alone but other compounds.

Furthermore, the correlation between the duration of marijuana use and the amount of activated monocytes suggests that the longer these individuals smoked marijuana, the more their monocytes were activated, creating a chronic immune activation state similar to that in chronic inflammatory disorders. Similar to the elevated activated monocytes in our MJ users, with or without HIV infection, the inflammatory activated monocytes (CD14+CD16+) are also elevated and contributes to the pathology of various chronic inflammatory diseases (multiple sclerosis, cardiovascular disease, and HIV-associated dementia, etc) (Carracedo et al 2006; Thieblemont et al 1995). The inflammatory CD14+CD16+ phenotype was also correlated with total lifetime amount of
marijuana and the average daily marijuana of marijuana smoked by the subjects suggesting that chronic marijuana smoking activated monocytes. Monocyte activation has been implicated in inflammatory diseases and has been associated with increase in CCR5 expression (Munsaka et al 2009).

We further analyzed CCR5 expression on monocyte populations from all the subject groups. CCR5 expression was higher in the HIV-negative marijuana users than in HIV- Seronegative controls suggesting that marijuana use may increase CCR5 expression in seronegative subjects. However, the expression pattern of CCR5 was not different between the two HIV groups, which may be due to HIV downregulation of CCR5 since we observed a trend of negative correlation between CCR5 expression and duration of marijuana use. CCR5 expression also correlated with duration of marijuana use, which suggested that the CCR5 chemokine co-receptors may progressively increase over time in those with longer duration of marijuana smoking.

Activated monocytes with increased CD16 and CCR5 expression play important roles in HIV neuropathology since they act as reservoirs of infection (Crowe 2007; Pulliam et al 1997; Pulliam et al 2004), traffic HIV into the brain (Fischer-Smith et al 2001) and produce neurotoxic inflammatory cytokines (Thieblemont et al 1995). Because previous studies showed THC as immunosuppressive, we had hypothesized that marijuana smokers would have lower activated CD16+ monocytes and lower CCR5, but our results showed the opposite outcome, suggesting that other compounds in marijuana might have activated the monocytes and cause upregulation of CCR5 or that the effects of marijuana on humans as a result of in vivo exposure may be distinct from the effects observed in vitro and in animal models. Since activated monocytes have been shown to be associated with increased CCR5 expression (Munsaka et al 2009), which renders the monocytes more permissive to HIV infection in vivo and in vitro (Crowe 2007), our finding suggests that the seronegative marijuana users with elevated CCR5 might be more susceptible to HIV infection if exposed. Future studies of subjects taking medications containing only THC are needed to further evaluate the role of THC on monocytes and CCR5 expression.
More than half of our HIV cohort used marijuana or have prescription marijuana. The effects of smoking marijuana or oral ingestion of dronabinol for enhancing appetite and treating neuropathic pain in HIV disease need to be studied further. Because this study has found that marijuana increases monocyte activation and CCR5 expression, which are key players in HIV disease pathogenesis, the risks of marijuana and cannabinoid based therapies in HIV disease need to be re-examined. Marijuana use may exacerbate the chronic immune activation in HIV disease. Persistent immune activation is an important factor in HIV pathology and progression to AIDS (Hazenberg et al 2003). In seronegative subjects, marijuana smoking may create a chronic immune activation state and higher susceptibility to HIV infections. Although HIV RNA load is the main factor in the transmission of HIV (Quinn et al 2000), transmission may be potentiated in the context of low HIV RNA loads if there are more activated, highly susceptible cells in the mucous epithelia during sexual transmission.

There are several limitations of this study. First, the seronegative marijuana users participating in this study were younger than the other subject groups. Immune phenotypes, especially CD16, may vary in expression across age and thus may affect the analysis. However, CD16 and CCR5 expression did not statistically correlate with age in these subjects. To account for this limitation, age was used as a covariate in the analysis. Secondly, the subjects were not genotyped for their CCR5 Δ 32 alleles. Individuals with CCR5 Δ 32 alleles (+/- or +/-) may have different expression levels of CCR5. However, this factor may not affect the results significantly since the proportion of individuals with the CCR5 Δ 32 allele is only 10% and is only found in people of northern European ancestry who were not highly represented in this study population (Hedrick & Verrelli 2006; Martinson et al 1997). Third, marijuana usage among the groups was not similar. HIV+MJ users smoked more marijuana and had a longer duration of marijuana use than MJ users. This in part is due to the age difference, since HIV+MJ were older than MJ and have smoked more marijuana for a longer period of time to manage their HIV disease. However, MJ users started smoking
in the early teens and have continued to smoke though in lower quantities than HIV+MJ users into their thirties. Cigarette smoking was not an exclusion criterion and was not uniform across all groups. Nicotine use (cigarette smoking) is known to be immunosuppressive (Carlson et al 1998). However, after controlling for nicotine (partial correlation), the correlations did not significantly differ implying that the nicotine effect was not significant. Lastly HIV positive subjects who recreationally or medically smoked marijuana in the past (more than 6 months prior to the study), and had negative urine toxicology for marijuana, were not excluded in the study. This may explain why there was no difference in CCR5 expression between HIV+MJ and HIV. The CCR5 expression may be affected by chronic marijuana use and thus past marijuana use may have skewed our CCR5 observation.

To conclude, we found elevated activated monocytes in HIV-infected marijuana users and marijuana users. CCR5 expression was also increased in marijuana users. The importance of this finding is that marijuana smoking may activate immune cells contrary to the prior reports of immune suppression. Immune activation may exacerbate HIV pathology or predispose seronegative marijuana users to HIV infection after exposure. Further studies to examine the effects of marijuana use on immune function and impact on HIV disease are warranted.
CHAPTER 4
MARIJUANA USE PROMOTES NEUROINFLAMMATION IN HIV-INFECTED SUBJECTS

4.1 Summary
Cannabinoids, including marijuana (MJ) and tetrahydrocannabinol (THC), modulate cellular immune responses and dampen inflammatory cytokine secretion in vitro. The goal of this study was to examine the effects of MJ smoking and HIV infection in the brain by measuring cerebral spinal fluid (CSF) cytokines using Luminex. We hypothesized that proinflammatory cytokines will be lower in MJ smokers, with or without HIV, than in non-MJ users, with or without HIV. Contrary to our hypothesis, inflammatory cytokines (fractalkine, IFN-α2 and IL-1α) and chemoattractants (MCP-1, IP-10 and IL-8) were all significantly higher in the HIV+ MJ smokers than in MJ and non MJ users (HIV and SN controls). The higher expression of inflammatory cytokines and chemokines in CSF of HIV+MJ and MJ users suggests that marijuana may promote neuroinflammation in MJ users.
4.2 Background and aim

Marijuana use for medical and recreational purposes dates back to ancient times and currently it is widely used around the world. Despite the fact that marijuana possession and use is illegal in most countries, it is regularly used by about 20 million people in the United States and Europe, and by millions more in many parts of the world (Iversen 2000). According to the United Nations’ 2010 world drug report, marijuana (MJ) is the most widely used illicit drug in the world. Marijuana is at times prescribed for nausea, neuropathic pain and HIV wasting syndrome. Epidemiological studies and animal studies identified MJ as a potential cofactor in the development and progression of HIV infection (Roth et al 2005). Marijuana preparations are from hemp (Cannabis sativa) which contains several cannabinoids of which THC is the main psychoactive and immunosuppressive constituent (Mechoulam & Gaoni 1965). Short term exposure of huPBL-SCID mice to THC suppressed immune cell function, increase HIV co-receptor expression, and acted as a cofactor to significantly enhance HIV replication (Roth et al 2005). However, the effect of long term exposure to cannabinoids remains unclear. Other studies have shown that higher doses of THC (>3nM) may promote cellular activation and cause secretion of proinflammatory cytokines (Berdyshev et al 1997). These studies were conducted in vitro and may not be comparable to the effects of chronic daily MJ smoking that occurs in human subjects.

Few studies have reported the effects of marijuana on immune function in chronic active MJ users and HIV-infected MJ users. Therefore, the aim of this study was to examine the effects of chronic active MJ use on CSF cytokine expression in both HIV-infected and HIV-seronegative subjects. Based on the evidence of THC mediation of immune suppression, we hypothesized that MJ use would suppress immune function and reduce secretion of proinflammatory cytokines in the CSF of MJ users and HIV-infected MJ users relative to non-MJ users.
4.3 **Human Subjects and Methods**

4.3.1 **Experimental Design**

CSF was collected from research subjects by research physicians via lumbar puncture (see appendix 1 for definitions and eligibility criteria). The subset of subjects who provided CSF across the four groups had similar age ranges and the two HIV-infected subject groups had comparable CD4 counts, nadir CD4, HIV plasma viral load and HIV dementia scale (see Table 3.1 in Chapter 3). CSF was centrifuged at 220 x g for 10 minutes at 4°C to pellet cells and then spun at high speed (10000 rpm for 10 minutes at 4°C) to remove cellular debris. The CSF was banked in 500µL aliquots at -80°C. Because not all the enrolled subjects consented to the lumbar punctures, CSF from a subset of 50 subjects from the subject groups (HIV+MJ, HIV, MJ and SN) was included in these analyses.

Microsphere beads were used to measure up to 42 cytokines in the CSF. Mean fluorescent intensity for each cytokine was converted to absolute values using a standard curve and cytokine expression from the 4 subject groups was assessed by two-way ANOVA, with post-hoc analyses. Multiple regression and nonparametric Spearman correlation analyses were performed to examine linear relationships between cytokine expression levels and MJ usage (lifetime dose, duration of MJ use and amount per use) and/or other clinical variables. Results were analyzed in SAS Enterprise v 9.1 (SAS Institute, Cary, NC). In all analyses, p-values <0.05 were considered significant. The data are presented as means ± standard error of the means. Two assays were performed using two commercially available kits from Millipore. The first kit used magnetic microsphere beads with capture antibodies bound to beads via magnetism and the second kit used polystyrene beads having capture antibodies covalently linked. The magnetic bead assay was developed more recently, and had not been tested on human CSF. Therefore, one of our aims was to compare its robustness with the older polystyrene bead assay.
4.3.2 Methods

Banked CSF samples collected by lumbar puncture were used for the Luminex assays. Luminex’s xMAP a microsphere based multiplexing Immunoassay (MBIA) using the LUMINEX platform (Millipore, USA) was used to measure 42 cytokines (EGF, Eotaxin, FGF-2, Flt-3 ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN-α2, IFN-γ, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IL-1Rα, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IP-10, MCP-1, MCP-3, MDC (CCL22), MIP-1α, MIP-1β, PDGF-AA, PDGF-AB/BB, RANTES, TGF-α, TNF-α, TNF-β, VEGF, sCD40L, and sIL-2Rα) in the CSF of our subjects.

Briefly, the wells of the 1.2-μm filter membrane 96-well microtiter plates were pre-wetted with assay buffer. 25μL of the CSF sample, as well as the standard and quality control preparations, were added to the relevant wells and incubated with pre-mixed microbeads for 1 hour on an orbital plate shaker at room temperature. The plates were washed twice with assay wash buffer and 25μL biotinylated detector antibody was added per well.

The samples were incubated for 30 minutes at room temperature or overnight at 4°C on the plate shaker. Without washing, 25μl/well streptavidin–phycoerythrin solutions was added, and plates incubated for a further 30 minutes at room temperature on a plate shaker, protected from direct light. Before analyzing, microbeads were washed twice in assay wash buffer and resuspended in 100μL/well of Luminex sheath fluid. The Luminex assay was acquired on a Luminex-200™ instrument using Exponent software (Invitrogen, Paisley, England). An acquisition gate of between 8000 and 13,500 was set to discriminate against any doublet events and ensure that only single microbeads were measured. 100 events per region were collected and median fluorescence intensity (MFI) measured. MFI were converted to concentrations using results from a standard cytokine preparation. The cytokine standards were diluted 1:5 with a starting concentration of 10,000 pg/mL, yielding a detection limit of 3.2 pg/mL.
Statistical analysis was performed using SAS version 9.1 and STAT View (SAS institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) and post-hoc test was used for group comparison. Multiple linear regression and non-parametric Spearman Rank Correlation Test were used to examine relationships between cytokine expression, amount of marijuana smoked, duration of marijuana use, amounts of activated monocytes, and HIV and marijuana status of the subjects. Data are represented as mean ± standard error of the mean (SEM). p values < 0.05 were considered statistically significant.
4.4 Results

The age distribution of the subjects who provided the CSF samples is shown in Figure 4.1 (see also Table 3.1, Chapter 3). The four groups were not different in their age distribution; hence, the cytokine measurements were not adjusted for age.

![Figure 4.1 Age distribution among study subjects](image)

The magnetic bead assay detected more cytokines in the CSF of the subjects. CSF from the same subjects were run on both plates and the cytokines measured are shown in Figure 4.2. The magnetic bead assay also had the higher average bead counts per analyte (>100) and had the best linear fit for the standards for the cytokines measured. More than half of the cytokines were not detected by the polystyrene beads assay and less bead counts were obtained. The polystyrene bead assay did not detect FGF-2, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-14, IL-15, MIP-3, PDGF, RANTES, MDC, TNF-α, and VEGF. However, not all cytokines and chemokines were detectable by magnetic bead assay either; these include: FGF-2, FLT-3L, GRO, MCP-3, IL-12, MDC, PDGF, IL-1RA, IL-1β, RANTES, TNF-α and VEGF. Therefore, both assays could not detect FGF-2, IL-1 β, IL-12, MDC, PDGF, RANTES, TNF-α and
VEGF. In contrast, fractalkine, MCP-1 and IP-10 were the major cytokines detected using both assays. The magnetic bead assay had more sensitivity than the polystyrene beads assay. For example, there was no significant difference in the MCP-1 detection in the CSF using the polystyrene assay but the magnetic beads assay showed a significant difference in MCP-1 concentration among the groups (Figure 4.3). Overall, based on the better bead counts, best linear fit for standards and the number and quantity of cytokines measured in the CSF, we chose to present only the results obtained from the magnetic bead assay.

Figure 4.2 Cytokine detection in the CSF using magnetic and polystyrene beads. Two Luminex assays were compared in the detection of cytokines in the CFS. The cytokines detected by both assays are in the intersection of the two circles and cytokines that could not be detected both assays are outside the circles.
Figure 4.3 Comparison of MCP-1 detection in the CSF between magnetic beads and polystyrene beads Luminex assays. MCP-1 was measured by magnetic (A) and polystyrene (B) beads assays and the results compared.

Inflammatory cytokines, fractalkine and interferon-alpha 2 (IFN-α2), were detected in higher amounts in the CSF of MJ smokers (with or without HIV, 2-way ANOVA, MJ effect: p=0.0035 (fractalkine) and p=0.014 (IFN-α2); Figure 4.4). Post-hoc analyses show that fractalkine levels were significantly higher in HIV+MJ than in HIV (+21%, p=0.04) and SN controls (+27%, p=0.035; Figure 4.4A). MJ users also had higher fractalkine levels detected in their CSF than SN controls (+20%, p=0.026; Figure 4.4A). The amounts of fractalkine in the CSF also correlated with the duration of marijuana use (r=0.42, p=0.028) by Spearman Rank Correlation Test. Similarly, post hoc analyses showed that IFN-α2 levels were higher in HIV+MJ users than HIV (+30%, p=0.014) and SN controls (+31%, p=0.006; Figure 4.4B). No statistically significant differences were observed in the levels of IFN-α2 in MJ compared to SN controls. IFN-α2 levels in the CSF were correlated with the duration of marijuana use (r=0.50, p=0.007) by Spearman Rank Correlation Test.
Figure 4.4 Expression of inflammatory cytokines from CSF measured by Luminex. CSF was obtained from study subjects by lumbar puncture and the amount of cytokines and chemokines was measured by multiplex Luminex. Inflammatory cytokines detected included (A) fractalkine and (B) IFN-α2.

Chemoattractant cytokines (chemokines) were also detected in the CSF of subjects. Interferon gamma-inducing protein-10 (IP-10) and macrophage chemoattractant protein-1 (MCP-1) were the most abundant chemokines detected. IP-10 expression displayed both an MJ and an HIV effect (p=0.01 for both; Figure 4.5A) by 2-way ANOVA. Post-hoc analyses show that HIV+MJ users expressed more IP-10 than HIV (3.6 vs. 1.9 ng/mL, p=0.027), MJ (3.6 vs. 1.9 ng/mL, p=0.021) and SN controls (3.6 vs. 1.2 ng/mL, p=0.0006; Figure 4.5A). IP-10 levels were correlated with the duration of marijuana use (r=0.52, p=0.006) and the amount of CD14+CD16+ activated monocytes (r=0.45, p=0.024) by Spearman Rank Correlation Test. Similarly, MCP-1 was detected in higher amounts in HIV+MJ than in either HIV (+28%, p=0.004) or MJ (+28%, p=0.0033; Figure 4.5B) and there was an interaction between MJ use and HIV infection (interaction-p=0.0022) in the expression of MCP-1 in the CSF.
Figure 4.5 Expression of chemoattractant cytokines (chemokines) from CSF measured by Luminex. CSF was obtained from study subjects via lumbar puncture and the amount of chemokines was measured by multiplex Luminex. (A) IP-10 and (B) MCP-1 were detected in the CSF of subjects.

Other cytokines detected in the CSF included IL-8 and IL-1α. IL-8 was also found in higher amounts in HIV+MJ than in either HIV (+22%, 0.033) and MJ (+28%, 0.0052), again showing to an interaction effect between MJ use and HIV infection (p-interaction=0.018; Figure 4.6A). HIV+MJ users also displayed higher amounts of IL-1α than MJ (+42%, p=0.025) and SN controls (+38%, p=0.0007), Figure 4.6B, which appears to be due to an additive effect of HIV (p=0.0083) and MJ (p=0.032). IL-1α levels in the CSF were correlated with the amount of CD14+CD16+ activated monocytes (r=0.44, p=0.035) by Spearman Rank Correlation Test.
Figure 4.6 Expression of other cytokines from CSF measured by Luminex. CSF was obtained from study subjects via lumbar puncture and the amount of cytokines was measured by multiplex Luminex. Other cytokines detected include; (A) Interleukin-8 and (B) Interferon1-alpha.
4.5 Discussion and Conclusion

Despite the CNS being a well regulated and secluded anatomical compartment, leukocytes and monocytes traffic back and forth from periphery as part of normal homeostasis. During acute HIV infection, activated monocytes traffic into the CNS (Fischer-Smith et al 2001) and HIV is thought to be introduced into the CNS during this phase of infection via a ‘Trojan horse’ mechanism (Nottet et al 1996). Monocyte and lymphocyte ingress into the CNS continues during the chronic phase of the disease. In the CNS, activated monocytes produce mediators including cytokines/chemokines that are toxic to neurons (Belge et al 2002; Thieblemont et al 1995). Since THC in marijuana can modulate cytokine secretion (Srivastava et al 1998), we compared cytokines in the CSF of marijuana users and non-marijuana users, with and without HIV infection.

Inflammatory cytokines, fractalkine and IFN-α2, were elevated in HIV+MJ users. Soluble fractalkine is a C-X3-C motif chemokine that potently attracts T cells and monocytes (Szekanecz & Koch 2001). This cell bound chemokine typically promotes strong adhesion of leukocytes to activated endothelial cells; therefore, the higher fractalkine observed in MJ users, with or without HIV, may promote leukocyte adhesion to the brain microvascular endothelial cells and trafficking into the CNS thus resulting in enhanced neuroinflammation. Fractalkine has also been implicated in many inflammatory diseases, such as rheumatoid arthritis (Ruth et al 2001) and atherosclerosis (Sugiura et al 2006b; Szekanecz et al 2006). The elevated fractalkine levels in MJ users (HIV+MJ and MJ) imply that marijuana smoking might increase inflammation in the CNS by recruiting peripheral immune cells. Fractalkine levels in the CSF were also correlated with the duration of marijuana use suggesting that chronic marijuana use may promote neuroinflammation.

The high levels of IFN-α2 in HIV+MJ may be attributable primarily to MJ use since non-MJ using HIV subjects had normal IFN-α2 levels while seronegative MJ users trended toward elevated levels of IFN-α2, although this increase was not statistically significant. However, IFN-α2 concentrations in the
CSF were correlated with duration of marijuana use suggesting that IFN-α2 levels were elevated due to chronic marijuana use.

Marijuana smoking and HIV infection both appear to induce higher levels of IL-1α in the CSF, leading to an additive effect in HIV+MJ users. IL-1α is produced by endothelial cells and induces TNF-α release (Imaizumi et al 2000). However, TNF-α could not be detected in CSF by either polystyrene or magnetic beads platforms in our studies. TNF-α typically induces proliferation of CD4+ cells, IL-2 production and co-stimulates CD8+/IL-1R+ cells, induces proliferation of mature B cells and immunoglobulin secretion. The higher levels of IL-1α in HIV+MJ and MJ smokers may suggest promotion of inflammation by marijuana and this is worse in HIV-infected marijuana users.

Chemokines including IP-10, MCP-1 and IL-8 were all detected in higher quantities in the CSF of HIV+MJ users. IP-10 is a C-X-C motif chemokine produced by monocytes and endothelial cells in response to IFN-γ. IP-10 is also a potent chemoattractant of monocytes, T cells, NK cells and dendritic cells and it promotes T cell adhesion to endothelial cells. Therefore, the high levels of IP-10 in HIV+MJ users suggest that MJ smoking may promote leukocytes and monocytes traffic into the CNS, especially in the setting of HIV infection. Recently, IP-10 and MCP-1 were correlated with neuroinflammatory cerebral metabolic patterns in HIV patients (Letendre et al 2011). MCP-1 is a C-C motif cytokine that primarily is secreted by monocytes, macrophages and dendritic cells. It is cleaved by matrix metalloproteinase (MMP)-12. MCP-1 recruits monocytes, memory T cells and dendritic cells to cells of tissue injury, infection and inflammation. MCP-1 has been implicated in the pathogenesis of many diseases characterized by monocyte infiltrates. For example, MCP-1 plays a key role in neuroinflammatory processes in many CNS diseases where neuronal degeneration is involved including HIV encephalitis, epilepsy, ischemia, Alzheimer’s disease, traumatic brain injury and autoimmune encephalitis. The higher MCP-1 levels in HIV+ MJ users suggest that marijuana may promote HIV neuropathogenesis through recruitment of monocytes into the CNS. IP-10 levels in the CSF were correlated with the amount of CD14+CD16+ activated...
monocytes of these same patients affirming that chronic marijuana use may drive leukocyte activation and promotion of inflammatory cytokine secretion. Chronic immune activation and production of inflammatory cytokines in the CNS has been implicated in the neuropathogenesis of HIV leading to cognitive dysfunction (Kaul et al 2001).

In our parallel study, HIV+MJ and MJ users had higher amounts of activated monocytes (CD14+ CD16+) in their peripheral blood. HIV chemokine co-receptor, CCR5, was also elevated in MJ smokers and both the CD14+ CD16+ and CD14+ CCR5+ correlated with the duration of marijuana use. The correlation of activated monocytes and CCR5 expression with duration of marijuana use suggests that marijuana smoking increases immune cell activation and causes CCR5 upregulation. Activated monocytes are more permissive to HIV infection and preferentially harbor the virus in-vivo (Crowe 2007) and in-vitro activated monocytes display elevated CCR5 surface expression and support macrophage-tropic (R5-tropic) HIV (Munsaka et al 2009; Tuttle et al 1998). Individuals with HIV-associated dementia have higher percentages of circulating activated monocytes/macrophages (CD14+/CD16+) (Fischer-Smith et al 2001; Pulliam et al 2004; Shiramizu et al 2005) and these activated monocytes infiltrate perivascular brain regions (Fischer-Smith et al 2001). An expanded population of CD14+ CD16+ monocyte subset producing IL-1α and TNF-α has been identified in HIV-infected individuals, which has been implicated in HIV neuropathogenesis (Thieblemont et al 1995). Therefore, our finding of elevated amounts of IL-1α in the CSF of HIV+ and MJ users, along with the higher percentages of peripheral CD14+/CD16+, suggest that IL-1α is produced by activated monocytes since they readily cross the blood brain barrier (Fischer-Smith et al 2001; Sonza et al 2001). The activation of immune cells and induction of proinflammatory cytokine secretion (IL-1α, IL-8, IFN-α2, IP-10, fractalkine and MCP-1) by marijuana in HIV-infected individuals suggest that marijuana may worsen HIV pathology.

IL-8 is also a major chemoattractant of neutrophils and macrophages. It is produced by macrophages and epithelial cells. IL-8 is associated with inflammation and is the major inflammatory mediator in gingivitis and psoriasis.
Elevated IL-8 in HIV+MJ also may be indicative of marijuana induced neuroinflammation.

Potent proinflammatory cytokines such as IL-1β, IL-2, IL-12, IFN-γ, TNF-γ and IL-1γ were not detected by our multiplex assays. We plan to measure these cytokines using single-plex ELISAs or using a TH-1 cytokine assay. An attempt was made to measure IL-1β by ELISA but only four samples out of forty had detectable IL-1β. The low detection of certain cytokines in the CSF may imply that CSF concentrations are much lower that what is detected in serum. We plan to optimize ELISA assays with longer incubation (overnight instead of 2 hours) and using more sensitive capture antibodies. None of the TH-2 (IL2, IL4, IL5, IL10 and IL13) or TH-17 cytokines (IL6, IL17A, TNF-α, G-CSF, and TGFβ1) were differentially expressed among the groups suggesting that marijuana shifted immune responses towards TH-1 with elevated IL-1, IL-8, fractalkine, MCP-1 and IP-10 secretion. TH-1 responses are associated with monocyte/macrophage activation, inflammation and tissue injury.

Finally, we used both of the two commercially available assays from the same vendor to conduct the experiments. The results reported here are from the magnetic bead capture assay from Millipore (Miliplex). Of note is that the same samples were run using the covalently linked polystyrene bead capture assay and the amounts of cytokine expression were much lower and did not show statistical differences among the groups. Interpretation of these results is cautioned because the magnetic bead assay concentrations for some cytokines were low and thus may have no biological significance despite the observed statistically significant differences in expression of the cytokines between HIV+MJ users and the other three groups. Recently, in a study that evaluated the relationship between CSF IP-10 and MCP-1 and cerebral metabolic patterns in HIV patients (Letendre et al 2011), the concentrations reported were in picogram range; therefore, the concentrations we obtained are consistent with those found in the CSF of HIV patients by others. We also decided to report the results from the magnetic bead assay only, because this assay detected more cytokines, had a higher sensitivity, had higher bead counts, had a better linear fit of the
standards, and the replicates had lower standard deviations compared to the polystyrene bead assay.

This study evaluated how marijuana smoking might influence in HIV associated neuroinflammatory response in the CSF. On-going studies are examining the effects of genetic factors, such as the Apolipoprotein E (APOE) ε4 allele, in HIV and healthy individuals (Andres et al 2011). Genetic factors may play a role or may have an interactive effect with marijuana use in the neuropathogenesis of HIV.

To conclude, contrary to our hypothesis, MJ smoking appears to elevate the levels of several pro-inflammatory cytokine and chemokines in the CSF of HIV subjects than in seronegative controls. Specifically, inflammatory cytokines including fractalkine, IFN-α2 and IL-1α, chemoattractants such as IP-10, MCP-1 and IL-8, were elevated in HIV+MJ and MJ users than non-MJ user controls. The higher expression of inflammatory cytokines in CSF of HIV+MJ and MJ users suggests that chronic active marijuana smoking may promote neuroinflammation. The correlation of the amounts of inflammatory cytokines/chemokines fractalkine, IFN-α2 and IP-10 in the CSF with the duration of marijuana use suggests that chronic marijuana use may further promote neuroinflammation.
CHAPTER 5
CHARACTERIZATION OF CANNABINOID RECEPTOR EXPRESSION IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF HIV-INFECTED AND HIV-NEGATIVE INDIVIDUALS WITH AND WITHOUT MARIJUANA USE

5.1 Summary
Marijuana (MJ) comprises several cannabinoids, which along with endogenous cannabinoids produced by the body, act through cannabinoid receptors. Cannabinoid receptors (CB) are found in the CNS and in peripheral organs and on immune cells. Others have reported that CB1 receptors are mainly expressed by neurons and microglia in the brain where as CB2 is expressed by immune cells and peripheral organs but mRNA for both CB1 and CB2 receptors is found throughout the body. We measured expression levels of both CB1 and CB2 receptors on peripheral blood mononuclear cells of HIV-infected individuals and HIV-seronegative subjects, with and without marijuana use, by flow cytometry. CB1 receptors were found on monocytes of these subjects but lower levels were observed in MJ users. CB1 receptor expression was also lower on CD4+ cells of HIV-infected subjects (HIV+MJ and HIV) than in HIV-seronegative groups (MJ and SN). In addition, CB1 receptor expression inversely correlated with the duration of marijuana use. Contrary to our hypothesis and the reported mRNA levels of CB2 receptors, expression of CB2 receptors were lower in lymphocytes and monocytes across all groups but the expression was higher in CD8+ T and CD19+ B cells of marijuana users than in SN controls.
5.2 Background

The main psychoactive constituent of marijuana (Δ9-Tetrahydrocannabinol or THC) has pharmacological and immunological effects and these are mediated by cannabinoid receptors (CB). The psychoactivity produced by THC is induced via CB1 receptors, which are widely expressed in the CNS (Matsuda et al 1990), while most of the effects of THC on immune cells are thought to be mediated through CB2 receptors (Kaminski et al 1992; Munro et al 1993). Immune cells have mRNA for both CB1 and CB2, and levels of CB2 expression in monocyte/macrophage are increased in inflammatory conditions (Davis et al 1995; Di Marzo et al 1994). The rank order of CB2 messenger RNA expression on human blood leukocytes is B cells >NK cells>monocytes>neutrophils>CD8 cells >CD4 cells (Bouaboula et al 1993) but the actual protein expression patterns in human cells has not been well characterized.

Cannabinoid receptors are G protein-coupled receptors modulating various signal transduction pathways involved in controlling cell proliferation, differentiation and survival (Howlett et al 2002). Their natural ligands include anandamide (CB1 agonist) and 2-arachydonoylglyceral (2-AG, CB2 agonist) (Devane et al 1992; Mechoulam et al 1995). Dendritic cells, as well as macrophages, generate anandamide and 2-AG in response to inflammatory conditions and express CB receptors and the enzyme responsible for endocannabinoid hydrolysis (Horswill et al 1994; Varga et al 1998), suggesting a physiological role of the endocannabinoid system in immune functions which may have important implications in pathological inflammatory conditions, including brain-immune related disorders such as HIV-associated neurocognitive disorder.

In the CNS, CB1 receptor activation is associated with neuronal protection from excitotoxicity and from reactive oxygen species (ROS) (Marsicano et al 2003; Marsicano et al 2002), whereas CB2 receptor activation has been linked to neuronal survival and protection through anti-inflammatory action (downregulation of IL-1β, TNF-α, reactive oxygen species, prostaglandins and up regulation of IL-4 and IL-10 from microglia and other antigen presenting cells)
(Ehrhart et al 2005; Fernandez-Ruiz et al 2007). CB1 activation by cannabinoids prevents glutamate excitotoxicity through activating cannabinoid receptors that reduce calcium influx (Hampson et al 1998). However, conflicting evidence has shown that activation of the CB1 receptor in cultured C6 glioma cells by THC in the presence of reagents generating reactive oxygen species leads to amplification of cellular damage from oxidative stress (Goncharov et al 2005).

In the periphery, CB2 activation by cannabinoids and endocannabinoids cause immunosuppression by interfering with humoral immunity, cell-mediated immunity and cellular defenses against infectious agents (Sanders et al 1991). Elevated CB2 receptor expression was found on peripheral blood mononuclear leukocytes obtained from marijuana smokers (Nong et al 2002). Because immune suppression by cannabinoids is mediated by CB2 it may be important to measure CB2 expression in HIV-infected individuals and HIV-infected marijuana users. While marijuana usage by HIV-infected individuals may result in changes in CB2 receptor expression that modulate the immune response to infection, no studies have examined the levels of cannabinoid receptor expression in HIV-infected individuals with and without marijuana use. Therefore the aim of this study was to characterize patterns of cannabinoid receptor expression in HIV-infected and HIV-negative individuals, with and without marijuana use. Because of the chronic immune activation and inflammation associated with HIV infection, we hypothesized that CB2 receptor levels would be higher in peripheral blood mononuclear cells of HIV-infected subjects (HIV+MJ and HIV) than in seronegative controls (MJ and SN). Concomitantly, we hypothesized that low levels or no CB1 receptors, thought to be primarily expressed in the CNS, would be detected on the peripheral cells of these subjects.
5.3 Methods
5.3.1 Human Subjects

A subset of fifty-four subjects (93% males, Table 5.1) over 18 years of age were consented, evaluated and blood was drawn by venipuncture. Each subject provided written informed consent approved by the University of Hawai‘i and Queens Medical Center Institutional Review Boards prior to the study and were evaluated by medical personnel to ensure they fulfilled eligibility criteria. Participants were recruited from local communities in Honolulu, Hawai‘i, and were enrolled in a longitudinal observational study of HIV associated brain injury (Chang et al 2008a; Ernst et al 2009). Seronegative (SN) controls included in the study were ≥18 years of age, negative for HIV by blood test (ELISA), had negative urine toxicology results and had no prior history of drug dependency except for nicotine.

HIV+ subjects (HIV) were recruited if they were ≥18 years of age, tested positive for HIV, had nadir CD4 count <500 cells/mL, were stable on combinational antiretroviral therapy (cART) for at least six months or were cART naïve, had no drug dependency except for nicotine and fulfilled other criteria (Chang et al 2008b). Marijuana users (MJ) were recruited if they were ≥18 years of age, used marijuana for at least 3 times per week for at least 2 years and within six months if abstinent, negative for HIV blood test, had negative urine toxicology results and had no previous drug dependency except marijuana and nicotine. HIV-positive marijuana users (HIV+MJ) were included in the study if they were ≥18 years of age, tested positive for HIV, used marijuana for at least 3 times per week for at least 2 years and within six months if abstinent, had negative urine toxicology results and had no prior drug dependency except marijuana and nicotine, had nadir CD4 count <500 cells/mL, were stable on combinational antiretroviral therapy (cART) for at least six months or cART were naïve and fulfilled other criteria (Chang et al 2008b) (See appendix 1 for detailed inclusion and exclusion criteria).
<table>
<thead>
<tr>
<th></th>
<th>HIV+MJ</th>
<th>HIV</th>
<th>MJ</th>
<th>SN</th>
<th>p Value</th>
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</thead>
<tbody>
<tr>
<td>n= 54 (male/female)</td>
<td>15/0</td>
<td>12/0</td>
<td>12/2</td>
<td>12/1</td>
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<td>Age (yrs)</td>
<td>38.4 ± 2.8</td>
<td>46.4 ± 2.3</td>
<td>30.7 ± 2.7</td>
<td>36.7 ± 2.5</td>
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<td>Education (yrs)</td>
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<td>15.6 ± 2.7</td>
<td>13.3 ± 1.3</td>
<td>18.8 ± 0.8</td>
<td>0.006</td>
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<tr>
<td>CES-Depression (0-30)</td>
<td>18.6 ± 3.7</td>
<td>12.9 ± 3.3</td>
<td>11.7 ± 5.7</td>
<td>5.5 ± 1.0</td>
<td>0.24</td>
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<tr>
<td>Estimated VIQ (WTAR)</td>
<td>97.5 ± 3.9</td>
<td>106.8 ± 3.2</td>
<td>106.0 ± 3.0</td>
<td>94 ± 4.0</td>
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<td>CD4 Count (mm$^3$)</td>
<td>543.1 ± 61</td>
<td>565.5 ± 80</td>
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<td></td>
<td>0.823</td>
</tr>
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<td>Nadir CD4 Count (mm$^3$)</td>
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<td>189.1 ± 39</td>
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<td>Plasma Viral Load (Log copies/mL)</td>
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<td>2.1 ± 0.4</td>
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<td>0.674</td>
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<td>Months since HIV Diagnosis</td>
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<td>124 ± 28</td>
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<td>0.091</td>
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<td>HIV Dementia Scale (0-16)</td>
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<td>Karnofsky Score (0-100)</td>
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<td>88.3 ± 2.8</td>
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<td>Life Time MJ use (Kg)</td>
<td>9.3 ± 3.6</td>
<td>1.4 ± 1.3</td>
<td>7.8 ± 3.3</td>
<td>0.001 ± 0.001</td>
<td>0.07</td>
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<td>Duration of MJ use (months)</td>
<td>155 ± 35</td>
<td>53 ± 42</td>
<td>85 ± 23</td>
<td>0 ± 0</td>
<td>0.006</td>
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<tr>
<td>Daily average MJ use (Kg)</td>
<td>1.9 ± 0.5</td>
<td>0.17 ± 0.14</td>
<td>2.0 ± 0.6</td>
<td>0.8 ± 0.8</td>
<td>0.06</td>
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<tr>
<td>Lifetime Nicotine use (g)</td>
<td>187 ± 74</td>
<td>101 ± 61</td>
<td>207 ± 82</td>
<td>4.9 ± 4.9</td>
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</table>
5.3.2 Experimental Design

Whole blood was collected from each participant in sodium Ethylenediaminetetraacetic acid (EDTA) tubes. PBMCs were isolated by Ficoll-paque centrifugation of whole blood. Isolated PBMCs were stained for human cannabinoid receptors and cell surface immune markers: CD4-FITC (eBioscience, clone RPA-T4) or CD14-FITC (eBioscience, clone 61D3), CD8-PE (eBioscience, clone RPA-T8), CD19-PerCP (Biolegend, clone HIB19), CB2-biotine + PE or APC (R and D Systems, Clone 352114) and CB1-APC (R and D Systems, clone 368302). Mouse IgG2A (R and D Systems, clone 20102) was used as the isotype control. The cells were fixed in 4% paraformaldehyde, PBS and stored at 4°C before running on the 2 laser, 4 color detection FACS Calibre flow cytometer (BD Biosciences, San Diego, CA).

Compensation was set using Calibrite beads and 50,000 events were collected. Unstained cells and control mouse IgG2A treated cells were used as negative controls. Analysis was performed using Summit V4.2 gating on lymphocyte and monocytes populations. The mean fluorescent intensity (MFI) and percentage of cells expressing respective CB and immune markers from the 4 subject groups were assessed by two-way ANOVA, with post-hoc analyses. Multiple linear regression analysis was used to examine linear relationships between immune markers, CB receptor expression, HIV duration, lifetime marijuana use (lifetime dose) or other clinical variables. Results were analyzed in SAS Enterprise v 9.1 (SAS Institute, Cary, NC). In all analyses, a p value <0.05 was considered significant.

5.3.3 CB2 Conjugation to Biotin

100µg lyophilized mouse IgG2A monoclonal anti-human cannabinoid receptor 2 (R and D Systems, Clone 352114) was conjugated to biotin by Sulfo-ChromaLink biotin™ technology (SoluLink, San Diego, CA) using the manufacturer specifications. Briefly, 100µg anti-Human CB2 was reconstituted in 100µL modification buffer at pH 7 to obtain a final concentration of 1mg/mL. The concentration was confirmed by UV spectrophotometry. The antibody was
equilibrated and buffer exchanged through a spin column and the concentration was measured by UV scan. The antibody was incubated for 1 hour at room temperature with Sulfo-ChromaLink™ biotin for labeling. The biotinylation reaction was quenched with 10µL 1M Tris (pH 8.7) and a second buffer exchange via spin column was performed to remove excess labeling reagent and unincorporated biotin. Biotin incorporation was confirmed by UV scan. Anti-Human CB2-biotin labeled cells were stained with streptavidin-phycoerythrin (PE) or- Allophycocyanin (APC) for flow cytometry analysis.

5.3.4 CB1 Conjugation to APC

100µg lyophilized mouse IgG2A monoclonal anti-human cannabinoid receptor 1 (R and D Systems, Clone 368302) was conjugated to Allophycocyanin using SoluLink™'s HydraLink bioconjugation technology. Briefly, 100µg IgG2A monoclonal anti-human CB1 receptor was reconstituted in 100µL PBS to make a final concentration of 1mg/mL. The reconstituted antibody was desalted by buffer exchange in 0.5mL 1x modification buffer using a Zeba column. The concentration was confirmed by a UV spectrophotometer. S-HyNic solution was added to the antibody mix and the mixture was incubated at room temperature for 2 hours. Linked antibody was desalted and buffer exchanged again in 0.5mL 1x conjugation buffer on a Zeba column. 2 equivalents of APC (173.3µL) were added to the antibody and the mixture was allowed to conjugate overnight at room temperature in the dark. The two equivalents of APC were enough to saturate all of the antibody and the excess APC stain was removed in washing steps during the staining procedure. The protocol was designed to have excess APC rather than to have unconjugated antibody.
5.3.5 **PBMC isolation and FACS Staining**

Whole blood was obtained from the research subjects via venipuncture in 2x 6mL Potassium EDTA tubes (BD Vacutainer, BD Franklin, Lakes, NJ). Whole blood was diluted 1:1 with 1x phosphate buffered saline (PBS) (Mediatech Inc, Manassas, VA) and mixed. 10mL of blood /PBS mixture was layered on 4mL Ficoll-paque (GE Healthcare, Uppsala, Sweden) and the gradient was centrifuged at 700g for 25 minutes at 4°C with the brake off to isolate PBMCs. Isolated buffy coat PBMCs were washed (300g for 25 minutes at 4°C) twice in 20mL 1x PBS supplemented with 2% fetal bovine serum (FBS) (Mediatech Inc, Manassas, VA). PBMCs were then be resuspended in 100µL PBS in 15ml BD FACS tubes and stained with CD4-FITC (eBioscience, clone RPA-T4) or CD14-FITC (eBioscience, clone 61D3), CD8-PE (eBioscience, clone RPA-T8), CD19-PerCP (Biolegend, clone HIB19), CB2-biotine + PE or APC (R and D Systems, Clone 352114) and CB1-APC (R and D Systems, clone 368302) according to manufacturer specifications.

The cells were incubated at 4°C in the dark for 30 minutes. The cells were then washed twice with up to 4mL PBS, 2% FBS to remove excess antibody stains. As negative control, unstained cells or cells stained with anti-mouse IgG2a isotype were used to set background fluorescent intensity. FACS analysis was done using Summit V4.2 software and results were analyses using SAS Enterprise v 9.1 (SAS Institute, Cary, NC) or Stat View Student t-test and analysis of variance (ANOVA). The results are reported as means and stand error of the mean with a p value <0.05 considered significant. Multiple linear regression analysis was used to examine relationship between cannabinoid receptor expression in different immune cells and marijuana use variables.
5.4 Results

PBMCs were obtained by Ficoll paque centrifugation of whole blood from the study population (Table 5.1) and stained for lymphocyte, monocyte and cannabinoid receptors. The expression of the surface markers was assessed by four color flow cytometry. The subjects were mostly males (93%) with a significant age difference among the groups; ANOVA p=0.007 (Table 5.1). Since some immune markers may vary by age, the expression profiles were co-varied by age. Marijuana users were younger than HIV (p=0.005) and HIV+MJ (p=0.04) whereas HIV subjects were older than HIV+MJ (p=0.05) and SN controls (p=0.02, Figure 5.1).

![Age distribution among study subjects](image)

Figure 5.1 Age distribution among study subjects
Forward and side scatter was used to discriminate between lymphocytes and monocytes. Two gates were drawn, one for lymphocytes and one for monocytes, as shown in Figure 5.2A. Individual populations of CD4, CD8, CD19 and C14 cells are shown in Figures 5.2B, 5.2C and 5.2D. CB1 expression on lymphocyte and monocyte populations was analyzed as shown in Figure 5.2 E, 5.2F, 5.2G and 5.2H. The monocyte population had the highest expression (85%) of CB1 as compared to lymphocytes (CD4+ cells 1%, CD8+ cells 1% and CD19+ cells 5%; Figure 5.2 E, 5.2F, 5.2G and 5.2H). Within the lymphocyte population, CD19+ B cells had a higher expression of CB1 than CD4 and CD8 cells (5% vs. 1% on CD4+ and 1% on CD8+ cells, Figure 5.2E, 5.2F, and 5.2G).

Figure 5.2 Dot plots showing gating strategies used to analyze CB1 expression on lymphocytes and monocytes. A; Forward and side scatter gating on lymphocytes and monocytes. Panel B, CD4 and CD8 cells in the lymphocyte gate, C; CD4 and CD19 cells from the lymphocyte gate. D; CD14+ monocytes staining with CD16. CB1 expression on lymphocytes and monocytes; E,CB1 expression on CD4+ cells , F; CB1 expression on CD8+ cells, G; CB1 expression on CD19+ cells and H, CB1 expression on CD14+ monocytes.
CB2 expression on lymphocytes and monocytes was lower than expected based on expression levels of CB2 mRNA reported in literature (Figures 5.3 A-H). Two CB2 antibody clones were used including a polyclonal and monoclonal anti-human CB2. Because these antibodies may bind to the C-terminal of CB2 which is embedded in the cell membrane or may be intracellular, we planned to permeabilize PBMCs using saponin-containing BD fix/perm solution and then stain with anti-CB2 (Please see Chapter 6).

Figure 5.3 Scatter dot plots showing gating and CB2 staining on different unpermeabilized PBMC populations. A: Forward and side scatter gating on lymphocytes and monocytes. Panel B, CD4 and CD8 cells in the lymphocyte gate, C; CD4 and CD19 cells from the lymphocyte gate. D; CD14+ monocytes staining with CD16. CB2 expression on lymphocytes and monocytes; E, CB2 expression on CD4+ cells, F; CB2 expression on CD8+ cells, G; CB2 expression on CD19+ cells and H, CB2 expression on CD14+ monocytes.
Lymphocyte gates were selected using forward and side scatter and the expression of CD4, CD8 and CD19 was analyzed for the four subject groups. There was a statistically significant difference in CD4 expression across all groups (p<0.0001, Figure 5.4A). HIV-infected subjects (HIV+MJ and HIV) subjects had lower CD4 expression compared to HIV-negative subjects (MJ and SN). There were no significant difference in CD4 expression between HIV-infected marijuana users and HIV-infected-non-marijuana users and between marijuana users and HIV-negative non-marijuana users. As expected CD8 expression was higher in the two HIV-infected groups compared to controls (p<0.0001, Figure 5.4B) and the expression was not different between HIV+MJ users and HIV, and between MJ and SN controls. Both HIV groups had a lower CD4/CD8 ratio (p<0.001) and this was a significant HIV effect (p<0.001) by 2-way ANOVA (Figure 5.4C). There was no significant difference in the CD19 expression observed among all the groups (Figure 5.4D).
CB1 receptor expression was detectable in a small percentage of lymphocytes, with similar levels seen for CD4+ T cells (4%) and B cells (5%) and lower levels in CD8+ T cells (3%). In contrast, a majority of monocytes expressed CB1; between 40 to 80% of monocytes expressed CB1 across all groups (Figure 5.5A).

There was a trend of lower CB1 expression by CD4 cells of HIV-infected subjects (2-way ANOVA $p=0.053$). Posthoc analyses show that HIV subjects had lower CD4+CB1+ than SN subjects ($p=0.043$) and HIV+MJ subjects also had lower CD4+CB1+ than SN subjects ($p=0.034$, Figure 5.5A). CB1 expression on CD4+ cells of HIV subjects, were lower than seronegative subjects, irrespective
of MJ use (Figure 5.5A). However, there was a general trend for persistent HIV infection and MJ usage to contribute independently to a decline in CD4+ CB1 expression over time, although this was statistically significant only for the seronegative MJ group (Figure 5.5B). No significant difference in the CB1 expression among the four groups was observed for CD8+ T-cells (p=0.24, Figure 5.6A) and CD19+ B-cells (p=0.71, Figure 5.6B).

There was no difference in the expression of CB1 in monocytes of HIV-infected subjects with or without MJ use, but CB1 was lower in non-HIV-infected MJ subjects compared to seronegative controls (p=0.0094, Figure 5.7A). There was a significant marijuana and HIV interaction effect in CB1 expression of monocytes (HIV x MJ interaction-p=0.038, Figure 5.7A) suggesting that HIV infection may obliterate the downregulation of CB1 in monocytes. There was also a trend of inverse correlation between expression of CB1 on monocytes and duration of marijuana use in seronegatives (r= -0.29, p=0.16, Figure 5.8).

Figure 5.5 Percentage of CD4 lymphocytes expressing CB1 and correlation with duration of marijuana use in the study population. PBMCs were isolated by Ficoll centrifugation of whole blood and stained with A; CD4-FITC and CB1-APC. B; The percentage of CD4+ cells expressing CB1 was plotted against the duration of marijuana use.
Figure 5.6 Percentage of CD8 and CD19 lymphocytes expressing CB1 in the study population. PBMCs were isolated by Ficoll centrifugation of whole blood and stained with A; CD4-FITC and CB1-APC and B; CD19-PerCP and CB1-APC.

Figure 5.7 CB1 and CB2 staining of CD14+ monocytes. PBMCs were isolated by Ficoll centrifugation of whole blood and stained with CD14-FITC and A; CB1-APC and B; CB2-PE
The detection levels of CB2 on PBMCs from the subjects were much lower than anticipated. The expression patterns of CB2 on CD4+, CD8+ and CD19+ cells was not significant across all groups by ANOVA. No significant differences in the expression of CB2 on CD4+ Th-cells were observed across all groups (p=0.18, Figure 5.9A). HIV-negative non-marijuana users had significantly lower CB2 on CD8+ Tc-cells compared to HIV-negative marijuana users (p=0.028, Figure 5.9B) and there were no significant differences in CB2 expression on CD8+ cells in the HIV+ groups by posthoc analysis. In the CD19+ B-cells, there were no group differences in the expression of CB2. However there was a trend for higher CB2 expression in the CD19+ cells of HIV-negative marijuana users compared to HIV-negative non-marijuana user (p=0.085 Figure 5.9C) by posthoc analysis.
Figure 5.9 CB2 expression on CD8+ and CD19+ cells. PBMCs were isolated by Ficoll centrifugation of whole blood and stained with A; CD8-PE and CB2-biotin-APC and B; CD19-PerCp and CB2-biotin-APC.
5.5 Discussion and Conclusions

In order to characterize the peripheral blood immune phenotypes of our study population, we initially evaluated standard lymphocyte and monocyte lineage markers. We found lower levels of CD4 T cells and higher CD8 T cells in HIV-infected subjects, resulting in lower CD4/CD8 ratios. The levels of CD19+ B cells and CD14+ monocytes were not different among the four study groups. This profile is consistent with well-documented clinical features of HIV patients who typically have lower CD4 and higher CD8 counts compared to healthy individuals. However, our findings also show that HIV+MJ users had the lowest CD4/CD8 ratios, indicating a greater degree of CD4 T cell depletion than HIV+MJ- subjects.

Because CB receptor expression can be influenced by the cell activation status (Derocq 2000), we compared CB1 and CB2 receptor expression on lymphocytes and monocytes from HIV-Infected and HIV-negative subjects, with and without marijuana use. We detected both CB1 and CB2 receptors on leukocytes; however the cellular distribution and level of expression of the two CB receptors were distinct. The most striking finding of this study was the high percentage of monocytes expressing CB1 receptors in all study groups. Lower percentages of CB1 positive cells were seen for CD4+ T cells, CD8+ T cells, and B cells. Prior studies reported that CB1 receptors are primarily expressed on neurons and microglia in the CNS (Matsuda et al 1990), while CB2 receptors are expressed in the periphery and on immune cells and other organs (Munro, Thomas et al. 1993). It has also been reported that while leukocytes possesses both CB1 and CB2 messenger RNA, they mainly express CB2 receptors which are associated with the immunomodulatory activities of cannabinoids (Kaminski et al 1992). Because immune cells have messenger RNA for CB1 receptors, these receptors could be expressed during altered physiological conditions.

Differences in CB1 receptor expression were observed in study groups distinguished by HIV status and MJ usage. Percentages of CB1 receptor positive cells were lower in CD4+ cells of HIV+ subjects regardless of marijuana use, suggesting that HIV infection may down-regulate CB1 receptor expression.
in these cells. There were no differences in CB1 receptor expression of CD8+ cells and CD19+ cells between groups. Marijuana usage also influenced CB1 receptor expression. The CB1+ monocyte population was significantly reduced in MJ users as compared to HIV-MJ- and HIV+MJ- subjects. We also observed a decline in CB1 positive CD4+ cells and B cells over time in seronegative marijuana users. Interestingly, this reduction was not observed for HIV+ MJ users, suggesting that there was a significant HIV and marijuana interaction in CB1 receptor expression. However, the lower CB1 levels in lymphocytes may be due to the already lower CD4+ population in the HIV groups.

CB1 receptor activation in the brain has been linked to neuronal protection from excitotoxicity through inhibition of and from reactive oxygen species (ROS) (Marsicano et al 2003; Marsicano et al 2002). However, activation of the CB1 receptor in cultured C6 glioma cells by THC lead to increase in oxidative stress and cell death (Goncharov et al 2005) suggesting that CB1 receptor activation in the periphery may promote inflammation and oxidative stress. The observed down regulation of CB1 receptor in marijuana users in the current study may be indicative of a cellular response to counter over-stimulation of CB1 by its agonists and the associated oxidative stress.

CB2 receptor expression levels of CD4+ cells were similar among all groups while CB2 receptor expression of CD8+ cells was higher in HIV-negative marijuana users compared to HIV-negative controls. The presence of CB2 receptor expression on leukocytes is consistent with prior reports although our detected levels were lower. Other studies showed that the levels of mRNA expression were higher in monocytes than B cells, an observation not confirmed at the protein level in our study and also contrary to a prior report (Bouaboula et al 1993), suggesting that not all CB2 mRNA detected in leukocytes is translated into protein. There are some remaining technical issues that need to be resolved to obtain optimal detection of CB receptor positive cells, particularly cells expressing the CB2 receptor. Additional studies needed include testing of different anti-CB2 monoclonal antibodies and performing whole cell staining by permeabilizing the cells (Harui A et al 2010 personal communication). CB
receptors are G protein-coupled transmembrane receptors which span the membrane seven times. Membrane-spanning domains may be difficult to stain since antibody-binding sites may be embedded in the membrane. Whole cell staining may provide better quantification of CB2 expression on leukocytes.

In conclusion, differential expression of cannabinoid receptors was observed among the four study groups, with HIV+ groups expressing notably lower amounts of CB1 receptors on CD4+ cells. Monocytes appeared to have the highest percentage of CB1 receptor positive cells although levels were lower in marijuana users than the other three groups. CB1 receptor expression by CD4+ cells and B cells correlated negatively with the duration of marijuana use. A significant interaction between HIV and marijuana usage in monocytes was observed in which dampening of CB1 receptor expression by marijuana usage appeared to be counterbalanced by increased CB1 receptor expression on monocytes (CD14+CD16+) activated by HIV infection. Overall levels of CB2 receptor positive cells were lower than anticipated. The monocyte population expressed CB2 receptors more than the lymphocyte population. CB2 receptor expression was slightly increased by marijuana use and may be an indication of activation or inflammatory processes since monocytes have elevated CB2 expression in inflammatory conditions (Davis et al 1995; Di Marzo et al 1994).
6.1 **Summary**

Cannabinoid receptor (CB) 2 expression on different peripheral immune cells has not been well characterized due to technical challenges and unavailability of fluorochrome-conjugated antibodies for CB2. Using a monoclonal anti-human CB2 antibody, we have developed and optimized a flow cytometry assay to stain peripheral blood mononuclear cells (PBMCs). PBMCs were permeabilized to target extracellular, transmembrane and intracellular regions of the CB2 receptor. The antibody stained the CB2 receptors of permeabilized PBMCs more than unpermeabilized PBMCs. Of all the cell populations in PBMCs, monocytes had the highest percentage of CB2 receptor positive cells, with only a minority of T cells and B cells expressing CB2 receptor.
6.2 Background

The identification of $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) as the main psychoactive compound in *Cannabis sativa* and the identification of receptors for the endogenous cannabinoid system have led to more research of the physiological roles of cannabinoids. Two cannabinoid receptors have been identified. Cannabinoid receptor (CB) 1 receptor (Matsuda et al 1990) is mainly found on neurons and microglia in the CNS and CB2 is expressed in peripheral organs and on immune cells (Munro et al 1993). White blood cells have both CB1 and CB2 receptors, but it is CB2 receptor that possesses most of the immunomodulatory activities of cannabinoids (Kaminski et al 1992).

Levels of CB2 receptor expression in cells of the myeloid lineage are affected by cell activation and increase during inflammation (Davis et al 1995; Di Marzo et al 1994). Cannabinoid receptor expression in different immune cell populations is not well characterized. Gene expression studies have evaluated cannabinoid receptor rRNA levels or total protein levels by immunoblot (Galiegue et al 1995; Klein et al 1998), but there are no studies that have assessed cannabinoid receptor expression on immune cell subpopulations. The major challenge has been to develop fluorescent-labeled antibodies against cannabinoid receptors that provide robust staining of cells analyzed by flow cytometry. Currently there are few commercially available anti-CB2 antibodies conjugated to fluorochromes.

We have developed a flow cytometry assay using a biotinylated monoclonal anti-human CB2 antibody detected with streptavidin phycoerythrin (PE) or Allophycocyanin (APC), and optimized it by staining permeabilized peripheral blood mononuclear cells (PBMCs) (Harui A et al 2010 personal communication). This assay will be useful in characterizing cannabinoid receptor expression in different immune cell populations from healthy subjects, marijuana users and HIV-infected individuals.
6.3 Methods

6.3.1 CB2 Conjugation

100μg lyophilized mouse IgG2A monoclonal anti-human cannabinoid receptor 2 (R and D Systems, Clone 352114) was conjugated to biotin using Sulfo-ChromaLink biotin™ technology (SoluLink, San Diego, CA) according to the manufacturer specifications. Briefly, 100μg anti-Human CB2 was reconstituted in 100μL modification buffer at pH 7 to obtain a final concentration of 1mg/mL as confirmed by a UV spectrophotometer. The antibody was equilibrated and the buffer exchanged by passing the reconstituted antibody through a resin filled spin column. The buffer exchanged antibody concentration was measured by UV scan. The antibody was incubated with Sulfo-ChromaLink™ biotin for labeling and was incubated for 1 hour at room temperature. The biotinylation reaction was quenched with 10μL 1M Tris (pH 8.7) and a second buffer exchange via spin column was performed to remove excess labeling reagent and unincorporated biotin. Biotin incorporation was confirmed by UV scan. Anti-Human CB2-biotin labeled cells were stained with streptavidin-PE or-APC for flow cytometry.

6.3.2 PBMC isolation and FACS Staining

Following informed consent whole blood was obtained from healthy research subjects by venipuncture in 6mL Potassium EDTA tubes (BD Vacutainer, BD Franklin, Lakes, NJ). Whole blood was diluted 1:1 with 1x phosphate buffered saline (PBS) (Mediatech Inc, Manassas, VA) and mixed. The blood/PBS mixture was layered on a Ficoll-paque (GE Healthcare, Uppsala, Sweden) gradient and centrifuged at 700g for 25 minutes with the brake off at 4°C to isolate PBMCs. Isolated buffy coat PBMCs were washed (300g for 25 minutes at 4°C) twice in 20mL 1x PBS supplemented with 2% fetal bovine serum (FBS) (Mediatech Inc, Manassas, VA). PBMCs were then resuspended in 100μL PBS in 15ml BD FACS tubes respectively and stained with CD4-FITC (eBioscience, clone RPA-T4), CD14-FITC (eBioscience, clone 61D3), CD8-PE (eBioscience, clone RPA-T8),
CD19-PerCP (Biolegend, clone HIB19), and CD16-PerCp (Biolegend, clone 3G8) according to manufacturer specifications. The cells were incubated at 4°C in the dark for 30 minutes.

Cells were then washed twice with up to 4mL PBS, 2% FBS to remove excess antibody stains. Washed cells were then permeabilized with 250µL saponin containing BD cytofix/cytoperm solution (BD Biosciences, San Diego, CA) and incubated for 20 minutes at 4°C in the dark. Cells were washed twice with 4mL 1x BD perm/wash buffer. Cells were again resuspended in 100µL 1x BD perm/wash buffer and stained with biotinylated CB2 (CB2-biotin) (R and D Systems, Clone 352114) or biotinylated anti-mouse IgG2A isotype (R and D Systems, clone 20102) as control according to the manufacturer specifications and incubated for 30 minutes at 4°C in the dark. Cells were washed twice with 4mL saponin containing 1x BD perm/wash buffer and resuspended in 100µL BD perm/wash buffer. The cells were stained with streptavidin APC or PE (R and D Systems) for 30 minutes at 4°C in the dark according to manufacturer specifications. Cells were washed twice with 4mL 1x BD perm/wash buffer and resuspended in 500µL BD perm/wash buffer. Cells were stored at 4°C in the dark and run on BD FACSCalibur (BD Biosciences, San Diego, CA) on the same day. For comparison, unstained cells and unpermeabilized stained with the following antibodies: CD4-FITC, CD14-FITC, CD8-PE, CD19-PerCP, CD16-PerCP, and CB2-biotin (PE or APC) and fixed with 500uL 4% paraformaldehyde were run on the FACSCalibur. Three experiments were set up in triplicate and 50,000 events were collected gating on lymphocyte and monocyte gates. FACS analysis was done using Summit V4.2 software and statistics was done by StartView student t-test comparing between permeabilized vs. unpermeabilized cells using unstained cells and isotype staining as background.
6.4 Results

Forward and side scatter was used to discriminate lymphocytes from monocytes (Figure 6.1A). A lymphocyte gate was drawn to analyze CD4, CD8 and CD19 cells (Figure 6.1B and C) and a monocyte gate to analyze CD14 cells (Figure 6.1D), and CB2 expression on CD4, CD8, CD19 and CD14 cells was assessed (Figure 6.1E-H). CB2 staining was significantly higher in permeabilized cells (Figure 6.2, E-H) compared to unpermeabilized cells (Figure 6.1, E-H). Similar results were obtained from three healthy donors in the three experiments performed in triplicate. Comparing populations of PBMCs permeabilized vs. unpermeabilized PBMCs, permeabilized monocytes stained the most compared to T cells and B cells (82% vs. 2%, p<0.0001, Figure 6.3B). Figure 6.3A presents a representative histogram in which the fluorescence intensity of permeabilized monocytes is shifted to the right relative to monocytes treated with the isotype control antibody or unpermeabilized anti-CB-2 stained monocytes (Figure 6.3A).

Permeabilization of lymphocytes after permeabilization did not result in as dramatic an increase in CB2 staining as monocytes. There was no statistical difference in the CB2 detection between permeabilized and unpermeabilized CD4+ T lymphocytes (ANOVA p=0.72, Figure 6.4) and CD19 B+ cells (ANOVA p=0.27, Figure 6.4). However after post-hoc analysis, permeabilized CD8+ T lymphocytes had a significant increase in CB2 detection over unpermeabilized CD8+ T cells (2.0% Vs. 0.40%, ANOVA p=0.028, Figure 6.4).
Figure 6.1 Scatter dot plots of CB2 expression on different unpermeabilized PBMC populations. A; Forward and side scatter was used to discriminate between lymphocytes and monocytes, B; CD4+ and CD8+ cells, C; CD4+ and CD19+, D; CD14+ and CD16+ cells. CB2 staining was analyzed in the E; CD4+ cells, F; CD8+ cells, G; CD19+ cells, and H; CD14+ cells. The mean fluorescent intensity was subtracted from unstained background mean fluorescence.
Figure 6.2 Scatter dot plots of CB2 expression on different permeabilized PBMC populations. A; Forward and side scatter was used to discriminate between lymphocytes and monocytes, B; CD4+ and CD8+ cells, C; CD4+ and CD19+, D; CD14+ and CD16+ cells. CB2 staining was analyzed in the E; CD4+ cells, F; CD8+ cells, G; CD19+ cells, and H; CD14+ cells. The mean fluorescent intensity was subtracted from unstained background mean fluorescence.
Figure 6.3 CB2 Expression on monocytes. A; Histograms representing CB2 staining in the CD14+ monocyte gate were overlaid to compare staining profiles between unstained permeabilized PBMC (Shaded red), isotype staining (open green), stained unpermeabilized PBMC (shaded blue), and permeabilized stained PBMC (shaded black). B; Graph representing the percentage of permeabilized vs. unpermeabilized CD14+ monocytes staining for CB2.
Figure 6.4 Expression of CB2 on PBMC populations. Permeabilized (perm) and unpermeabilized (unperm) PBMCs from healthy controls were stained with anti-CB2. Expression of CB2 was examined in permeabilized versus unpermeabilized CD4+, CD8+ and CD19+ cells.
6.5 Discussion and Conclusion

Flow cytometry is a powerful tool for studying receptor expression by cell populations. In pilot studies, we had attempted to evaluate the expression of CB1 and CB2 receptors by peripheral blood mononuclear cells using flow cytometry but were unable to detect significant levels of CB2 cell surface expression. Using biotinylated IgG2A monoclonal anti-human CB-2 Clone 352114, we have developed a flow cytometry assay using permeabilized PBMC to detect CB receptor positive cell populations in immune cells. Our approach used either streptavidin-PE or streptavidin-APC for fluorescent staining. CB2-specific antibody stained permeabilized cells more effectively than unpermeabilized cells. Permeabilization enables the detection of both intracellular and surface membrane-associated antigen. In addition, it may also expose antigenic domains of the CB2 protein which are not accessible using surface staining techniques. This assay can also be used to study intracellular trafficking of CB2 from vesicles to the cell membrane. PBMCs treated with mouse IgG2A isotype control did not bind to the streptavidin-PE or streptavidin-APC above background levels, validating the specificity of the assay.

Of all the cell populations examined, monocytes had the highest density of CB2 receptors in permeabilized PBMCs. Gene expression studies have ranked the order of CB2 RNA expression of human blood leukocytes as B cells > NK cells > monocytes > neutrophils > CD8 cells > CD4 cells (Bouaboula et al 1993). The differences in CB2 receptor expression observed in those gene expression studies and our flow cytometry experiments suggest that CB2 expression of immune cells may be post-transcriptionally regulated. It is also interesting to note that in parallel studies we have found that expression of the CB1 receptor is highest in monocytes as compared to T and B lymphocytes. Thus, monocytes may be the primary target of immune modulation by CB receptor agonists.
This assay will help in characterizing CB2 receptor expression in different immune cells of healthy individuals or individuals undergoing infectious or immunopathological processes. For example, we are currently measuring CB2 expression on PBMCs from HIV-infected subjects and marijuana users, and seronegative controls to determine whether CB2 expression is altered in HIV-infection or in marijuana users. Because cannabinoids exert immunosuppressive effects through CB2, elevation of CB2 expression in HIV positive marijuana users may further weaken their immune system and predispose them to opportunistic infections and neurocognitive impairment.
CHAPTER 7

7.1 CONCLUSIONS AND FUTURE DIRECTION

HIV-associated brain injury is characterized by neuronal apoptosis or dysfunction, glial activation and inflammation in the brain as a result of the direct effects of HIV or the indirect effects by neurotoxic or inflammatory substances released by HIV-infected cells. Oxidative stress due to inflammation may also lead to glial dysfunction. In addition, illicit substance dependency including chronic active marijuana abuse may contribute to inflammation as shown in the work presented in this dissertation. Persistent immune activation, inflammation and resultant oxidative stress may be caused by marijuana and may further exacerbate neuronal injury in HIV-infected patients leading to cognitive dysfunction. We therefore implicate marijuana in playing a key role in driving inflammation in HIV-marijuana smokers.

The major findings of this study may be summarized as follows: (1) Activated monocytes were elevated in HIV+MJ and MJ users and HIV co-receptor for macrophage strains, CCR5, was upregulated in MJ users. (2) The activated monocyte phenotype (CD14+CD16+) and CCR5 expression were correlated with duration of marijuana use, amounts of average daily marijuana use and lifetime total amount of marijuana smoked. (3) Activated monocytes were found to have high CD16, had higher CCR5 than non-activated monocytes and were more readily infected with a macrophage tropic (R5) HIV strain (BAL). (4) Several inflammatory cytokines (IL-1α, IL-8, fractalkine, IFN-α2, IP-10 and MCP-1) were found in higher amounts in HIV+MJ users than HIV, MJ and SN controls. (5) Elevated inflammatory cytokines in HIV+MJ were correlated with the duration of marijuana use and the amount of activated monocytes. (6) CB1 receptors were found on a majority of monocytes and a smaller number of lymphocytes. CB1 receptors are mainly expressed on neurons and microglia in the brain. CB1 levels were lower on CD4+ cells of HIV-infected subjects (HIV+MJ and HIV) than on CD4+ cells of HIV seronegative groups (MJ and SN). CB1 levels in monocytes were similar across all groups but tended to be lower in MJ users and there was an inverse correlation between expression of CB1 and the
duration of marijuana use suggesting that marijuana may down regulate CB1 on monocytes. However, this trend was only seen in seronegative groups (MJ and SN). CB1 expression was not significantly different in CD8+ and CD19 cells of the subjects. (7) CB2 receptor was detected after permeabilization of PBMCs. Monocytes had highest density of CB2 as well as a high percentage of CB2 receptor positive cells as compared to lymphocytes (CD4, CD8 and CD19 cells). There were no significant differences in CB2 expression among lymphocyte populations.

In conclusion, contrary to our hypothesis that marijuana is immunosuppressive and would lower cell activation, marijuana smoking in our study was associated with increased monocyte activation and elevated CCR5 in seronegative marijuana users. CCR5 is the HIV chemokine co-receptor for macrophage tropic strains. The HIV strains recovered from HAD patients that have been associated with neuronal injury are mainly macrophage tropic (Cheng-Mayer et al 1989). Chronic immune activation is implicated in driving the pathogenesis of HIV and (Kaul et al 2001) we found that marijuana use caused immune activation. Therefore, we conclude that chronic marijuana use by HIV-infected patients leads to further immune activation and worsen HIV pathology. Immune activation particularly monocyte activation has been associated with enhanced HIV susceptibility in vivo (Crowe 2007). HIV-infected activated monocytes have increased trafficking into the CNS and have been found to cross into perivascular brain regions (Fischer-Smith et al 2001) thus transporting HIV. Activated monocytes have also been found in HAD patients and harbor HIV proviral DNA and serve as reservoirs of HIV infection (Pulliam et al 1997; Shiramizu et al 2005; Shiramizu et al 2007; Shiramizu et al 2009).

Further, we found higher expression of CCR5 on seronegative marijuana users than controls. Elevated CCR5 in seronegative marijuana users would make them more susceptible to HIV infection when exposed. We also found higher amounts of inflammatory cytokines were found in the CSF of HIV+MJ users than in the other groups. Inflammatory cytokines produced by chronically infected immune cells particularly monocytes (Thieblemont et al 1995) in the brain may
influence neuropathogenesis of HIV (Griffin 1997). Therefore, we suggest that marijuana smoking may exacerbate HAND. Having observed immune activation and enhanced cytokine secretion in HIV+MJ and MJ users questions about the mechanism of this activation arise. One possible mechanism may be systemic immune suppression by marijuana and the resultant disruption of mucosal immunity. Reduced gut immunity causes translocation of microbes and microbial products including LPS into systemic circulation (Brenchley et al 2006). Elevated LPS levels have been reported in the plasma of HIV infected individuals and this is thought to be the likely cause of systemic chronic immune activation (Ancuta et al 2008). Since we have banked serum samples from these subject, we plan to measure plasma LPS and correlate the amounts with activation phenotypes to establish whether there is a relationship between marijuana use, plasma LPS concentration and immune activation.

Several questions remain unanswered by this dissertation. For examples; (1) How does marijuana activate immune cells when the main psychoactive component of marijuana, THC, is an immune suppressor? What are the mechanisms and pathways of immune activation and enhanced inflammatory cytokine secretion by marijuana? (2) Are these mechanisms mediated by cannabinoid receptors or other immune cell receptors? What cellular signaling pathways do compounds in marijuana use to induce proinflammatory cytokine expression? (3) What component of marijuana activates immune cells? (4) Is the downregulation of CB1 seen in MJ users a compensatory effect to avoid constant stimulation of cells by marijuana? (5) Finally, because transcription of genes that code for proinflammatory cytokines is tightly regulated, it may be important to study whether marijuana has epigenetic effects (e.g. DNA methylation) on transcription of cellular activation markers and inflammatory cytokines or if marijuana activates cellular pathways that induce transcription of genes coding for inflammatory cytokines like the NF-κβ pathway.
These legitimate questions could be answered in-part by *in-vitro* studies therefore the one future direction of this project is to explorer mechanisms of marijuana induced cell activation and enhanced inflammatory cytokine secretion. Because marijuana contains several cannabinoids and alkaloids, some of these purified compounds will be tested *in vitro* to determine which constituents may activate immune cells. It is also important to study the direct effects of marijuana smoking in controls and HIV infected individuals. Numerous methods have been developed in our laboratory to address neurological effects including neuropsychological tests as well as *in-vivo* imaging techniques (Principal investigators; Dr Linda Chang and Dr. Thomas Ernst).

Proton magnetic resonance spectroscopy (\(^1\)H MRS) has shown significant metabolic abnormalities that reflect neuronal or glial abnormalities in the frontal white matter, basal ganglia and parietal cortex of HIV patients with cognitive dysfunction compared to controls (Chang et al 2004b), as well as in HIV subjects with or without chronic marijuana use (Chang et al 2006). Serum glutathione, a major antioxidant, has been shown to be decreased in patients with HIV (Gil et al 2003); however, it is not known if glutathione levels are also altered in the brains of HIV patients and marijuana users.

Advanced \(^1\)H MRS techniques can measure brain glutamate (using TE-average PRESS) and glutathione (using MEGA-PRESS or BASING) levels in various brain regions. Brain injury from inflammation and or oxidative stress also may lead to cognitive deficits which can be assessed using detailed neuropsychological tests. Furthermore, inflammation and oxidative stress in the brain may cause changes in microstructure and water movement (diffusion) in the brain. Diffusion tensor imaging (DTI) may be used to evaluate diffusion changes in HIV and marijuana users. All of these imaging data are being collected from ongoing studies led by Dr. Linda Chang. Therefore, one possible future direction of this study is to correlate immune markers with imaging data from HIV-infected subjects and marijuana users.

Another future direction of this project is to examine the possible effects of monocyte activation, both in the periphery and CNS, on brain dysfunction.
Recent studies indicate that plasma viral loads no longer provide a good predictor of cognitive impairment or brain abnormalities in neuroimaging studies in HIV patients who are treated with cART (Price et al 1999). However, because activated monocytes are more susceptible to HIV infection and may harbor HIV both in vitro and in-vivo (Crowe 2007), we would like to measure HIV reservoirs (HIV DNA) in the monocytes from peripheral blood of HIV-infected subjects and HIV-infected marijuana users. We hypothesized that HIV+marijuana users having more CD16+ cells would have more HIV DNA than HIV subjects without MJ use. We further would like to measure HIV DNA levels in CSF cells of the same subjects. If higher amounts of HIV DNA are found in the CSF cells of HIV+ marijuana users, this could explain the observed trends of poor cognitive performance and altered brain metabolites in HIV+ marijuana users compared to HIV+ non-marijuana users (see below). HIV infection in monocytes tends to be latent (as HIV DNA) and because monocytes are long lived or differentiated into macrophages/microglia, they may serve as important reservoirs of HIV. An earlier study indeed found that HIV-1 proviral DNA copy number in activated mononuclear cells correlated with HAND (Shiramizu et al 2005), and the severity of HAND is proportional to the amount of circulating proviral HIV DNA in activated monocytes (Shiramizu et al 2007; Shiramizu et al 2009); therefore, HIV DNA may play a key role in the neuropathogenesis of HIV, leading to ongoing re-infection of the brain, both in the periphery and CNS.

This study will also explore the impact of inflammation in HIV-infected subjects and marijuana users on brain metabolites and cognitive function. We conducted a pilot study using 20 HIV seropositive (HIV+) cognitively normal subjects, who were current or recent marijuana users (within last 6 months; HIV+MJ, n=7), or abstinent (last use >1 year) or non-users (HIV+, n=13) (Table 7.1). Each subject underwent neuropsychological testing that involves 7 cognitive domains (Table 7.2) and localized brain $^1$H MR Spectroscopy. Localized $^1$H MRS was performed on a 3 Tesla Siemens Trio MR scanner in four brain regions: medial frontal gray matter, right frontal white matter, medial parietal grey matter and right basal ganglia (Figure 7.2 B) using a Point...
RESolved Spectroscopy (PRESS) acquisition sequence (TR/TE=3000/30ms, 64 averages, 3.5 min per location). LCMModel analysis in conjunction with additional water, T2 measurements allowed for determination of metabolite concentrations.

All the subjects underwent detailed neurocognitive assessment for Fluency: Controlled Oral Word Test (FAS), Design Fluency Test (Ruff Figure Fluency); Executive Function: Stroop Interference, Trail Making Test Part B Learning: Rey Auditory Verbal Learning (Trial 1-5), Rey-Osterreith Complex Figure Test Speed of Information Processing: Symbol Digit Modalities Test, Trail Making Test-Part A, Stroop Color Naming, CalCAP Simple RT; Memory: Rey Auditory Verbal Learning Test Delayed Recall (Trial 7), Rey Complex Figure Delayed Recall Motor Skills: Grooved Pegboard Test-Dominant hand, Grooved Pegboard Test-Non dominant hand Attention/Working Memory: WAIS-III Digit Span, WAIS-III Letter-Number Sequencing, WAIS-III Arithmetic, Paced Auditory Serial Addition Test (PASAT). z-scores for all domains will be calculated by adjusting a subject's score with mean scores of age-and-education matched controls in our normative database.

<table>
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<tr>
<th>Table 7.1 Subjects characteristics (mean ± SEM)</th>
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<tr>
<td>HIV+MJ</td>
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<td>--------</td>
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<tr>
<td>n= 20 (m/f)</td>
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<tr>
<td>Age (yrs)</td>
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<td>Education (yrs)</td>
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<td>ARV Meds (+/-)</td>
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<td>ARV duration (mo)</td>
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<td>Log viral load</td>
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<td>Lifetime MJ joints</td>
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HIV+MJ had significantly lower z-scores in the cognitive domains of learning (-0.13±0.25 vs. 0.47±0.15, p=0.040), memory (-0.148±0.27 vs. 0.56±0.16, p=0.050) and motor function (-0.148±0.27 vs. 0.46±0.16, p=0.023) (Figure 7.1). In addition, consistent with prior findings, compared to HIV subjects without MJ use, HIV+MJ subjects also had lower glutamate in the basal ganglia (5.46±0.41 vs. 6.34±0.22 mM, p=0.05) but higher glutamate+glutamine in the frontal white matter (6.97±0.322 vs. 6.12±0.21 mM, p=0.03) on MR spectroscopy (Figure 7.2).

![Figure 7.1 Neuropsychological Test Performance in the Two HIV Subject Groups](image-url)
Figure 7.2 Brain glutamate levels in basal ganglia and frontal white matter as measured by $^1$H MR Spectroscopy. A. Brain glutamate and glutamine were measured by proton MR Spectroscopy in for brain regions. B. Locations of the four brain regions where glutamate was measured.

These findings suggest that chronic active marijuana use may lead to poorer cognitive performance (learning, memory and motor function) in HIV-infected marijuana users. We plan to measure brain metabolites using a larger sample population and also to include HIV sero-negatives and HIV sero-negative marijuana users to delineate the effect of marijuana on brain metabolites and cognition among the four groups.
Finally the alterations in brain metabolites (glutamate in the basal ganglia and glutamate+glutamine in the frontal white matter) suggest that marijuana can alter glial (astroglial or microglial) activities, which in turn may affect neuronal function. Further studies are warranted to study the effects of marijuana of cognitive function and on brain metabolites using larger population sizes. The neurocognitive data and $^1$H MR Spectroscopy data demonstrate that our laboratory can perform such studies with the hypothesis that brain glutamate (neuronal marker) and glutathione (antioxidant) will be lower in HIV+marijuana users compared to controls and HIV+MJ will have more cognitive deficits followed by MJ compared to their respective controls.
Marijuana users were recruited from local HIV support organizations and drug treatment programs including Hina Mauka, Gregory House, Life Foundation, Salvation Army ETS, and through advertisements from Institutional Review Board-approved flyers and palm cards. HIV-infected subjects were also recruited from local hospitals and community support centers. Subjects already enrolled in our ongoing studies that meet eligibility criteria and provided consent were co-enrolled in this study. Approval from the University of Hawai‘i institutional review board was obtained for this study to co-enroll from the following already approved studies: 'Brain Activation in Patients with Early HIV Dementia' UH CHS # 12833, PI: Dr. Linda Chang; 'Brain Glutamate and Glutathione as Oxidative Stress Markers in Patients with HIV and Methamphetamine Abuse' UH CHS # 14740 PI: Dr. Linda Chang; and 'Effects of Dopamine Agonists on Brain Activation in HIV Patients and Nicotine Smokers' UH CHS # 17692, PI: Dr. Linda Chang and 'Investigating Brain Function in Active Cannabis Users', PI Dr. George King. Because these studies involved collection, processing and measuring immune markers and cytokines in peripheral blood and CSF of potentially HIV-infected patients, a full biosafety protocol also was submitted and was approved by the University of Hawai‘i Biosafety Committee; protocol number:11-08-511-01, Principal Investigator: Dr. Linda Chang.

Subjects fulfilling study criteria underwent baseline evaluation which included a medical examination, baseline blood tests, electrocardiogram, urine toxicology tests and lumbar puncture. Detailed consent forms and inclusion and exclusion criteria are provided in the appendix. Protection of human subjects, privacy and confidentiality were all done in accordance to all applicable local, state and federal laws. Subjects were recruited from all racial/ethnic groups, including Native Hawai‘ians and Pacific Islanders, as well as women. The study enrolled healthy or HIV-infected men and women of any race and ethnicity older than 18 years of age.
7.2.1 Risks

The protocol was designed to minimize potential risks and we used standard protocols including medical staff available in an event for an adverse reaction. The risks to participants were reasonable in relation to the compensation and anticipated benefit to the contribution of valuable knowledge to how drug abuse can affect HIV. The risks from the examination and medical interview were very small. Rarely, these interviews caused anxiety or concern and because some personal questions were asked, some subjects might have felt embarrassed. There was very little risk from the blood draw (venipuncture) since disposable sterile needles were used. There was however a risk of bruising, soreness, or bleeding where the needle entered the arm. Only one subject had light headedness and no one fainted. The risk of infection at the area of blood draw was minimal since only sterile one-time equipment was used. Lumbar punctures also present the possibility of bruising, soreness, infection or bleeding, and rarely, feeling light-headed or fainting. In addition, back pain may occur at or near the site where the needle entered the back since bruising, or rarely a larger hematoma, may occur around that location. Another risk of a lumbar puncture is a postural headache after the procedure, which can last up to a few days; prolonged headaches (lasting a few days) occurred in less than 5% of cases in our experience. More information regarding the lumbar punctures was made available to the subjects and this procedure was voluntary. The subjects were advised to minimize vigorous activities after the lumbar puncture. All information in this study was kept confidential, including the results from the urine drug screen.
7.2.2 Benefits

There were no direct benefits for participating in this study. The subjects, however, were compensated for their time and they were contributing valuable information to the understanding of how HIV and marijuana use might affect the immune system. If abnormal vital signs and or blood/CSF test results were observed, the subjects were informed and referred to their primary care physicians. Because this study was an add-on study to other adult clinical studies in the research program, no children were recruited.
Appendix A

Eligibility Criteria:

(i) HIV-infected marijuana users were enrolled if they fulfilled the following inclusion criteria:
   a. HIV seropositive male or female of any ethnicity, over 18 years old
   b. Nadir CD4 ≤500/mm³
   c. Stable on HIV antiretroviral therapy for more than 6 months or was HAART naïve
   d. History of marijuana use defined as at least 3 times a week for at least 2 years and if abstinent within 6 months of last marijuana use.
   e. Negative urine toxicology screen for other drugs of abuse (cocaine, methamphetamine and opiates)
   f. Willingness to participate in the study and give informed consent

(ii) Marijuana users were enrolled if they fulfill the following inclusion criteria
   a. HIV seronegative male or female of any ethnicity, over 18 years old
   b. History of marijuana use defined as at least 3 times a week for at least 2 years and if abstinent within 6 months of last marijuana use.
   c. Negative urine toxicology screen for other drugs of abuse (cocaine, methamphetamine and opiates)
   d. Willingness to participate in the study and give informed consent

(iii) HIV-infected subjects were enrolled if they fulfill the following inclusion criteria
   a. HIV seropositive male or female of any ethnicity, over 18 years old
   b. Nadir CD4 ≤500/mm³
   c. Stable on HIV antiretroviral therapy for more than 6 months or was HAART naïve
   d. No history of marijuana dependency or any other drug dependency with the exception of nicotine
e. Negative urine toxicology screen for other drugs of abuse (cocaine, methamphetamine and opiates)
f. Willingness to participate in the study and give informed consent

(iv) HIV-seronegative control subjects were enrolled if they fulfill the following criteria
   a. HIV seronegative male or female of any ethnicity, over 18 years old
   b. No history of marijuana dependency or any other drug dependency with the exception of nicotine
   c. Negative urine toxicology screen for other drugs of abuse (cocaine, methamphetamine and opiates)
   d. Willingness to participate in the study and give informed consent

Exclusion Criteria
1. History of co-morbid psychiatric illnesses (e.g. bipolar, schizophrenia and major depression)
2. Cofounding neurological disorders (e.g. multiple sclerosis, ADHD, degenerative brain diseases, other brain infections, such as hepatitis C, excluding HIV for HIV+ subjects)
3. History of drug dependency as defined by DSM-IV (including cocaine, methamphetamine, alcohol, opiates, barbiturates, and marijuana in non-marijuana user groups) except nicotine
4. Inability to provide informed consent.
5. Any contraindications for lumbar puncture (e.g., coagulopathy with platelets <100K or severe liver disease).
Appendix B

Consent Forms

UNIVERSITY OF HAWAI'I
THE QUEEN'S MEDICAL CENTER
HONOLULU, HAWAI'I

INFORMED CONSENT TO TAKE PART IN A
CLINICAL RESEARCH STUDY

TITLE OF STUDY

Brain Glutamate and Glutathione as Oxidative Stress Markers in Patients with HIV and Methamphetamine Abuse

PRINCIPAL INVESTIGATOR

Linda Chang, M.D.
Queen’s Medical Center, UH Tower
1356 Lusitana Street, 7th Floor
Honolulu, HI 96813

Office/voice mail: (808) 586-7467

SPONSOR

National Institute of Neurological Disorders and Stroke (NINDS)
6001 Executive Blvd., Bethesda, MD 20892
INFORMED CONSENT

Abbreviations Used

MR: Magnetic Resonance (also known as MR imaging or MRI)

HIV: Human Immunodeficiency Virus

This is a research study that will use several new brain scanning techniques, magnetic resonance (MR) spectroscopy (MRS), to measure changes in brain chemicals and structure in patients with HIV, methamphetamine dependence, and both or neither conditions. We aim to determine how HIV and methamphetamine dependence change cognition (e.g. attention, thinking and memory), brain chemistry and brain structure. This will involve a complete study of your physical health, cognition, and a set of three different MRS scans.

You have been asked to take part in this study because you are in one of the following categories:

- An adult infected with HIV.
- An adult infected with HIV, and current methamphetamine dependence.
- An adult with methamphetamine dependence, without HIV infection.
- A healthy adult volunteer without HIV infection or methamphetamine dependence.

Before you decide whether or not to take part in this study, you must understand the purpose, how it may help, any risks, and what you have to do. This process is called informed consent. This consent form gives you information about the study that will be talked about with you by your doctors. It also gives you information about what health information will be collected as part of the research study and how that information will be used or disclosed. Once you understand the study, and if you agree to take part, you will be asked to sign this consent form. If you sign this form you are agreeing to take part in this study and to allow the use and disclosure of your study records and health information collected in connection with your participation in this study. You will be given a signed copy to keep. If you do not sign this consent form, you may continue to receive care, but not as part of this study.
Before you learn about the study, it is important that you know the following:

- Taking part in this study is of your own free will.
- You may decide not to take part in the study or stop being in the study at any time without it making any difference to your care now or in the future, or to any benefits that you are allowed.
- If the study changes in any way which could make a difference to your taking part, you will be told about the changes and may be asked to sign a new consent form.

EXPECTED LENGTH OF INVOLVEMENT IN STUDY

Depending on the scheduling and your availability, the study can be completed in 2-4 visits. Even though we expect the study to continue for its entire length, things may come up causing us to end early (such as a loss of funding). You may also be discontinued from the study if we are unable to contact you, or a change in your condition makes it not possible or unsafe for you to continue with the study. Whenever possible, you will be told about this change.

During the initial visit you will have medical and neuropsychiatric evaluations (during which you will be asked about any use of prescription, over the counter, herbal, and illegal drugs), blood samples taken (about 5-6 teaspoons), a urine toxicology test to find out the presence of any illegal drugs [e.g. methamphetamine (ice, meth, crystal, speed), marijuana (pot, weed, hashish), cocaine (coke, crack), opiates (morphine, heroin) etc.], EKG, neuropsychological testing, and mood scale ratings. The total time required for the screening visit and neuropsychological testing will be up to 5 hours. After the initial evaluation, you will also have a spinal tap and a set of 3 different MRS scans. The total scan time for each of the MR scans typically will not be more than 2 hours.

If you were enrolled in another study that had a recent physical examination, neuropsychological test, drug history, blood tests, or lumbar puncture, we may request your permission to obtain the information from the appropriate source, rather than asking you to repeat everything.

We may need to contact you between visits for scheduling or after your tests if the doctors have any concerns. Please initial if we may contact any of the following people.

_________ It is OK to call your home number about issues related to this project.

(your initials)
It is OK to call your personal contact (listed on contact sheet) and ask about (your initials) where you are and how you are doing. This may be done between exams and if the study staff is unable to contact you. Even though no information will be directly given to these people during the interview, they may question your HIV status or methamphetamine dependence when the research staff identifies him/herself. (We recommend that you tell these people about taking part in the study now to decrease any confusion.)

It is OK to provide information about study results to your primary care doctor. (your initials) This does not include information about the urine drug screen. The urine drug screen information will not be given to your doctor.

PURPOSE OF THE STUDY

The purpose of this research study is to find out whether HIV and methamphetamine abuse can change the brain chemicals and structure using structural MRI and MR spectroscopy.

PROCEDURES

If you decide to take part in this study, you will be asked to sign this consent form. The research project will include the following research procedures:

1. An interview and evaluation to collect information on demographic background (who you are, ethnicity, age, etc) and medical history, as well as to check your mood and your nerves and muscles (physical, psychiatric, and neurological examination), and drug use history. If you have a history of drug use this would include questions on how it has affected your life, family, health, and any resulting legal difficulties.
2. A set of memory and thinking tests (neuropsychological testing), questions about mood, and questions about ability to function in day-to-day activities. During the neuropsychological testing, you will be asked to remember things, do easy tasks as fast as you can (such as put pegs in holes), and draw objects.
3. We will perform a spinal tap and take a blood sample [about thirty (30) milliliters (6 teaspoons) for basic health measures and for chemical
measurements specific for this study]. If you have recent blood tests from your doctor, we can use those to reduce the amount we need.

4. A urine drug test and an EKG will be performed as well.

5. A set of MR scans will be done. The MR scans are used to take pictures of your brain using magnetic rather than x-ray energy. You will need to lie still on a table inside of a machine shaped like a tunnel. All procedures will be done at the Queen’s Medical Center.

RISKS

The risks to you from the exam, neuropsychological tests, and medical interview are very small. Rarely, they can cause anxiety or concern and because some personal questions are asked some people may feel embarrassed. Some of the neuropsychological tests can also be frustrating. You do not have to answer any questions or complete any tests that you feel uncomfortable with.

There is very little risk from the blood draw (venipuncture). There is the possibility of bruising, soreness, or bleeding where the needle enters the arm. Rarely, it could cause fainting. Risk of infection at the area of blood draw is slight since only sterile one-time equipment is used.

Spinal taps also present the possibility of bruising, soreness, infection, or bleeding, and rarely feeling light-headed or fainting. In addition, back pain may occur at or near the site where the needle enters the back since bruising, or rarely a larger hematoma, may occur around that location. Another risk of a spinal tap is a headache after the procedure, which can last a few days; however, this risk is low since prolonged headaches (lasting a few days) occur in less than 5% of cases in our experience. You should stay at home to rest on the days this will be done. More information about this procedure and any associated risks will be available to you.

The only common risks associated with the MR scans are a feeling of anxiety, claustrophobia (feeling enclosed), and potential danger associated with the magnet when having metallic parts in the body, which will be minimized by careful screening before scans. While the MR scanner makes a “knocking” noise, you might feel a tingling sensation in your arms or legs, but it is unlikely that this will occur. The noise made by the machine can be very loud and you will wear earplugs to prevent damage to your hearing. The radio waves used in the MRI have given burns in about one in a million tests (most of those minor). People with back problems may feel some pain and soreness from lying on their back during the MRI scan. We will try to make you as comfortable as possible with
pillows. People who are claustrophobic (afraid of being in a closed space) may feel anxious about lying down in the MRI machine for the time of the test. If you are claustrophobic and don’t feel you could stand the test without a mild sedative, you will be offered a mild sedative such as Valium (for the brain chemistry study only). You will need to arrive at the imaging facility about one hour before the test and take the pill when you get there. You will need to arrange for a ride home, as you will not be able to drive or operate any machinery for several hours after taking the pill. The most common side effects of Valium include tiredness/fatigue, ataxia (not being able to walk a straight line, as if you were drunk), confusion, slurred talking, and blurred vision.

Any metal object placed in the strong magnetic field of the MRI scanner will be pulled by the magnet and may cause injury. In order to avoid this type of accidents, we keep large metal objects away from the scanner area, and we ask you to leave as many metal objects as possible at home, and to remove all metallic objects including jewelry, denture, glasses, watches, and any artificial removable body parts before the MRI. Injury may also happen if you have a metal device or metal particles in your body located near a sensitive organ. Examples include a heart pacemaker, aneurysm clips, or metal pieces from work exposure (metal shavings, shrapnel) in the eyes. For your safety, before the scan is done, you will be asked questions about your risk of having these dangerous pieces of metal in your body. If you have any of these metal objects in your body, you will be evaluated by a physician to determine whether you could have the MRI done. You must tell the technicians and study staff of any situations described above that may put you at risk.

All information in this study is private and will be kept confidential, including the urine drug screen. The information that we gather will not be given to anyone, including your doctor, without your written permission, within the limits of the law.

**BENEFITS**
The possible benefits of your being in this study are:

1. There is no direct benefit to you. If any abnormal findings are detected, we will talk to you, and with your permission, we can talk to your primary care doctor. These may be caught earlier if you take part in the study.
2. You will be contributing useful information about how HIV and/or methamphetamine dependence affect certain brain chemicals.
3. You will be contributing useful information for people infected with the HIV virus or with methamphetamine abuse or both.

OTHER TREATMENT

This study is voluntary and does not include any drug treatments. You may choose not to take part in it. Your choice not to take part in this study will not make any difference in your taking part in other studies you may be in now or may want to enter in the future. Your decision will not change the medical care you receive now or in the future.

CONFIDENTIALITY

**Federal Privacy Regulations provide safeguards for privacy, security, and authorized access to health information.** The confidentiality of all study-related records will be kept according to all applicable laws. Although we will take all measures to maintain the privacy of your study records, we cannot be held responsible for or guarantee that other institutions will do the same. For study purposes, the results of this research may be presented at meetings and/or in publications; however, your identity will not be disclosed. There is also a possibility that your study records, including identifying information may be inspected by officials of regulatory agencies such as the Institutional Review Boards (IRB) of the Queens Medical Center or the Committee on Human Studies (CHS) at the University of Hawai‘i. They look at research subjects to make sure their rights are being protected ethically.

A code, which will be known only to study personnel, will be used instead of your name on study records in this study. The code will be stored in a locked place. If you agree, we will give clinical information to your doctor to help in your medical care. However, if we feel that the information you give us will put you or someone else at risk for personal harm, it may be necessary to break confidentiality and inform your doctor, police, or other relevant authorities even if you have asked us not to. This would be done to protect you or others from harm.

To help us protect your privacy, we have obtained a Certificate of Confidentiality from the National Institutes of Health. With this Certificate, the researchers cannot be forced to disclose information that may identify you, even by a court subpoena, in any federal, state, or local civil, criminal, administrative, legislative, or other proceedings. The researchers will use the Certificate to resist any demands for information that would identify you, except as explained below. The Certificate cannot be used to resist a demand for information from personnel of the United States Government that is used for auditing or evaluation of federally
funded projects or for information that must be disclosed in order to meet the requirements of the federal Food and Drug Administration (FDA). You should understand that a Certificate of Confidentiality does not prevent you or a member of your family from voluntarily releasing information about yourself or your involvement in this research. If an insurer, employer, or other person obtains your written consent to receive research information, then the researchers may not use the Certificate to withhold that information.

USE AND DISCLOSURE (RELEASE) OF YOUR HEALTH INFORMATION

By signing this form you are authorizing the collection, use and release of your personal health information in study records and diagnostic imaging and any health information gathered about you as part of this study. Your information will only be used/disclosed as described in this consent form and as permitted by state and federal laws. Your personal health information is health information about you that could be used to identify you. This information may include information about AIDS or HIV infection, treatment for alcohol and/or drug abuse, or mental health or psychiatric services.

The purposes of releasing your protected health information are to collect the data needed to complete the research, to properly monitor (watch) how the study is done, and to answer research questions related to this study.

There is no expiration date to this authorization.

Who may receive, use or release information:

Your study records and any health information related to this study may be used or released in connection with this research study to the following:

- Linda Chang, M.D., and her research staff for the purposes of conducting this research study.
- The UH cooperative institutional review board and staff members of the Research Regulatory Office for purposes of overseeing the research study and making sure that your ethical rights are being protected.
- Providers and other healthcare staff of QMC involved in your care.
Who may receive the information by the above groups:

The individuals or groups named above may release your study records, this consent form and the information about you created by this study to:

- The sponsor of this study, National Institute of Neurological Disorders and Stroke/ National Institutes of Health, and their designees (if applicable)
- Federal, state and local agencies having oversight over this research, such as The Office for Human Research Protections in the U.S. Department of Health and Human Services, Food and Drug Administration, the National Institutes of Health,
- Representatives of outside groups hired by QMC Research Department for audits to make sure studies are done as required.

There is a possibility that your information may be released again by the sponsor of the study or governmental agencies described above and no longer covered by federal privacy rules.

RIGHT TO WITHDRAW OR STOP TAKING PART IN THE STUDY

You may refuse to sign this authorization. If you refuse to sign the authorization, you will not be able to take part in this study. If you choose not to be in the study, or choose to withdraw from the study, or if you refuse to sign the authorization, it will not make a difference in your usual treatment, or your payment, and it will not change your eligibility for any health plan or health plan benefits that you are allowed.

If you decide to end your taking part in the study or you are removed from the study by the researcher (study doctor), you may revoke (take away) your authorization. In order to take away this authorization, you must send a letter/notice to the researcher in charge of this study. Send the written notice to the researcher to the address listed on the front page of this consent form.

If you take away your authorization, your part in the study will end and the study staff will stop collecting medical information from you and about you. The researchers and sponsor will continue to use information that has already been collected, but no new information about you will be collected unless the information is about an adverse event (a bad side effect) related to the study or to keep the scientific integrity of the study. If an adverse event happens, we may need to review your entire study record.

ACCESS TO YOUR INFORMATION

As is usually the case, you may see the information in your study record; however, the records and the information related only to the study that are kept separately will not be available to you until the study is finished. If you wish to
review your study records after the completion of the study, you should request this from the study doctor.

COSTS/COMPENSATION

There will be no cost to you for the examination and tests in this study. You will be paid for the time and effort of taking part, as follows:

$25 for the medical examination, urine toxicology screen and bloodwork

$50 for the neuropsychological testing ($25 for paper & pencil tests, $25 for computer tests)

$100 for the lumbar puncture

$75 for the set of MR scans ($25 for each of the three different MR scans)

Taking part in this research might not necessarily benefit your present health condition nor increase the length of your life.

TREATMENT AND COMPENSATION FOR INJURY

Information that may be gained from the study and made available to your primary care doctor may lead him or her to order more tests as part of your overall health care. We will not typically be involved in this and will not be able to pay for these tests.

If you have an injury or illness (get sick) as a result of being in this study, immediate emergency medical care and treatment, which may be needed, will be available at the usual charge. The sponsor of the study and the study doctor do not have any funding (money) to pay for treating the injury or illness. Your insurance company may not pay for some (or all) of the treatment of the injury or illness as a result of being in this study. If your medical insurance does not pay for these medical costs, you alone will be responsible for payment. There is no way of knowing what the costs will be. You should talk about the kind of insurance coverage you have with your doctor and insurance company before you decide to take part in this study. You can have financial counseling to go over your insurance coverage.
REMOVAL FROM THE STUDY

You take part in this study of your own free will. You may be taken off the study without your consent for any of the following reasons:

- You are unable or unwilling to comply with study procedures;
- You get pregnant;
- You are unable to have a MRI done (for example, after you have received surgical implants, such as a pacemaker or certain metallic objects)

NEW FINDINGS

You will be told of any important new information learned during the study.

WHO TO CONTACT

If you feel that you have been injured as a result of taking part in this study or if you have any questions about your treatment, your rights as a volunteer or any other matter relating to this study, you may call Dr. Linda Chang at (808) 586-7467 and talk about any questions that you might have.

If you cannot get satisfactory answers to your questions or you have comments or complaints about your treatment in this study, you may contact:

Multi Institutional Cooperative Committee on Human Studies at
1960 East-West Road Phone: 808. 956.5007
Rm. B-104 Email: uhirb@hawaii.edu
Honolulu, HI 96822
I, or my legally authorize representative (the legal person who cares for me) have read and understand the description of this study such as the purpose and nature of this study, its expected length, the procedures to be done, reasonably known risks and discomforts, benefits to expect, other treatments I may have, release of my study records, payment and medical treatment for injury, and removal without my consent for this research study.

I am taking part in this study of my own free will. I may withdraw (stop taking part) and/or withdraw my authorization for use and release of protected health information at any time after signing this consent form without it making a difference to my care now or in the future or any loss of benefits that I am allowed. My consent does not take away my legal rights in case of carelessness or negligence of anyone connected with this study. My signature means that I have read the information above or that it has been read to me, my questions have been satisfactorily answered, and at any time I have other questions, I can contact the researcher listed on the first page.

Specially Protected Health Information
I agree to the release of the following information should it be contained in my study records: Acquired Immune Deficiency Syndrome (AIDS or HIV), alcohol and/or drug abuse treatment, or behavioral or mental health services.

cc: Signed copy of consent to patient

__________________________    ____________________________
Subject’s Name (Print)        Subject’s Signature        Date/ Time

__________________________    ____________________________
Witness’ Name (Print)         Witness’ Signature        Date/ Time
Witnessing Signature Only

***************
I have explained this research to the above subject. In my judgment, the subject is voluntarily and knowingly giving informed consent and has the legal capacity to give informed consent to take part in this research study.

_________________________  ___________________________
Investigator’s Name (Print)   Investigator’s Signature   Date/ Time
(Individual obtaining Subject’s consent)

_________________________  ___________________________
Translator’s Name (if appropriate) Translator’s Signature   Date/ Time
(Print)
Additional Requests

Banking additional samples:

Our research group would like to take an extra 2 tablespoons of blood and 1 tablespoon of spinal fluid for future research. If you agree, this blood/spinal fluid will be stored in a locked freezer and may be used in future research to learn more about HIV and/or methamphetamine dependence. For example if a new test is discovered in the future, then we can use your samples for that new test without needing another sample from you or someone else. Your sample will be kept until it is used up or as long as it still may be useful.

__________ You agree to bank additional blood/spinal fluid for possible future research. (If you change (your initials) your mind, you may revoke (take away) your authorization. To revoke this authorization, send a letter to the researcher in charge of this study at the address listed on the first page of the consent form.

__________ You do not agree to bank additional blood/spinal fluid for possible future research.

(your initials)

Using data for comparison in other studies

Our research group would like to use the research data we are collecting from you for comparison purposes in future studies. We would only be using data that we have already obtained from you in this study.

__________ You agree to allow my research data to be compared or combined with other studies. (If you (your initials) change your mind, you may revoke (take away) your authorization. To revoke this authorization, send a letter to the researcher in charge of this study at the address listed on the first page of the consent form.

__________ You do not agree to allow my research data to be compared or combined with other studies.

(your initials)
Your samples/data will be used only for future research that is covered by a research protocol that has been reviewed and approved by an Institutional Review Board (IRB). New analysis of your samples/data may involve other researchers, in which case your samples/data could be sent to scientists who are affiliated with the University of Hawai‘i and with other institutions, but your samples/data will never be sold to anyone. The research done with your samples/data may help identify genetic and protein markers and help develop new medical knowledge and technologies in the future. The greatest risk to you is the release of information from your records. Our research group has taken steps to ensure that the chance of this happening is very small and only a code number and the date will be on any samples/data, not any personal information.

If you do not agree to one or both of these requests, you can still take part in the study.

cc:  Signed copy of consent to patient

________________________  _______________________
Subject’s Name (Print)    Subject’s Signature       Date/ Time

________________________  _______________________
Investigator’s Name (Print) Investigator’s Signature Date/ Time

(Individual obtaining Subject’s consent)
REFERENCES


Caldwell DJ, Evans JD. 2008. Developing clinical role of a CCR5 co-receptor antagonist in HIV-1 infection. Expert Opin Pharmacother 9:3231-42
Chiang CW BG, Brine D. 1983. Systemic absorption of delta 9-tetrahydrocannabinol after ophthalmic administration to
the rabbit. *J Pharm Sci* 72 (2): 136-8


Hazenberg MD, Otto SA, van Benthem BH, Roos MT, Coutinho RA, et al. 2003. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. *AIDS* 17:1881-8


Hooker WD, Jones RT. 1987. Increased susceptibility to memory intrusions and the Stroop interference effect during acute marijuana intoxication. *Psychopharmacology (Berl)* 91:20-4


Klein TW, Newton CA, Nakachi N, Friedman H. 2000. Delta 9-tetrahydrocannabinol treatment suppresses immunity and early IFN-
gamma, IL-12, and IL-12 receptor beta 2 responses to Legionella pneumophila infection. *J Immunol* 164:6461-6


distribution of the CCR5 gene 32-basepair deletion. Nat Genet 16:100-3
Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. 1990. Structure of
a cannabinoid receptor and functional expression of the cloned cDNA.
Nature 346:561-4
medical students’ frequently asked questions about family medicine. Am
Fam Physician 76:99-106
Identification of an endogenous 2-monoglyceride, present in canine gut,
that binds to cannabinoid receptors. Biochem Pharmacol 50:83-90
Mechoulam R, Gaoni Y. 1965. Hashish. IV. The isolation and structure of
cannabinolic cannabidiolic and cannabigerolic acids. Tetrahedron
21:1223-9
Merrill JE, Chen IS. 1991. HIV-1, macrophages, glial cells, and cytokines in AIDS
nervous system disease. Faseb J 5:2391-7
Mravec B, Gidron Y, Kukanova B, Bizik J, Kiss A, Hulin I. 2006. Neural-
endocrine-immune complex in the central modulation of tumorigenesis:
facts, assumptions, and hypotheses. J Neuroimmunol 180:104-16
renal tumor antigens by human dendritic cells activates tumor-infiltrating
lymphocytes against autologous tumor: implications for live kidney cancer
Characteristics of Activated Monocyte Phenotype Support R5-Tropic
Human Immunodeficiency Virus. Immunology and Immunogenetics
Insights 2009:15


Ruitenberg JJ, Mulder CB, Maino VC, Landay AL, Ghanekar SA. 2006. VACUTAINER CPT and Ficoll density gradient separation perform equivalently in maintaining the quality and function of PBMC from HIV seropositive blood samples. *BMC Immunol* 7:11


Sugiura T, Yoshikawa H, Awaga K. 2006b. 1D helical polymeric chain with a pseudo-5(3) screw axis formed by cuprophilicity. synthesis and crystal structure of copper(I) pivalate. *Inorg Chem* 45:7584-6


Williams K, Alvarez X, Lackner AA. 2001. Central nervous system perivascular cells are immunoregulatory cells that connect the CNS with the peripheral immune system. *Glia* 36:156-64