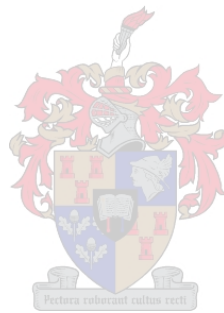# HIV-1-associated Neuroinflammation: Effects of Two Complimentary Medicines Illustrated in an *in vitro* Model of the Blood-Brain Barrier

Luan Dane Africa

Dedicated to

Marlon le Roux

DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Background: Neuroinflammation is central to the aetiology of HIV-associated neurocognitive disorders (HAND) that are prevalent in late stage AIDS. ARV treatments are rolled out relatively late in the context of neuroinflammatory changes, so that their usefulness in directly preventing HAND is probably limited. It is common practice for HIV+ individuals in developing countries to make use of traditional/complimentary medicines. One such medicine is *Sutherlandia frutescens* - commonly consumed as a water infusion. We have also identified a new candidate complimentary medicine for use in this context - grape seed-derived proanthocyanidolic oligomers (PCO) have significant anti-inflammatory action in the peripheral compartment in the context of e.g. skeletal muscle injury, but have not been investigated in the context of either neuroinflammation or HIV/AIDS. Here the efficacy of these two substances as an anti-inflammatory modality in this context was investigated in an *in vitro* co-culture model of the blood-brain barrier (BBB).

Methods: Single cultures of human astrocytes, HUVECs and primary human monocytes, as well as co-cultures (BBB), were stimulated with HIV-1 subtype B & C Tat protein and/or HL2/3 cell secretory proteins after pre-treatment with *S. frutescens* or PCO extracts. Effects of this pre-treatment on pro-inflammatory mediator expression and monocyte migration across the BBB were assessed.

Results: In accordance with others, B Tat was more pro-inflammatory than C Tat, validating our model. *S. frutescens* decreased IL-1β secretion significantly (P<0.0001), but exacerbated both monocyte chemoattractant protein-1 (P<0001) – a major role player in HIV-associated neuroinflammation – and CD14+ monocyte infiltration across the BBB (P<0.01). PCO pre-treatment resulted in a significantly dampened IL-1β (P<0.0001) response to stimulation with HIV-associated proteins. In contrast to *S. frutescens,* PCO modulated monocyte chemoattractant protein-1 (P<0001) response and decreased capacity for CD14+ monocytes to migrate across the simulated

BBB (P<0.0001). Additionally, PCO pre-treatment decreased both GFAP (P<0.001) and HSP-27 (P<0.001) expression in the astrocytes of the BBB.

Conclusions: Current data illustrates that the combined use of HL2/3 cells and the simulated BBB presents an accurate, disease relevant *in vitro* model with which to study neuroinflammation in the context of HIV/AIDS. In addition, our results caution against the use of *S. frutescens* as anti-inflammatory modality at any stage post-HIV infection. Novel data presented here illustrate that PCO is able to blunt the MCP-1 and IL-1β response to HIV-1 proteins in single cultures of human astrocytes and HUVECs, as well as in an *in vitro* simulation of the BBB. In addition, PCO was able to limit monocyte transmigration across the simulated BBB in response to HIV-1 proteins generated by HL2/3 cells. This suggests that grape seed-derived PCO could be considered as complimentary anti-neuroinflammatory drug in the context of HIV/AIDS.

UITTREKSEL

Agtergrond: Neuroinflammasie staan sentraal in die ontwikkeling van MIV-verwante toestande wat gekenmerk word deur neurokognitiewe afteruitgang, veral in die later stadia van die siekte. Aangesien anti-virale middels relatief laat toegedien word in die konteks van neuroinflammasie, is hul rol in die voorkoming van neuroinflammatoriese veranderinge heel moontlik weglaatbaar.

MIV+ individue, veral in ontwikkelende lande, gebruik algemeen natuurlike medisinale preparate. *Sutherlandia frutescens* is een so 'n middel wat as 'n tee ingeneem word. Verder het ons ook 'n nuwe kandidaat komplimentêre medisyne identifiseer – druiwepitekstrak wat polifenole bevat (PCO) het aansienlike anti-inflammatoriese eienskappe in die periferie, bv. in die konteks van skeletspierskade, maar die middel is nog nie voorheen in die konteks van neuroinflammasie of MIV/VIGS ondersoek nie. Hier word die anti-inflammatoriese effektiwiteit van beide middels in hierdie konteks ondersoek deur gebruik te maak van 'n *in vitro* simulasie van die bloedbreinskans (BBS).

Metodes: Kulture van menslike astrosiete, menslike naelstring endoteelselle (HUVECs) en primêre menslike monosiete, sowel as gesamentlike kulture (BBS) is met MIV-1 subtipe B en C Tat proteïen en/of HL2/3 selprodukte gestimuleer na voorafbehandeling met *S. frutescens* of PCO ekstrakte. Effekte op pro-inflammatoriese mediator uitdrukking sowel as monosiet migrasie oor die BBS is ondersoek.

Resultate: In ooreenstemming met die literatuur was B Tat meer inflammatories as C Tat, wat die akkuraatheid en gepastheid van ons model bevestig. . *S. frutescens* het afskeiding van IL-1β betekenisvol verminder (P<0.0001), maar het afskeiding van beide monosiet chemoaantrekkingsproteïen-1 – 'n groot rolspeler in MIV-verwante neuroinflammasie – en CD14+ monosiet migrasie oor die BBS vererger (P<0.0001 en P<0.01 onderskeidelik). PCO behandeling het 'n betekenisvolle demping van die IL-1β reaksie (P<0.0001) op stimulasie met MIV-geassosieerde proteïene tot gevolg gehad. Anders as *S. frutescens* het PCO die MCP-1 reaksie,

asook CD14+ monosiet migrasie betekenisvol inhibeer. Verder het PCO ook beide GFAP en HSP-27 uitdrukking in astrosiete van die BBS verminder (beide P<0.001).

Gevolgtrekkings: Huidige data wys dat die gekombineerde gebruik van HL2/3 selle en die gesimuleerde BBS 'n akkurate en fisiologies relevante *in vitro* model daarstel, waarmee neuroinflammasie in die konteks van MIV/VIGS bestudeer kan word. Ons resultate waarsku verder teen die gebruik van *S. frutescens* as anti-inflammatoriese middel in selfs die vroeë stadium na MIV infeksie. Oorspronklike data wat hier aangebied word illustreer dat PCO die pro-inflammatoriese reaksie op MIV-proteïene in kulture van astrosiete en HUVECs, asook die *in vitro* simulasie van die BBS, effektief demp. Verder het PCO die vermoë getoon om monosiet migrasie oor die BBS, in reaksie op MIV-1 proteïene wat hul oorsprong uit HL2/3 selle het, te beperk. Hierdie bevindings beteken dat PCO dus eerder as *S. frutescens* oorweeg moet word as komplimentêre anti-inflammatoriese medisyne in die konteks van MIV/VIGS.

RESEARCH OUTPUTS

1. Submitted manuscripts:

- **Africa LD,** Smith C. *Sutherlandia frutescens* may exacerbate HIV-1-associated neuroinflammation. Journal of Neuroimmunology (Manuscript submitted)

- **Africa LD,** Smith C. Using a simulated blood-brain barrier to investigate potential modulators of HIV-1-associated neuroinflammatory processes *in vitro.* Neurotoxicity Research (Manuscript submitted)

2. Conference contributions

➤ **International invited seminar**

- **Africa LD**, Smith C. HIV-1 Associated Neuroinflammation: Modulatory Effects on Cellular Role Players Illustrated *In Vitro.* Oral presentation presented at the World Congress on Inflammation in Exercise, Health and Disease, 65th Annual Meeting of the American College of Sports Medicine, Orange County Convention Centre, Orlando, Florida, USA

➤ **National poster presentation**

- Smith C, **Africa LD**. HIV-Associated Neuroinflammation in HIV-1 Subtypes B and C: Leucocyte Migration Across the Blood Brain-Barrier. Poster presented at the Annual Meeting of the Physiological Society of Southern Africa (PSSA), Gateway Hotel & Conference Centre, Umhlanga, Kwazulu Natal,14-17 September 2014

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LIST OF ABBREVIATIONS

AAN - American Association of Neurology

ACTG – Aids Clinical Trials Group

AIDS – Acquired Immunodeficiency Syndrome

ANI – Asymptomatic Neurocognitive Impairment

ARV – Anti-Retroviral

BBB – Blood-Brain Barrier

BMEC – Brain Microvascular Endothelial Cells

CAM – Cell Adhesion Molecule

CCR - C-C chemokine Receptor

CD – Cluster of Differentiation

CSF – Cerebrospinal Fluid

CNS – Central Nervous System

CO_2 – Carbon Dioxide

COX – Cyclo-Oxygenase

CX3CL1 – C-X3-C motif Ligand 1

CXCR – C-X-C chemokine Receptor

DMEM – Dulbecco's Modified Eagle's Medium

Egr-1 – Early Growth Response protein -1

ERK - Extracellular signal-Related Kinase

FITC - Fluorescein Isothiocyanate

GFAP – Glial Fibrillary Acidic Protein

GM-CSF – Granulocyte/ Macrophage – Colony Stimulating Factor

gp120 – Glycoprotein 120

HAART – Highly Active Anti-Retroviral Therapy

HAD – HIV-Associated Dementia

HAND – HIV-1-Associated Neurocognitive Disorders

HIV-1 – Human Immunodeficiency Virus type-1

HIV-2 - Human Immunodeficiency Virus type-2

HIVE – HIV Encephalitis

HLA-DR – Human Leucocyte Antigen – Death Receptor

HSP-27 – Heat Shock Protein 27

HUVECs – Human Umbilical Vein Endothelial Cells

ICAM-1 – Intercellular Adhesion Molecule 1

IL-1β – Interleuken 1β

JNK - c-Jun N-terminal Kinase

LPS - Lipopolysaccharide

MAPK - Mitogen-Activated Protein Kinase

MCMD – Mild Cognitive Motor Disorder

MCP-1 – Monocyte Chemo-attractant Protein 1

MIP-1α – Macrophage Inflammatory Protein 1α

MIP-1β – Macrophage Inflammatory Protein 1β

MMP – Matrix Metalloprotease

Nef – Negative factor

NF-κB – Nuclear Factor κB

NLRP3 - – NOD-Like Receptor family, Pyrine domain containing 3

NMDA - N-Methyl-D-aspartic Acid

NO – Nitric Oxide

OD – Optical Density

PBS – Phosphate Buffered Saline

PCO - Proanthocyanidolic Oligomers

PECAM -1 - Platelet Endothelial Cell Adhesion Molecule

PI-3K – Phosphoinositide 3-kinase

RANTES – Regulated upon Activation Normal T cell Expressed presumably Secreted

ROS – Reactive Oxygen Species

SDF-1 – Stromal cell-Derived Factor 1

SIV – Simian Immunodeficiency Virus

Tat – Transactivator of transcription

TJ – Tight Junction

TNF – Tumour Necrosis Factor

TPA - 12-O-tetradecanoylphorbol-13-acetate

VCAM-1 – Vascular Cell Adhesion Molecule 1

Vpr – Virus protein R

ZO-1 - Zonula Occludens 1

ZO-2 - Zonula Occludens 2

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data illustrating the effects of PCO extract pre-treatment on GFAP expression in primary human astrocytes of the *in vitro* BBB (4.5b)

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CHAPTER 1

Introduction

It is common knowledge that infection with the human immunodeficiency virus type 1 (HIV-1) ultimately leads to the obliteration of the host immune system and that in addition to this, it may induce a host of neurological disorders. Early on in HIV-1 infection the virus establishes itself in the central nervous system (CNS), which may well result in the development of a number of neurological conditions. Such neurological maladies associated with HIV-1 infection have been reported to have a prevalence of between 40% and 70% within the infected population, affecting both the CNS and the peripheral nervous system (McArthur *et al.* 2005). For the purposes of this body of work, only CNS infection and dysfunction will be discussed.

While manifestation of neurological disorders may occur at any point in the progression of HIV-1 infection, the vast majority of cases are reported in patients who present with an advanced stage of HIV disease (Acquired Immunodeficiency Syndrome - AIDS). Since the advent of highly active antiretroviral therapy (HAART), the clinical picture of neuropathology of primary HIV-1 associated CNS disorders – differentiated from conditions associated with opportunistic infections or neoplasia within the CNS - has changed considerably. These anomalies are now referred to as HIV-1 associated neurocognitive disorders (HAND), an umbrella term that encompasses a broad spectrum of neurocognitive dysfunction. The most severe form of cognitive impairment in this context is known as HIV-1-associated dementia (HAD) (Anderson *et al.*, 2002), typified by marked interference in day-to-day functioning. At the midpoint of the spectrum is mild cognitive motor disorder (MCMD), characterized by milder cognitive impairment which only hampers normal daily functioning to a moderate degree. The mildest form of HAND is asymptomatic neurocognitive impairment (ANI) (Gandhi *et al.* 2011), which is set apart from HAD and MCMD in that although mild cognitive impairment is present, it does not impact on the patient's normal daily functioning. ANI is a relatively recent sub-group

addition under HAND, and although quite common, its impact and outcome have yet to be defined (Antinori *et al.* 2007)

HAD is considered to be one of the most devastating outcomes of HIV-1 infection, its characteristic features being neurocognitive impairment, typified by forgetfulness and lack of concentration, emotional disturbances such as apathy and social withdrawal, and atypical motor functioning (weakness, ataxia, clumsy gait and tremor) (Boisse´ *et al.* 2008). In the pre-HAART era, the diagnosis of HAD was a common occurrence with an annual incidence of 7% or more in patients with full blown AIDS, but since the introduction of the treatment regimens, fewer and fewer cases of HAD are being diagnosed. According to the Multicenter AIDS Cohort study there has been a marked decline in HAD incidence when comparing the period 1990-1992 to 1996-1998. In spite of all this, loss of cognitive functioning is still a persistent feature of HIV-1 infection, with one study reporting a 37% prevalence of HAD and MCMD in advanced AIDS patients irrespective of HAART (Sacktor *et al.* 2001). Reports from the AIDS Clinical Trials Group (ACTG) Longitudinal Linked Randomized Trials study of patients on HAART indicated a baseline prevalence of mild to moderate cognitive impairment of 26% and a collective incidence of 21% in patients deemed neurologically normal at baseline (Robertson *et al.* 2007). While comparing the standard American Association of Neurology (AAN) criteria with AAN criteria adjusted to include ANI, a study of HIV-1 infected women reported a noteworthy discrepancy between the two. When using the standard criteria, 54% of subjects were regarded as neurocognitively normal, 19% were diagnosed with MCMD and 23% of subjects were identified as HAD sufferers. According to the modified AAN criteria, only 31% of subjects were considered to be cognitively normal whilst 20% had ANI (Rosca *et al.* 2012).

Histologically, the characteristic neuropathology of HAND include infiltration and accumulation of monocyte/macrophages, microglial nodule and multinucleated giant cell formation in central white matter and deep gray matter (indicative of virus-induced fusion of

microglia and/or monocytes/macrophages), widespread reactive astrogliosis (which is suggestive of astrocyte activation and damage), specific neuron subpopulation loss (mainly those in the hippocampus and basal ganglia involved in cognition and motor function), a reduction in the number of synaptic connections and myelin pallor (the loss of myelin surrounding neuronal axons, indicating damage to oligodendrocytes) (Lawrence & Major 2002). All the aforementioned pathologies are collectively termed HIV-1 encephalitis (HIVE).

While severe HAD is by and large observed only during the late stages of infection, it is apparent that moderate neuropsychological, neurophysiological and neuroimaging irregularities can arise prior to end stage AIDS. These observations indicate that neurological injury induced by HIV-1 infection occurs gradually, and more than likely begins in the initial stage of infection. As with the clinical picture of the disease, HAART has altered the way in which these neuropathologies present (Boisse´ et al. 2008). The activation of macrophages/microglia is still observed. However the extent to which these cells are activated, as well as the sites where activated macrophage/microglia infiltrates are seen, appear to have changed. Strong involvement of the basal ganglia has been reported in cases from the pre-HAART era, whereas post-HAART cases report inflamed hippocampi and adjacent regions of entorhinal and temporal cortices (Anthony & Bell 2008). Moreover, variant patterns involving lymphocytic infiltration and what is described as a "burn-out" form of HIVE, as well as an increased presence of amyloid-β plaques akin to those seen in Alzheimer's disease, are additional neuropathologies observed in patients on HAART (Neuenburg et al. 2002; Gray et al. 2003). Consequently, though HAART is capable of slowing down the development of HAD, it fails in combating all forms of HAND.

In recent years evidence has been brought forward to suggest that in patients with no neurocognitive disease, the majority of the virus present in the cerebrospinal fluid (CSF) is of systemic origin, whereas in the case of HAD patients, the virions present in CSF are derived

from a genetically compartmentalized population of virus indicative of replication within the brain. Therefore, viral replication within the brain seems to be one of the hallmarks in HAD pathogenesis. In contrast to other forms of viral encephalitis, productive infection of neurons does not occur in the case of HIVE. As an alternative, productive viral replication within the brain is restricted to perivascular monocytes/macrophages and microglia (Williams *et al.* 2001). In spite of this, viral CNS infection does not seem to be the primary cause for the induction of disease. Instead of using the amount of virus and infected cells within the CNS as an indicator of disease severity, there is a greater association between the number of brain mononuclear phagocytes, this includes perivascular macrophages and microglia, their activation status and the severity of clinical disease. During the early stage of infection, resident monocytes/macrophages and microglia play a neuroprotective role in the context of HIV-1 infection, but their predominant function in the pathogenesis of HAND seems to be neuroinflammatory and neurotoxic (Yadav & Collman 2009) and is the result of increased migration of monocytes (both healthy and virally infected cells) from the circulatory compartment. Therefore, therapy effectively inhibiting migration of monocytes across the blood brain barrier (BBB) may benefically affect longer term prognosis in the context of neuroinflammation, neurodegeneration and, more specifically, HAND.

The aim of this thesis was to examine aspects of HIV-associated neuroinflammation by setting up an *in vitro* model of the BBB, using cells that play a pivotal role in progression of the disease. A further aim was to evaluate the potential benefit of two known and commonly used natural products, in this context.

Prior to any further examination, the current knowledge on the topic needs to be reviewed, and for this reason the following chapter will give insight into the inflammatory milieu of HAND, as well as describe each role player involved in this life altering condition. At the end of the review, I will present my hypothesis and main aims for the thesis. Chapters 3 and 4 will

describe two studies to investigate potential immune modulators in the context of HIV-neuroinflammation. This will be followed by a synthesis, final conclusions and recommendations for future research in Chapter 5.

CHAPTER 2

Literature Review

The purpose of this chapter is to give insight into the general inflammatory milieu associated with HIV-1 infection, and more specifically, the inflammatory aspects of HAND, outlining key role players and mediators, both host and viral, in disease progression and outcome.

2.1 HIV – Origin & Epidemiology

A few decades after its first description in 1981, the human immunodeficiency virus type - 1, the aetiological agent of AIDS, stands tall as one of the most devastating infectious agents known to mankind. According to the UNAIDS global report published in 2013, approximately 35.3 (32.2 - 38.8) million people worldwide were living with HIV at the end of 2012, with a reported infection incidence of 2.3 (1.9 – 2.7) million and 1.6 (1.4 – 1.9) million AIDS related deaths for the same year. It has been estimated that 0.8% of adults aged 15-49 worldwide are living with HIV, although disease burden still varies considerably between countries and regions. Sub-Saharan Africa remains the region hardest hit by the pandemic, with nearly one in every 20 adults (4.9%) living with HIV, accounting for approximately 69% of the global HIV infected population (UNAIDS 2013).

HIV, a diploid primate retrovirus, belongs to the *Lentivirinae* family of viruses, and these are able to persistently infect their hosts, leading to the progressive damage of the host immune system. The origins of HIV lie in the cross-species or zoonotic transmission of the simian immunodeficiency virus (SIV), also a member of the Lentivirus family, from non-human primates to humans. HIV comprises 2 viral types, namely HIV-1 and HIV-2, presumably transmitted to humans from the chimpanzee (*Pan troglodytes troglodytes)* and the sooty mangabey (*Cercocebus atys)* respectively (Hahn 2000).

At the onset of the HIV pandemic, Central Africa and West Africa were the primary nodes of HIV-1 and HIV-2 infection respectively. Unlike HIV-2, which is still principally found in individuals

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from West Africa (De Cock *et al.* 1993), HIV-1 has run rampant all across the globe. Also, HIV-1 is further divided into four divergent lines, designated as group M (major), N (non-M or non-O) and O (outlier) and P (non-M, non-N or non-O) respectively, with group M primarily responsible for the global pandemic. It has been proposed that each of the aforementioned lines represent a separate transmission event from chimpanzees to humans in Central Africa. One should note that the contribution of the latter 2 lineages to global infection and mortality rates is negligible as their occurrence, which is very rare, is restricted, by and large, to Cameroon and Central Africa (McCutchan, 2006).

It has been speculated that the genetic diversity of HIV has a major influence on epidemiologic transmission patterns, and also it has been proposed that HIV genetic and phenotypic variation, as well as recombination between viruses from different lineages, impacts on the transmissibility, the pathogenicity of the virus, as well as how patients respond to vaccines and therapies.

In the context of South Africa and sub-Saharan Africa at large, there has been a shift in the prevalence of HIV-1 subtypes. Initially the epidemic was dominated by sub-type B and D viruses, spread through a homosexual mode of transmission. Currently, the epidemic is, for the most part, dominated by viruses from the sub-type C lineage and spread through a heterosexual mode of transmission, as well as an increase in the prevalence of recombinant strains.

The purpose of the following sections is not to focus on the HIV-1 infectious cycle and additional aspects of general HIV-1 pathogenesis, but rather to shed light on CNS infection and the subsequent HIV-1-associated inflammatory milieu within this compartment.

2.2 HIV-1-Associated inflammation in the CNS

2.2.1 The role of microglia & macrophages in the neuropathogenesis of HIV-1

There is general consensus that HIV-1 neuroinvasion occurs via transmigration of infected cells derived from the monocyte-macrophage lineage from peripheral circulation, crossing the BBB, (discussed in more detail in section 2.2.3) and into the CNS. The primary targets for viral infection in the CNS are macrophages and microglia. Microglia are subdivided into two groups, namely, parenchymal microglia, and perivascular microglia (Cosenza *et al.* 2002). Notably, viral infection of long lived macrophages and microglia is thought not only to make a contribution to neurological dysfunction, but also to act as an enduring reservoir resulting in viral persistence within a sanctuary site. It is unclear whether the increase in the number of HIV-1 infected cells within the CNS is due to increased viral replication and subsequent infection of resident cells or due to increased migration of infected cells from peripheral circulation (Yadav & Collman 2009).

2.2.2 Accumulation of monocytes/macrophages in the CNS in HIVE

The perivascular macrophage population within the CNS is replenished by monocytes in peripheral circulation. These cells migrate into the brain, hereby allowing HIV-1 entry into the CNS via infected monocytes. The monocytes differentiate into macrophages after entering the brain, and this enables them to support viral replication to a far greater extent. This mode of viral neuroinvasion has been deemed the "Trojan horse" mechanism (Meltzer *et al*, 1990). Once the virus has taken a firm hold on the susceptible cells of the CNS, it elicits a chemokine and inflammatory mediator response, hereby recruiting and activating additional monocytes/macrophages. This mechanism of monocyte/macrophage recruitment could be considered as a "pull" mechanism. Additionally there are factors which appear to play a part in the accumulation and activation of monocytes/macrophages in the CNS: an expanded population of activated blood monocytes develops in certain patients – this is thought to point towards an invasive cellular phenotype prone to accumulation in the brain. The accumulation of this so-called invasive population of monocytes,

referred to as the "push" mechanism, may also assist progression of HIV-1 infection, hereby adding fuel to the proverbial fire. Both the "push" and "pull" mechanisms are non-mutually exclusive, and seem to play a considerable role in the accumulation and activation of monocyte/macrophages in the CNS (Yadav & Collman 2009), the details of which follow in the sections to come.

2.2.3 HIV-1 & the blood-brain-barrier

2.2.3.1 The normal physiology & HIV-associated pathophysiology of the blood-brain-barrier

By definition, the BBB (see figure 2.1) (Netter's Atlas of Human Physiology) is a barrier of critical importance involved in providing vital biological, physiological and immunological partitioning between peripheral circulation and the CNS (Strazza *et al.*, 2011). In order to gain a greater understanding of the effects of HIV-1 on the BBB, one first needs to gain a greater understanding of the molecular structure and regulation of its permeability.

The barrier comprises brain microvascular endothelial cells (BMECs), a distinct subset of endothelial cell, underlined by basal lamina, followed by other cell types which include astrocytes, pericytes, neurons, perivascular macrophages and parenchymal microglia. All of these play a role in the maintenance of a specific microenvironment which promotes and preserves BMEC functionality. On the whole, this neurovascular unit is responsible for the clear-cut homeostasis between the CNS and peripheral circulation. The role of the astrocytes in the BBB is indispensable in that they support the endothelial cells biochemically via physical interaction with their foot processes which cross the basal lamina to come in contact with BMECs (Ivey *et al.* 2009). It is widely accepted that astrocytes are required for endothelial cell differentiation into BMECs, as well as maintaining the BMEC state of differentiation. For example, when co-culturing BMECs with astrocytes, removal of the astrocytes alone is sufficient to result in the opening of tight junctions (TJs) and an increase in the barrier's permeability (Hamm *et al.* 2004). However, astrocytes, though important in the maintenance of the BBB integrity, are not the only role players involved in its upkeep. Pericytes, which are connective tissue cells, play a role in BMEC proliferation and survival.

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These cells physically interact with BMECs and neurons via cellular projections that cross the basal lamina and form gap junctions. It has been speculated that extracellular communication between astrocytes and pericytes occurs (Bonkowski,*et al.*, 2011), but this is very poorly understood. Although it has been shown that pericytes are susceptible to HIV-1 infection *in vitro,* the implications are not fully understood. Considering that these cells play a key role in the optimal functioning of the BBB and that their loss results in increased focal permeability (Bonkowski *et al.*, 2011), the effect of HIV-1 on pericytes, both directly and indirectly, may be an important area for future study.

BMECs form an unfenestrated endothelium, which, in combination with the TJs and the low level presence of intracellular vesicles under normal conditions, result in the BBB's selectively permeable nature to proteins and other molecules in peripheral circulation. In contrast to other TJs, these provide high trans-endothelial electrical resistance, placing additional limitations on the movement of a host of macromolecules across the barrier endothelium (Lossinsky & Shivers 2004). The regulation of substances able to pass this barrier is facilitated by multiple transporters expressed on BMECs. These include the blood-to-brain influx transport system, the brain-to-blood efflux transport system as well as efflux pumps on the luminal membrane of BMECs. The BMECs create a precisely regulated permeability system (Ueno *et al.*, 2010). By way of facilitated transport, saturable transport systems, secondary active transport or adsorptive transcytosis, molecules from peripheral circulation are able to enter BMECs, including the HIV-1 proteins Tat (Trans-activator of transcription), gp120 (glycoprotein 120), Nef (Negative effector) and Vpr (Viral protein R). In spite of this, small lipophilic molecules, able to passively diffuse across the BMEC plasma membrane, may serve as substrates or partial substrates for primary active or secondary efflux transporters, and for this reason, may not enter the CNS or pass from the central compartment to peripheral circulation, making the BBB not completely impermeable, but rather highly selective in permeability.
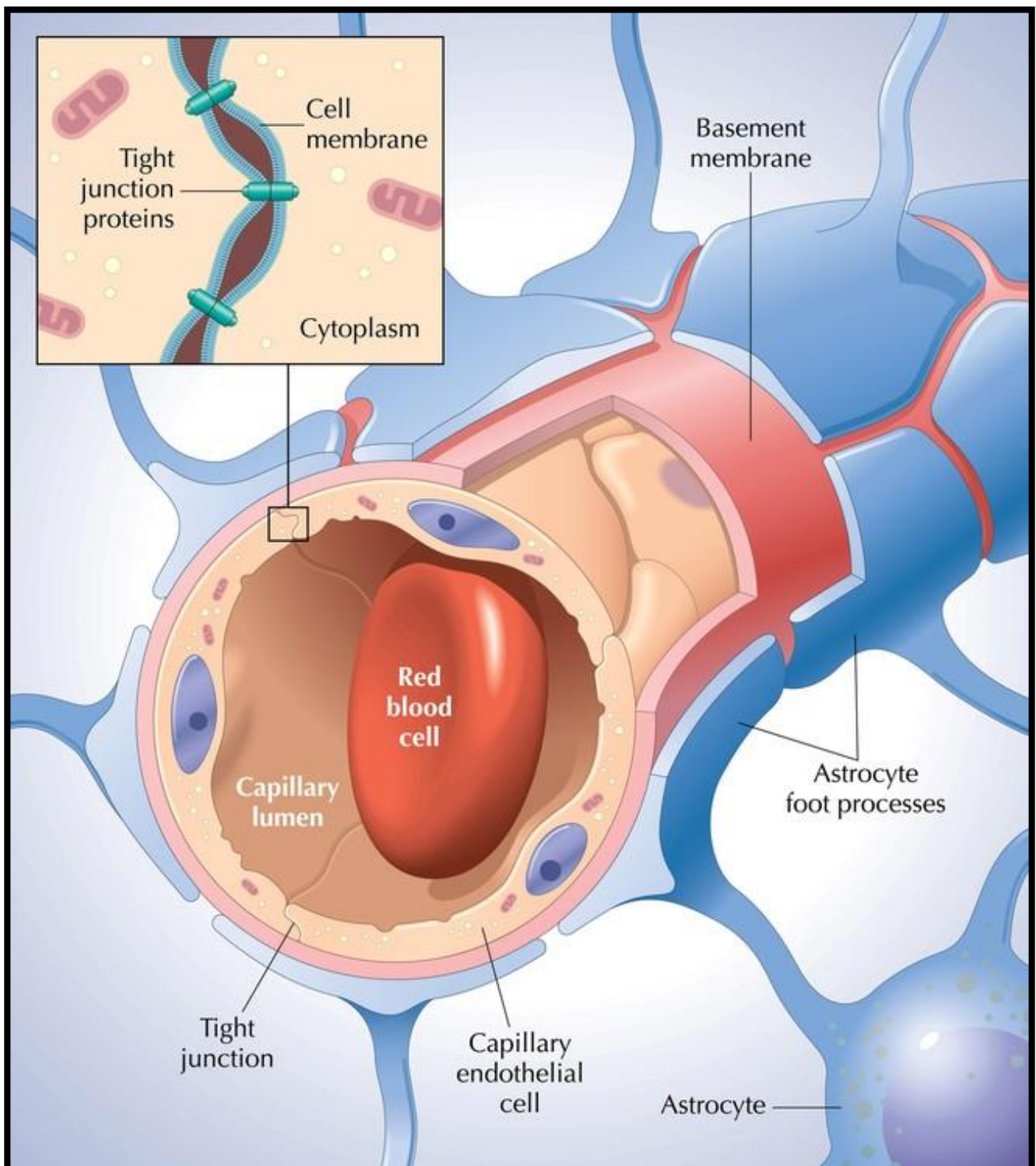
Figure 2.1 Cellular constituents of the blood-brain barrier

Consequently, size and lipophilicity of molecules are not enough to guarantee passage into, or even being kept out of the CNS. Disruption of this precisely orchestrated chain of events which control the permeability of the BBB has a significant impact on the neuropathogenesis of HIV-1. The vast majority of studies on this topic have suggested that the disruption of TJ protein expression is a

major role player in HIV-1-mediated BBB permeability increases (Andra *et al.*, 2003; Pu *et al.*, 2005; Toborek *et al.*, 2005; Avraham *et al.*, 2004).

2.2.3.2 The "Push" Mechanism of HIV-1 Neuroinvasion

More than a decade ago it was discovered that patients who presented with symptoms of HAND exhibited an expanded population of CD (Cluster of Differentiation) 14+ monocytes co-expressing CD16. In addition to CD16, these cells express high levels of additional markers of monocyte/macrophage activation which include Human Leucocyte Antigen – Death Receptor (HLA-DR), CD40 and CD86, monocyte chemoattractant protein-1 (MCP-1) and sialoadhesin. Moreover these cells have a greater capacity to produce interleukin (IL)-1β and tumor necrosis factor-alpha (TNF-α) (Pulliam *et al.* 1997). These altered protein expression and cytokine secretion patterns of these cells is a particularly interesting find as the CD14 low/CD16+ monocyte subset in healthy individuals do not share these traits (Strazza *et al.* 2011). This subset of cells is thought to have invasive properties allowing them to cross the BBB and enter the CNS in HAD in response to chemotactic factors such as monocyte chemoattractant protein-1 (MCP-1), fractalkine (CX3CL1) and others. Brain specimens derived from patients with HIVE have shown that most of the accumulated perivascular macrophages and cells in microglial nodules are CD14+/CD16+ (Ancuta *et al.* 2004). This is an important observation due to the fact that interaction between circulating immune cells and BMECs - mediated by cytokines, chemokines, and adhesion molecules - plays a crucial role in the regulation of diapedesis under both normal and inflammatory conditions. Paracellular as well as transcellular diapedesis mechanisms are used by immune cells to cross the BBB (Carman, 2009; Carman and Springer, 2004; Engelhardt and Wolburg, 2004). It has been put forward that carefully balanced regulation of these two processes that determines the mechanism of entry. Paracellular diapedesis is regulated through the interaction of cell surface molecules on immune cells and BMECs and is enhanced through joint activation of these cells that up-regulate surface molecules such as Vascular Cell Adhesion Molecule 1 (VCAM-1) and various classes of integrins (Ley *et al.*, 2007; Nourshargh *et al.*, 2010). Immune surveillance of the CNS under normal

conditions occurs to a far lesser extent when compared to that in other tissues, such as lung and spleen, owing to restricted expression of cell adhesion molecules (CAMs) on BMECs (Carrithers *et al.*, 2000; Raine *et al.*,1990). Passage across the BBB is mediated by the activation of intracellular pathways within BMECs that transiently up-regulate surface expression of CAMs (Alvarez *et al.*, 2011; Bo *et al.*, 1996; Cayrol *et al.*, 2008; Sobel *et al.*, 1990; Steffen *et al.*, 1994). BMEC activation can be as a result of activated leukocyte binding or as a reaction to leukocyte-secreted activating cytokines, which include IL-1β, IL- 17, IL-22, TNF-α, and interferon-gamma (IFN-γ), (Alvarez *et al.*, 2011; Bo *et al.*, 1996; Cayrol *et al.*, 2008; Sobel *et al.*, 1990; Steffen *et al.*, 1994). Therefore, the degree of diapedesis is greatly influenced by the activation state of both the aforementioned cell types. The increased recruitment of CD14+ monocytes expressing high levels of CD16, C-C Chemokine Receptor (CCR) 5, MCP-1, and sialoadhesin (Pulliam *et al.*, 2004) in individuals infected with HIV-1 leads to enhanced activation of BMECs. This serves as a possible explanation for increased numbers of CD14low/CD16+ monocytes transmigrating into the CNS (Fischer- Smith *et al.*, 2001). After initial interaction of activated immune cells with BMECs, intracellular signalling cascades are triggered within the BMECs that communicate back to the engaged leukocytes, resulting in changes in surface adhesion molecules that allow firm adhesion at high-avidity patches along the endothelium (Carman and Springer, 2003) and control the slow-crawling movement of the leukocytes that delivers the cells to these patches (Phillipson *et al.*, 2006; Schenkel *et al.*, 2004). The BBB can also be activated through stimulation of BMECs by cytokines or other factors not originating from leukocytes. In this situation, activated BMECs can in turn activate circulating leukocytes. Chemokines upregulated on the surface of BMECs will interact with receptors on circulating leukocytes, leading to the activation of integrins and subsequent strengthening of leukocyte adhesion (Ley *et al.*, 2007). Expression levels of Stromal Cell Derived Factor 1 (SDF-1) on BMECs and its cognate receptor C-X-C Chemokine Receptor (CXCR) 4 on circulating immune cells mediate activation of infiltrating cells, as demonstrated in models of inflammatory disease (Holman *et al.*, 2011; Krumbholz *et al.*, 2006; McCandless *et al.*, 2006). This has important

implications for HIV due to the role CXCR4 plays as a co-receptor in viral entry. Therefore, changes in CXCR4 levels by virus or viral proteins can influence the activation state of the BBB and the general control of diapedesis.

These double positive monocytes seem to be at a greater predisposition to infection and they can act as viral reservoirs (Ellery *et al.* 2007). Also, CD16+ monocyte-derived-macrophages are particularly efficient at forming conjugates with T cells that promote activation of the latter, virus transfer and efficient HIV-1 replication (Ellery *et al.* 2007). It has been suggested that the CD14+CD16+ macrophage is the major virus reservoir within the CNS due to the fact that the viral p24 antigen co-localizes with both CD14 and CD16 (Fischer-Smith *et al.* 2001). A possible explanation for this increase in viral replication could be a result of the increased cytokine secretion having an autocrine effect, up-regulating transcription factors that promote viral replication. The fact that infection in these cells is productive leads to a double assault on BBB integrity, since these monocytes/macrophages are likely secreting viral proteins in addition to pro-inflammatory cytokines (Strazza *et al.* 2011)

The exact reason behind the expansion of the CD14+CD16+ monocyte subset has not come to light, and two possible explanations offered in the literature for this are either production of these cells is increased in the bone marrow or that CD14+CD16- monocytes are more inclined to differentiate into the double positive phenotype (Hume 2008). According to a recent study, CD14+CD16+ monocytes differentiate from CD14+CD16- monocytes following migration out of the bone marrow or that double positive cells traffic out of the bone marrow at a later time. The increase in the invasive monocyte/macrophage pool could be as a result HIV-1 induced changes in cytokine production, as previously mentioned, which could possibly have an impact on haematopoiesis. This could be suggestive of the fact that phenotypic changes in the monocyte population, in addition to the altered cytokine secretion, precede expansion. There is growing evidence that suggests that an early event in HIV-1 infection is injury to the gastrointestinal mucosa, including preferential loss of

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mucosal lymphocytes, resulting in the translocation of bacterial lipopolysaccharide (LPS) into circulation (Lackner *et al.* 2009). LPS and other translocation products induce activation of monocytes via CD14 and Toll-like receptor signalling, resulting in the release of soluble CD14 and pro-inflammatory mediators that worsen chronic systemic immune activation, believed to be a principal driver of immunopathogenesis in AIDS (Miedema *et al.* 2013). Recently, microbial translocation from the gut has also been correlated specifically with increased monocyte activation in the development of dementia (Ancuta *et al.* 2008), hereby suggesting a mechanism driving expansion of this population. Furthermore, integrity of the BBB can be compromised *in vivo* by exposure to eincreasing levels of LPS in the bloodstream. In fact, LPS compromise of BBB integrity and HIV-1 infection of monocytes may act in synergy to augment BBB disruption and monocyte transmigration across the barrier.

2.2.3.3 The "Pull" Mechanism of HIV-1 Neuroinvasion

In addition to the invasive activated monocyte phenotype, the accumulation of monocytes/macrophages in the CNS is in part due to recruitment of cells along a chemotactic gradient. Of particular importance is the chemokine MCP- 1, which is increase in the cerebrospinal CSF and brain tissue in HAD. Brain macrophages are significant contributors to elevated MCP-1 and other pro-inflammatory mediators that recruit T cells and additional monocytes into the CNS and promote neuronal cell death (discussed further, below in section 2.3.4). MCP-1 is also produced by astrocytes in response to (among others) quinolinic acid, which is elevated in HAD. Additionally, MCP-1-dependent disruption of the BBB, as seen by increased permeability, reduction of tight junction proteins, and expression of matrix metallo-proteinases (MMP)-2 and MMP-9 has also been demonstrated. MCP-1 is also produced by astrocytes in response to the viral proteins Tat (Weiss *et al*. 1999) and Nef (Lehmann *et al*. 2006). For this reason MCP-1 acts as amajor chemoattractant in the recruitment of monocytes across the BBB. Another chemokine that is up-regulated in brain tissue and CSF of HAD patients is fractalkine. Fractalkine exists in both membrane-bound and soluble forms. In the CNS, it is constitutively expressed by neurons and up-

regulated by inflammatory stimuli. The receptor for fractalkine, CX3CR1, is expressed on monocytes and brain microglia, T cells, Natural Killer cells, dendritic cells, neurons, and astrocytes.

Cellular transmigration requires precisely regulated reversible disengagement of TJs. Intercellular CAM-1 (ICAM-1) has been well characterized to mediate TJ changes during lymphocyte migration into the CNS (extensively reviewed in Greenwood *et al.* 2011). Individual CAMs are believed to play different roles in mediating diapedesis of specific immune cell populations, with VCAM-1 and platelet endothelial CAM-1 (PECAM-1) playing central roles in monocyte migration into the CNS (Floris *et al*. 2002; Graesser *et al*. 2002; Muller *et al*. 1993). These CAMs also play important roles in returning BMECs to an inactivated state following stimulation (Couty *et al*. 2007; Graesser *et al*. 2002; Muller *et al*. 1993). Consequently, interruption of the inactivation mechanisms serves as a different explanation for the increased migration observed in HIV-infected individuals. Understanding the details of regulation of transmigration across the BBB, mechanisms of both activation and inactivation, sheds light on the multitude of targets for viral proteins to alter the process and disrupt peripheral-CNS homeostasis. The constitutive production of pro-inflammatory cytokines in the HIV disease state is likely to play an important role in dysregulation of these processes because the constitutive production of IL-1 and TNF-α at the BBB alters permeability ((Farkas *et al*. 2006; Wong *et al*. 2004). An experimental knockout of TNF-α in mice displayed decreased BBB leakage after treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, which is known to cause BBB leakage and boost TNF-α expression (Zhao *et al*. 2007). An additional possible explanation for increased migration of this monocytic subset is increased or constitutively high activation and expression levels of adhesion molecules on the monocyte cell surface, causing increased interaction with and activation of BMECs, changing the regulation of normal extravasation. Investigation of this possibility is an important area of future research concerning this monocytic subset. Figure 2.2 illustrates a mechanistic model of HIV-1 neuroinvasion (Gras & Kaul 2010).

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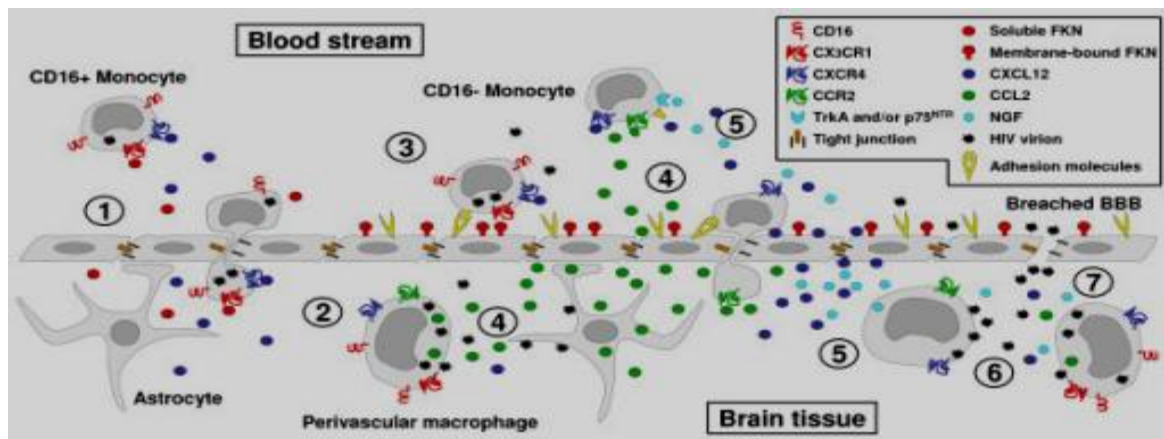
Figure 2.2 Mechanistic model of HIV-1 neuroinvasion. (1) The physiological expression of chemokines by brain cells, among which are soluble fractalkine (Fkn) and CXCL12, supports a slow but continuous entry of monocytes and macrophages into the central nervous system. Due to their expression of CX3CR1, CD16 positive, activated monocytes are the preferential targets for such attraction. These CD16 positive monocytes are the main reservoir of monocyte/macrophage-harbored virus and are thus likely to be the predominant cell type carrying HIV into the brain. (2) Infiltrated HIV infected monocytes locally produce HIV and inflammatory mediators in perivascular areas. This activates neighbouring astrocytes as well as the blood brain barrier (BBB) endothelium. (3) In response, endothelial cells up-regulate adhesion molecules, enhancing monocyte recruitment. However, membrane-bound Fkn is also induced on endothelial cells and can arrest CD16 positive monocytes at the endothelium thus inhibiting their further infiltration.(4) CCL2 is overexpressed by infected, HIV-stimulated macrophages and activated astrocytes, attracting CD16 negative, CCR2 positive monocytes toward the perivascular area. (5) Both CXCL12 and nerve growth factor (NGF) are overexpressed in the inflamed brain. NGF increases CXCR4 expression and promotes uninfected monocyte attraction by CXCL12. At the same time it limits entry of infected monocytes into the brain. (6) Activated uninfected perivascular macrophages may be targets for *de novo* infection by locally produced HIV, amplifying the activation - attraction – infection cycle. (7) Local inflammation as well as HIV products induce tight junction disorganization and lead to breaches in the BBB. Toxic serum proteins and free virions may enter the brain, favouring more infection and further amplifying inflammation.

2.3.4 Brain monocyte/macrophage & glial activation by HIV-1

As mentioned before, microglial activation and macrophage infiltration, rather than viral load, correlate best with clinical disease in HAD. Moreover, infected as well as uninfected monocytes/macrophages are involved, as the extent of monocytes/macrophages activation is often greater than the extent of direct infection. Monocytes/macrophages activation within the CNS seems to be a consequence of both direct infection and indirect mechanisms. Several viral proteins have been identified which activates monocytes/macrophages both directly and in trans, resulting in the production of inflammatory mediators which are responsible for amplifying and perpetuating the cascade.

Changes in BBB permeability during HIV-1 infection are probably as a consequence of multiple intra- and intercellular events involving multiple cell types as well as both viral and host derived proteins. In 2004, Maclean and colleagues reported that the presence of virions at the BBB is not

necessarily a sufficient stimulus for activation. However, virus-infected mononuclear cells were consistently able to bring about activation (MacLean *et al.*, 2004), suggesting that a factor secreted from infected cells is crucial to activate the BBB. Many studies have been undertaken to examine the effects of viral proteins such as Tat, Nef, and gp120 on the BBB, and combined evidence identifies these factors as essential mediators in causing breakdown of the BBB. While much focus has been placed on assessing the roles of these proteins on neural degeneration, the purpose of this body of work is to put the spotlight on their effects on inflammation in the context of the BBB and CNS.

2.3.4.1 Neurotoxic effects of HIV-1 gp120

The HIV-1 envelope glycoprotein is composed of two sub-units, namely a trans-membrane gp41 domain and a non-covalently associated surface sub-unit gp120, which facilitates infection of target cells through interaction with cellular CD4 plus one of two chemokine receptors, i.e. CCR5 or CXCR4 (with CCR5 used by most HIV-1 primary isolates and most of those that enter the CNS). In addition, gp120 is also released by infected cells and is shed from virions. Gp120 has been detected in the brain of HIV-1-infected individuals, localized to multinucleated giant cells and microglia (Scorziello *et al*. 1998). Exposing monocytes/macrophages to natural and recombinant gp120 induces a broad range of activation responses which include the release of pro-inflammatory cytokines, granulocyte- macrophage colony-stimulating factor (GM-CSF) as well as the production of reactive oxygen species (ROS). Not only does it elicit a cytokine response, but also a chemokine response by stimulating the production of MCP-1, macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and RANTES (Regulated upon Activation Normal T cell Expressed presumably Secreted) in monocytes/macrophages(Louboutin *et al*. 2010). Moreover, monocytes/macrophages populations treated with gp120 present with an increased amount of CD14+CD16+ cells (Zembala *et al*. 1997). From this it is obvious that gp120 does not only play an essential role in mediating target cell infection, but also appears to act as an important monocytes/macrophage activating factor.

The activation of monocytes/macrophages by gp120 seems to be associated with the chemokine receptors that act as viral entry receptors. Apart from their role in viral entry, CCR5 and CXCR4, which are G-protein coupled receptors, are able to activate multiple signalling pathways, and multiple signalling events have been identified in primary human macrophages in response to gp120/chemokine receptor interactions (Scorziello *et al*. 1998). These signalling events include increasing intracellular Ca2+ and activation of protein kinases including the focal adhesion-related kinase Pyk2, members of the mitogen-activated protein kinase (MAPK) family, phosphoinositol-3 (PI-3) kinase, and Lyn kinase (Cheung *et al*. 2008, Corno 2003). Collectively, these mechanisms seem to be responsible for cytokine and chemokine release following gp120/CCR5 interactions, predominantly TNF-α and IL-1β production (Corno 2003). Binding of HIV-1 gp120 to monocyte/macrophage CCR5 triggers PI-3K and Pyk2 re-localization to the membrane and formation of a signalling complex with Lyn. Activation of this complex leads to IL-1β production, possibly via the action of downstream MAPKs and nuclear transcription factors. These gp120-triggered signalling pathways could be to blame for the abnormal production of pro-inflammatory cytokines by monocytes/macrophages, which then contribute to the immunopathogenesis of HAD (Cheung *et al*. 2008). Gp120 from CXCR4-using (X4) strains of HIV-1 can be directly neurotoxic by inducing neuronal apoptosis, mainly through interactions with the chemokine receptor CXCR4, as inhibition of CXCR4 activity blocks gp120-mediated toxicity in rat neurons (Casanova *et al*. 2000).

2.3.4.2 Neurotoxic effects of HIV-1 Tat

An additional HIV-1 protein strongly implicated in HIV-1 neuropathogenesis is Tat, the virally encoded trans-activator of transcription. HIV-1 infected cells in culture release this protein into the culture media, and it is also detectable within the extracellular space and in peripheral circulation of infected individuals, as well as in CSF and the brains of people with HIVE. While it is unclear

whether the majority of Tat protein in the CNS is released by infected cells locally or is transported across the BBB from the periphery, it seems to contribute to HIV-1 neuropathogenesis via numerous mechanisms.

The HIV-1 Tat protein is encoded for by the bi-exonic *tat* gene. The full-length HIV-1 Tat protein consists of 101-amino acid residues, with residues 1–72 encoded by a first exon and residues 73–101 encoded by a second exon (Jeang *et al.*, 1999). This is an important point due to the fact that a truncated, 86 amino acid protein, derived from a few laboratory passaged virus strains (e.g., LAI, HXB2, pNL4-3), has been frequently used and is considered a full-length protein. In fact, more than 90% of the more than 100 independently characterized HIV-1 Tat proteins maintain the 101- (and not the 86-) amino acid configuration (Myers *et al.*, 1996). Nevertheless, the functions of cellular uptake (Vives *et al.*, 1997), trans-activation (Churcher *et al.*, 1993), neurotoxicity (Sabatier *et al.*, 1991), and immune activation (Buonaguro *et al.*, 1992; Li *et al.*,2005) are all present within the first 72 amino acids encoded by the first exon. For this very reason, many studies use the Tat 72 amino acid protein encoded by the first exon of the *tat* gene, or the truncated Tat 86 amino acid protein rather than the 101 amino acid full-length protein. However, the functional properties contained within the second Tat exon are being explored in more detail (Jeang *et al.*, 1999; Lopez-Huertas *et al.*, 2010). Because of its protein transfer domain, it has been shown to be able to cross membranes of uninfected cells, inducing a number of different effects depending on cell type (Ensoli *et al.*, 1993; Magnuson *et al.*, 1995). Tat protein crosses the BBB at a rate comparable to passage of IL-1 and TNF-α, possibly through a mechanism of adsorptive endocytosis (Banks *et al.*, 2005). In addition to influx, brain efflux of Tat was also observed; suggesting that Tat produced on either side of the BBB can impact cellular function within the opposite compartment (Banks *et al.*, 2005).

It has been observed that Tat decreased expression of occludin and zonula occludens (ZO) -1 in the TJs of the BBB (two proteins essential for BBB functional integrity), following Tat injection into

the tail veins of C57BL/6 mice to model Tat secreted from circulating infected cells (Pu *et al.*, 2007). The mechanism of the decreased expression occurred through activation of cyclo-oxygenase (COX)-2, an enzyme induced during inflammation, specifically in response to TNF-α (Pu *et al.*, 2007) and known to be elevated in patients infected with HIV-1.

Treatment with Tat also induces oxidative stress in a number of cell types, including BMECs (Price *et al.*, 2005; Price *et al.*, 2006; Shiu *et al.*, 2007), which is well known to affect TJs (Plateel *et al.*, 1995).

As indicated earlier, circulating monocytes in patients infected with HIV-1 often constitutively express TNF-α, which has been shown to induce endothelial activation, oxidative stress, and the activation of multiple pro-inflammatory factors, such as p38 MAPK, Nuclear Factor (NF) – kappa B (κB), COX-2, and prostaglandin E2 (Wang *et al.*, 2008). Further investigation into the mechanisms associated with the impact of TNF-α on endothelial cells has revealed the necessity for caveolin-1 (Wang *et al.*, 2008), a scaffolding protein that is a major component of caveolae and necessary for the formation of caveolae in endothelial cells. Caveolae are specialized lipid rafts where many signalling molecules localize, and they are especially abundant on endothelial cells. Proteins involved in pro-inflammatory signal transduction, such as activators of the extracellular-signal-regulated kinase (ERK) pathway and inhibitor of κB, the upstream regulator of NF-κB, and elements of the Ras signaling cascade are localized to caveolae. Tat activates Ras signaling in BMECs and decreases the amount of occludin, ZO-1, and ZO-2 in caveolae (Zhong *et al.*, 2008). The Tat-induced decrease in these TJ proteins was abolished with loss of caveolin-1 (Zhong *et al.*, 2008). Importantly, these studies demonstrated a possible synergy between Tat and host pro-inflammatory factors while both molecules are circulating throughout HIV-1 infection, with respect to the down-regulation of TJ proteins through similar mechanisms ultimately leading to disruption of the BBB.

As indicated above, the migration of HIV-1-infected monocytes to the BBB increases during the course of HIV disease; additionally, Tat acts as a chemo-attractant for monocytes (Albini *et al*. 1998). Based on these observations, it has been proposed that infected monocytes in circulation accumulate at the BBB and secrete Tat, thereby recruiting more monocytes to the site possibly leading to increased Tat secretion if these newly recruited monocytes are also productively infected. The question remaining centers on whether there is sufficient Tat present at the BBB to induce changes in permeability. Most of the previous studies have shown dose-dependent results with increasing concentrations of Tat, including 5, 10, and 25 μg of Tat in 100 μL phosphate-buffered saline (Pu *et al.*, 2005), 2–100 nM (Zhong *et al.*, 2008), or 500 ng/mL (Ju *et al.*, 2009). These results demonstrate that the impact of Tat is concentration-dependent, but it remains questionable whether the threshold concentration to achieve a biological affect *in vitro* is reached in circulation during the course of a natural HIV-1 infection. In this regard, experiments involving the injection of Tat into circulation through the mouse tail vein have not yielded information concerning the concentration of Tat reaching the BBB, although additional evidence has indicated that pathological concentrations of Tat in patients infected with HIV-1 can reach the range of nanograms per milliliter of serum (Xiao *et al.*, 2000).

Tat has been extensively studied for toxic effects on CNS cells, often resulting in apoptosis, especially of the neurons (Giacca 2005). The toxic effect of Tat on neurons is, in part, a result of the cytokines, chemokines, and nitric oxide (NO) released by microglia. In addition, Tat appears to activate the neuronal excitatory N-methyl-D-aspartate (NMDA) receptor, leading to excitotoxicity and consequent apoptosis (Eugenin *et al*. 2007). Astrocytes and endothelial cells are also susceptible to Tat-induced inflammatory responses, thereby further augmenting infiltration of monocytes/macrophages into the brain. Finally, Tat may also contribute to the disruption of the BBB by altering the distribution of endothelial cell-tight junction proteins such as claudin-1, claudin-5, zonula ocludens (ZO) 1 and 2, which may lead to enhanced transmigration of monocytes and lymphocytes (Strazza *et al*. 2011).

2.3.4.3 Neurotoxic effects of the HIV-1 protein Vpr

Vpr is a 96 amino acid HIV-1-encoded virion-incorporated protein that is essential for HIV-1 replication in macrophages. Vpr is present in detectable amounts in both the basal ganglia and frontal cortex, mainly in the macrophages and neurons of HIVE patients (Dale & Wheeler 2005). Soluble HIV-1 Vpr protein is also detected in the CSF and serum of infected patients with neurological disorders. Vpr treatment of glial cells induces the secretion of cellular neurotoxins Furthermore, expression of HIV-1 Vpr in mouse brain monocytoid cells results in abnormalities in motor tasks and neuronal injury (Jones *et al*. 2007). Treatment of neurons with soluble Vpr leads to changes in neuronal membrane potentials, with the induction of apoptosis. These observations suggest that Vpr in the CNS may play a role in neuronal injury, including both direct cytotoxic actions on neurons but also activate glia resulting in the release of neurotoxic molecules (Guha *et al*. 2012).

2.3.4.4 Indirect neurotoxic effects of HIV-1

HIV-1 does not infect neurons directly, and the end result of neurotoxicity is brought about by factors released by activated and infected monocytes/macrophages. The two components responsible appear to be HIV-1 encoded proteins derived from infected monocytes/macrophages that themselves injure neurons directly, and cellular products released by the activated monocytes/macrophages (both of which also serve to amplify the cascade of monocytes/macrophages activation, discussed above).

Multiple pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6, GM-CSF, and macrophage colony-stimulating factor (M-CSF) are elevated in the CNS and/or CSF of HAD patients. Increased expression of these pro-inflammatory cytokines can be a result of direct viral infection or an effect of shed viral proteins which stimulate uninfected mononuclear phagocytes to express elevated levels of cytokines. Once initiated, many of these cytokines can act in an autocrine or paracrine manner and augment the expression leading to a pro-inflammatory environment in the CNS. In

addition, several of these have direct or indirect neurotoxic properties and appear to also contribute to neuronal injury (Tasca *et al*. 2012).

TNF-α is elevated both in the brain and CSF in HAD patients. Monocyte/macrophage exposure to gp120 or Tat results in elevated TNF-α expression. TNF-α damages the BBB and also induces the expression of the adhesion molecules ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and E-selectin on astrocytes and endothelial cells, resulting in HIV-1-infected M/M transmigration into the CNS. Additionally, TNF-α upregulates the expression and release of various chemokines in the CNS such as MCP-1(Lee *et al*. 2011). By increasing the BBB permeability and inducing adhesion molecule and chemokine expression, TNF-α plays an important role in facilitating the entry of HIV-infected cells into the brain. TNF-α also has toxic effects on human neurons. It causes the over-stimulation of the glutamate receptors such as the NMDA receptors which are expressed on neurons (Campbell *et al*. 2011). Along with SDF-1α, TNF-α also increases release of the excitotoxic neurotransmitter glutamate from astrocytes and microglia. HIV-1-infected macrophages are a source of extracellular glutamate, and glutamate concentrations in the CSF of HIV-1-infected patients are higher as compared to uninfected controls. Production of excess glutamate by HIV-infected macrophages in HAD may contribute to neuronal cell death. TNF-α also inhibits glutamate uptake by astrocytes, resulting in increased extracellular levels. These multiple effects of TNF-α cause over-stimulation of the NMDA receptor, resulting in Ca2+ mobilization , formation of NO, and superoxide toxicity.. Another cytokine that appears to have a central role in both the inflammation and neurotoxicity associated with HAD is IL-1β. In the CSF and brains of HIV-1-infected patients with HAD, IL-1β is significantly increased compared to non-demented HIV-1-infected patients. IL-1β shares many neurotoxic properties with TNF-α. Like TNF-α, IL-1β induces the expression of ICAM-1, VCAM-1, and E-selectin on endothelial cells (McHale *et al*. 1999) and astrocytes, facilitating monocyte infiltration into the CNS. IL-1β induces the release of L-cysteine from macrophages and microglia.

2.4 Summary

Much focus has been placed on teasing out the mechanism of action, in the context of HIV-1-associated neuroinflammation, of the HIV-1 encoded proteins, i.e. Tat, gp120 and Nef proteins. Although findings from these studies have provided greater insight into the molecular mechanisms involved in HIV-1 associated neuroinflammation, they are not necessarily disease relevant. This is due to the fact that, in an *in vivo* infection scenario, these HIV-1 encoded proteins may act in synergy to induce a greater inflammatory response than stimulation with an individual protein could achieve. Thus it is of the utmost importance to select the most disease representative model of HIV-1 infection when looking to assess the neuroinflammatory events associated with infection. Therefore it would be advisable to either employ an infectious model using HIV-1 infected cultures, or use alternative non-infectious methods. One such method is the use of the HL2/3 cell line, which is a HeLa derived cell line that has been stably transfected with a clone of the HXB2 strain that lacks the reverse transcriptase coding sequence. This consequently results in high level production of the HIV-1 Gag, Env, Tat, Rev and Nef proteins but no shedding of infectious viral particles. For this very reason the HL2/3 cell line is an ideal research tool for those wanting to investigate the inflammatory effects of this greater repertoire of viral proteins, but are limited by a lack of high bio-safety level infrastructure needed to conduct infectious work.

2.5 Hypothesis

S. frutescens and PCO may be able to modulate HIV-1-associated neuroinflammatory processes *in vitro*

2.6 Aims

We aimed to investigate the potential of grape seed-derived polyphenols and extract from *Sutherlandia frutescens subsp. microphylla* to limit or delay the progression of HIV-associated neuroinflammation in an *in vitro* simulated BBB, via limiting inflammatory responses to viral

proteins. Specifically, these treatments are investigated in the context of a preventative, rather than curative model, since both extracts are currently consumed as daily supplements.

2.6.1   Specific objectives:

2.6.1.1 To assess effects of grape seed extract and *Sutherlandia* extract on mono cultures of primary human astrocytes, primary human monocytes and human umbilical vein-derived endothelial cells (HUVECs) in the presence and absence of HIV viral proteins.

2.6.1.2 To simulate the blood-brain-barrier by co-culture of primary astrocytes and HUVECs, to assess anti-inflammatory effects of *Sutherlandia* and grape seed extracts the presence and absence of HIV viral proteins

CHAPTER 3

SUTHERLANDIA FRUTESCENS MAY EXACERBATE HIV-ASSOCIATED NEUROINFLAMMATION

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(This chapter is presented in the prescribed format required by the Journal of Neuroimmunology. The manuscript is currently under review.)

3.1 ABSTRACT

Background: Neuroinflammation is central to the aetiology of HIV-associated neurocognitive disorders (HAND) that are prevalent in late stage AIDS. ARV treatments are rolled out relatively late in the context of neuroinflammatory changes, so that their usefulness in directly preventing HAND is probably limited. It is common practice for HIV+ individuals in developing countries to make use of traditional medicines. One such medicine is *Sutherlandia frutescens* - commonly consumed as a water infusion. Here its efficacy as an anti-inflammatory modality in this context was investigated in an *in vitro* co-culture model of the blood-brain barrier (BBB).

Methods: Single cultures of human astrocytes, HUVECs and primary human monocytes, as well as co-cultures (BBB), were stimulated with HIV-1 subtype B & C Tat protein and/or HL2/3 cell secretory proteins after pre-treatment with *S. frutescens* extract. Effects of this pre-treatment on pro-inflammatory cytokine secretion and monocyte migration across the BBB were assessed.

Results: In accordance with others, B Tat was more pro-inflammatory than C Tat, validating our model. *S. frutescens* decreased IL-1β secretion significantly (P<0.0001), but exacerbated both monocyte chemoattractant protein-1 (P<0001) – a major role player in HIV-associated neuroinflammation – and CD14+ monocyte infiltration across the BBB (P<0.01).

Conclusions: Current data illustrates that the combined use of HL2/3 cells and the simulated BBB presents an accurate, disease relevant *in vitro* model with which to study neuroinflammation in the context of HIV/AIDS. In addition, our results caution against the use of *S. frutescens* as anti-inflammatory modality at any stage post-HIV infection.

Keywords: blood-brain barrier, HL2/3, NLRP3 inflammasome, monocyte migration, astrocyte, HUVEC

3.2 INTRODUCTION

Neuroinflammation in the context of HIV/AIDS is known to have onset soon after infection with the virus and is associated with the HIV-associated neurocognitive disorders (HAND) that are prevalent in late stage AIDS (Yao & Bethel-brown 2010). Conventional anti-retroviral treatments are rolled out relatively late in the context of neuroinflammatory changes, so that their usefulness in directly preventing HAND is probably limited. A recent multi-centre study in more than 800 HIV+ patients (Heaton *et al.* 2011) reported that high rates of neurocognitive impairment persist at all stages of HIV infection, despite modern anti-retroviral (ARV) treatment and immune reconstitution regimes. Furthermore, in the same study, neurocognitive impairment was consistently associated with lowest CD4 counts. From this, it is clear that early prevention strategies to limit the extent of neuroinflammation, is required to positively influence the longer-term prognosis in terms of not only HAND, but also disease progression.

Traditional medicine is commonly used by those living with HIV infection, particularly in developing countries. One such complementary medicine that is widely used is *Sutherlandia frutescens* – a herb that is commonly consumed in the form of a tea. Several beneficial effects relevant to HIV/AIDS have been reported for this herbal remedy. Firstly, it was shown to directly inhibit activity of HIV-target enzymes (Harnett *et al.* 2005). Secondly, the use of *S. frutescens* has been associated with benefits in the peripheral compartment - decreased psychological stress levels (Smith & Myburgh 2004; Prevoo *et al.* 2004) and preservation of skeletal muscle mass (Engelbrecht *et al.* 2010) suggest a less catabolic state and maintenance of overall body strength. Thirdly, central uptake and effect was reported in separate studies reporting anxiolytic (Smith & van Vuuren 2014) and anti-convulsant (Ojewole 2008) effects of *S. frutescens* via modulation of GABAergic neurotransmission.

As a result of these promising data, the use of *S. frutescens* in the context of HIV/AIDS is currently endorsed by the Ministries of Health of several African nations (Mills *et al.* 2005). Even though *S. frutescens* was recently implicated in herb-drug interactions which may lead to

therapeutic failure and/or increased drug toxicity in the context of HIV ARVs specifically (Fasinu *et al.* 2013; Prevoo *et al.* 2008; Minocha *et al.* 2011; Müller *et al.* 2013) clinic staff continue to recommend its use to HIV+ patients still waiting for ARV roll out, for management of secondary symptoms of HIV/AIDS in otherwise untreated patients (personal communications to CS).

From the literature reviewed, *S.frutescens* is known to be absorbed and have central activity, but its potential role in modulation of neuroinflammation has not been assessed. This fact, together with the fact that it is currently being used widely in the time frame of disease progression where treatments for neuroinflammation should be applied, warrants investigation into the potential of *S. frutescens* as anti-inflammatory modality in the context of HIV-associated neuroinflammation. Therefore, the effect of *S. frutescens* treatment on monocyte migration across a simulated blood-brain barrier was assessed *in vitro*. In addition, effects on the secretion of inflammatory modulators by various cell types were investigate

3.3 METHODS

3.3.1 Cell culture

Single cultures of primary human cerebrocortical astrocytes (Sciencell, USA) and Human Umbilical Vein Endothelial Cells (HUVECs) (Lonza, Germany) were maintained at 37°C in a humidified 5% CO_2 in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies Corp., USA) supplemented with 10% Fetal Calf Serum (FCS) (Biochrom, Germany)and 1% N2 Supplement (Life Technologies Corp., USA) and complete Endothelial Growth Medium (EGM) (Lonza, Germany) respectively. HL2/3 cells (obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HL2/3 from Dr. Barbara K. Felber and Dr. George N. Pavlakis), HeLa derived cells producing high levels of Gag, Env, Tat, Rev and Nef proteins, were maintained at the conditions mentioned above in high glucose DMEM (Life

Technologies Corp., USA) supplemented with 10% FCS (Biochrom, Germany). Cells were routinely sub-cultured before reaching confluency.

Cell numbers were determined using a haemocytometer following trypsinization and trypan blue staining. For 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assays, human astrocytes and HUVECs were seeded in 6 well cell culture plates (500000 cells/well). For all other single culture experiments all cell types were seeded in 6 well cell culture dishes at the aforementioned cell density.

In order to simulate the blood-brain barrier, co-cultures of human astrocytes and HUVECs were established on opposite sides of fibronectin (BD Biosciences, USA) coated 3 µm pore size tissue culture inserts (BD Biosciences, USA) (Eugenin & Berman 2003) .

All cell culture experiments were done in triplicate and repeated a mimimum of three times.

3.3.2 Preparation of *S. frutescens* aqueous extract

Sutherlandia frutescens plants were cultivated from seed, taxonomically verified as *S. frutescens SU1*, harvested (well after flowering and seeding phase) and dry milled (leaves and stems only) by Parceval Pharmaceuticals Pty Ltd (Wellington, South Africa) using proprietary procedures. A warm water extract of dry-milled *S. frutescens* (moisture content 16.41%) was prepared in boiling distilled water (25 mg/ml) using methods previously described for *in vivo* treatment (Smith & van Vuuren 2014; Smith & Myburgh 2004) and then sterile filtered using filter pore size 0.22 µm.

3.3.3 *S. frutescens* dose response cell viability assay

In order to determine the highest dose of *S. frutescens* tolerated with the least amount of cell death, human astrocytes, HUVECs and primary human monocytes were incubated with 50, 500 and 5000µg/ml *S. frutescens* extract for 24 hours.

Cell viability was assessed using a modified version of the MTT assay described by Gomez and colleagues (Gomez *et al.* 1997). The assay is based upon the principle of reduction of MTT into

blue formazan pigments by viable mitochondria in healthy cells. At the end of the experiment, the medium was removed from the 6 well plates and the cells washed twice with PBS. MTT (0.01 g/ml) was dissolved in PBS, and 500 µl was added to each well dish. Cells were subsequently incubated for 1 h at 37°C in an atmosphere of 5% CO_2. After the incubation period, cells were washed twice with PBS, and one ml of HCl–isopropanol–Triton (1% HCl in isopropanol; 0.1% Triton X- 100; 50:1) was added to each well and gently agitated for 5 min. This lysed the cell membranes and liberated the formazan pigments. The suspension was then centrifuged at 131 x g for 2 min. The optical density (OD) was determined spectrophotometrically at a wavelength of 540nm and the values expressed as percentages of control.

3.3.4 Full length HIV-1 subtype B & C Tat protein stimulation

Full length synthetic Tat proteins were kindly provided by Professor Ranga Udaykumar of the Jawaharlal Nehru Centre for Advanced Scientific Research (Bangalore, India) and were synthesized and purified as previously described (Siddappa *et al.* 2006). Tat proteins were reconstituted and subsequently diluted in Tris-Cl buffer (20mM, pH8) supplemented with 1 mM DTT.

Human astrocytes, HUVECs, primary human monocytes as well as simulated BBB co-cultures were stimulated with either protein (10 ng/ml) for 2.5h and 24h, after which culture media was collected and stored at -80°C for further downstream analyses. In order to test the efficacy of *S. frutescens* as a modulator of neuroinflammatory processes, cells were pre-treated for 4h and 24h respectively prior to HIV-1 Tat protein stimulation. Following stimulation culture supernatants were collected and stored at -80°C for further downstream analyses.

3.3.5 HL2/3 Cells – A more representative *in vitro* model of HIV-1 infection

As previously mentioned, HL2/3 cells produce and secrete high levels of most HIV-1 proteins into their culture media, and for this reason it was decided to co-culture these cells with the simulated BBB cultures in order to mimic the neuroinflammatory milieu at the interface between

the infected central nervous system (represented by the HL2/3 cells seeded into the wells of a 24 well culture plate into which the tissue culture inserts, on which the simulated BBB has been constructed, are placed) and the neurovasculature (i.e. the BBB represented by the *in vitro* simulated BBB cultures). Firstly, to assess the effect of the HL2/3 derived HIV-1 proteins on the individual cell types used to construct the *in vitro* BBB, HL2/3 cells were seeded into 6 well plates at 200 000 cells per well and allowed to adhere to the culture surface. Once the HL2/3 cells had adhered, culture media was replaced. HL2/3 conditioned media was collected at 2.5h and 24h from separate cultures, and this media was then used to stimulate human astrocytes, HUVECs and primary human monocytes for either 2.5h or 24h. Also, as in the aforementioned section outlining the HIV-1 Tat experiments, cells were pre-treated with *S. frutescens* for either 4h or 24h prior to stimulation. Culture supernatants were collected post stimulation and stored at -80°C for further downstream analyses.

The aforementioned experiment was repeated in the co-culture system, with the omission of the 24h time point. HL2/3 cells were seeded into 24 well plates at 50 000 cells/well and allowed to adhere. Culture media was refreshed after which the BBB co-cultures were transferred to the wells containing the HL2/3 cells. BBB co-cultures were exposed to the HL2/3 cells for a period of 2.5h, after which culture supernatants were collected and stored at -80°C for further downstream analyses. Additional BBB co-cultures were treated with *S. frutescens* for 4h prior to stimulation.

3.3.6 Pro-inflammatory cytokine & chemokine analysis

Monocyte chemoattractant protein-1 (MCP-1), a key role player in HIV-1-associated neuroinflammation, was measured in all supernatants by a conventional ELISA kit (Biolegend, San Diego, CA), used according to the manufacturer's instructions.

IL-1β was measured in all co-culture supernatants by AlphaLISA (PerkinElmer, Waltham, MA), according to the manufacturer's instructions.

3.3.7 Monocyte/macrophage transmigration

Transmigration of both infected and uninfected inflammatory cells, in particular those from the monocyte macrophage lineage, play a major role in the aetiology of HIV-1-associated neuroinflammation. For this reason monocyte transmigration was assessed in the BBB co-cultures by adding primary human monocytes to the top of the insert, allowing the cells to migrate in response to the various stimuli for 2.5h, after which the BBB inserts and cells in the bottom of the well were fixed in 4% paraformaldehyde and stained with a FITC-anti-human CD14 antibody (Biolegend, San Diego, CA). CD14 is a specific marker of cells from the monocyte/macrophage lineage. All CD14+ monocytes on top of the entire insert (unmigrated) and on the bottom of the culture well (migrated) were counted using a fluorescent microscope (Leica, Germany). Cells in suspension were not quantified, since we have previously shown that cell counts in these compartments are independent of interventions/treatments.(Kruger *et al.* 2014)

3.3.8 Statistical analysis

All statistical analyses were performed using Graphpad Prism Version 5 software (Graphpad Software, La Jolla, CA, USA). Results are expressed as mean ± SD. One- or two- way analysis of variance (ANOVA) as relevant, followed by a Bonferroni *post hoc* test, was used to assess differences between experimental groups and/or time points. Differences were considered to be of statistical significance when *P* value < 0.05.

<div align="center">

3.4 RESULTS

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3.4.1 *S. frutescens* dose-dependent cell viability

Due to the fact that *S. frutescens* had not, up until this point, been tested on the cell types used in this study, it was important to first establish the optimal dose for use *in vitro*, prior to assessment of *S. frutescens* as an effective anti-inflammatory modality. We defined this optimal experimental dose as the highest dosage which does not result in a significant reduction in cell viability, represented by % MTT reductive capacity, Across all three cell types, a marked,

statistically significant reduction in % MTT reductive capacity was observed in the group treated with 5000μg/ml *S. frutescens* at all time points (P<0.0001 when compared to control; see graphs, Supplemental Content 1). No statistically significant changes in cell viability were observed for any of the other treatment doses at any time point, and thus the highest of these - 500μg/ml - was selected as the optimal experimental dose.

3.4.2 *S. frutescens* does not have desirable effects on HIV-1 protein-induced MCP-1 response

MCP-1 responses were evaluated in human astrocytes, HUVEC, primary human monocyte and BBB co-cultures following stimulation with HIV-1 subtype B & C Tat protein, and also HL2/3 conditioned media in the case of astrocytes, HUVECs and monocytes, and co-culturing BBB cultures with HL2/3 cells. The potential of *S. frutescens* as a modulator of inflammation was evaluated by pre-treating cells with *S. frutescens* prior to introduction of the inflammatory stimulus.

In the absence of HIV-associated proteins, control astrocytes secreted low basal levels of MCP-1 at 6.5 hours, but no MCP-1 was detectable after 24 hours in culture (Fig.3.1a). After exposure to either B Tat or HL2/3 cell products, MCP-1 secretion increased significantly over time up to the 24 hour point (all P<0.0001 when compared to control). As expected, C Tat elicited no MCP-1 response. Pre-treatment with *S. frutescens* in absence of HIV proteins seemed to reduce basal secretion of MCP-1. However, it exacerbated the response to B Tat in stimulated cells at both 6.5 and 24h (all P<0.0001 when compared to control) and failed to improve the response to HL2/3 cell products.

In HUVECs (Fig.3.1b), basal MCP-1 secretion followed a similar pattern to that seen in astrocytes at 6.5 hours, with the exception of C Tat, which also elicited a basal response in this cell type (P<0.0001 when compared to control). In contrast to astrocytes, this basal secretion was maintained and even relatively enhanced at 24 hours. In the absence of HIV proteins, HUVECs responded similarly to astrocytes when *S. frutescens* pre-treated, showing smaller increases in basal MCP-1 levels. In the presence of all HIV protein stimuli employed, MCP-1 secretion increased

continuously up to 24 hours. Although *S. frutescens* did not exacerbate the response in this cell type, the *S. frutescens*-associated inhibition of the MCP-1 response seen in non-HIV conditions, was insufficient to restore the response after exposure to HIV proteins at 24 hours to control levels.

When repeating the intervention protocols in a co-culture simulation of the BBB (consisting of astrocytes, HUVECs and monocytes, Fig. 3.1c), the net effect of *S. frutescens* that may be expected in an *in vivo* situation becomes more evident. Pre-treatment with *S. frutescens* had no beneficial effect on the HIV Tat (both B and C) associated MCP-1 response and exacerbated the HL2/3-induced response (P<0.0001). (Monocyte cultures did not secrete detectable levels of MCP-1 under any of the experimental conditions, so most probably did not contribute significantly to this outcome.) These results suggested that HIV Tat proteins are not a therapeutic target of *S. frutescens*. Also, treatment with HL2/3 conditioned media or co-culture with these cells (which contain Tat as well as other HIV proteins) resulted in the most pronounced inflammatory response. Therefore, HL2/3 cells were selected as pro-inflammatory stimulus for all further experiments. Additionally, the fact that these cells secrete a greater repertoire of HIV-1 proteins makes them a more disease representative model of infection.
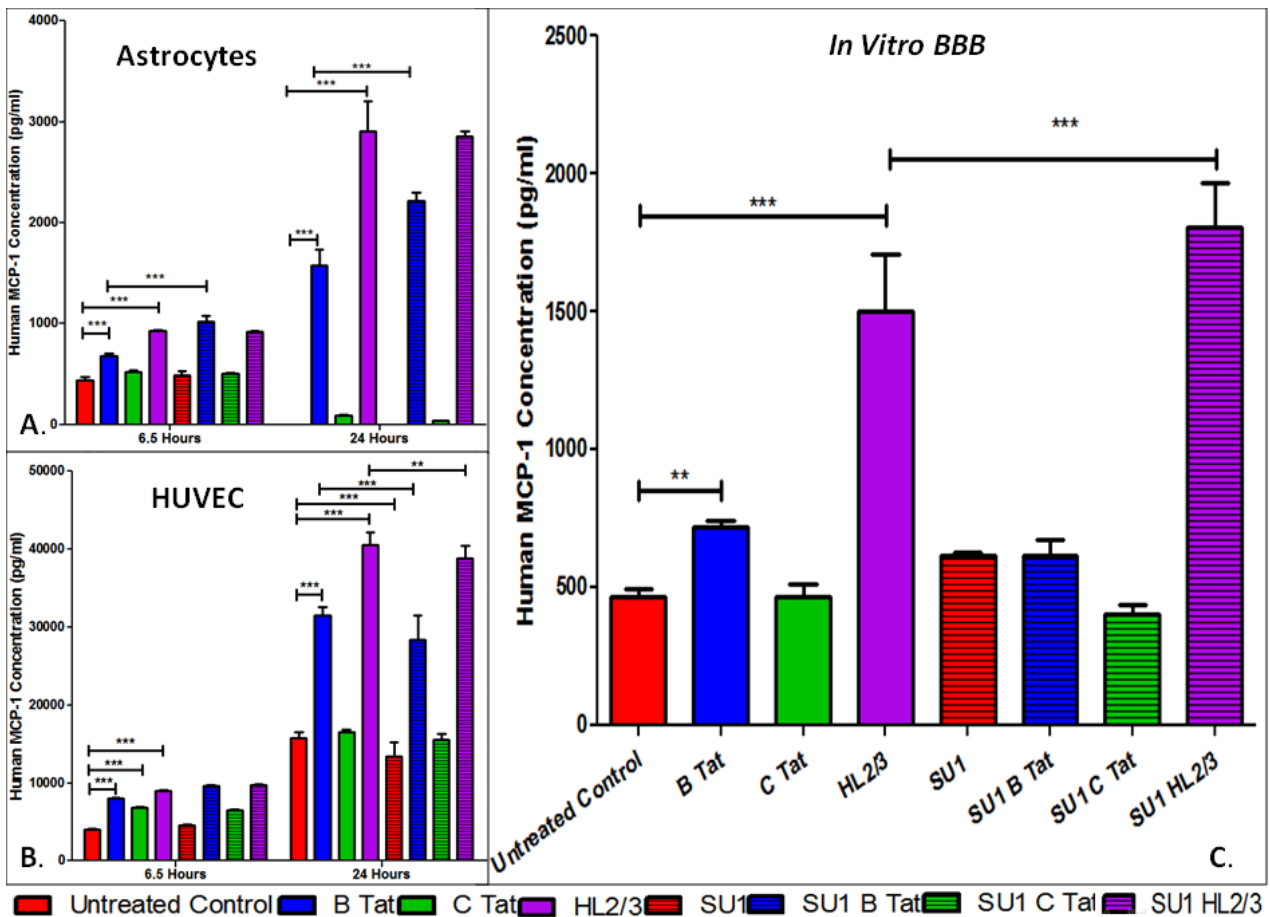
Figure 3.1. MCP-1 response to HIV-1 proteins in human astrocytes (1a), HUVECs (1b) and BBB co-cultures (1c), with or without pre-treatment with *S.frutescens* extract.

*** = P < 0.0001; ** = P < 0.001; * = P < 0.05

3.4.3 *S. frutescens* attenuates the HL2/3 induced IL-1β response in *in vitro* BBB cultures

IL-1β levels were non-detectable in single cultures of HL2/3 cells (data not shown), so that any IL-1β detected originated from the BBB. IL-1β secretion was evaluated in BBB cultures stimulated by co-culture with HL2/3 cells. Pre-treatment of the BBB cultures with *S. frutescens* was able to effectively inhibit the IL-1β response following co-culture with HL2/3 cells, so that the response was similar to basal secretion levels, both of which did not reach the lower threshold for detection (Fig.3.2).
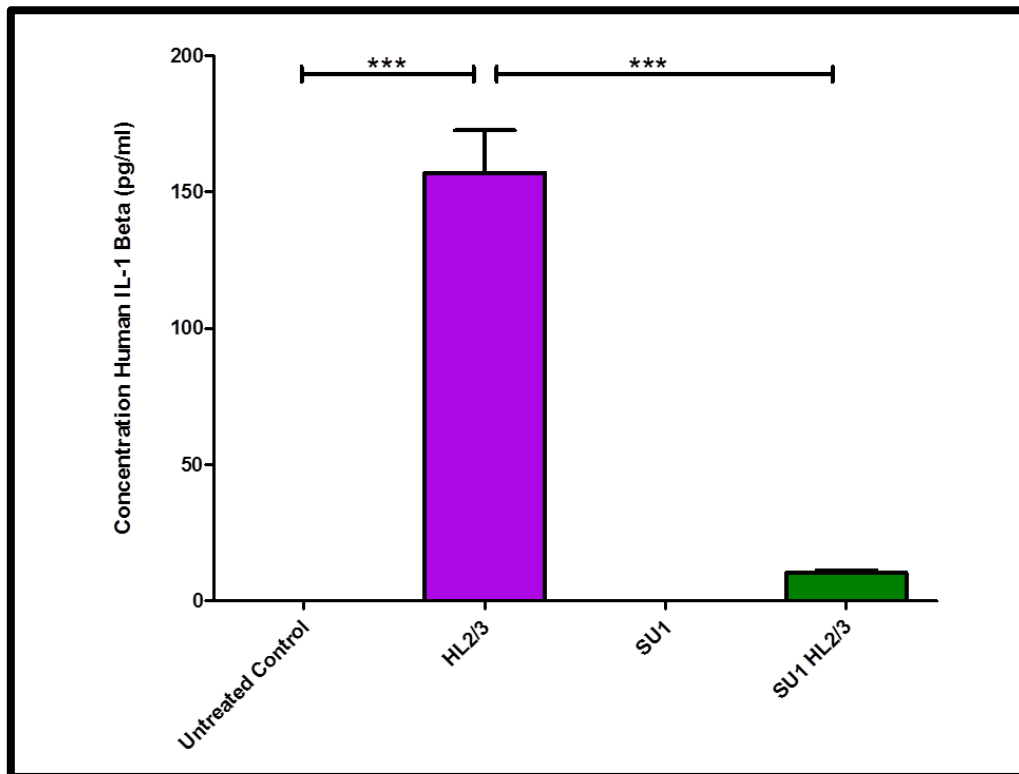
Figure 3. 2. IL-1β response to co-culture exposure of BBB co-cultures to HL2/3 cells, in the presence or absence of *S.frutescens* extract.

*** = P < 0.0001; ** = P < 0.001; * = P < 0.05

3.4.4 *S. frutescens* increases HL2/3 induced CD14+ monocyte transmigration across BBB

Migration of primary human monocytes across the BBB was assessed, as well as the role of *S. frutescens* as modulator of this process. The number of monocytes remaining on top of the transwell filter insert (containing BBB) was named unmigrated cells, while those collecting in the bottom of the well are referred to as the migrated cells. Representative images of immunocytochemistry used to visualise monocytes for the purpose of quantification, are presented in Fig. 3.3 and illustrate the marked differences in CD14+ monocyte counts between the experimental groups.
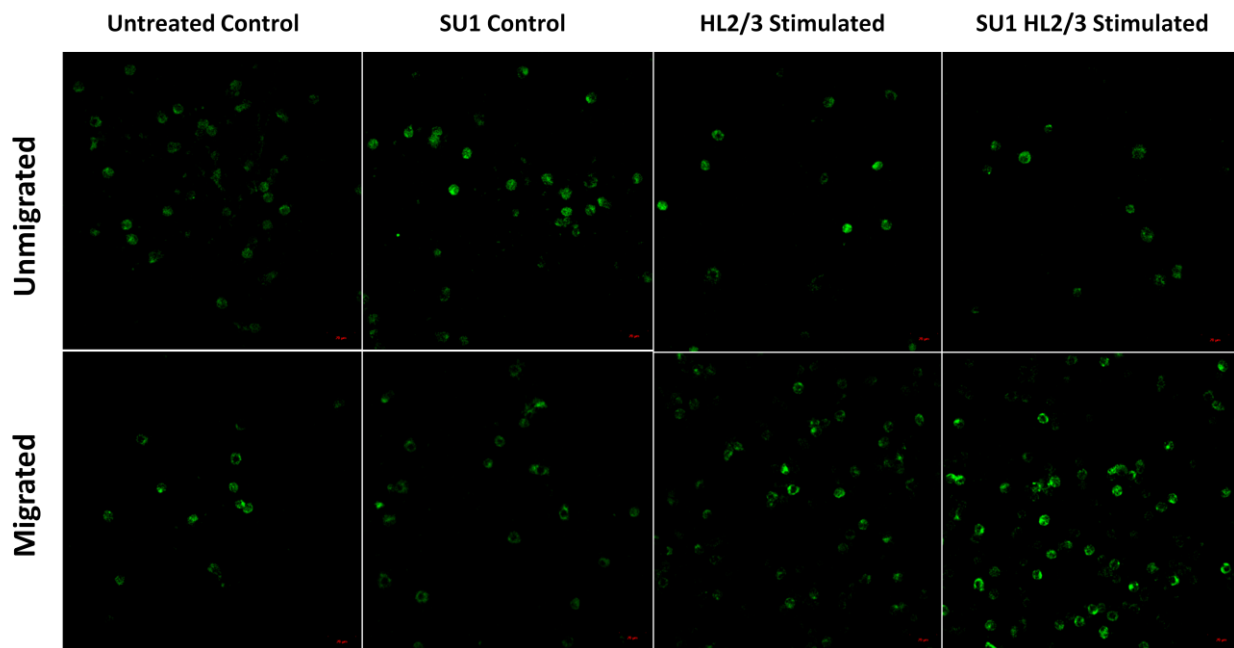
Figure 3.3. Representative images indicating the effect of *S.frutescens* extract on migration capacity of CD14+ (FITC) primary human monocytes across a simulated BBB. Magnification: 40x objective

Numerical data are presented in Fig. 3.4. As anticipated, HL2/3 stimulation resulted in a significant increase in monocyte migration across the *in vitro* BBB (ANOVA main effect P<0.0001). Pre-treatment with *S. frutescens* had no effect on migration in absence of HIV proteins, but exacerbated the monocyte migration capacity in response to HL2/3 stimulation significantly
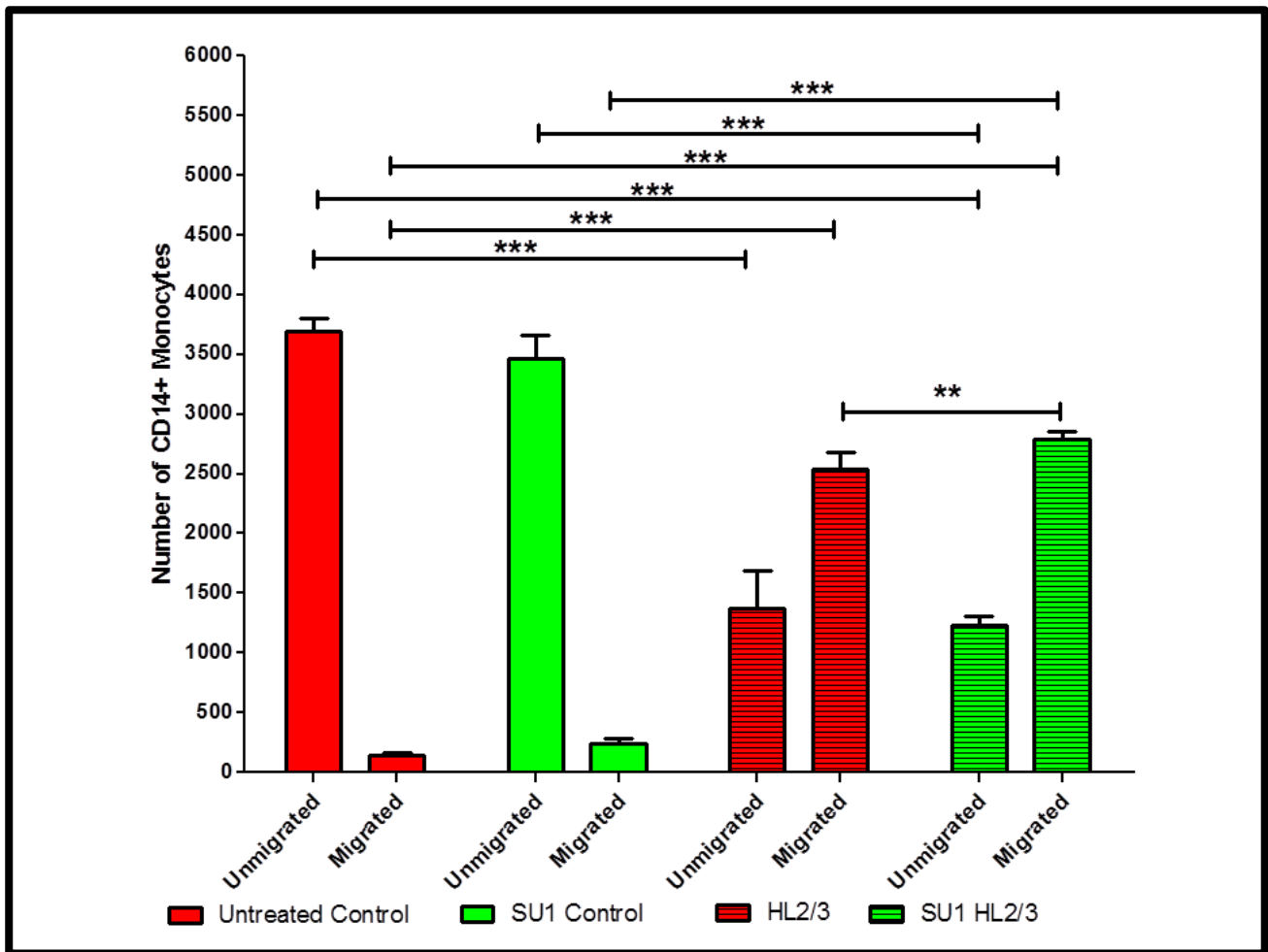
Figure 3.4. Effects of *S.frutescens* extract pre-treatment on monocyte migration across an in vitro BBB.

*** = P < 0.0001; ** = P < 0.001; * = P < 0.05

3.5 DISCUSSION

Neuroinflammation is central to the aetiology, progression and prognosis of neurocognitive disorders associated with HIV infection. In addressing this problem, it is of paramount importance to not only search for potential therapeutic modalities, but also to invest in development of the best investigative models with which to evaluate these potential therapies. We believe that our data, presented here, contribute significantly to the advancement on both these fronts. Firstly, a novel aspect of our study is the use of HL2/3 cells to simulate in a more disease relevant manner than other non-infectious methods commonly employed, conditions following HIV 1 subtype B

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infection. Furthermore, to our knowledge this is the first study employing this model to test the efficacy of a complimentary medicine that is currently recommended for use in a HIV population at risk of neuroinflammation.

In terms of the model, HL2/3 cells are mostly used in research focused on studying viral fusion mechanisms (Wexler-Cohen & Shai 2007). Here, we have used this cell type in a novel application, utilising its high-level production of a variety of HIV-associated proteins, including Gag, Env, Tat, Rev and Nef, to stimulate neuroinflammation *in vitro*. The simulated BBB co-culture, originally used for testing chemokine and monocyte migration responses to HIV-1, (Weiss *et al.* 1999) was used here in a broader application to investigate efficacy of a natural extract. Proof of the accuracy of the model is the fact that data generated from the HIV-1 subtype B & C Tat stimulation experiments – showing that subtype B is more inflammatory than subtype C – are congruent with previous reports in the literature (Mishra *et al.* 2007). The combined use of the BBB model and HL2/3 cells substantially improved the outcome of our investigation. For example, results suggesting that *S. frutescens* aggravates the MCP-1 response is independent of Tat, may have been missed using stimulation with single proteins alone. We confidently recommend the use of this model to evaluate modulatory effects of various compounds/drugs/medicines which may have the potential to modulate HIV-1 induced inflammatory processes within and surrounding the neurovasculature.

Turning our attention to the complementary medicine evaluated, at first glance results may seem contradictory, with both HIV protein-stimulated MCP-1 production and monocyte migration suggesting a pro-inflammatory effect of *S. frutescens*, while the decreased IL-1β levels after pre-treatment seems to argue against this interpretation. However, these results may be explained by delineating the signalling pathways involved in pro-inflammatory cytokine production, which are affected by the plant. A simplified signalling pathway is presented in Fig. 5, illustrating three main avenues by which the production of MCP-1 and IL-1β are affected.

HIV-1 and its associated proteins set off an inflammatory cascade, activating all three these signal transduction pathways, one via direct activation of the NLRP3 inflammasome, (Walsh *et al.* 2014) and the other two by activation of either of ERK1/2 or JNK (Yao *et al.* 2010). *S. frutescens* has been the subject of research for a number of years due to its claimed effectiveness against cancer, stress and cachexia – this research elucidated some signalling targets of the plant relevant to the current study. For example, *S. frutescens* has been reported to inhibit activation of ERK1/2 (Lei *et al.* 2014. Jiang *et al.* 2014. Kundu *et al.* 2005) and p38 MAPK (Engelbrecht *et al.* 2010), albeit in non-HIV models. In the context of our data, inhibition of the ERK1/2-activated Egr-1 and p38MAPK pathways did not eliminate signalling to achieve MCP-1 production, which was increased after *S. frutescens* pre-treatment, suggesting a relative up-regulation of the activation of these pathways via JNK. The fact that this upregulated MCP-1 response did not translate to increased IL-1β levels, suggests that *S. frutescens* may also inhibit the NLRP3 inflammasome, which is the predominant pathway responsible for conversion of pro-IL-1β to IL-1β by caspase-1 (also called IL-1 converting enzyme) (Schroder *et al.* 2010). The latter may also explain the anti-inflammatory function of *S. frutescens* reported here under non-HIV basal conditions. This suggests that while *S. frutescens* may well have an anti-inflammatory effect useful under basal conditions, its use should be avoided in the presence of HIV infection. This interpretation of the signalling targets of *S. frutescens* is supported by our data showing increased monocyte infiltration across the simulated BBB after *S. frutescens* pre-treatment.
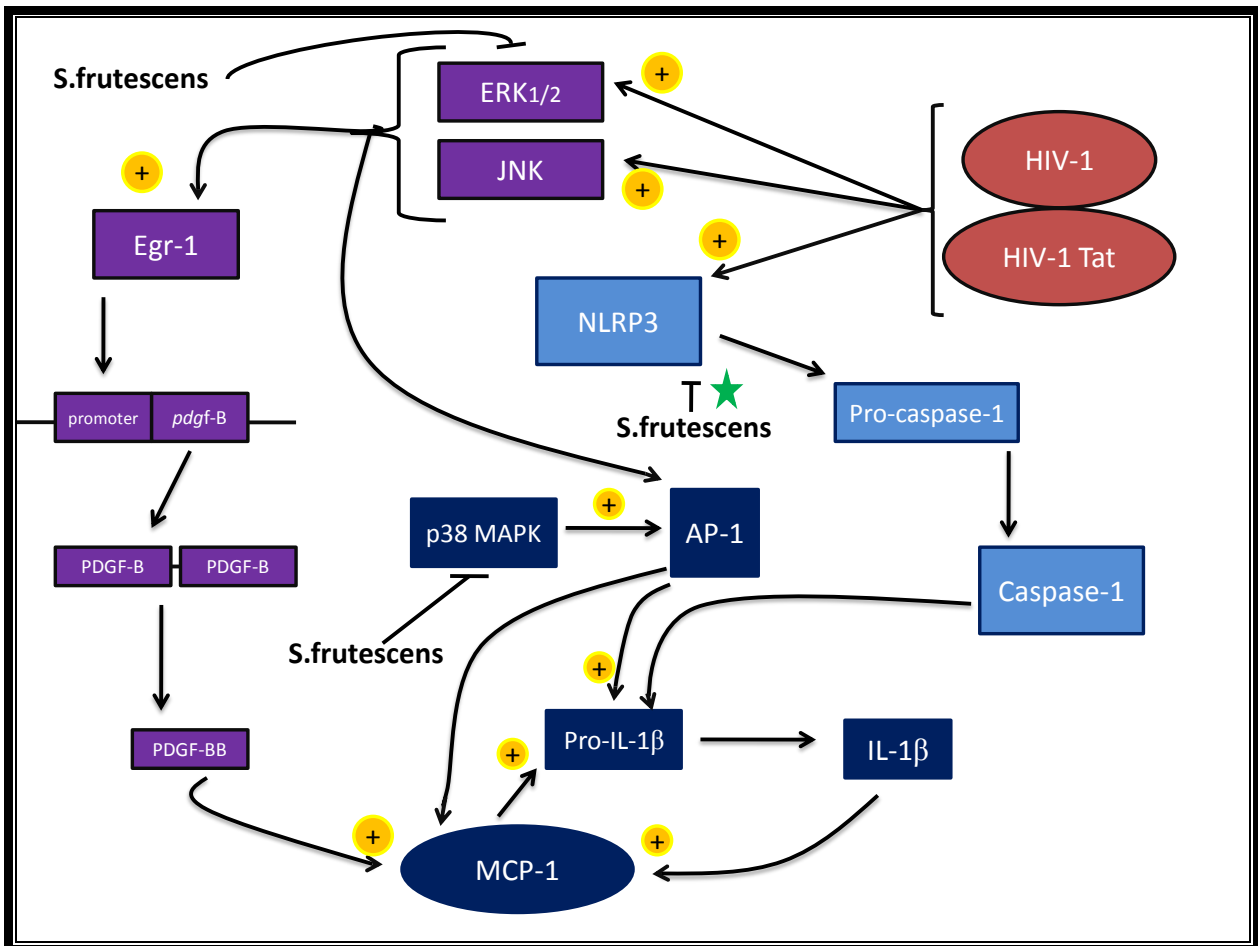
Figure 3.5. Schematic representation of inflammatory signalling pathways associated with HIV-1 infection. Novel mechanism of action elucidated by current data is indicated with a green star. Abbreviations: Mitogen activated protein kinase (MAPK); Extracellular signal-regulated kinase 1/2(ERK1/2); c-Jun N-terminal Kinase (JNK); Early Growth Response protein-1 (Egr-1); NLRP3 – NOD-like receptor family, pyrine domain containing 3

Extrapolating our data to a clinical application, CNS infiltration of both HIV-1 infected and uninfected monocytes is one of the main routes by which the virus enters and seeds the CNS as a viral reservoir to initiate neuroinflammatory processes. Thus, in order for any anti-inflammatory modality to be useful in this context, it would need to modulate this response, which *S. frutescens* does not, and which it in fact seems to aggravate. Staying with clinical application, a recent study by Fasinu *et al.* (Fasinu *et al*. 2013) has cautioned the use of *S. frutescens* by HIV+ patients on ARVs due to the fact that the herb is able to inhibit enzymes involved in the metabolic clearance of

these drugs. Given this data, the use of *S. frutescens* as complimentary medicine to patients already on ARV has been discouraged. However, use of medicinal plants by HIV-patients waiting for roll-out, is currently recommended by the Ministries of Health in several African countries – in South Africa, *S. frutescens* is recommended in this context (Mills *et al.* 2005) for prevention of cachexia, a beneficial effect of the plant previously reported by our group, as well as to prevent neuroinflammatory changes reported to occur in the early phases after infection, i.e. prior to ARV roll-out (Yao & Bethel-brown 2010). Our data now suggests that *S. frutescens* may in fact promote development of neurocognitive disorders by exacerbating inflammation and not inhibiting it in the context of neuroinflammation specifically. We urge policy makers to incorporate this evidence in their education of this at risk population as well as the health care practitioners providing primary care to them.

In conclusion, current data illustrates that the combined use of HL2/3 cells and the simulated BBB presents an accurate, disease relevant *in vitro* model with which to study neuroinflammation in the context of HIV/AIDS. In addition, our results caution against the use of *S. frutescens* as anti-inflammatory modality at any stage post-HIV infection.

Supplemental content
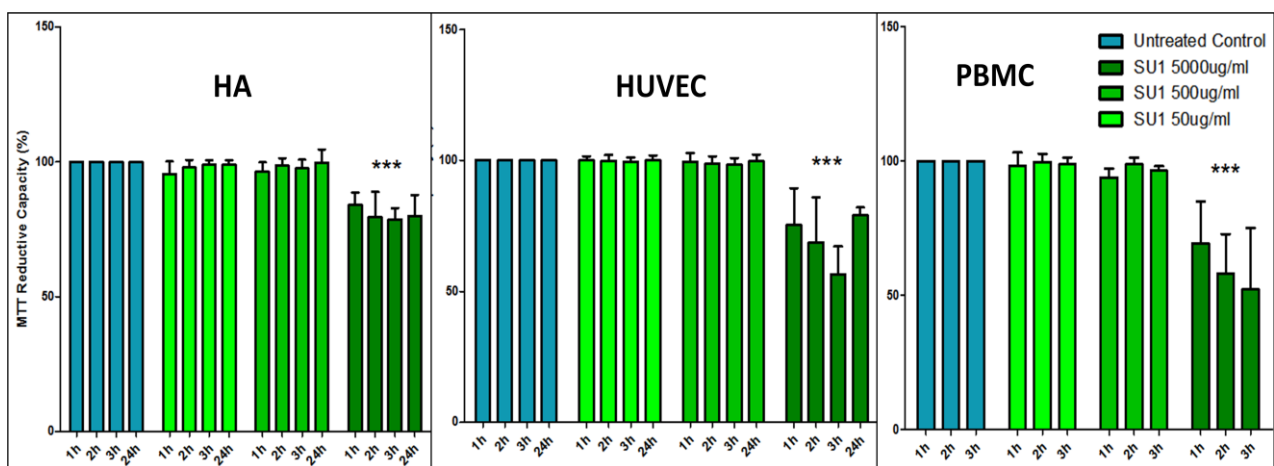
Supplemental Content 1: Effects of the *S. frutescens* extract used on cell viability in astrocytes (1a), HUVECs (1b) and monocytes (1c)

*** = P < 0.0001; ** = P < 0.001; * = P < 0.05

CHAPTER 4

USING A SIMULATED BLOOD-BRAIN BARRIER TO INVESTIGATE POTENTIAL MODULATORS OF HIV-1-ASSOCIATED NEUROINFLAMMATORY PROCESSES *IN VITRO*

LUAN D AFRICA, CARINE SMITH

(This chapter is presented in the format required by the Neurotoxicity Research journal. The manuscript is currently under review.)

4.1 ABSTRACT

Neuroinflammation is a central feature of HIV-1 infection and HIV-1 Associated Neurocognitive Disorders. Standard anti-retroviral (ARV) and accompanying therapies are limited in their capacity to limit neuroinflammation. We have identified a new candidate complimentary medicine for use in this context. Grape seed-derived proanthocyanidolic oligomers (PCO) have significant anti-inflammatory action in the peripheral compartment in the context of e.g. skeletal muscle injury, but have not been investigated in the context of either neuroinflammation or HIV/AIDS. Here its efficacy as an anti-inflammatory modality in this context was investigated in an *in vitro* co-culture model simulating the blood-brain barrier (BBB). Single cultures of human astrocytes, HUVECs and primary human monocytes, as well as co-cultures (BBB), were stimulated with HIV-1 subtype B & C Tat protein and/or HL2/3 cell secretory proteins after pre-treatment with PCO extract. In accordance with existing literature, B Tat was more pro-inflammatory than C Tat, validating the accuracy of our model. PCO pre-treatment resulted in a significantly dampened IL-1β ($P<0.0001$) response to stimulation with HIV-associated proteins, as well as a modulated monocyte chemoattractant protein-1 ($P<0001$) response and decreased capacity for CD14+ monocytes to migrate across the simulated BBB ($P<0.0001$). Additionally, PCO pre-treatment decreased both GFAP ($P<0.001$) and HSP-27 ($P<0.001$) expression in the BBB astrocytes. We conclude that PCO is able to blunt HIV-associated neuroinflammation in vitro. Current data suggests that grape seed-derived PCO should be studied further as potential complimentary anti-neuroinflammatory drug in the context of HIV/AIDS.

Keywords: monocyte, migration, inflammation, proanthocyanidin, HL2/3, Blood-brain barrier, Grape seed extract, polyphenol, MCP-1

4.2 Background

Neuroinflammation plays a key role in the neuropathogenesis associated with the Human Immunodeficiency Virus type 1 (HIV-1), the causative agent of Acquired Immune Deficiency Syndrome (AIDS). The virus establishes itself within the central nervous system (CNS) soon after an individual becomes infected (Yao & Bethel-Brown 2010), initiating an inflammatory cascade which eventually results in the development of a broad spectrum of neurological disease states collectively referred to as HIV-1 associated neurocognitive disorders (HAND). Data presented in a recent multi-centre cohort study describe persistently high rates of neurocognitive impairment at all stages of HIV-1 infection irrespective of whether a patient is on modern anti-retroviral treatment and immune reconstitution regimes (Heaton *et al*. 2011). Thus it is evident that an early preventative approach to limiting the extent of neuroinflammation, is essential to positively influence the longer-term prognosis in terms of not only HAND, but also HIV-1 disease progression.

The utility of standard anti-retroviral (ARV) treatments in directly preventing HAND is limited. Apart from the fact that they were not intended or designed for this purpose, their relative ineffectiveness in this context is mainly due to two reasons: firstly, they are administered relatively late in the context of neuroinflammatory changes associated with HIV-1 infection and secondly, different ARV drugs differ in their ability to cross the blood-brain barrier (BBB) and penetrate the CNS effectively (Strazielle & Ghersi-Egea 2005). While some ARV drugs are able to do so and reduce viral replication - reducing neuroinflammation in an indirect manner - these may be associated with adverse side effects such as neurotoxicity (Cavalcante *et al*. 2010). Thus, although certain classes of ARV drugs are very effective in reducing viral load within the CNS, the consequent inflammation associated with CNS infection cannot be effectively treated with ARV drugs alone. Additionally, astrocytes, which are non-productively infected with the virus, still shed a number of neurotoxic viral proteins e.g. Tat, Rev, and Nef, without infectious virion production (Williams *et al*. 2009). ARV therapy does not target these accessory proteins, and therefore is not

able to reduce their neurotoxic/ neuroinflammatory effects. As result, much attention has been given to the identification of possible adjuvant therapies that can either be administered concurrently with ARV drugs or given much earlier, before ARV roll-out. For example, chloroquine, simvastatin and minocycline has recently been shown to attenuate HIV-1 glycoprotein 120 (gp120)-mediated brain inflammation by modulating the interleukin-1β (IL-1β) and inducible nitric oxide synthase (iNOS) responses in an *in vivo* rat model (Ashraf *et al*. 2014). However, more widespread use of some of these pharmaceutical preparations may result in drug resistance in the context of their original application – e.g. exacerbating drug-resistant malaria – so that not all of these are ideal solutions to the problem. It is therefore warranted that not only conventional Western medicine is considered to address the problem of HIV-associated neuroinflammation, but also traditional therapies. The use of complementary medicines especially within the HIV+ population is widespread (Mills *et al*. 2005; Morris 2001) especially in developing countries (Smith & van Vuuren 2014) although few have been investigated for efficacy of these therapies specifically in the context of neuroinflammation, while others have been shown to be potentially detrimental in this context (Africa and Smith, manuscript under review). We have identified a commercially available plant-derived product which may have the potential to alleviate both the chronic systemic inflammation and neuroinflammation associated with HIV infection. Grape seed-derived proathocyanidolic oligomers (PCO) comprised of catechin or epicatechin monomers, have been shown in a rat model of skeletal muscle injury, to improve antioxidant status and modulating inflammatory cytokine responses, in both tissue and circulatory compartments (Myburgh *et al*. 2012; Kruger & Smith 2012). In the same model, PCO supplementation was shown to substantially reduce neutrophil infiltration into injured muscle and to facilitate an earlier switch in monocyte phenotype from a pro- to an anti-inflammatory phenotype, which was associated with faster resolution of inflammation and faster tissue regeneration (Kruger & Smith 2012; Kruger *et al*. 2014). Additional beneficial effects within the CNS that have been reported for PCO include protection against 12-O-tetradecanoylphorbol-13-acetate (TPA) induced lipid peroxidation and

DNA fragmentation in the brain (Bagchi *et al*. 1998), reduction in brain oxidative stress in adult and middle-aged rats as well as well as inhibition of peritoneal macrophages (Devi *et al*. 2011) and protection of primary glial cells against nitrosative/oxidative stress (Roychowdhury *et al*. 2001). An additional benefit of the *in vivo* studies mentioned above, as well as a number of other *in vivo* studies and toxicity studies (Bagchi *et al*. 2000; Bagchi *et al*. 2002; Kim *et al*. 2006) is that they provide proof that PCO indeed crosses the blood-brain barrier – an important consideration in the research approach, since many anti-inflammatory/antioxidant compounds are limited in their therapeutic use for the very reason that they do not cross the BBB (Gilgun-Sherki *et al*. 2001).

Indeed, more evidence of beneficial effects of PCO on inflammatory processes can be found in mechanistic studies, albeit not in the context of HIV-neuroinflammation specifically. For example, PCO inhibited the mitogen-activated protein kinase (MAPK) pathway's extracellular signal-related kinase 1/2 (ERK1/2), c-Jun N-terminal kinases (JNK) and p38 kinases, which are involved in the generation of an inflammatory response (Kim *et al*. 2011). Also PCO was reported to inhibit the activation of caspase-1 (also known as interleukin-1 converting enzyme) (Zhang *et al*. 2013).

The above mentioned effects on signalling pathways and *in vivo* processes – although not assessed in the context of HIV-associated neuroinflammation - are all relevant to inflammation and thus HIV-associated neuroinflammation (Yao & Bethel-Brown, 2010; Bethel-Brown *et al*. 2011; Yadav *et al*. 2010; Walsh *et al*. 2014), suggesting potential benefits for PCO in this context. Furthermore, both *in vivo* toxicity studies (Bentivegna & Whitney 2002) and *in vitro* cytotoxicity studies (Bagchi *et al*. 2002) have proven PCO consumption to be safe, with no reports of adverse effects.

Therefore, the current study aimed to investigate the efficacy of PCO as an anti-neuroinflammatory therapy, utilizing individual human cell lines as well as an *in vitro* simulation of the BBB. In addition to investigating the effect of PCO pre-treatment on inflammatory cytokine responses to HIV-associated proteins, the ability of PCO to limit HIV-1-induced monocyte transmigration across the simulated BBB was assessed. To our knowledge we are the first to report significant anti-

inflammatory effects of PCO in the context of HIV-associated neuroinflammation and elucidate potential mechanisms by which this is achieved.

4.3 Methods

4.3.1 Materials

Full length synthetic HIV-1 transactivator of transcription (Tat) (derived from clinical isolates of HIV-1subtype B [GenBank: M93258.1] & C [GenBank: FJ765005] proteins were synthesized and purified as previously described (Siddappa *et al*. 2006) and provided by Professor Ranga Udaykumar of the Jawaharlal Nehru Centre for Advanced Scientific Research (Bangalore, India). Legend MAX ELISA kits to detect monocyte chemoattractant protein-1 (MCP-1) in cell culture supernatants and FITC-conjugated anti-human CD14 were purchased from Biolegend (USA), and AlphaLISA kits to assess IL-1β secretion were purchased from PerkinElmer (Waltham, MA, USA),. 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide for the MTT assay was purchased from Sigma-Aldrich (South Africa). Commercially available grape seed-derived PCO (Oxiprovin^TM) was obtained from Brenn-O-Kem Pty Ltd (Wolseley, South Africa). Grape seed-derived PCO aqueous solution was prepared by dissolving a commercially available PCO powder in warm distilled water (1 mg/ml) and then sterile filtered using filter pore size 0.22 μm.

4.3.2 Cell culture

Single cultures of primary human cerebrocortical astrocytes (Sciencell, USA) and human umbilical vein endothelial cells (HUVECs) (Lonza, Germany) were maintained at 37°C in a humidified 5% CO_2 in high glucose Dulbecco's modified eagle's medium (DMEM) (Gibco, Life Technologies Corp., USA) supplemented with 10% foetal calf serum (FCS) (Biochrom, Germany), with added 1% N2 Supplement (Gibco, Life Technologies Corp., USA) and complete endothelial growth medium (EGM) (Lonza, Germany) for astorcytes and HUVECs respectively.

HL2/3 cells are HeLa derived cells producing high levels of Gag, Env, Tat, Rev and Nef proteins and were obtained through the NIH AIDS Reagent Program (Division of AIDS, NIAID, NIH:

HL2/3 from Dr. Barbara K. Felber and Dr. George N. Pavlakis). HL2/3 cells were maintained in high glucose DMEM (Life Technologies Corp., USA) supplemented with only 10% FCS (Biochrom, Germany). Cells were routinely sub-cultured before reaching confluence and all experiments were conducted on low passage counts.

Cell numbers were determined using a haemocytometer following trypsinization and trypan blue staining. For all single culture experiments and MTT assays, all cell types were seeded in 6-well cell culture plates at a density of 500, 000 cells per well.

In order to simulate the blood-brain barrier, co-cultures of human astrocytes and HUVECs were established on opposite sides of fibronectin (BD Biosciences, USA) coated 3 μm pore size tissue culture inserts (BD Biosciences, USA), using the method described earlier (Eugenin & Berman 2003) .

All cell culture experiments were done in triplicate and repeated a mimimum of three times.

4.3.3 PCO Dose Response Cell Viability Assay

Considering that our specific PCO compound had not been tested on the cell types used in this study before, the optimal dose for *in vitro* use – defined as the highest dosage which does not result in a significant reduction in cell viability as assessed by % MTT reductive capacity – was determined. Briefly, HUVECs and primary human monocytes (selected from human PBMCs by adherence) were incubated with 50, 100 and 200μg/ml PCO extract for 1,2,3,4 and 24 hours. At the end of the incubation period, the medium was removed from the 6 well plates and the cells washed twice with PBS. Cell viability was assessed using a modified version of the MTT assay described by Gomez and colleagues (Gomez *et al*. 1997). MTT (0.01 g/ml) was dissolved in PBS, and 500 μl was added to each well dish. Cells were subsequently incubated for 1 h at 37°C in an atmosphere of 5% CO_2. After the incubation period, cells were washed twice with PBS, and 1 ml of HCl–isopropanol–Triton (1% HCl in isopropanol; 0.1% Triton X- 100; 50:1) added to each well and gently agitated for 5 min. This lysed the cell membranes and liberated the formazan pigment. The

suspension was then centrifuged at 131 x g for 2 min. The optical density (OD) was determined spectrophotometrically at a wavelength of 540 nm and the values expressed as percentages of control.

4.3.4 Full length HIV-1 subtype B & C Tat protein stimulation

Tat proteins were reconstituted in Tris-Cl buffer (20mM, pH8) and diluted supplemented with 1 mM DTT. Human astrocytes, HUVECs, primary human monocytes and co-cultures were pre-treated for 4h and 24h respectively prior to HIV-1 Tat protein stimulation. After pre-treatment, all cells were stimulated with B Tat and C Tat (in separate wells) (10 ng/ml) for 2.5h and 24h, after which culture supernatants was collected and stored at -80°C for subsequent batch analyses. Appropriate controls were included for all conditions and treatments.

4.3.5 HL2/3 cell stimulation

Firstly, HL2/3 cell conditioned media was prepared: HL2/3 cells were seeded into 6 well plates at 200 000 cells per well and allowed to adhere to the culture surface. Once the HL2/3 cells had adhered, culture media was refreshed. HL2/3 conditioned media was collected at 2.5h and 24h from separate cultures. Secondly, the method described above for B and C Tat stimulation with and without PCO pre-treatment was then repeated, using HL2/3 cell conditioned media collected at 2.5 and 24 h as inflammatory stimulus instead of added B or C Tat.

This experiment was repeated in the co-culture system, but only with a final 4h incubation – the 24h incubation was omitted due to lowered HUVEC cell viability at this time point in the single culture experiments (refer to results). Briefly, HL2/3 cells were seeded into 24 well plates at 50 000 cells/well and allowed to adhere. Simultaneously, BBB cultures were treated with PCO for 4h prior to stimulation. Culture media was then refreshed, after which the BBB co-culture inserts were transferred to the wells containing the adherent HL2/3 cells. BBB co-cultures were exposed to the HL2/3 cells for a period of 2.5h, after which culture supernatants were collected and stored at -80°C until subsequent batch analyses.

4.3.6 Pro-inflammatory cytokine & chemokine analysis

MCP-1 concentration was measured in all supernatants by a conventional ELISA kit (Biolegend, USA), used according to the manufacturer's instructions. IL-1β concentration was measured in all co-culture supernatants by AlphaLISA (PerkinElmer, Waltham, MA, USA) according to the manufacturer's instructions.

4.3.7 Assessment of *in vitro* monocyte migration capacity

Monocyte transmigration was assessed in the BBB co-cultures by adding human PBMCs ($3x10^5$ cells) to the top of the insert, allowing the cells to migrate in response to the various stimuli (i.e. HL2/3 cell products, of which some are well-known chemotactic agents (Albini *et al*. 1998) for 2.5h, after which the BBB inserts and cells in the bottom of the well were fixed in 4% paraformaldehyde and stained with a FITC-anti-human CD14 antibody, which is a monocyte specific marker. All CD14+ monocytes on top of the entire insert (unmigrated) and on the bottom of the culture well (migrated) were counted using a fluorescent microscope (Leica, Germany). The ability of PCO to modulate this response was investigated by again pre-treating BBB co-cultures for 4h prior to stimulation. Cells in suspension were not quantified, since we have previously shown that cell counts in these compartments are a constant for the model and independent of interventions/treatments (Kruger *et al*. 2014).

4.3.8 Immunocytochemistry

Additional membranes containing the BBB cells (i.e. treated the same as the migration assay inserts but not the same ones that were stained for CD14) were fixed in 4% paraformaldehyde for 15 minutes at room temperature. Membranes were simultaneously incubated with an appropriate dilution (1:10000) of a chicken polyclonal antibody to glial fibrillary acidic protein (GFAP) (Abcam, UK) and a mouse monoclonal antibody to heat shock protein 27 (HSP-27) (Abcam, UK) overnight at 4°C, followed by 5 washes with phosphate buffered saline (PBS). Cells were then incubated simultaneously with anti-chicken FITC (Abcam, UK) and anti-mouse Pacific Blue (Molecular Probes, Life Technologies Corp,. USA) secondary antibodies for 1 hour at room

temperature, followed by 5 washes with PBS. Fluorescent images were captured at constant sensitivity setting to standardise fluorescent imaging between samples, using a Carl Zeiss Confocal Laser Scanning Microscope (LSM) 780 Elyra S1 with Super-Resolution (SR) - Structured Illumination Microscopy (SIM) super resolution platform. Results are expressed as mean fluorescent intensity.

4.3.9 Statistical analysis

All statistical analyses were performed using Graphpad Prism Version 5 software (Graphpad Software, La Jolla, CA, USA). Results are expressed as mean ± SD. One- or two- way analysis of variance (ANOVA) as relevant, followed by a Bonferroni *post hoc* test, was used to assess differences between time points and/or experimental groups. Differences were considered to be of statistical significance when $P < 0.05$.

4.4 Results

4.4.1 PCO Dose-Dependent Cell Viability

For monocyte mono cultures, no difference in % MTT reductive capacity between control and treatment groups was evident (Figure 1c), suggesting that PCO had no effect on viability of this cell type. In astrocytes there was a moderate, yet statistically significant reduction in cell viability in the 200µg/ml treated group at the 24h time point only (Figure 1a). For HUVECs, a marked reduction in %MTT reductive capacity was observed across all treatment dosage groups at the 24h time point (Figure 1b). Although this may suggest increased sensitivity to PCO in this cell type specifically, further investigation indicated that the reduction in %MTT reductive capacity was probably not due to an increase in cell death, but rather due to a significant number of non-adherent living cells that were lost during the washing steps in the MTT assay protocol.
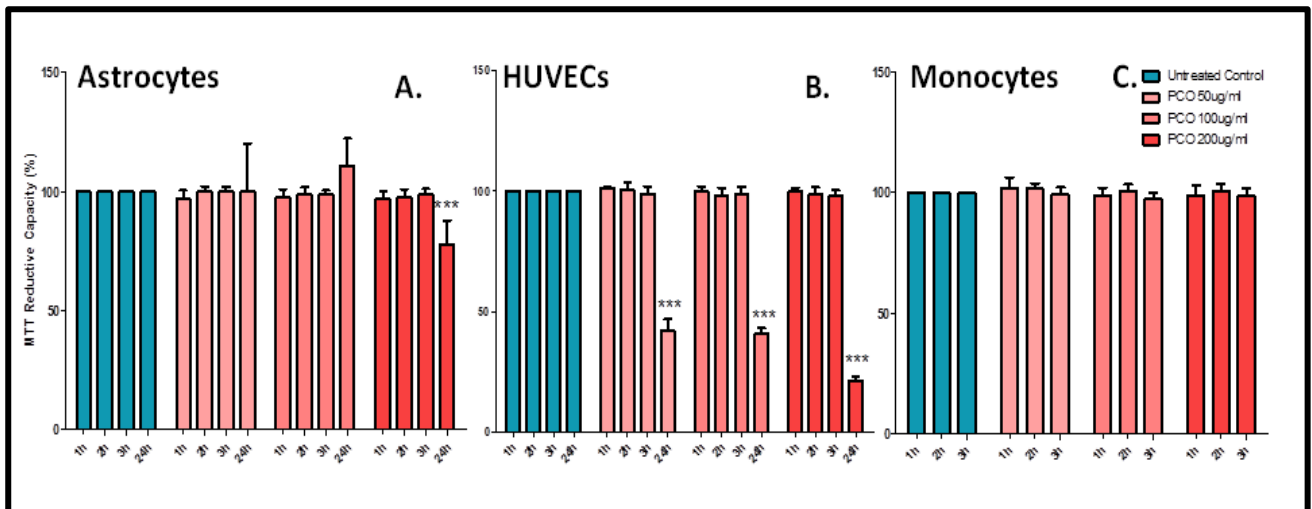
Figure 4.1. Effects of the PCO extract used on cell viability in astrocytes (1a), HUVECs (1b) and monocytes (1c).

*** = P < 0.0001; ** = P < 0.001; * = P < 0.05

4.4.2 Anti-inflammatory effects of PCO elucidated in mono cultures

In the absence of HIV-associated proteins, control (unstimulated) astrocytes secreted low basal levels of MCP-1 at 6.5 hours, but no MCP-1 was detectable after 24 hours in culture (Figure 4.2a). The discrepancy between the 6.5 hr and 24 hr time points are not unexpected, given the relatively short half-life reported for MCP-1 *in vivo* (10 minutes)(Smith *et al*. 2010). This result may thus indicate that the MCP-1 response in absence of HIV-associated proteins was only transient in nature. In contrast, after exposure to either B Tat or HL2/3 cell products, MCP-1 secretion increased significantly over time and was maintained up to the 24 hour point (all P<0.0001 when compared to control). As expected, C Tat elicited no MCP-1 response. Pre-treatment with PCO in absence of HIV proteins reduced basal secretion of MCP-1. Additionally, PCO was able to blunt the response to B Tat and HL2/3 cell products in stimulated cells at both 6.5 and 24h (all P<0.0001 when compared to control).

In HUVECs (Figure 4.2b), basal MCP-1 secretion followed a similar pattern to that seen in astrocytes at 6.5 hours, with the exception of C Tat, which also elicited a basal response in this cell type (P<0.0001 when compared to control). In contrast to astrocytes, this basal secretion was

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maintained and even relatively enhanced at 24 hours. In the absence of HIV proteins, HUVECs responded similarly to astrocytes when PCO pre-treated. In the presence of all HIV protein stimuli employed, MCP-1 secretion increased continuously up to 24 hours. PCO was able to blunt the response in this cell type, reducing the MCP-1 response to non-detectable levels.

4.4.3 PCO modulates mediators of inflammation in a simulated BBB

When repeating the intervention protocols in a co-culture simulation of the BBB (consisting of astrocytes, HUVECs and monocytes) using the EGM media to ensure optimal survival of all cell types, the net effect of PCO that may be expected in an *in vivo* situation becomes more evident (Figure 4.2c). In the absence of HIV-1 protein stimuli, PCO was able to reduce basal MCP-1 secretion by the BBB. Pre-treatment with PCO resulted in an attenuated MCP-1 response to HIV-1 B Tat (as anticipated C Tat did not elicit a response) ($P<0.05$) and significantly limited the HL2/3-induced response, so that MCP-1 levels did not differ from basal levels ($P<0.0001$). Monocyte cultures did not secrete detectable levels of MCP-1 under any of the experimental conditions, so most probably did not contribute significantly to this outcome.

Since treatment with HL2/3 conditioned media or co-culture with these cells (which contain Tat as well as other HIV proteins) resulted in the most pronounced inflammatory response in all experimental conditions up to this point, and since HL2/3 stimulation was deemed more disease relevant, HL2/3 cells were selected as only pro-inflammatory stimulus for all further experiments.

IL-1β levels were non-detectable in single cultures of HL2/3 cells (data not shown), so that any IL-1β detected originated from the BBB. IL-1β secretion was evaluated in BBB cultures stimulated by co-culture with HL2/3 cells. Pre-treatment of the BBB cultures with PCO was able to effectively inhibit the IL-1β response following co-culture with HL2/3 cells, so that the response was similar to basal secretion levels (Figure 4.2d). TNF-α levels in co-culture supernatants were also assessed, however this cytokine did not prove to be a reliable marker of inflammation as all samples assayed came up negative (data not shown)
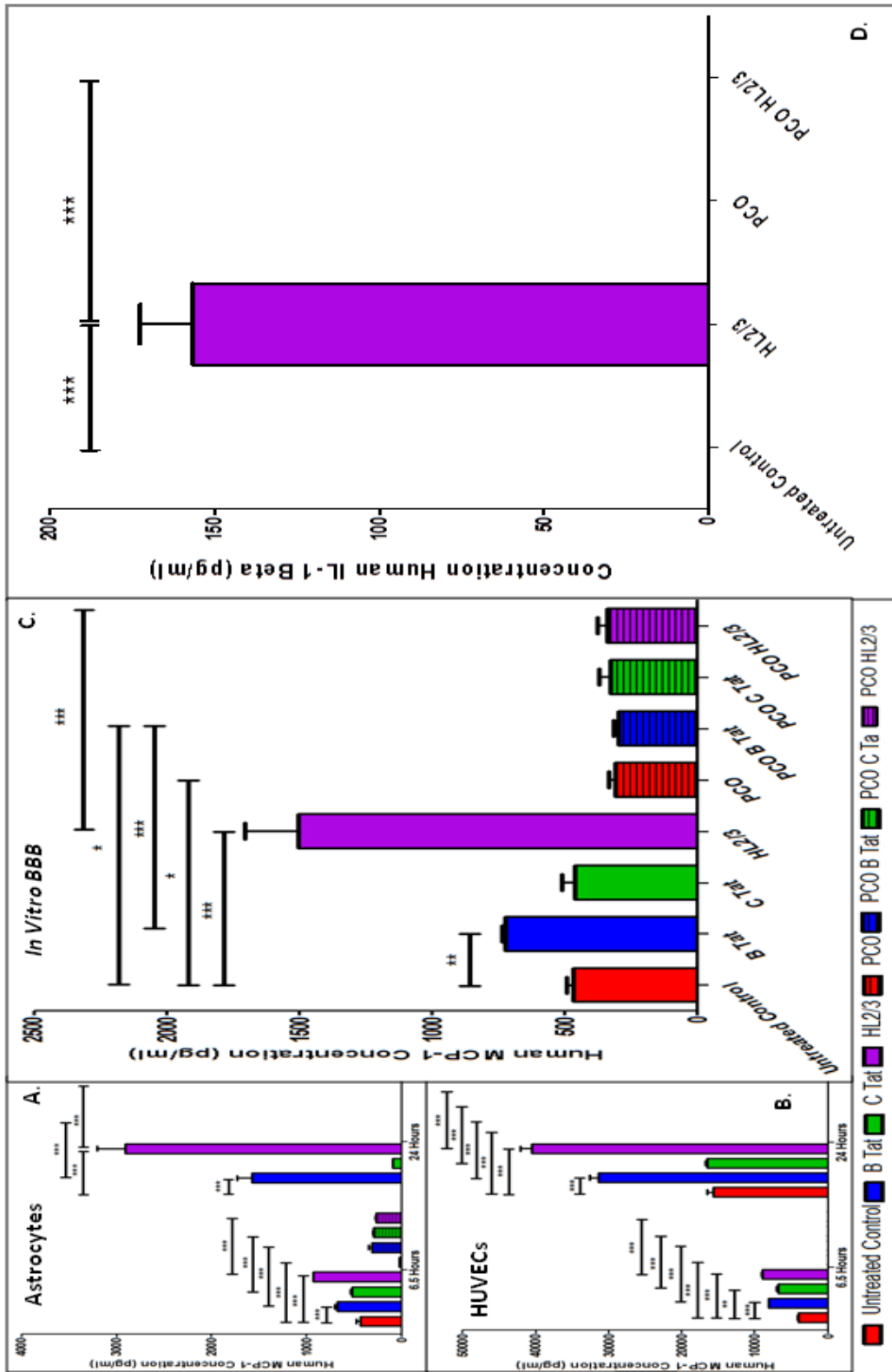
Figure 4.2. MCP-1 response to HIV-1 proteins in human astrocytes (2a), HUVECs (2b) and BBB co-cultures (2c), and IL-1β response to co-culture exposure of BBB co-cultures to HL2/3 cells in the presence or absence of PCO extract. *** = P < 0.0001: ** = P < 0.001: * = P < 0.05

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4.4.4 Monocyte migration across the BBB is effectively inhibited by PCO

Migration of primary human monocytes across the BBB was assessed, as well as the role of PCO as modulator of this process. The number of monocytes remaining on top of the trans-well filter insert (containing BBB) was named unmigrated cells, while those collecting in the bottom of the well are referred to as the migrated cells. Representative images of immunocytochemistry used to visualise monocytes for the purpose of quantification (Figure 4.3a) illustrate the marked differences in CD14+ monocyte counts between the experimental groups. Numerical data are presented in Figure 3b. As anticipated, HL2/3 stimulation resulted in a significant increase in monocyte migration across the *in vitro* BBB (ANOVA main effect P<0.0001). Pre-treatment with PCO had no effect on migration in absence of HIV proteins, but was able to effectively reduce the monocyte migration capacity in response to HL2/3 stimulation, maintaining it at basal levels.
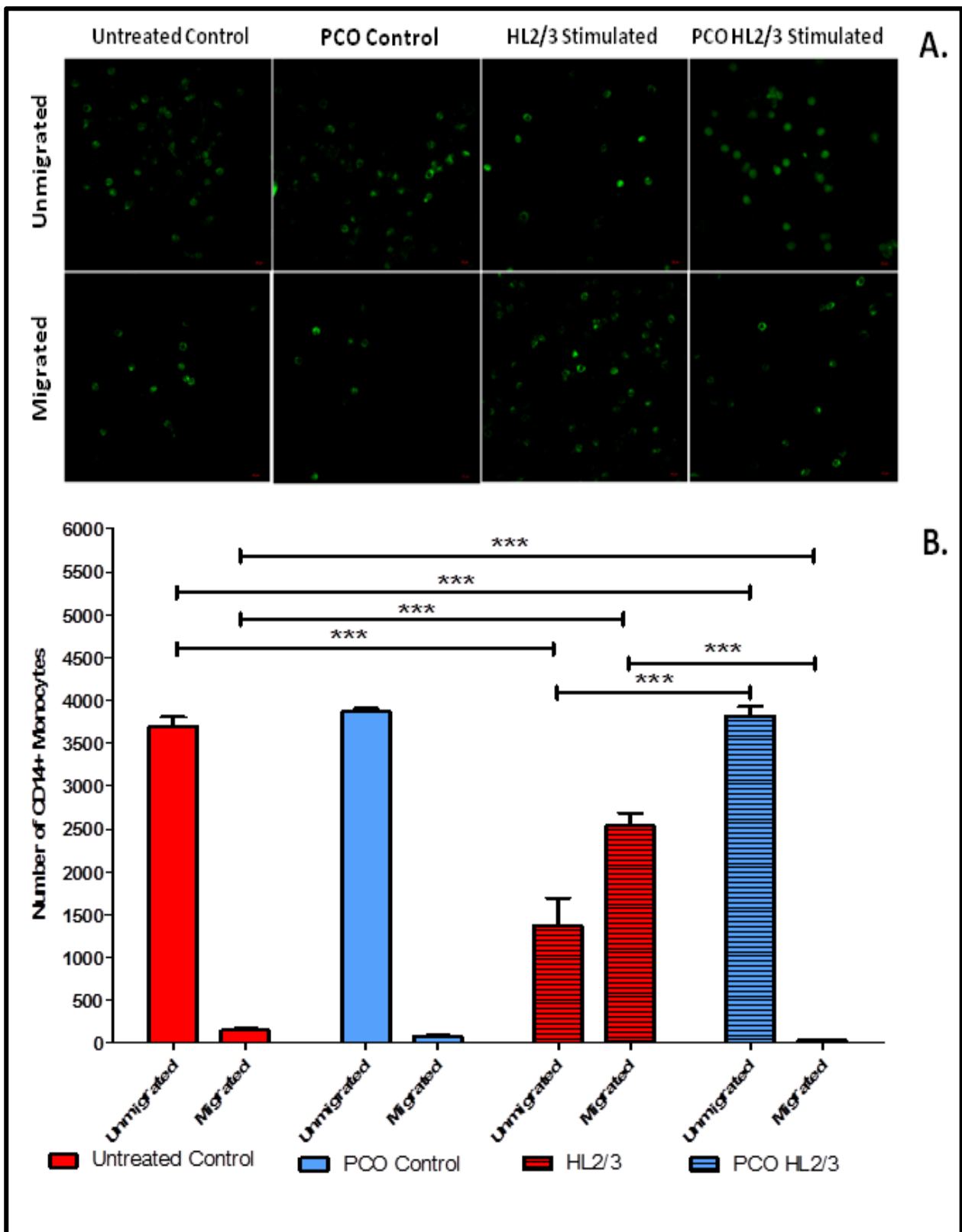
Figure 4.3. Representative images indicating the effect of PCO extract on migration capacity of CD14+ (FITC) primary human monocytes across a simulated BBB (3a). Magnification: 40x objective. Numerical data illustrating the effects of PCO extract pre-treatment on monocyte migration across an *in vitro* BBB (3b).

*** = P < 0.0001; ** = P < 0.001; * = P < 0.05

Astrocyte intercellular signalling most affected by PCO

The expression of GFAP, an intermediate filament protein associated with the cytoskeletal protein network, as well as the small heat shock protein HSP-27, were measured by immunocytochemical staining of the astrocytes on the BBB membranes. PCO pre-treatment on its own resulted in a statistically significant increase in HSP-27(Figure 4). Expression of GFAP and HSP-27 was significantly increased post HL2/3 stimulation (P<0.001). PCO pre-treatment was associated with a reduction in GFAP expression to control levels and limited increases only in HSP-27 expression (when compared to baseline) post HL2/3 stimulation (Figures 4.4 & 4.5).
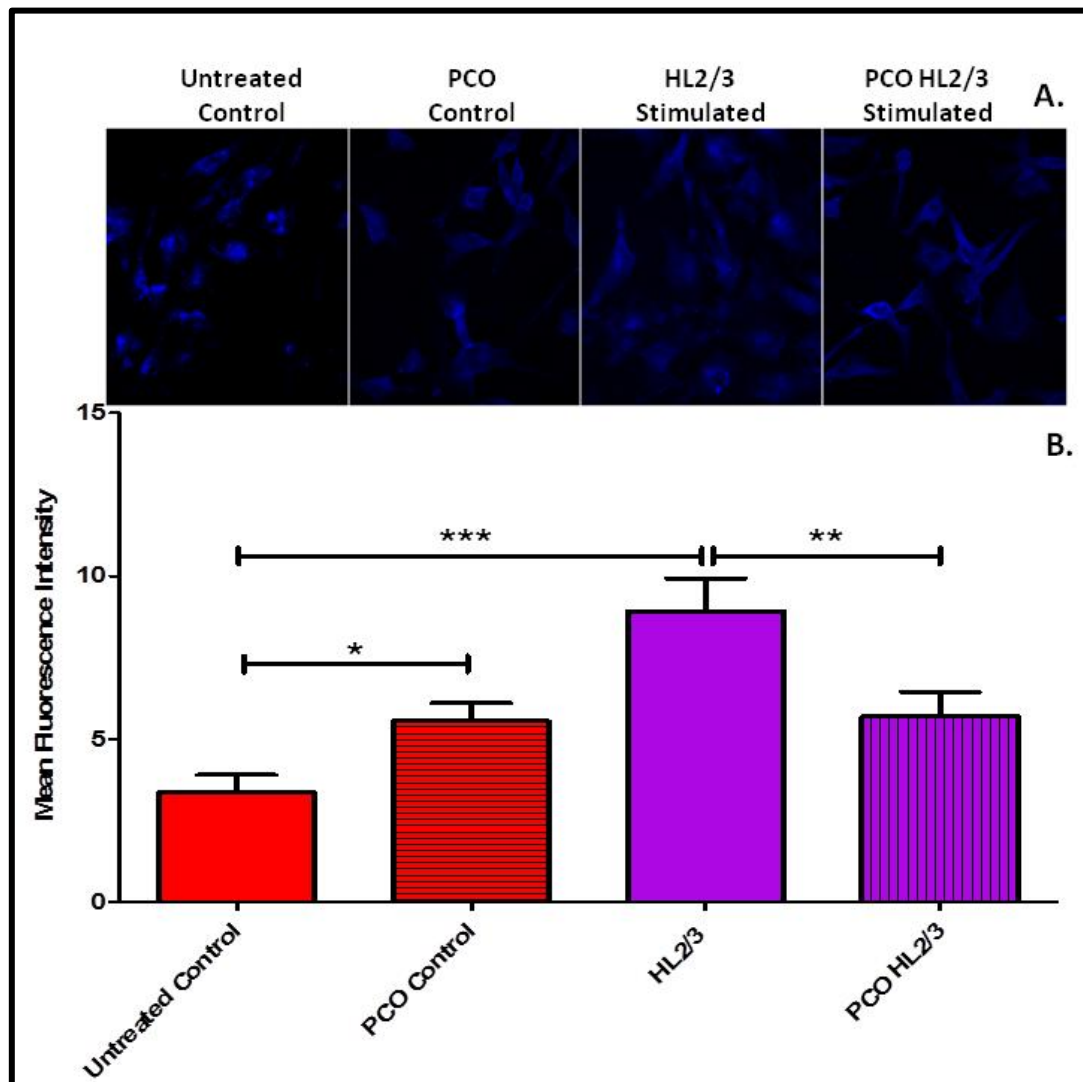
Figure 4.4. Representative images indicating the effect of PCO extract on HSP-27 expression (Pacific Blue) in primary human astrocytes of the simulated BBB (4a). Magnification: 40x objective. Numerical data illustrating the effects of PCO extract pre-treatment on HSP-27 expression in primary human astrocytes of the *in vitro* BBB (4b).

*** = P < 0.0001; ** = P < 0.001; * = P < 0.05
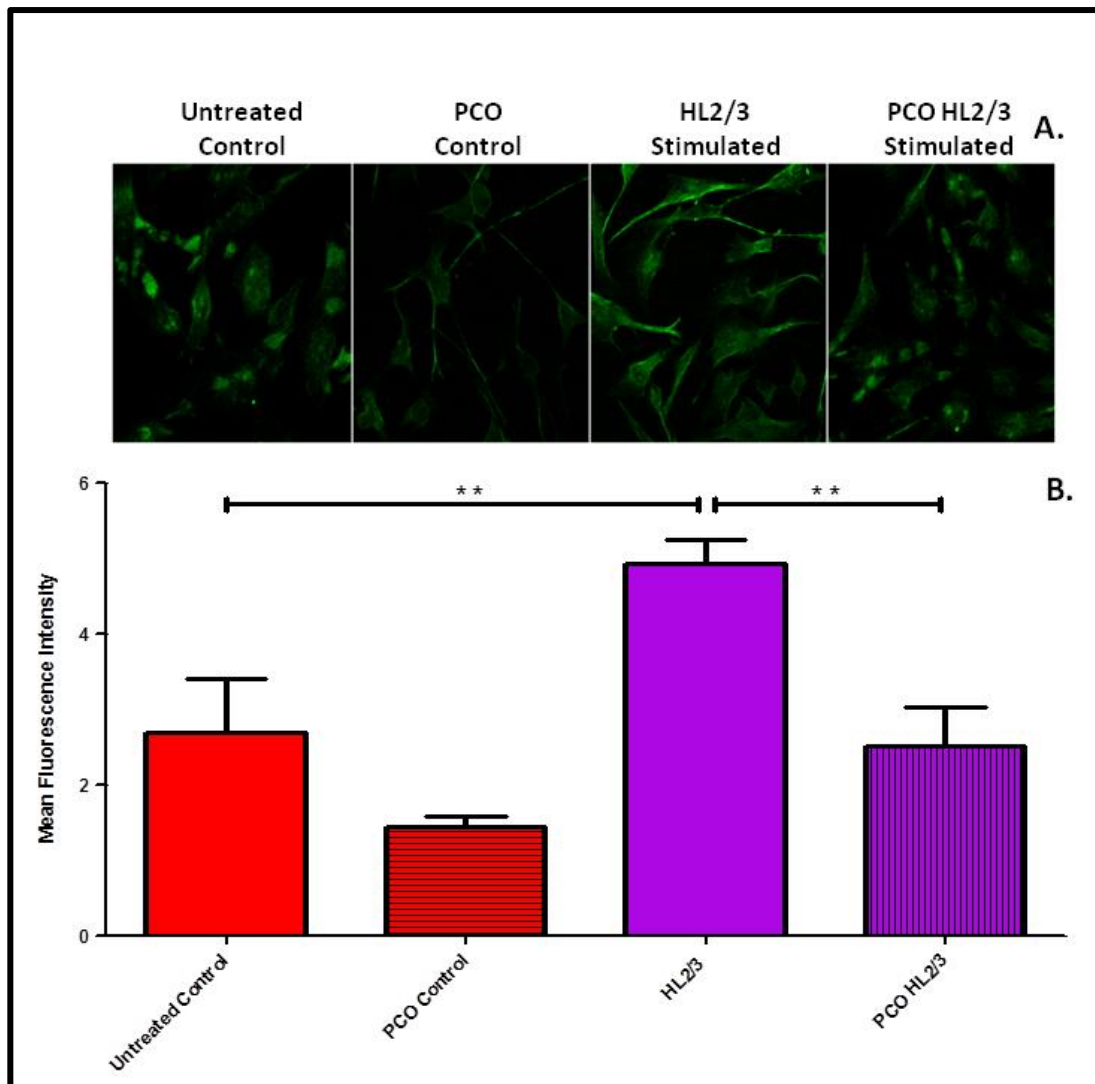
Figure 4.5. Representative images indicating the effect of PCO extract on GFAP expression (FITC) in primary human astrocytes of the simulated BBB (5a). Magnification: 40x objective. Numerical data illustrating the effects of PCO extract pre-treatment on GFAP expression in primary human astrocytes of the *in vitro* BBB (5b).

*** = P < 0.0001; ** = P < 0.001; * = P < 0.05

4.5 Discussion

Neuroinflammatory processes are central to the aetiology, progression and prognosis of HAND, and considering the fact that standard ARV therapies are not able to effectively reduce neuroinflammation associated with HIV-1 infection, identification of alternative strategies have become a focus. In particular, natural products should be considered, given their widespread use. We are of the opinion that data generated in this study make a substantial contribution to this area.

Current data presents two major novel findings. Firstly, we illustrate that PCO largely inhibits monocyte infiltration across the BBB, by down-regulation of the MCP-1 and IL-1β responses. Secondly, an innovative feature of this study is the use of HL2/3 cells, in combination with a well-established *in vitro* model of the BBB, to simulate – in a more disease relevant manner than other non-infectious methods commonly employed – conditions at the neurovascular interface following HIV-1 subtype B infection. To our knowledge, we are the first to utilize this simulated BBB to assess (and successfully demonstrate) the efficacy of a complimentary medicine as an anti-inflammatory modality.

4.5.1 HL2/3 cells mimic HIV-1 neuroinfection to produce a more HIV-relevant model

Up to this point, HL2/3 cells have most commonly been used to investigate viral fusion dynamics in the context of HIV (Wexler-Cohen & Shai 2007). *In vitro* studies investigating neuroinflammation commonly use either infectious cultures or stimulation with single HIV-associated proteins or cells secreting single proteins. Here, we describe utilisation of the ability of HL2/3 cells for high-level production of a variety of HIV-associated proteins, including Gag, Env, Tat, Rev and Nef, to stimulate *non-infective* neuroinflammation *in vitro.* (We did not assess the exact concentration of HIV-associated proteins in the co-culture, since we could not find much literature reporting on physiological levels or *in vitro* levels of these proteins secreted; thus such a measurement would not assist in placing our results in context.) Our model is also more disease relevant than those using only single HIV-associated proteins, as evidenced by comparative data on responses to the Tat proteins we included in the first experiments of the current study. The simulated BBB co-culture, originally used for testing chemokine and monocyte migration responses to HIV-1 (Eugenin & Berman 2003), was used here in a broader application to investigate efficacy of a natural extract as anti-inflammatory modality. Proof of the accuracy of the model to assess pro-inflammatory signalling is the fact that data generated from the HIV-1 subtype B & C Tat stimulation experiments – showing that subtype B is more inflammatory than subtype C – are congruent with previous reports in the literature (Mishra *et al*. 2007; Rao *et al*. 2008).

The combined use of the BBB model and HL2/3 cell stimulation – although technically complex – considerably improved the disease specific relevance and thus practical applicability of our results. For instance, using this model we can conclude that the effects of PCO reported here, are not limited to modulation of Tat-specific events only, but that it applies to inflammation associated with a much larger repertoire of HIV-1 proteins.

We would therefore like to recommend the use of this model for first-line *in vitro* evaluation of modulatory effects of newly discovered compounds and newly developed pharmaceuticals which may have the potential to modulate HIV-1 induced inflammatory processes within and surrounding the neurovasculature.

4.5.2 PCO modulates HIV-1 – associated neuroinflammation via the ERK1/2/JNK, p38 MAPK and NLRP3 pathways

Turning our attention to the complementary medicine evaluated, to our knowledge our study is the first to provide scientific support for an anti-inflammatory effect of PCO in the context of HIV-associated neuroinflammation. Given the fact that the natural product currently endorsed by developing country governments for supplementation in people living with HIV, have been shown to be detrimental in this context (Fasinu *et al*. 2012; Müller *et al*. 2013) – leaving a gap in terms of adjuvant therapy for these patients – the current results have far reaching implications. Since many studies have been conducted on the effect of this particular PCO in the context of other diseases (Engelbrecht *et al*. 2007) and non-HIV models of inflammation (Myburgh *et al*. 2012; Kruger & Smith 2012; Kruger *et al*. 2014), showing only positive effects and no undesired ones, our expansion of the knowledge highlights the need to focus resources on the continued development of this particular product.

In terms of potential mechanisms of action – which may be more widely applicable – available data and literature allows for discussion of potential mechanisms of PCO. Here we propose avenues by

which PCO may modulate the pro-inflammatory signalling pathways in a simplified schematic presentation (Figure 4.6).
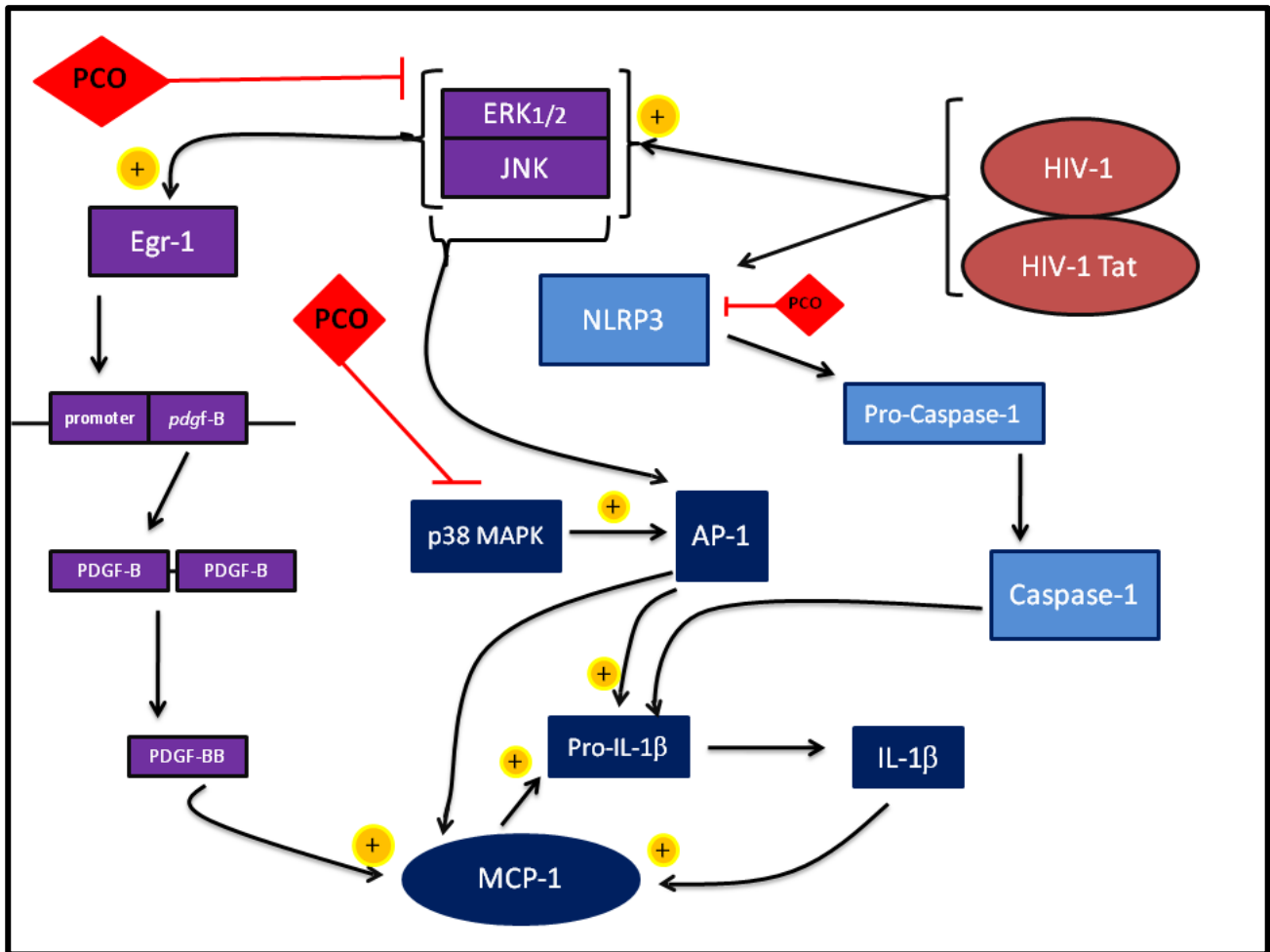
Figure 4.6 Schematic representation of inflammatory signalling pathways associated with HIV-1 infection. Abbreviations: Mitogen activated protein kinase (MAPK); Extracellular signal-regulated kinase 1/2(ERK1/2); c-Jun N-terminal Kinase (JNK); Early Growth Response protein-1 (Egr-1); NLRP3 – NOD-like receptor family, pyrine domain containing 3.

The β-chemokine MCP-1 is a central role player in HIV-associated neuroinflammation. MCP-1 production in response to HIV-stimulation is facilitated via three routes, namely a) the ERK1/2/JNK pathway (Bethel-Brown *et al*. 2011) , b) the p38 MAPK pathway (Fan *et al*. 2011) and c) caspase-1 activation via the NLRP3 inflammasome (Walsh *et al*. 2014). From the non-HIV literature, PCO is known to suppress the p38 MAPK pathway as well as the ERK1/2/JNK pathway (Kim *et al*. 2011), which is in agreement with our interpretation. However, the fact that all

inflammatory responses to HL2/3 stimulation assessed in the current study seemed to be completely inhibited after PCO pre-treatment, suggests that it prevented HIV-associated activation of all three pathways. In particular the inhibition of the IL-1β response suggests that PCO also inhibited the NLRP3 inflammasome – caspase-1 pathway, which is the predominant pathway responsible for conversion of pro-IL-1β to IL-1β by caspase-1 (also called IL-1 converting enzyme) (Schroder *et al*. 2010). Inhibition of this pathway may also explain the anti-inflammatory function of PCO reported under non-HIV basal conditions, both here and by others (Kim *et al*. 2011; Sakurai *et al*. 2010; Terra *et al*. 2011).

Our data is in agreement with other studies elucidating potential mechanisms for PCO, but adds more mechanistic insight. For example, monocytes in circulation have been reported to switch to an anti-inflammatory phenotype which does not readily leave circulation to infiltrate tissue, after PCO treatment (Kruger *et al*. 2014) – our data shows that this phenomenon is also applicable to extravasation across the BBB. Others report downregulation of adhesion molecules (VCAM-1) by PCO (Sen & Bagchi 2001). Our observations of decreased attachment of specifically HUVECs after PCO pre-treatment, supports this notion. In addition to these two mechanisms described in the literature, our data propose a third target – prevention of the inflammatory cytokine signalling cascades related to MCP-1. Interestingly, our data is supported by relatively unexplained reports of decreases in pro-inflammatory cytokine levels in *in vivo* models after PCO treatment (Myburgh *et al*. 2012; Kruger & Smith 2012). Our data thus not only adds to the knowledge base related to HIV and neuroinflammation, but also contributes to interpretation of non-HIV results on inflammation and PCO.

4.5.3 PCO modulates astrocyte inflammatory processes

The intermediate filament protein GFAP is a widely recognised marker for astrocytes and its upregulation is used by many researchers to indicate reactive astrocytosis. In the context of HIV-1 Tat associated neurotoxicity, GFAP was identified as predictor of neurotoxicity. In the same study,

downregulated expression of GFAP in astrocytes was indicative of modulation of the inflammatory processes in this cell type and thus increased neuronal cell survival (Zou *et al*. 2010). Our result of decreased GFAP expression after PCO pre-treatment, both in the presence and absence of HIV-1 associated proteins, is in agreement with this and further strengthens our data indicating an anti-inflammatory effect for PCO.

The small heat shock protein HSP-27 has been reported to increase as part of the cellular stress response and its overexpression is associated with activation of protective mechanisms during neuronal injury. For example in neuronal injury specifically, constitutive overexpression of HSP-27 has been linked to reduced apoptosis and caspase-3 induction (Vidyasagar *et al*. 2012). Thus, our observation that PCO pre-treatment, in the presence of HIV-1 associated proteins, is associated with a blunted HSP-27 response to HL2/3 stimulation may indicate that less cell damage was present, due to the anti-inflammatory effects already elucidated, so that a high degree of protection against e.g. apoptosis was not required. In addition, the PCO-induced increased HSP-27 expression in non-stimulated astrocytes suggests that PCO may confer resistance to apoptosis in these cells under basal conditions. Further investigation considering allostasis is required to confirm the longer term benefit of this effect in non-pathological states.

4.6 Conclusions

Current data illustrate that the combined use of HL2/3 cells and the simulated BBB presents an accurate, disease relevant *in vitro* model with which to study neuroinflammation in the context of HIV/AIDS.

CNS infiltration of both HIV-1 infected and uninfected monocytes is one of the main methods by which the virus enters and seeds the CNS as a viral reservoir to initiate neuroinflammatory processes. Thus, in order for any anti-inflammatory modality to be useful in this context, it would need to modulate this response, which PCO does very effectively by targeting multiple processes. Thus, extrapolating our data to a more clinical application, the overwhelming body of literature

pointing towards several anti-inflammatory benefits of PCO, warrants further development of this extract as adjuvant therapy in the context of HIV-associated neuroinflammation in particular, but also as general anti-inflammatory supplement.

CHAPTER 5

SYNTHESIS & FUTURE PROSPECTS

In my opinion, my thesis makes the following contributions:

- Establishing a disease relevant non-infectious *in vitro* model of HIV-1-associated neuroinflammation in the context of the BBB. The model has been validated in terms of the literature using HIV-1 subtype B and C Tat proteins to induce an inflammatory response, and our results are congruent with previous studies using this *in vitro* model of the BBB. Additionally the results show that the model is accurate and sensitive enough to reflect effects on neuroinflammatory processes – both positively and negatively. It is widely accepted that the main route by which HIV-1 enters the CNS is via infected monocytes/macrophages migrating from peripheral circulation to the CNS. This model is particularly useful in that it allows one to assess leucocyte migration, making it useful to investigate possible interventions aimed at limiting HIV-1 neuroinfection via the transmigration of infected cells from the monocyte/macrophage lineage from peripheral circulation which cross the BBB.

- It has broadened the understanding of the anti-inflammatory capacity of PCO. This is the first study to test the anti-inflammatory effects of PCO in the context of HIV-1-associated neuroinflammation. This body of work lays the foundation for future basic, mechanistic and clinical studies. Examples of such studies include assessment of the anti-oxidant capacity of PCO *in vitro, in vivo* as well as in human subjects in the context of HIV infection. Also, the effect of PCO on the modulation of a larger panel of cytokines should be assessed as well as a number of cell adhesion molecules (e.g. ICAM-1, PECAM-1, VCAM-1) involved in leucocyte migration in the context of HIV infection. Moreover, in order to validate our interpretation of the cytokine data generated in this study, *in vitro* as well as *in vivo* studies

should be undertaken in order to confirm PCO's ability to regulate the specific signalling pathways implicated in this study

- I envisage that the findings of the *Sutherlandia* study will make a significant impact on primary care in the HIV+ population. Considering that the South African Ministries of Health are advocating the use of traditional herbal medicines such as *S. frutescens* to manage HIV disease, it is of the utmost importance that this information be disseminated to clinical communities. There is a relative lack of empirical data to support the use of traditional medicines in the management of HIV disease; thus it is paramount that such studies be undertaken to assess the effects of various traditional medicines used by this vulnerable population group. Such availability and widespread dissemination of such information would help to ensure that protective measures are in place to prevent early neuroinflammation that will have longer term detrimental effects on both prognosis and the burden on the public health sector.

CHAPTER 6

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