

**Investigations into the mechanisms underlying HIV-1
gp120-associated neurotoxicity**

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Abstract

HIV-associated distal sensory polyneuropathy is a frequent (~40% prevalence) complication of HIV infection and treatment. It is characterised by a dying back pattern of axonal degeneration, predominantly of nociceptors usually accompanied by neuropathic pain. The HIV envelope glycoprotein, gp120, has recently been identified as a key mediator of axonal degeneration both *in vitro* and *in vivo*. We hypothesised that gp120 interacts, in a chemokine receptor-dependant manner, with primary sensory neurons either directly or indirectly via macrophages and/or Schwann cells. Neurite outgrowth of cultured adult rat dorsal root ganglia (DRG) cells was used to assess direct or indirect neurotoxicity. Gp120 induced concentration-dependent neurite degeneration 24h after exposure, which was not restricted to phenotypic subsets of DRG cells. However, gp120 localisation studies indicated that only a minority (approx. 10%) of neurons internalised gp120 before the onset of neurite degeneration suggesting that the direct toxicity was not a predominant mechanism. Therefore, indirect mechanisms of neurotoxicity were investigated. Application of gp120-conditioned macrophage or Schwann cell media to DRG neuronal cultures revealed that gp120-conditioned media also had the capacity to induce neurite degeneration. Using qPCR it was shown that 4hr exposure to gp120 increased transcription of cytokine-related genes, in cultured Schwann cells and macrophages. These gp120-mediated effects were then explored *in vivo*. The cytokine expression profiles 4hrs following intradermal gp120 injection in rats were similar to the gene upregulation observed *in vitro*.

These findings highlight the complexity of gp120-mediated mechanisms and indicate that macrophages and Schwann cells may play a key indirect role in the pathogenesis of HIV-associated peripheral neuropathy.

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Declaration of Originality

I hereby declare that this thesis and the work reported herein was composed by and originated entirely by me. Information derived from the published and unpublished work of others has been cited within the text and the full references can be found in the bibliography at the end of the thesis.

Signed: Philippa Moss

A handwritten signature in black ink, appearing to read 'PJMoss', is enclosed within a light gray rectangular box.

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Abbreviations

AIDS	Acquired immune deficiency syndrome
AraC	Arabinofuranosyl cytidine (Cytosine arabinoside)
Arg1	Arginase-1
ATN	Antiretroviral toxic neuropathy
ATP	Adenosine-5'-triphosphate
B-gp120	Biotinylated-gp120
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
C.DMEM	Complete Dulbecco's Modified Eagle's Medium
CDK5	Cyclin-dependent kinase 5
CNS	Central nervous system
COX	Cyclo-oxygenase
CSF	Cerebral spinal fluid
d4T	Stavudine (a NRTI)
DHA	Docosahexaenoic acid
DIV	Days <i>in vitro</i>
DRG	Dorsal root ganglion
DSP	Distal sensory polyneuropathy
DTAF	Dichlorotriazinyl amino Fluorescein (a fluorochrome)
EPA	Eicosapentaenoic acid
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HIV-ATN	HIV-associated antiretroviral toxic neuropathy
HIV-DSP	HIV-associated distal sensory polyneuropathy
HIV-SN	HIV-associated sensory neuropathy
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IMEM	Iscoe's Modified Eagle's Medium
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1 (CCL2; chemokine (C-C motif) ligand 1)
MIP-1 α	Macrophage inflammatory protein-1 α (CCL3)
MIP-1 β	Macrophage inflammatory protein-1 β (CCL4)
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
n.s.	Normal solution
NRTI	Nucleoside reverse transcriptase inhibitor
PG	Prostaglandin
PI	Propidium iodide
PNS	Peripheral nervous system
PUFA	Polyunsaturated fatty acid
QUIN	Quinolinic acid

RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted (CCL5)
R.T.	Room temperature
SDF-1	Stromal cell-derived factor-1 (CXCL12; chemokine (C-X-C motif) ligand 12)
SIV	Simian immunodeficiency virus
TNF	Tumor necrosis factor

Chapter 1: General Introduction

1.1. HIV

1.1.1 HIV-1 Biology

Acquired immune deficiency syndrome (AIDS) was officially classified as a disease on June 5th 1981 and has continued to grow ever since to become a worldwide health and humanitarian crisis. AIDS is a human disease state that results from infection with a lentivirus known as the human immunodeficiency virus (HIV). Lentivirae fall into the genus classification of retroviridae due to their RNA based genetic material, and are characterised collectively by a long incubation period prior to clinical symptoms, resistance to vigorous host immune response and survivability.

Two known types of HIV exist today: HIV-1 and HIV-2. Both types are transmitted by sexual contact, and through cross contamination of blood and cause clinically indistinguishable AIDS. However HIV-1 is the predominant virus type found worldwide, and is most generally referred to in connection with AIDS. This appears to be due to the greater transmissibility of HIV-1 compared to HIV-2 (Kanki *et al.*, 1994) and as such, HIV-2 infection is rare and cases tend to be concentrated in West Africa. With HIV-1 being the predominant virus that underlies most of the HIV-associated conditions researched; the content on this thesis from here-on will relate only to HIV-1.

The structural basis of HIV-1 is illustrated in Figure 1.1.1. The HIV virion comprises two copies of single-stranded RNA enclosed by a conical capsid formed by the viral protein p24. In addition to viral RNA the capsid contains three enzymes essential for the successful maturation and replication of the virus; namely, reverse transcriptase (RT; p61/p52 heterodimer); integrase (IN; p31) and protease (p10). The capsid is surrounded by a viral membrane layer called the matrix, which comprises the protein p17. As the newly synthesised, and mature, p17-bound virion buds from the infected cell, it becomes encapsulated by the host cell's lipid membrane thereby giving HIV virions an exterior lipid envelope of host cell origin (Aloia *et al.*, 1988). Protruding from the viral envelope are polyglycoprotein "spikes" derived from the precursor protein gp160 that is enzymatically cleaved, after release from the Golgi apparatus, into gp120 and gp41 glycoproteins. There are suggested to be 72 spikes on HIV-1 (Gelderblom *et al.*, 1987; Ozel *et al.*, 1988), each comprising of a trimeric composition formed by three trans-membrane gp41 stalks (Pinter A *et al.*, 1989) that act as anchors for three exterior gp120s, non-covalently attached at their ends (Wyatt R *et al.*, 1998). Gp120 is host to most of the surface-exposed elements; containing protein sequences essential for binding with the primary receptor and secondary co-receptors utilised by HIV virions for host cell binding. Gp41,

on the other hand, contains domains that are critical for catalyzing the membrane fusion reaction between viral and host lipid bilayers during virus entry.

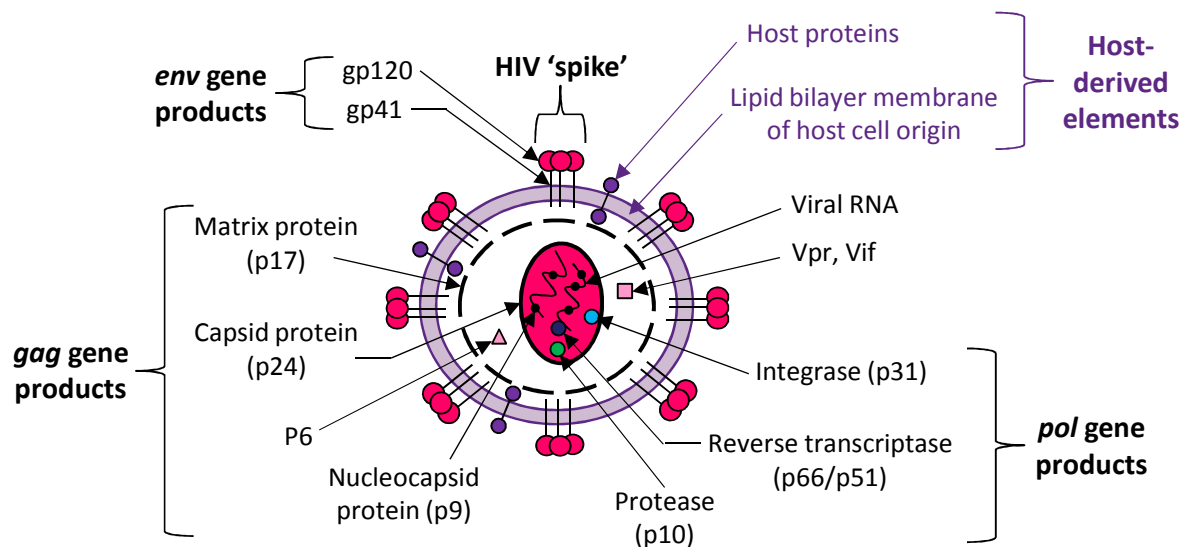


Figure 1.1.1) Schematic depicting the structural basis of an HIV virion its associated viral proteins, adapted from Joshi *et al.* (Joshi *et al.*, 1996).

HIV viral genes can be categorised broadly into three functionally distinct groups; structural genes, transactivation genes and accessory genes. The early gene products resulting from HIV-host genomic integration include 1) those of the transactivation group; trans-activator of transcription (*Tat*) and regulator of expression of viral proteins (*Rev*), both essential for viral replication, and 2) an accessory gene; negative factor (*Nef*), which regulates viral replication. In the absence of *Tat*, viral mRNA transcription is slow, inefficient and strongly inhibited by cellular barriers (Kao *et al.*, 1987). RNA polymerase II remains able to bind to the HIV-1 promoter region however, in the absence of *Tat*, is unable to efficiently elongate and terminates transcription soon after passing the promoter region. *Tat* not only inhibits the repression on the viral promoter region, through the recruitment of cellular proteins (Richman *et al.*, 2009), but additionally causes the phosphorylation of RNA polymerase II affording it the ability to efficiently and completely perform transcriptional elongation (Herrman CH *et al.*, 1995; Reines *et al.*, 1996), thereby producing full length viral transcripts. These functions optimise viral replication, increasing the production of viral mRNA by ~100 fold (Frankel *et al.*, 1998) and clarify why *Tat* is the first protein synthesised following first introduction of viral DNA into a host (Li *et al.*, 2009). Following efficient transcription, HIV-1 *Rev* protein ensures the HIV-coding mRNA is protected from host splicing machinery by rapidly exporting it from the nucleus into the cytoplasm. These mechanisms allow the full HIV genome to be transcribed and exported to the ribosomes where translation of all HIV viral proteins can now occur. The *Nef* and *Vpu* viral proteins encompass

similar functionality to each other in that both downregulate the surface expression of MHC class I proteins, which protect the infected cell from target killing by cytotoxic T cells (Le *et al.*, 1997; Kerkau *et al.*, 1997). In addition they lead to the degradation (Vpu) and downregulation (Nef) of CD4 molecules (Schubert *et al.*, 1998; Piguet *et al.*, 1998) thereby preventing inappropriate interactions with Env and assists in avoiding instigation of an immune response. Together these mechanisms allow for correct transportation and sorting of Env glycoproteins, gp120 and gp41, to the cell membrane, aiding in the assembly of new virus particles.

A summary of these functional roles of the HIV-1 viral proteins and further explanation of additional viral proteins can be found in Table 1.1.1.

Table 1.1.1) Table of HIV-1 viral genes and associated functions.

HIV-1 Viral Genes

Proteins encoded		Function
Structural Genes		
Gag	Interior: Virus core	
	capsid (p24)	Forms the viral core that holds viral RNA and enzymes
	matrix (p17)	p17 units line the inner-side of the HIV envelope helping to anchor the gp41-gp120 spikes into the envelope
	nucleocapsid (p9)	Binds to HIV RNA, retaining it within the viral core
Pol	HIV Viral enzymes	
	reverse transcriptase (p61/52)	Transcribes HIV genomic RNA into double-stranded DNA
	RNAseH	Degrades the RNA following transcription by reverse transcriptase
	integrase (p31)	Integrates viral-coding DNA into the host cell's DNA
	protease (p10)	Cleaves long polypeptide chains of gag and gag-pol proteins into their individual components allowing formation of the interior structure of HIV
Env	Exterior: Virus envelope	
	gp120	Exterior glycoprotein required for binding HIV virions to host cells; undergoes conformation change on binding to CD4 allowing more efficient binding to coreceptors CCR5 and/or CXCR4 and subsequent gp41 membrane insertion
	gp41	Anchors gp120 to the viral envelope and aids in the fusion of viral and host cell membranes, allowing HIV entry
Transactivation Genes		
Tat	Trans-activator of viral transcription (Tat)	Dramatically increases viral replication by phosphorylating cellular factors involved in transcription
Rev	Regulator of viral expression (Rev)	Regulates the expression of HIV proteins by controlling the export of newly synthesised mRNA from the nucleus into the cytoplasm
Regulatory/ Accessory Genes		
Nef	Negative factor (Nef)	Positively regulates viral replication; downregulates CD4 receptors; interferes with normal signal transduction machinery; downregulates markers targeted by host immune response increasing survival of infected cell
Vpu	Viral protein, unknown (Vpu)	Aids in the correct sorting and packaging of viral envelope proteins; degrades CD4 receptors; downregulates markers targeted by host immune response increasing survival of infected cell
Vif	Virion infectivity factor (Vif)	Restraints the inhibitory host defence mechanism that normally degrades newly synthesised viral DNA; allowing provirus to be formed and infection to be established
Vpr	Viral protein, regulatory (Vpr)	Essential for viral survival; facilitates the nuclear import of viral genomic content and increases HIV replication by arresting host cell cycle and concomitant transactivation of the HIV promoter region.

1.1.2 The HIV-1 Life Cycle

Virus attachment requires an interaction between HIV-1 gp120 and specific host cell receptors now identified as the CD4 receptor and members of the chemokine receptor family (Berger *et al.*, 1999). CD4 is expressed on the surface of T lymphocytes, macrophages, dendritic cells and brain microglia that are, in addition, the main target cells for HIV. This initially highlighted the importance of the CD4 receptor for the infection of host cells and it was believed that infection occurred solely via this glycoprotein. All HIV virus isolates can infect and replicate in CD4+ cells however, whilst the interaction between gp120 and CD4 is required for viral fusion, this interaction alone was found to be insufficient to allow infection of these cells. Binding of the envelope protein with the CD4 receptor induces a conformational change within the gp120 protein structure that exposes a secondary binding site, allowing an interaction between gp120 and a co-receptor to occur with higher affinity. These co-receptors have since been identified as the chemokine receptors, CCR5 and CXCR4 (Broder *et al.*, 1997). This is an essential step as it draws the virus closer to the cell membrane allowing it to subsequently anchor gp41 into the host membrane, which assists the fusion and entry of the HIV virus (Figure 1.1.2).

As HIV enters a cell the outer viral lipid envelope fuses with the lipid membrane of the host cell allowing the capsid containing the viral genetic material to be injected into the cell, leaving the viral envelope incorporated into the host membrane. The capsid is rapidly digested within the cytoplasm, thereby freeing the viral material following which HIV reverse transcriptase begins transcribing its single-stranded RNA into double-stranded DNA using host nucleotides. The lack of proof reading activity of reverse transcriptase leads to frequent mutations of the viral genome (Coffin, 1986; Bakhanashvili *et al.*, 1993). Typically it is believed one mutation occurs with every replication of the genome and is speculated to determine the ability of HIV to continually evade the host immune response and additionally allow resistance to drug therapies to occur within months of treatment initiation. Following reverse transcription, HIV integrase shuttles the viral HIV DNA into the nucleus where it is integrated into the host chromosome. The cell will continue to transcribe its DNA into mRNA as normal and in doing so will synthesise viral proteins along with its own. One such protein produced is the glycoprotein, gp160, which is cleaved after leaving the Golgi apparatus into gp41 and gp120. These two smaller glycoproteins make up the trimer spike formation and are inserted into the host cell membrane where they wait till the viral interior protein polypeptide chains reach the cell membrane. As the interior protein chains bud out of the host cell, as described earlier, they carry with them host cell membrane and at this point will become coated with the HIV spikes that were pre-inserted into the membrane (Manninen *et al.*, 2001). The final step in the HIV life cycle is performed by HIV protease, which is responsible for the maturation of new virions. Through

cleavage of the newly synthesised gag and gag-pol polypeptide chains (including p24, p17 and p7) into their mature monomeric and active states, HIV protease allows formation of the interior viral core, thereby converting the virion into an infectious entity (Moulard *et al.*, 2000).

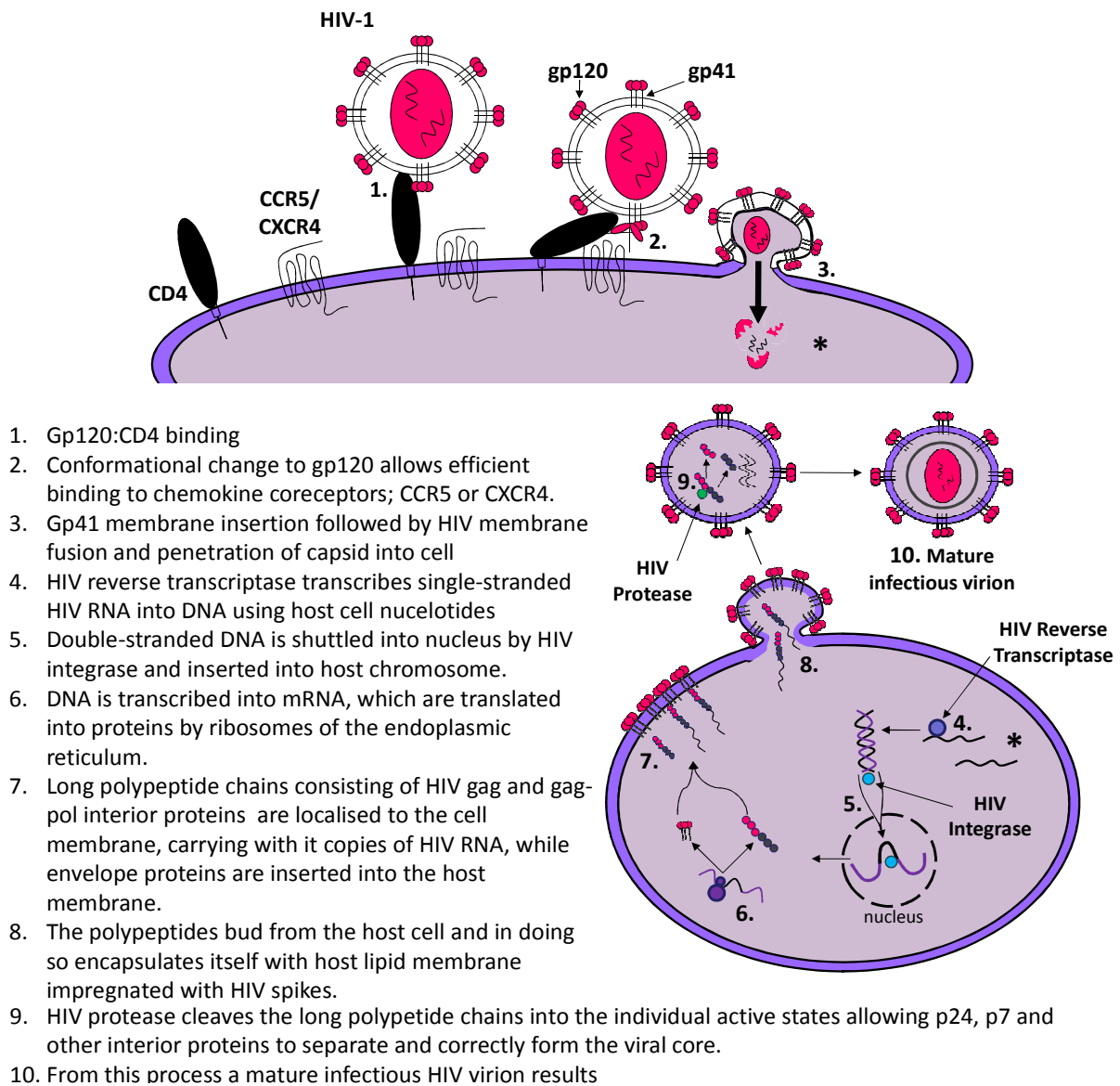


Figure 1.1.2) Schematic diagram depicting the HIV life-cycle; from first infection to replication and virus release.

1.1.3 HIV-1 Gp120

i Structure and binding kinetics

Gp120 has a molecular mass of 120 kDa, 50% of which comes from the N-linked and O-linked glycans attached to over 20 potential glycosylation sites determined by specific serine or threonine residues (Leonard *et al.*, 1990). This makes gp120 one of the most highly glycosylated proteins in nature.

Extensive glycosylation occurs to gp160 in the endoplasmic reticulum and Golgi complex during synthesis and post-translational processing. On exiting the Golgi pathway, the cellular proteases furin and plasmin, of the pro-protein convertase family, cleaves gp160 into gp120 and gp41 (Decroly *et al.*, 1997; Okumura *et al.*, 1999; Moulard *et al.*, 2000). The major consequence of the highly glycosylated state of gp120 is it acts as a 'glycan shield'. This disguises the gp120 surface from recognition by the host immune system and neutralising antibodies (Wyatt *et al.*, 1998) because the glycans themselves are of host-cell origin and are therefore less susceptible to targeting (Wang, 2006). In addition the complexity of gp120 glycosylation, afforded by varying combinations of N-glycans, O-glycans, of the complex and hybrid type at different glycosylation sites (Geyer *et al.*, 1988; Mizuochi *et al.*, 1988; for review see Marth *et al.*, 2008), allows evolution of gp120 throughout the course of infection in response to the continuous adaptive immune response.

The glycan moieties are not believed to be directly involved in the binding of gp120 to CD4 as suggested by Fenouillet *et al.* who demonstrated that enzymatic removal of N-linked glycans from gp120 reduced but did not abolish CD4 binding (Fenouillet *et al.*, 1990). However, there is now growing evidence to suggest that, while not directly involved in CD4 binding, glycan moieties influence viral tropism, ensure correct folding and may play a direct role in coreceptor binding and the necessary conformational change in gp120 required post CD4-binding. The gp120 coding domain can be divided into 5 conserved regions (C1-C5) and 5 variable regions (V1-V5) (Starcich *et al.*, 1986) determined from the comparison of gp120 glycoproteins derived from different HIV isolates. The CD4 binding is dependent on discontinuous conserved sequences found within the third and fourth conserved regions (C3/C4) (Kowalski *et al.*, 1987; Olshevsky *et al.*, 1990). The variable V1/V2 loop provides the greatest shielding of the coreceptor binding sites and following binding with CD4, conformational repositioning of these regions allows access to be gained for subsequent coreceptor binding (Kwong *et al.*, 1998). Glycan binding sites flank the crucial binding sites involved in CD4 and CXCR4/CCR5 binding, but not within it, therefore glycosylation at these sites can greatly influence the folding of the gp120 structure and likely influence binding affinity (Borbe *et al.*, 1995; Pollakis *et al.*, 2001).

On complete formation of the mature virus, gp120 is exposed on the surface of the viral envelope where it is non-covalently bound to the gp41 stalks meaning it has just a loose connection with the envelope. This results in gp120 being easily shed, whether during HIV entry, during viral circulation or from infected cells (Gelderblom *et al.*, 1987). Monomeric gp120 may therefore roam free in the blood, allowing it to interact with and activate CD4+/ coreceptor-expressing cells. Virus-bound gp120 is essential for initiating HIV-host cell binding but monomeric gp120 also maintains the ability to

target, bind and activate CD4+ cells in the absence of virus or infection (Doranz *et al.*, 1999; Dey *et al.*, 2009). In addition, in the absence of CD4, certain cell types expressing the chemokine coreceptors CCR5 and or CXCR4, exhibit the ability to respond to HIV and viral proteins. Evidence for this is shown by 1) immunohistochemical staining and receptor-binding studies (Hesselgesser *et al.*, 1997; Broder *et al.*, 1997) and 2) calcium transients in primary cultured DRG cells on exposure to HIV-1 gp120 and the natural ligands of CCR5 and CXCR4; CCL5 (also known as RANTES: Regulated Upon Activation, Normal T-cell Expressed, and Secreted) and CXCL12 (also known as SDF-1 α : stromal cell-derived factor-1 α) respectively (Oh *et al.*, 2001). This indicates that CD4-independent pathways can be elicited by monomeric gp120. Based on affinity binding studies the K_d for gp120-CD4 binding is reported to be within the range of 1–10 nM (Ashkenazi *et al.*, 1990; Moore *et al.*, 1990; Ivey-Hoyle *et al.*, 1991; Moebius *et al.*, 1992), although others have reported it to be higher, around 30nM (Turville *et al.*, 2001). While in the absence of CD4, as on neuronal cells and Schwann cells, the K_d for monomeric gp120 binding to CXCR4 and CCR5 is much more controversial at 70–200nM (Hesselgesser *et al.*, 1997; Babcock *et al.*, 2001) and 4–300nM (Doranz *et al.*, 1999; Liu *et al.*, 2000), respectively. Conversely other reports identify, in the absence of CD4, no CXCR4 binding at gp120 concentrations as high as 0.5 μ M (Mondor *et al.*, 1998; Doranz *et al.*, 1999) and only minimal binding with CCR5 (Trkola *et al.*, 1996; Wu *et al.*, 1996). Clearly great discrepancies regarding gp120 binding kinetics remain and likely result from the broad differences in experimental model, the strain of gp120 tested, glycosylation state of gp120 and the analysis protocol.

The occurrence of free-floating monomeric gp120 has given researchers the rationale to use recombinant monomeric gp120, in the absence of HIV virions. However this has lead to potential problems in the development of effective recombinant gp120 glycoproteins, which have demonstrated variable physiological activity when expressed in different cellular systems. Glycan complexity has increased with evolution so that there are now broad differences in the glycosylation pathway between prokaryotes and eukaryotes (Marth *et al.*, 2008; Dell *et al.*, 2010). The glycosylation state therefore greatly influences the binding kinetics of gp120 for CD4 and CCR5/CXCR4 and likely explains some of the contradictory results obtained from studies using different forms of gp120.

ii Viral tropism

All HIV isolates can infect and replicate in CD4⁺ T-lymphocytes, however different HIV strains specifically target and infect certain cell types via the selectivity of gp120 to one or both of CCR5 or CXCR4. The virus' preference for one co-receptor versus the other is known as "viral tropism" and is determined by a specific sequence within the V3 variable loop of gp120 as identified by single amino acid substitutions (Takeuchi *et al.*, 1991; Isaka *et al.*, 1999). One of the most commonly used tests to determine viral tropism in the clinical setting is known as the 11/25 rule, used in combination with viral sequencing and statistical prediction paradigms to enhance precision. The 11/25 rule is based on mutagenesis studies by de Jong *et al.* who identified specific "hotspots" of aminoacidic residues (De Jong *et al.*, 1992). This has since been further supported by others, demonstrating that positively charged amino acid residues at positions 11 and / or 25 within the V3 loop are highly associated with CXCR4-utilising viruses, while those targeting CCR5 have negatively charged amino acids in these positions (Cordonnier *et al.*, 1989; Shioda *et al.*, 1992; Cann *et al.*, 1992). However, differences in glycosylation sites between R5 and X4-utilising viruses have also been postulated to be important for viral tropism (Pollakis *et al.*, 2001). The majority of viral strains, especially early on during infection, target CCR5 and are known as either CCR5 (R5)-tropic or M-tropic viruses as they preferentially infect and replicate in macrophages. Many fewer patients have CXCR4 (X4)-tropic viral strains that target CXCR4 and preferentially infect and replicate in CD4⁺ T-cells, thereby giving them their alternative name of T-tropic viruses. Viral strains that have the capacity to bind to both CCR5 and CXCR4 are known as X4/R5 or dual-tropic viruses. Interestingly, while the majority of viruses, during the early stages of HIV infection utilise CCR5, in approximately half of HIV patients a tropic switch from R5- to X4-tropic viruses, requiring changes in the envelope sequence, is known to occur (Delobel *et al.*, 2005). Whether this is a result of selective pressure from HIV therapy (Gunthard *et al.*, 1999) or the host immune response (Wodarz *et al.*, 1998), or an active process is still unclear. However, much evidence now presents a strong correlation between the R5 to X4 tropic switch and acceleration in the pathogenesis of HIV and progression to AIDS (Koot *et al.*, 1993; Hartley *et al.*, 2005).

iii Cellular targets of HIV and gp120

Like all lentivirae, HIV targets and infects cells of monocyte/ macrophage lineage (Clapham *et al.*, 2002); (Clements *et al.*, 1996; Miller *et al.*, 2000), only evolving to additionally infect CD4⁺ T-cells most likely as a result of host adaptation (Kuroda, 2010). As stipulated earlier, virus attachment requires an interaction of gp120 with specific host cell expressed CD4 receptors and members of the chemokine receptor family, CCR5 and/ or CXCR4. CD4 is expressed on the surface of T lymphocytes,

macrophages, dendritic cells and brain microglia that are, in addition, the main target cells for HIV infection. Neurons do not express the CD4 receptor required for viral entry. Several groups have demonstrated the presence of HIV-1 transcripts and proteins in peripheral nerve and DRG (Yoshioka *et al.*, 1994; Brannagan, III *et al.*, 1997). However, it is generally held that due to the lack of CD4 receptors, there is only limited HIV-1 replication in neurons, further supported by a study by Jones *et al.* who identified only low level detection of HIV-1 RNA in peroneal nerve of patients with neuropathy (Jones *et al.*, 2005). CCR5 and CXCR4 are both members of the G-protein-coupled chemokine receptor family and are expressed on numerous cells types including Schwann cells (Bhangoo *et al.*, 2007), macrophages (Sorensen *et al.*, 1999; Simpson *et al.*, 2000) and neurons (Oh *et al.*, 2001; Bhangoo *et al.*, 2007), in addition to other infective cells, with particularly high CCR5 expression on T cells (Wu *et al.*, 1997). In the presence of CD4, these chemokine coreceptors are essential for the fusion and entry of HIV into a permissive cell. In the absence of CD4 however, CCR5 and CXCR4-expressing cells remain a target for gp120 binding from both virion-associated and monomeric free-floating gp120 leading to activation of these receptors and induction of signalling pathways distinct to HIV infection. Interestingly, where both receptors are expressed on the cell surface, gp120 is known to target and activate just one of these receptors. Although macrophages and T-cells express both coreceptors gp120 preferentially targets and utilises CCR5 mainly on macrophages and CXCR4 predominantly on T-cells, but what determines this preference remains unclear. In addition specific cell types demonstrate enhanced expression levels of one receptor over the other. In the CNS, for example, CCR5 expression occurs predominantly on microglia (Ghorpade *et al.*, 1998; Albright *et al.*, 1999) while CXCR4 expression is found predominantly on glial cells and neurons (Lavi *et al.*, 1997; Horuk *et al.*, 1997; Hesselgesser *et al.*, 1999). A similar distribution pattern occurs in the peripheral nervous system where Schwann cells, representing glia of the periphery, and peripheral neurons show strong predominance for CXCR4 expression (Oh *et al.*, 2001; Keswani *et al.*, 2003; Bhangoo *et al.*, 2007). The mechanisms and pathways associated with gp120-binding will be reviewed in greater detail later.

CCR5 and CXCR4 are both G-protein coupled receptors, which typically internalise following activation (Kelly *et al.*, 2008). This raised the question as to whether gp120 is also internalised following CD4-coreceptor or coreceptor-only binding. Experiments using radioiodinated (¹²⁵I)-labelled or gold-conjugated gp120, and confocal or electron microscopy, have since demonstrated that gp120 is rapidly internalised following CD4 and/or CCR5/ CXCR4 binding into CD4+ T-cells (Cefai *et al.*, 1992), langerhan cells (Schmitt *et al.*, 1990), CHO (chinese hamster ovary) and Jurkat cell lines (Misse *et al.*, 1999), dendritic cells (Turville *et al.*, 2001) and neurons in the central nervous system (Bachis *et al.*, 2003; Bachis *et al.*, 2004; Bachis *et al.*, 2006). Furthermore, correlation studies have

indicated that gp120 internalisation is a critical step in the cytotoxicity of gp120 in both neurons (Bachis *et al.*, 2003) and non-neuronal cells (Cefai *et al.*, 1992; Misse *et al.*, 1999). Research by Bachis *et al.* using gp120 conjugated to colloidal gold particles, assessed by electron microscopy, has since elucidated the path and temporal course of the T- or CXCR4-tropic gp120, gp120_{IIIB}, in the CNS following coreceptor binding (Bachis *et al.*, 2006). Internalisation of gp120 into synaptic terminals of cerebellar granule cells was shown to occur within 15 min of gp120 exposure. By 30mins it was suggested that gp120 had been axonally transported by the observation of gold particles on microtubules. Gp120 activity appeared to be terminated by incorporation into lysosomes, which was evident 1hr following exposure. However, discrepancies exist in this topic and in fact some studies have suggested that gp120 does not internalise into glial cells (Bachis *et al.*, 2003; Bachis *et al.*, 2006) despite published data suggesting gp120 binding and activation of Schwann cells via CXCR4 (Keswani *et al.*, 2003; Melli *et al.*, 2006). The use of varying experiment models; different gp120 strains, cell lines versus primary tissue *in vitro*, *in vitro* versus *in vivo*, neuronal and non-neuronal cells in the CNS and systemic and tissue-specific differences within the CNS and PNS, likely explain the contradictory data. Therefore, gp120 dynamics, kinetics and the temporal course require further investigation to clarify tissue-specific functions and may elucidate mechanisms underlying associated cytotoxicity (to be reviewed later).

1.1.4 HIV and AIDS: prevalence

According to the WHO/UNAIDS 2010 Global Report, an estimated 33.3 million people (31.4–35.3 million) were living with HIV by the end of 2010, 92.5% of which are adults (aged >15 years). In 2009, approximately 2.6 million more people had become newly infected and 1.8 million died from AIDS (World Health Organization (WHO), 2010). Fortunately access to treatment has also expanded dramatically, from 240,000 people in 2001 receiving antiretroviral therapy in low- and middle-income countries to 1.3 million in 2005. However Sub-Saharan Africa remains the worst affected area with 67.6% (22.5 million; 20.9–24.2 million) of HIV positive people residing in this area, equating to an adult prevalence of 5.0% (4.7–5.2%) within this population, and 0.8% (0.7–0.8%) worldwide. The increasing number of HIV cases each year puts an even higher demand on current treatments available. Results from a recent epidemiological study that reviewed national HIV surveillance data across 25 states in North America, highlighted that the average life expectancy after HIV diagnosis had risen from 10.5 years in 1996 to 22.5 years in 2005 (Harrison *et al.*, 2010). Further studies now indicate an average total life-expectancy around 58 years with nearly 50% of patients, diagnosed above 40 years old, dying from non-AIDS-related causes (Braithwaite *et al.*, 2005). However, as HIV patients live longer, the prevalence of HIV complications, either from

infection *per se* or antiretroviral therapy has also increased making the need for improved therapy and efficacious analgesics even more vital.

1.1.5 HIV and AIDS: co-morbidities and complications

HIV infection is currently a non-curable chronic condition that leads to the eventual suppression of the hosts' immune system and development of AIDS. In adults, progression from HIV infection to AIDS is characterised by 1) the presentation of any clinical stage 4 condition (e.g. HIV wasting syndrome, recurrent severe pneumonia, extrapulmonary tuberculosis, HIV encephalopathy or HIV-associated cancer, for review see (World Health Organization (WHO), 2007) and/ or 2) a CD4+ cell count less than 200 cells/ mm³ or a CD4+ percentage less than 15. Pathological deaths associated with AIDS are the result of related co-morbidities that take hold following immune suppression after chronic infection with the virus; exacerbated by comorbid behaviours. Some of the most prevalent co-morbidities associated with HIV include hepatitis C infection (Operskalski *et al.*, 2011), herpes simplex and zoster (Freedman *et al.*, 2004; Rodgers S *et al.*, 2011), diabetes (Samaras, 2009), tuberculosis (Toossi, 2003; Diedrich *et al.*, 2011) and numerous cancers such as Kaposi's sarcoma, B-cell lymphoma and Hodgkin's disease (Tulpule *et al.*, 1999; Gucalp *et al.*, 2010). Co-morbid behaviours include alcohol and drug abuse, which have been shown to synergise the negative effects of HIV infection and increase the progression to AIDS (Cabral, 2006; Hahn *et al.*, 2010; Rogers, 2011).

In addition to these co-morbidities highlighted many pathological complications are associated with HIV infection and lead to additional disease states, including the highly researched HIV-related dementia and HIV-associated sensory neuropathies (HIV-SN). HIV-dementia will not be discussed in this project but has been reviewed previously (Kaul *et al.*, 2001).

HIV-SN is one of the most common neurological complications of HIV infection and its therapy, affecting on average around 36% of people living with HIV (Keswani *et al.*, 2002; Wright *et al.*, 2008; Affandi *et al.*, 2008). HIV-SNs represent a broad collection of different pathological processes and clinical presentations, which may result from one or a combination of factors including, direct exposure to HIV *per se*, HIV related infections or iatrogenic effects associated with antiretroviral therapy. Two of the most frequent and distressing HIV-SNs are known as antiretroviral toxic neuropathy (ATN) and HIV-associated distal sensory polyneuropathy (HIV-DSP). Both are small fibre distal symmetrical sensory polyneuropathies characterised by a 'die back' pattern of axonal degeneration of predominantly small diameter neurons and are often characterised by neuropathic pain; recently re-defined as 'pain arising as a direct consequence of a lesion or disease affecting the somatosensory system' (Treede *et al.*, 2008), which will be reviewed later. Defining symptoms of

HIV-SN include disabling pain with paraesthesiae, gait instability and autonomic dysfunction (Pardo *et al.*, 2001). However, with either condition, sub-clinical neuropathy may also occur in the absence of any such symptoms (Tagliati *et al.*, 1999). The clinical phenotype of ATN and HIV-DSP are clinically indistinguishable and yet they are believed to arise from and exhibit, two distinct aetiologies and pathological features. HIV-DSP results as a consequence of HIV infection *per se* while ATN is a frequent complication of antiretroviral therapy, predominantly as a consequence of the nucleoside-analogue reverse-transcriptase inhibitors (NRTIs) used as part of highly active antiretroviral therapy (HAART). ATN is more frequently observed in developed countries where treatment is more readily available. While NRTIs have radically reduced the morbidity and mortality rate of HIV sufferers the ATN that can result can sometimes be just as debilitating as the disease itself and is believed to be a mitochondrial neuropathy (Lewis *et al.*, 1995; Dalakas, 2001). HIV-DSP, in contrast, is hypothesised to result, not from direct infection of neurons, as neurons are believed not to express the CD4+ receptor required for cell entry, but rather via interaction of the HIV envelope glycoprotein, gp120, with neuronally expressed chemokine coreceptors; CXCR4 and CCR5. HIV-DSP is most prevalent in developing countries where little drug therapy is available. A recent cross-sectional comparative study by Smyth *et al.* assessed the prevalence of and risk factors for HIV-associated neuropathies in 100 patients from Melbourne, Australia in 2006 and compared their findings to the prevalences in 2001 and 1993 (Smyth *et al.*, 2007; Table 1.1.2). In 1993, the era before use of the neurotoxic antiretroviral drugs, the prevalence for DSPs was recorded as 13%, lower than present day figures however this low statistic is a result of early deaths in patients, as a result of insufficient treatment available at that time, and consequently fewer were living long enough to develop DSP. Around 2001 saw the highest use of the neurotoxic antiretroviral drugs (stavudine, didanosine, zalcitabine), and in this time the prevalence increased to 44%, with approximately 37% of these patients experiencing a painful neuropathy. This study subsequently showed that in 2006, when the use of these neurotoxic antiretrovirals had been significantly reduced and replaced with new non-toxic therapies, the prevalence of DSPs was unaltered, with the proportion developing painful neuropathies maintaining around 43%. This implied that the neurotoxic neuropathies diagnosed in people on antiretrovirals were not solely due to the therapies with identified toxic properties and that there could be an underlying cause driven by HIV *per se*. Furthermore, whether new drugs continue to produce unidentified toxic effects on neurons, to induce ATN, remains unclear at the moment. While both ATN and HIV-associated neuropathies are clinically important and require further research, this project will focus only on HIV-DSP, to understand what role HIV has in contributing to these neuropathies, and will be reviewed in greater detail in section 1.3.

Table 1.1.2) DSP prevalence in HIV; taken from a cross-sectional comparative study in 2007. *Smyth et al. HIV Medicine 2007; 8: 367–373, (n=100).*

	<u>1993</u> pre HAART	<u>2001</u> HAART- dNRTI era	<u>2006</u> HAART – post dNRTI era
DSP Prevalence	13%	44%	42%
Any Pain	?	74%	93%
Pain > 5/10	?	37%	43%
Using NRTI	-	62%	8%
Ever used NRTI	-	82%	53%

1.2. HIV and Immunity

1.2.1 Immunity and cell targets in HIV Infection

Immunity can be defined as the ability of a host to resist against the toxic actions of specific foreign entities and to avoid unwanted invasion by biological pathogens. Immunity has been categorised into two broad components; innate and adaptive. Innate immunity provides a rapid and maximal but non-specific response on first exposure to a new pathogen. This occurs not through an immunological memory of the pathogen but by recognition of conserved molecular markers unique to microorganisms that are expressed on its cell surface, using pathogen recognition receptors such as Toll-like receptors (TLRs). Innate mechanisms include both physical and chemical barriers provided by the skin or chemicals in the blood such as cytokines and chemokines, and function to alert the host to infection before an adaptive response takes hold. Adaptive immunity on the other hand is more delayed in its activation but provides a pathogen- or antigen-specific response on re-exposure to that element. Within adaptive immunity there is also passive immunity, whether natural or artificial, whereby antibodies directed against a particular pathogen are passively transferred to the host, whether during pregnancy from the mother to the fetus via the placenta or by means of vaccination. The converse of this is active adaptive immunity, which results in activated immune cells acquiring a ‘memory’ of the live pathogens it encounters via specific antigens presented on its surface. This allows adaptive immunity to evolve over the course of the hosts’ life as it accumulates ‘memories’ of a diverse range of pathogens.

Viral infections initiate a strong innate response that is followed by a gradual development in adaptive immune responses. In the context of HIV much evidence indicates a typical innate response

however, other reports have demonstrated that within 7 days of first exposure to HIV infection only a weak innate response is evident (Yan *et al.*, 2011). It is hypothesised that this is the result of the complex and dense shielding provided by attached glycans of host origin that disguise the recognisable antigens on the virion from the hosts' immune system.

Most HIV infections (>80%) occur via exposure of mucosal surfaces to the virus usually following sexual transmission (Joint United Nations Programme on HIV/AIDS (UNAIDS), 2008; Shattock *et al.*, 2003), although childbirth from an infected mother can also lead to mucosal transmission (Thorne *et al.*, 2000). The remaining 20%, in general, occur as a result of percutaneous or intravenous inoculation. It can take between 2 to 6 hours for the HIV virus to cross the mucosal barrier, following which the first cells believed to be targeted for infection are CD4 T cells, macrophages and Langerhan cells (epidermal dendritic cells) (Boggiano *et al.*, 2007; Hladik *et al.*, 2009). Dendritic cells (DCs), which are considered the first cells exposed to HIV, have been implicated in the dissemination of the virus and particularly suggested to explain sexual transmission in an intact undamaged vagina that is protected by numerous layers of squamous epithelium (Hu *et al.*, 2000; Miller *et al.*, 2005). However intraepithelial dendritic cells do not express CD4, CCR5 or CXCR4 and viral replication has been shown to be inefficient in DCs, therefore uniquely they appear to facilitate transmission independently of these receptors (Hu *et al.*, 2000; Shattock *et al.*, 2003). Further research has clarified that C-type lectin receptors and Toll-like receptors allow DCs to engulf the virion and then migrate to lymphoid tissues where they present antigen-presentation signals to T cells. Work by Tsunetsugu-Yokota *et al.* using co-cultures of HIV-infected DCs with resting CD4+ T cells first demonstrated that activated infected-DCs induce T cell migration, bringing them into close proximity, which in turn allows a 'virological synapse' to form between the two (Tsunetsugu-Yokota Y, 1997). This focal adhesion and mutual activation causes the virus to bud through the synapse and infect the T cells and is now a widely accepted mechanism for HIV transmission and dissemination following first infection (McDonald D, 2003; Arrighi JF, 2004; Zhou *et al.*, 2011).

Lymphocytes represent the largest population of white blood cells and can be sub-divided broadly into two categories 1) large granular lymphocytes, including natural killer cells (NK cells) and 2) small lymphocytes consisting of the T cells and B cells. Within the population of T cells further sub-categories exist, determined by a selection process in the thymus that aims to remove cells that could main target and attack 'self' or host peptides and allocates a particular function to the T cells that passed the selection process (Bevan MJ, 1997; Baldwin TA, 2004). Such subsets include the helper and cytotoxic T cells however, of particular interest to this review, are the memory and naïve T cells. Naïve and memory CD4+ T cells represent the main targets and source of HIV replication and

dissemination due to their widespread distribution, in almost every extra-lymphoid tissue, particularly in intestinal and genital mucosa, lung, skin and liver tissue (Douek DC *et al.*, 2003; Picker LJ, 2006), and their expression of CD4 and CCR5 receptors. However, they are also highly susceptible to HIV-induced apoptosis and have weak regenerative ability therefore their half-lives are relatively short. CCR5-utilising HIV strains are believed to underlie the majority of new infections, an insight that highlighted CCR5 to be critical for the transmission of HIV. This became widely accepted following the identification of the CCR5-Δ32 deletion mutation inherited by a small population of Caucasians (5–14%); predominantly in Europeans and is virtually absent among east Asians, native Americans and Africans (Liu *et al.*, 1996; Stephens *et al.*, 1998; Sabeti *et al.*, 2005). The CCR5-Δ32 allele constitutes the deletion of 32 base-pairs in the CCR5 gene (Dean *et al.*, 1996)) and offers almost complete protection from infection by CCR5-tropic HIV (Samson *et al.*, 1996), revealing CCR5 as a powerful therapeutic target (Lederman MM *et al.*, 2006). CD4+ T cell targets must also express the CCR5 receptor to maintain HIV infection however fortunately CCR5+ CD4+ T cells represent a minority of the T cell compartment (Picker *et al.*, 2004; Brenchley *et al.*, 2004), with only 5% of CD4+ T cells, present in peripheral blood, demonstrating CCR5 expression (Veazey *et al.*, 2000), although higher expression levels of CCR5 are evident in intestinal CD4+ T cells. Therefore it has been suggested that the dominant presence of CCR5- CD4+T cells, which remain uninfected and free of cell death, allows the persistence of the virus as they replenish the CD4+ T cell population following rapid death of infected CCR5+ T cells and in turn maintain a level of immunity within the host (Grossman Z *et al.*, 2006). Therefore while CD4+ T cells may act as the main target and source of HIV replication, they are also a relatively unstable source. In contrast monocytes are also considered a primary source of HIV replication however, unlike CD4+ T cells, they show great resistance to HIV-mediated apoptosis particularly on differentiation into macrophages (Busca A *et al.*, 2011) as a result of an upregulation of antiapoptotic molecules (Lum JJ *et al.*, 2003). Therefore macrophages represent stable and long-lived reservoirs of HIV replication and in turn their circulation and movement around the circulatory and lymphatic system, and ability to efficiently infiltrate tissue allows for rapid and widespread dissemination of the virus.

Macrophages are derived from monocytic cells generated in the bone marrow and distributed throughout the peripheral system, where they reside in periphery tissues. As resident tissue macrophages, or infiltrating monocytes that differentiate into macrophages, they play a key role in both innate and cell-mediated immunity. Macrophages derive their name from the Greek for big eaters; *makros* to denote large and *phagein* to mean eat, which perfectly describes their function as phagocytic cells that engulf and digest pathogens. They have evolved with the ability to target and eliminate debris and foreign microorganisms from the system by phagocytosis and endocytosis,

through presentation of antigens they stimulate T lymphocytes and other immune cells to respond to pathogens, contribute to tissue repair, as well as being capable of extracellular killing of infected and altered self target cells (Elhelu MA, 1983; Mosser *et al.*, 2008). Different macrophage activation states and function in immunity will be reviewed in greater detail in Section 3.1.2.

1.2.2 Cytokines, chemokines and their receptors: role in HIV

Cytokines are small regulatory proteins that have a diverse array of functions, particularly in response to inflammatory disease and infection. Cytokines are produced by white blood cells and a variety of other cells including macrophages, lymphocytes, Schwann cells and neurons. Cytokines make up a large group of proteins but structural homology has been able to partially categorise them into four families; 1) the four α -helix bundle family, which is further divided into three sub-families: IL-2, interferons (IFNs) and IL-10; 2) the IL-1 family (primarily including IL-1 and IL-18); 3) the IL-17 family (not fully characterised but members have a specific effect promoting proliferation of T-cells that cause cytotoxic effects) and finally 4) chemokines.

Chemokines are a family of small chemotactic cytokines produced by many kinds of leukocytes (white blood cells) and other cell types. They are considered to have either a pro-inflammatory function, chemotactic properties to recruit leukocytes to sites of infection, or a homeostatic role and are involved in controlling cell migration during tissue maintenance and lymphocyte trafficking. Aberrant regulation of chemokine activity, however, is now poised as a strong candidate for various disease states and has been implicated in the pathogenesis of several inflammatory and pain-related disorders, which include multiple sclerosis and rheumatoid arthritis (reviewed by Gerard C *et al.*, 2001; Trebst C *et al.*, 2001), in addition to HIV-related diseases (Lee B *et al.*, 1999; Lusso P, 2006).

For the purpose of this project only the chemokines and their receptors, directly related to HIV will be discussed in further detail here but the roles of other cytokines will be discussed in Chapter 3.

The chemokines actions are mediated through a family of 7 transmembrane G-protein coupled receptors (GPCR) that are expressed on a variety of different cell types. CCR5 and CXCR4 are two such GPCR chemokine receptors that have now been identified to also act as coreceptors for the HIV-1 virus allowing it to gain entry into permissive cells. They are expressed on numerous cells types but of particular importance to this project is their expression on neurons, macrophages and Schwann cells. GPCRs make up the largest group of transmembrane-spanning receptors and allow signals to be transduced from the extracellular domain to intracellular region of a cell. Their complexity and ability to recruit different proteins allows vast intracellular signalling pathways to be

activated, which in turn mediate a multitude of varying cellular responses. GPCR are first activated by the binding of a selective agonist, which may include anything from a photon of light, as with the rhodopsin receptor (Filipek *et al.*, 2003; Ahuja *et al.*, 2009), to entire proteins as with gp120 and the CCR5 or CXCR4 coreceptors. Binding initiates a conformational change that alters the orientation of the cytoplasmic loops of the receptor, exposing a portion previously hidden that allows selective intracellular residing G proteins to bind. Unbound G proteins exist as a trimeric complex comprising two subunits; $G\alpha$ and $G\beta\gamma$. Activation of GPCRs recruits these trimeric G proteins, which induces the exchange of GDP for GTP bound to the $G\alpha$ -subunit on binding to the GPCR intracellular loop. In turn, this causes the dissociation of the $G\alpha$ and $G\beta\gamma$ subunits, allowing each to depart to activate separate cellular effector molecules and initiate differential signalling pathways (Marinissen *et al.*, 2001). Such pathways can lead to the production of numerous second messengers such as cAMP cGMP, diacylglycerol (DAG), inositol (3,4,5)-triphosphate (IP3) and in turn, to increased intracellular Ca^{2+} levels and modulation of ion channel activation. Termination of the signalling is gained through GPCR desensitisation by a combination of mechanisms, including dissociation of the GPCR from its coupled G-protein through a process of phosphorylation steps (Bouvier *et al.*, 1988; Hausdorff *et al.*, 1989), GPCR internalisation facilitated by arrestin into clathrin-coated pits for membrane recycling and resensitisation (Lohse *et al.*, 1990; Oakley *et al.*, 1999) or sortment into lysosomal compartments from degradation (Trejo *et al.*, 1998) and downregulation of its components at both a transcriptional and translational level (Hadcock *et al.*, 1988; Hermans *et al.*, 1997).

SDF-1, also known as CXCL12, is the endogenous ligand for CXCR4 and has been reported to offer a degree of protection against T-tropic or CXCR4-tropic HIV infection or inhibition of gp120 activation. SDF-1 and CXCR4 are constitutively expressed, albeit at low expression levels, in the developing and mature CNS (Tham *et al.*, 2001; Banisadr *et al.*, 2003) in particular on neurons, microglia and astrocytes, where they play a key role in CNS development. In addition they have been found in a wide range of other tissues including heart, kidney, liver, lung and spleen (Zou *et al.*, 1998). Binding of SDF-1 to CXCR4 initiates a multitude of downstream cascades including 1) activation of phospholipase C (PLC), diacylglycerol (DAG) and inositol 1, 4, 5 trisphosphate (IP3) generation resulting in the release of Ca^{2+} from intracellular stores (Boutet *et al.*, 2001); 2) activation of phosphoinositide-3 kinase (PI3K) that activates downstream AKT, PyK2 and nuclear transcriptional factor NF- κ B signaling pathways (Bajetto *et al.*, 2001; Fernandis *et al.*, 2004; Florio *et al.*, 2006) and 3) induces several MAPKs signaling pathways such as ERK, JNK and p38 (Fernandis *et al.*, 2004). The day-to-day function of these CXCR4-CXCL12 signalling pathways revolves around mediating migration, proliferation and neuronal and lymphocyte survival (Lee *et al.*, 2004a) and regulate hematopoiesis and trafficking (Lapidot *et al.*, 2005), and the absence of such signalling has

detrimental effects as evidenced by knockout mice that show significant deficits in the development of blood cells, heart and CNS circuitry (Zou *et al.*, 1998; Tachibana *et al.*, 1998). While expression levels of both are generally low under pathological conditions either one or both may be briefly or chronically upregulated such as in AIDS. Quinolinic acid (QUIN), is an important neurotoxin associated with AIDS dementia complex (ADC) and up-regulates the expression of CXCL12 and CXCR4 in astrocytes (Guillemin *et al.*, 2003). Aberrant activation of CXCR4 on certain cell types leads to the abnormal function the cell and often secretion of bioactive products that in turn induce alterations in membrane integrity, blood brain barrier (BBB) function and/or neuronal viability.

CCR5 basal expression levels on neuron are lower than that of CXCR4 but show more significant upregulation after nerve injury. The receptor is expressed on subpopulations of lymphocytes and monocytes/macrophages in blood, primary and secondary lymphoid organs, and non-inflamed tissues. In the CNS, CCR5 is expressed on neurons, astrocytes, and microglia (Bajetto *et al.*, 2002) and signalling through this receptor regulates trafficking and functions of memory and T-lymphocytes, macrophages, and immature dendritic cells. Similar to CXCR4 signalling, and typical of a receptor coupled to $G_{i/o}$ proteins, CCR5 activation leads to inhibition of cAMP production, stimulation of Ca^{2+} ion release, and activation of MAP and Jun-N-terminal kinases (MAPK/ JNK, respectively) (Thelen, 2001). Whereas CXCR4 proves to have a fundamental role in CNS development, homeostasis and immune function, CCR5 appears to be less critical as CCR5 deficient mice demonstrate only limited immune deficits (Liu *et al.*, 1996). The importance of CCR5 in HIV infection was highlighted though by resistance to HIV-1 infection in individuals homozygous for the defective $\Delta 32$ -CCR5 allele, which results in a complete absence of CCR5 expression. Furthermore decreased CCR5 expression caused by heterozygosity for the $\Delta 32$ -CCR5 allele reduces the rate of disease progression in HIV-1-infected people (Liu *et al.*, 1996; Samson *et al.*, 1996). Unlike CXCR4, which has just one known natural ligand (SDF-1), several endogenous ligands have been reported to bind to CCR5, including MIP-1 α (macrophage inflammatory protein-1 α , also known as CCL3), MIP-1 β (macrophage inflammatory protein-1 β , or CCL4) and RANTES (regulated upon activation, normal T cell expressed and secreted, or CCL5) (Lederman *et al.*, 2006). Of these MIP-1 β and RANTES possess equivalent highest binding affinities; $IC_{50} < 1nM$ (Blanpain *et al.*, 1999). In turn these ligands have been shown to provide protection against HIV binding and infection through direct competition with gp120 for access to the CCR5 receptor (Simmons *et al.*, 1997). The CCR5 ligands are a mix of high- and intermediate-affinity ligands and in addition possess either full or partial agonist properties (Olbrich *et al.*, 1999). However activation by either type, in addition to the direct binding competition against gp120, may further protect cells from infection as they initiate the

desensitisation and subsequent internalisation of the receptor, thereby reducing cell surface expression and availability (Alkhatib *et al.*, 1997; Mack *et al.*, 1998; Navenot *et al.*, 2001).

Interestingly, there has been much debate surrounding the similarities and differences between RANTES and SDF-1 signalling, versus that induced by gp120 through the same receptors. Many studies have highlighted the ability of gp120 to activate similar signalling pathways as those activated by RANTES and SDF-1. Such measurable outcomes include Pyk2 phosphorylation, (Davis *et al.*, 1997), MAPK and functional PI-3K activation (Lee *et al.*, 2003). These second messengers have been reported to be activated via recruitment of $G_{\alpha i}$ as indicated by the ablation of these effects following incubation with pertussis toxin (Davis *et al.*, 1997; Blanpain *et al.*, 1999). Interestingly calcium mobilisation from intracellular stores (Oh *et al.*, 2001) has also been observed following gp120 exposure and is likely mediated via a $G_{\alpha q}$ pathway (Maghazachi *et al.*, 1998). This demonstrates the complex signalling pathways that can occur from activation of the same modulated by the cell type and the mode of cellular activation (Bleul *et al.*, 1998; Guinamard *et al.*, 1999). Furthermore for the first time Oh *et al.* demonstrated that both RANTES and SDF-1 induced mechanical hypersensitivity behaviour in rats following intradermal injection (Oh *et al.*, 2001). However, the observed effects were less pronounced than those induced by intradermal injection of gp120 and the erythema and swelling following gp120 injection was not observed after either RANTES or SDF-1 (Oh *et al.*, 2001). This highlights that, although similar signalling is activated both by gp120 and the endogenous ligands, they are not identical. Application of RANTES and SDF-1 α to primary cultures has been shown to potentially inhibit viral replication *in vitro* (Kelly *et al.*, 1998) and release of endogenous release of RANTES *in vivo* was demonstrated to be, in fact, protective against HIV infection (Cocchi *et al.*, 1995) in both cases by blocking and downregulating the chemokine receptors. Despite the apparent similarities, intriguingly, little data has suggested a neurotoxic effect of RANTES and SDF-1, while many studies have reported potent neurotoxicity induced by gp120, as previously outlined. Overexpression of RANTES has been identified in various chronic pathologies such as, advanced breast carcinoma (Luboshits *et al.*, 1999) and experimental autoimmune encephalomyelitis, in which RANTES overexpression was associated with the appearance of infiltrating cells in the central nervous system (Godiska *et al.*, 1995; Rodriguez-Frade *et al.*, 1999). Initial investigations into RANTES levels in HIV found that higher levels of RANTES and MIP-1 α were evident in AIDS patients than in non-progressors (Clerici *et al.*, 1996). However, high expression of RANTES has also been observed in inflammatory brain lesions both in HIV-positive and HIV-negative patients (Vago *et al.*, 2001) and no difference in β -chemokine secretion by peripheral blood monocytes was observed between those derived from AIDS patients and those from nonprogressors (Scala *et al.*, 1997). While few of these assess whether RANTES expression was a cause or a result of

the neuropathology present, this highlights a role that appears to be independent of HIV infection and associated signalling. The differential signalling between the endogenous CCR5 and CXCR4 ligands and gp120 is most likely a result of their overlapping but non-identical binding sites (Wu *et al.*, 1997; Dragic *et al.*, 1998; Lee *et al.*, 1999). In fact it is understood that while RANTES has a single binding site on the CCR5 receptor, gp120 utilises multiple binding sites (Bieniasz *et al.*, 1997; Lu *et al.*, 1997). However, further investigations are required to determine the relevance and importance of each of these different extracellular binding domains. At 120 kDa, gp120 is also 12–15 times the size of SDF-1 α or RANTES, which have molecular weights of 10 kDa and 8 kDa, respectively, which affords gp120 the ability to cover a larger binding area. Gp120 predominantly activates susceptible cells in a CD4-dependent manner to form a CD4: gp120: chemokine coreceptor complex (Wu *et al.*, 1996; Hill *et al.*, 1997), which again may result in different combinations of activated signalling pathways (Atchison *et al.*, 1996; Gosling *et al.*, 1997).

1.2.3 T cell immune responses and their role in HIV infection

Over the last decade or so, views on the immune system have changed dramatically; from originally classifying cells as active or inactive and the response as innate or adaptive, to the realisation that a broad spectrum of responses exist and can be driven by the same cells but in different ‘activation’ states. T cells and other immune cells are now understood to control the type and degree of immune response through the generation and secretion of specific profiles of cytokines and immune mediators (Mosmann *et al.*, 1989; Street *et al.*, 1991; O’Garra *et al.*, 1994; Mosmann *et al.*, 1996). These responses of T helper cells, until recently, had been broadly classified as Th1, which typically generate interleukin 2 (IL-2) and interferon- γ (IFN- γ) and induce ‘classical’ macrophage activation, or Th2 immune responses, which demonstrate secretion of IL-4, IL-5 and IL-10, and in contrast to Th1, inhibit classical macrophage activation and instead induce antibody production. As stipulated earlier the specific cytokine profiles determine the function of these responses therefore the immune responses have since been characterised functionally by investigations on these profiles; Th1-responding cells are involved with cell-mediated inflammation and the eradication of intracellular pathogens, while Th2 cells are associated with adaptive immunity and are important for the generation of antibodies to protect against or eliminate infectious microbes. Dysregulation of Th1 responses have been associated with cell and tissue destruction and chronic inflammation such as in multiple sclerosis (Giovannoni G, 1997; Weiner, 2008), diabetes (Sia C, 2005) and rheumatoid arthritis (Szekanecz *et al.*, 1994), while aberrant Th2 responses have been implicated in allergic disorders such as asthma (Melgert *et al.*, 2011). Recent identification of a third subset of helper T cells, the Th17 lineage (Harrington *et al.*, 2006), has further highlighted the complexity of immunity

and how broad classification is restrictive in its definition. The Th17-type response is now defined by the generation of IL-17 following IL-23-induced polarisation. Th17 responses had been previously hidden among Th1 responses due to common co-induction of IFN- γ (Th1) and IL-17, structural similarities between IL-12 and IL-23 (Oppmann *et al.*, 2000) and the weak IL-17 induction ability of IL-23 on naïve T cells (Aggarwal *et al.*, 2003; Langrish *et al.*, 2005; Harrington *et al.*, 2006). However, through use of *IL12p40*- or *IL23p19*-deficient mouse models (Cua *et al.*, 2003; Murphy *et al.*, 2003) and primary CD4⁺ T cell cultures (Harrington *et al.*, 2005) it has now been elucidated that IL-12 underlies polarisation of Th1 cells, preferentially inducing expression of cytotoxic-related genes including IFN- γ , FasL and granzymes (Langrish *et al.*, 2005), which are essential for host defense and tumor suppression. In contrast, IL-23, an IL-12 family member, polarises cells to the Th17-type, inducing gene expression associated with chronic inflammation, such as IL-17, IL-6, TNF- α and other pro-inflammatory chemokines (Langrish *et al.*, 2005), important for the development of tissue-specific autoimmune inflammation.

The immune responses of T cells are probably the most important aspect of HIV infection because the dysregulation in their function is associated with HIV-associated co-morbidities and complications, including the eventual loss of CD4⁺ T cells that defines the progression to AIDS and underlies morbidity. Complete understanding of the varying and switching in responses will provide greatest opportunity to intervene therapeutically and eventually offer preventative action. The current viewpoint regarding HIV and immunity suggests that a switch from a Th1-mediated response to a Th2-type underlies the progression to AIDS and onset of associated pathologies (Clerici *et al.*, 1993). As mentioned previously the ability of antibodies to target HIV virions for destruction is limited by the protection afforded to it by the heavily glycosylated envelope shielding any potential foreign antigens. Therefore adaptive immune responses and antibody-mediated immunity is unlikely to offer strong protection against infection. Cell-mediated innate immunity and the Th1-type immune response, in contrast, appears to offer far greater protection in the control and even prevention of HIV infection through the release of cytotoxic and pro-inflammatory mediators and the subsequent destruction of HIV-infected cells. This mechanism is effective, providing HIV infection does not overtake cellular immunity. This hypothesis is based on the original work of Clerici *et al.* who studied and compared the immune responses of uninfected, HIV seronegative and HIV seropositive with or without symptoms, using primary cultures of peripheral blood mononuclear cells (PBMCs) (Clerici *et al.*, 1993). They made the observation that individuals who did not seroconvert demonstrated a potent Th1 response (increased IL-2, the antigen-recall signal), similar to uninfected individuals, on exposure to HIV antigens. In contrast, the response of PBMCs from symptomatic HIV seropositive patients indicated loss of the normal Th1 IL-2 antigen-recall response

and instead a prominent Th2-type, indicated by an increase in IL-4 production. Th2-type responses function to inhibit inflammation and immune responses therefore this switch from a Th1 to Th2 immune response became highly correlated with the sudden loss of CD4+ T cells and progression to AIDS, which is defined by immune suppression and onset of opportunistic diseases. In fact another function of Th2 responses is antibody production, which is now used as key marker for disease prognosis following the association between an increase in anti-allergen IgE content and development of AIDS (Wright *et al.*, 1990; Ferrazzi *et al.*, 1993).

1.3. HIV-Associated Sensory Neuropathy (HIV-SN)

1.3.1 Clinical features and risk factors

As outlined earlier, HIV-associated sensory neuropathy (HIV-SN) is one of the most common neurological complications of HIV infection and its therapy, affecting on average around 36% of people living with HIV (Keswani *et al.*, 2002; Wright *et al.*, 2008; Affandi *et al.*, 2008). In the era before prominent use of toxic highly active antiretroviral therapy (HAART) the prevalence of HIV-SN was reported by So *et al.* to affect 35% (13/37) of patients (So *et al.*, 1988a), similar to figures reported in 2002 by Schifitto *et al.* who identified asymptomatic HIV-SN in 20% of patients and 35% with symptomatic HIV-SN (Schifitto *et al.*, 2002). With the introduction of nucleoside reverse transcriptase inhibitors (NRTI) around the beginning of the 21st century, we saw a dramatic reduction of mortality associated with HIV infection; however the prevalence of HIV-SN has remained the same. In fact, toxicity of early NRTI drugs, including stavudine (d4T), didanosine (ddI) and zalcitabine (ddC), introduced a new aetiology behind HIV-SN, classified as antiretroviral toxic neuropathy (ATN) that was clinically indistinguishable from the HIV-associated distal sensory polyneuropathies (HIV-DSP) present prior to therapy. A recent study by Evans *et al.* has reviewed the current prevalence of HIV-SN amongst ART-naïve patients and identified risk factors associated with the development of peripheral neuropathy (Evans *et al.*, 2011). They reported an HIV-SN prevalence of 22.6% amongst treatment-naïve patients, while patients who were currently receiving ARTs, or had been exposed to ARTs within 6 months of evaluation, showed a prevalence of 32.1%; 8.6% of whom exhibited a symptomatic phenotype (experiencing pain, 'pins and needles' sensation and/ or numbness). They demonstrated that with the effective control of viral replication, HIV-SN no longer correlates with viral load as indicated by the low viral loads (HIV RNA 400 copies/ml) in the majority (87.1%) of HIV-SN+ patients. Instead, age and current neurotoxic ART (nART) use were correlated with increased risk of HIV-SN and these factors, in addition to diabetes mellitus, were also correlated with increased

prevalence of accompanying negative symptoms including pain. However, the key point to highlight is that, while HAART therapy appears to increase the risk of developing HIV-SN, a significant prevalence still exists in its absence in treatment-naïve HIV patients highlighting significant toxicity remains to be driven by HIV infection and associated viral proteins *per se*. An additional interesting finding was that withdrawal from nART therapy, following the development of HIV-SN, did not allow recovery in 54.1% of patients who continued to display peripheral neuropathy. That acute neurotoxicity can prevail, even once an initiating factor has been removed, provides some insight into the underlying mechanisms and highlights the importance of preventing or at least recognising developing or underlying toxicities and adapting therapy accordingly so as to minimise the risk of developing HIV-SN. It has been suggested that HIV viral proteins establish an asymptomatic underlying toxicity that becomes unmasked on the initiation of ART therapy (McArthur *et al.*, 2005). This insight demonstrated the importance of investigating, in isolation of the ART drugs, the HIV-mediated mechanisms relating to nerve damage, despite few HIV+ individuals, at least in developed countries, particularly in European countries and America, living without some therapeutic intervention.

The clinical features are currently distinguished by a characteristic length-dependent degeneration of sensory neurons, initiating distally and progressing proximally (Verma, 2001; Keswani *et al.*, 2002; Pardo *et al.*, 2001). Degeneration occurs predominantly in unmyelinated fibres although some small and large myelinated fibres are also affected. This can be indicated by the reduction of intraepidermal nerve fibre (IENF) density (Holland *et al.*, 1997; Polydefkis *et al.*, 2002), a feature now used to assist in the diagnosis of degenerative 'die-back'-type neuropathies using PGP 9.5 (protein gene peptide 9.5) pan-neuronal labelling of skin punch nerve biopsies and subsequent quantification of fibres present in the epidermal layer. This can be accompanied by macrophage infiltration (Pardo *et al.*, 2001) with the local release of proinflammatory cytokines in areas of axonal degeneration. In fact the severity of HIV-SN has been highly correlated with the degree of inflammatory cell infiltration indicating macrophages to be potential be essential mediators (McArthur *et al.*, 1989; Griffin JWCT *et al.*, 1998). One of the most consistent pathologies in the DRG appears to be the activation of inflammatory mechanisms that leads to injury of DRG neurons. The presence of proinflammatory cytokines, such as TNF- α , IFN- γ and IL-6, as well as other mediators of inflammation such as nitric oxide, has been consistently demonstrated in the DRG of HIV patients (Yoshioka *et al.*, 1994; Nagano *et al.*, 1996) and together, in a minority of HIV-SN+ patients, these pathological processes manifest as a painful sensory neuropathy. Defining clinical features of symptomatic HIV-SN include disabling pain with paraesthesiae, gait instability and autonomic dysfunction (Pardo *et al.*, 2001) however, with both HIV-DSP and ATN, sub-clinical neuropathy may

also occur in the absence of any such symptoms (Tagliati *et al.*, 1999). While HIV-DSP is hypothesised to result from aberrant non-neuronal cell cytokine signalling and neuronal calcium dysregulation mediated by viral proteins, ATN is considered to be a result of mitochondrial toxicity (Wendelsdorf *et al.*, 2009). For the purpose of this review only mechanisms relevant to HIV-DSP will be reviewed but pathways involved in ATN have been reviewed previously by others (see Kohler *et al.*, 2007; Koczor *et al.*, 2010).

An increasing number of risk factors are being identified which, not only provide guidance to clinicians on the most appropriate treatment regimen, but additionally highlight to researchers key targets and mechanisms that may underpin the development of HIV-associated neuropathies, thereby offering hope for therapeutic prevention. Many recent advances have come with the development and increased accessibility to advanced genotyping technology. Using samples from HIV-1 infected individuals with and without neuropathy and additionally with or without painful symptoms, genotyping studies have highlighted positive correlations between the presence of specific TNF- α polymorphisms and neurological pathologies associated with HIV. The first indication came following a study in 2001 by Quasney *et al.* who quantified the expression of the TNF- α -308 site polymorphism that is associated with elevated TNF- α production (Louis *et al.*, 1998; Quasney *et al.*, 2001) in HIV-infected adults with and without dementia and control uninfected individuals. They demonstrated that the presence of the TNF- α -308 A allele was more prevalent in HIV+ adults with dementia than HIV+-undemented or uninfected healthy control individuals (Quasney *et al.*, 2001). Further TNF- α polymorphisms, TNF- α -1021*2, in addition to TNF- α -308, have since been correlated with other aspects of HIV infection including HIV-associated and ATN neuropathies (Cherry *et al.*, 2008; Chew *et al.*, 2011). This has highlighted a key role for aberrant cytokine signalling in the development of HIV pathologies.

Another risk factor associated with increased prevalence of HIV-SN, following the onset of stavudine (d4T) use, is intriguingly, but very logically, height, along with increased age (Cherry *et al.*, 2009). Among 294 subjects assessed, regardless of whether they received d4T, the risk of developing neuropathy was 6% among the population classified as younger than 40 years and shorter than 170 cm. In contrast, in individuals older than 40 years old and taller than 170 cm, the prevalence of HIV-SN rose to 22% (Cherry *et al.*, 2009). Initiation of d4T use further increased the risk of developing HIV-SN but independently of height and age, as shown by prevalence from 20% in younger, shorter patients, up to 66% in older and taller patients. Height as a risk factor is suggested to highlight the importance of the replenishment of essential compounds via active transport from the DRG in order to meet the high-energetic requirements of the nerves. Disruption in protein synthesis, active

transport or mitochondrial activity could interfere with this delivery thereby depriving areas furthest from the source first and would correlate with the length-dependency demonstrated by nerve degeneration.

1.3.2 Gp120-associated toxicity: implications for HIV-DSP

The nerve damage behind HIV-associated neuropathies is not believed to be a result of HIV viral infection because, although controversial, neurons are believed to lack the CD4 receptor required for HIV entry (Harouse *et al.*, 1989). The role of CD4 in the CNS remains elusive, and in the PNS relatively unexplored, however CD4 expression on brain-derived neuronal cells has been demonstrated (Funke *et al.*, 1987; Omri *et al.*, 1994) and viral transcripts and antigens have been identified within neuronal cells (Gabuzda *et al.*, 1986; Pumarola-Sune *et al.*, 1987). However viral replication within nerves is reported to be limited by currently unknown factors therefore only minimal, if any, HIV replication within nerves exists (Jones *et al.*, 2005).

The knowledge that gp120 maintains agonist abilities at CD4 and chemokine receptors, both when virion-bound and free-floating as monomeric protein, implicated this glycoprotein in the mechanisms underlying HIV toxicity. The ability of gp120 to bind to cells expressing CD4 and/ or CCR5 and CXCR4 in turn leads to the stimulation of the cells expressing these receptors, which includes macrophages and microglia (predominantly CCR5) (Lee *et al.*, 2003), Schwann cells and astrocytes (predominantly CXCR4) (Tanabe *et al.*, 1997; Bezzi *et al.*, 2001; Keswani *et al.*, 2003) and low expression of both (CXCR4>CCR5) on peripheral and central neurons (Oh *et al.*, 2001; Bachis *et al.*, 2003; Bachis *et al.*, 2004; Bhangoo *et al.*, 2007).

Some of the earliest indications of gp120-mediated toxicity came from *in vivo* studies where systemic and intracerebral injection in adult rats resulted in morphological damage to neurons and evidence of retardation from complex behaviour testing (Kimes *et al.*, 1991; Glowa *et al.*, 1992; Hill *et al.*, 1993). Furthermore an *in vivo* transgenic mouse model that sees gp120 env protein sequence under the GFAP promoter, causing chronic release of gp120 in the brain by astrocytes (the predominant expresser of GFAP in the CNS), elicited degeneration of neurons, loss of dendritic markers and other severe abnormalities (Toggas *et al.*, 1994). Since then further *in vivo* models have been established that utilise different delivery mechanisms, perineural, intrathecal or transgenic, however all have demonstrated a degenerative phenotype that further implicates gp120 in HIV-SN (Herzberg *et al.*, 2001; Wallace *et al.*, 2007a).

The strongest indication that gp120 may specifically initiate the degenerative phenotype associated with HIV-SN comes from *in vitro* studies assessing reductions in neurite length as an assay for degeneration. *In vitro* studies using primary cultures of embryonic or neonatal dorsal root ganglia have reported gp120-mediated neurite reduction and caspase-dependent apoptosis (Keswani *et al.*, 2003; Melli *et al.*, 2006). Direct actions of gp120 on neuronal cells have been highlighted *in vitro*, to include the activation of calcium signals in embryonic and adult primary neuronal cultures indicated by increased $[Ca^{2+}]_i$ (Oh *et al.*, 2001). In addition Oh *et al.* observed DRG neuronal excitation, either through the lowering of action potential thresholds without altering membrane potential or reversible depolarisation following independent exposure of neonatal DRGs to chemokine receptor agonists and gp120 glycoproteins. In addition it is important to note that no negative controls were performed in these studies therefore specificity of the responses was not verified. Gp120 exposure also resulted in substance P release in primary neonatal DRG neurons cultures (Oh *et al.*, 2001) however while the source was implied to occur from the neuronal cells this was not confirmed and may have been released by contaminating non-neuronal cells within the culture. Gp120 actions on non-neuronal cells in fact have been the focus of most gp120-related research. Acting through CXCR4, gp120 induces the release of TNF- α from both Schwann cells (Keswani *et al.*, 2003) and astrocytes (Bezzi *et al.*, 2001) and mononuclear phagocytes (Lee *et al.*, 2005), having implications in both the peripheral and central nervous systems.

The current consensus on gp120-mediated signalling via non-neuronal cells, in particular macrophages, includes the activation of K^+ , Cl^- and non-selective cation channels, activation of protein kinases including Pyk-2, MAPK p38 and Jun kinase pathways. These in turn lead to stimulation of transcription factors, such as NF- κ B and AP-1, induction of cytokine and chemokine gene expression and an increase in the synthesis and release of pro-inflammatory and stimulatory proteins (Briant *et al.*, 1998; Del *et al.*, 2001; Lee *et al.*, 2005; Cheung *et al.*, 2008).

Therefore neuronal toxicity is hypothesised to occur by two cellular-derived mechanisms, and have been outlined in Figure 1.3.1. One mechanism is neuronally-mediated in which HIV viral proteins directly activate CCR5 and/ or CXCR4 chemokine co-receptors expressed on neurons. This leads to increases in intracellular calcium levels and eventual axonal degeneration, although the pathways linking the activation and axonal degeneration remain obscure. The other mechanism is initiated indirectly via non-neuronal cells as a result of aberrant cytotoxic immune cell signalling and mediator release. Many of the pathways activated may converge together at some point however despite these insights it remains unclear what final processes initiate the axonal degeneration. Also it remains to be determined whether one pathway is dominant or in fact synergy of multiple pathways

is required. In addition clarification is required on whether gp120, at physiologically relevant levels, can directly activate macrophages or whether they prime macrophages. Priming of macrophages would see an exaggerated production and release of cytokines and excitotoxic mediators when activated by a secondary insult.

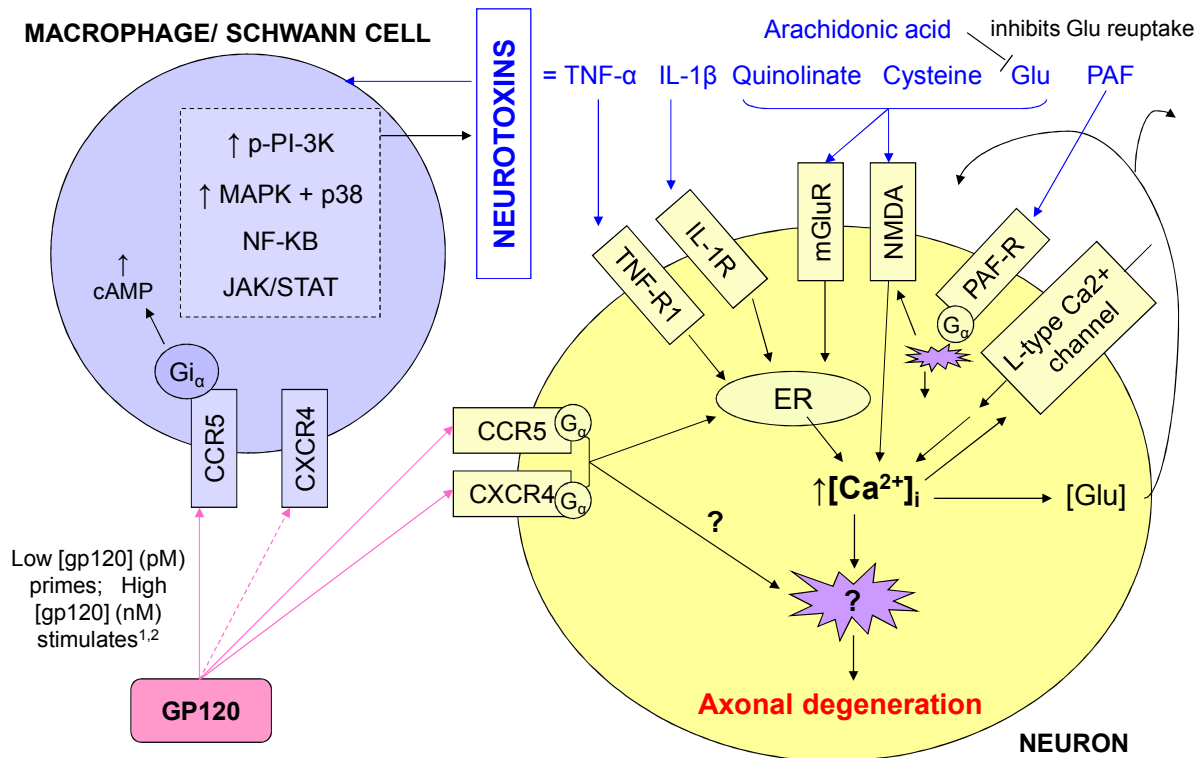


Figure 1.3.1) Schematic outlining the current hypothesis on gp120-mediated neurotoxicity and axonal degeneration. Citations: ¹ = (Freedman *et al.*, 2003); ² = (Klasse *et al.*, 2004)

1.3.3 Role of immunity in HIV-SN

Following extensive comparative research into the similarities between HIV and simian immunodeficiency virus (SIV) infection and studies interested in highlighting the origin and evolution of HIV, it has become clear that the pathology associated with HIV infection is largely a result of the highly evolved and powerful immune system present in humans. The prevalence of naturally occurring SIV_{sm} in sooty mangabeys (SM; hypothesised origin of HIV-1) and SIV_{agm} in African green monkeys (AGM; hypothesised origin of HIV-2; for review see (Apetrei *et al.*, 2004) is relatively high at between 30 and 60% (Heeney *et al.*, 2006). However studies on these natural hosts indicate they primarily do not develop AIDS; despite efficient SIV replication and high plasma levels of viremia, SMs and AGMs among other natural hosts, show relatively few neurological complications of the viral infection (Hirsch, 2004; Chakrabarti LA, 2004). This is evident by the maintenance of CD4+ T-cell

levels over the course of infection, normal lymph node morphology, little immune activation and normal life-expectancy. However intriguingly, experimental studies on these hosts have indicated that SIV and associated proteins can induce AIDS-like diseases (Hirsch *et al.*, 1995). Therefore it has been largely hypothesised that the asymptomatic affect of SIV infection in non-human primates is due to the lack of intense hyper-immune activation in natural infection and, in contrast to humans, SMs and AGMs mount a strong antibody response that targets the envelope proteins rather than gag proteins as in humans (Norley *et al.*, 1990). How and why these natural hosts lack the abnormal immune activation present in humans remains unclear however this, in addition to their ability to maintain thymic and bone marrow function, most likely explains their ability to resist cytopathology as a result of SIV infection. In turn, this highlighted how the disease progression in humans is likely the result of the abnormal immune activation and the importance of immune cells and their signalling in underlying the pathology associated with HIV infection.

Resident macrophages are evident in peripheral nerves and dorsal root ganglia (Moalem *et al.*, 2006). In times of nerve injury or inflammation the resident macrophages are assisted by haematogenous macrophages that influx into peripheral nerves during clearance of cellular debris (Bendszus *et al.*, 2003); a requirement if peripheral nerve regeneration is to occur (Perry *et al.*, 1987; Salegio *et al.*, 2011). While infiltration of immune cells holds a physiological role, the degree of infiltration has also been highly correlated with the severity of axonal degeneration and neuronal pathology indicating a potential pathological role of immune cells. In the presence of HIV infection, activation of the cytokine network has become a frequent finding in both the CNS and PNS of HIV-patients. CSF levels of TNF- α in healthy controls are typically < 10 pg/ml however increasing severity of AIDS-related dementia has been correlated with increased CSF levels of TNF- α , with early stages (stage 0.5) showing CSF levels of 7.5–40 pg/ml, while CSF levels during mid to late stages (stage 2-3) reach up to 190 pg/ml (Mastroianni *et al.*, 1992). In contrast HIV-seronegative subjects with other neurological diseases show no detectable increase in CSF levels of TNF- α (Perrella *et al.*, 1992). Increased TNF- α production by HIV-1 or gp120 has been demonstrated in primary human monocyte-derived macrophages (MDM) (Mabondzo *et al.*, 1991; Karsten *et al.*, 1996; Lee *et al.*, 2005). Stimulated macrophages from healthy donors produce < 5 pg/ml TNF- α however MDM exposure to recombinant macrophage-tropic (R5) gp120 led to dose- and donor-dependent release of TNF- α with a peak that ranged from 30–3000 pg/ml (Lee *et al.*, 2005). In addition to TNF- α , increased production of other pro-inflammatory cytokines has also been reported in response to HIV infection in cultured macrophages, including IL-1 β and IL-10 (Valentin *et al.*, 1992; Akridge *et al.*, 1994; Yoo *et al.*, 1996; Gessani *et al.*, 1997). Therefore HIV infection and viral proteins such as gp120 may directly mediate the aberrant cytokine network associated with HIV-associated neuropathology.

In addition other excitotoxic mediators released from immune cells have been implicated in neurologic disorders. CSF concentrations of quinolinic acid, the excitotoxic metabolite of L-tryptophan and endogenous agonist of NMDA receptors, were significantly correlated to the severity of the neuropsychological deficits (Heyes *et al.*, 1991). Established excitotoxic effects and magnitude of increases in CSF raise the possibility that quinolinic acid plays a direct role in the pathogenesis of neuronal dysfunction associated with HIV-1 infection.

It is well established that nerve injury and Wallerian degeneration result in the infiltration of macrophages and local release of multiple mediators. In the case of HIV-SN, as outlined above, many studies report macrophage infiltration at sites of pathology however what initiates the nerve injury or macrophage infiltration to begin with is unclear and likely implicates multiple pathways. The neurotoxic drugs are directly toxic to neurons therefore the neurotoxicity that follows may be exacerbated by the immune response. However, it has also been outlined that neurotoxic antiretroviral therapy does not explain all prevalences of HIV-SN and most likely unmasks a sub-clinical neuropathy (Phillips P *et al.*, 1997; Forrest *et al.*, 1998; McArthur *et al.*, 2005). Therefore viral proteins, in particular gp120, which also has chemotactic properties, may allow us to explain the initiation of nerve damage and maintenance by immune cells. Gp120 can bind to neurons, glia and macrophages locally to where it is released. This activation may prime the susceptible cells, in particular immune cells, to respond with greater potency to subsequent activation (Klasse *et al.*, 2004). Alternatively it may be sufficient to activate them to release mediators that subsequently recruit new immune cells that may be subsequently activated either by gp120 or by released mediators (Oh *et al.*, 2001; Toggas *et al.*, 1994; Kaul M *et al.*, 1999; Milligan *et al.*, 2001; Keswani *et al.*, 2003; Lee *et al.*, 2003).

1.3.4 Underlying mechanisms and relation to Wallerian degeneration

The mechanisms that directly underlie the process of axonal degeneration remain obscure. Until recently it was believed that axonal degeneration and apoptosis derived from the same mechanism and were directly connected however recent studies have suggested otherwise. Three factors have brought researchers to this conclusion, 1) it was shown that caspase-3, of the family of cysteine proteases normally indicative of apoptosis in neurons (Porter *et al.*, 1999), were not activated axonally during axonal degeneration (Finn *et al.*, 2000), 2) caspase inhibitors prevent the cell death associated with NGF deprivation but not axonal degeneration (Finn *et al.*, 2000), and 3) the gene for slow Wallerian degeneration (*Wld^S*) protects against axonal degeneration but not cell apoptosis following insult (Lunn *et al.*, 1989). Together these data provide evidence that axonal degeneration

and apoptosis are mechanistically distinct therefore when understanding the pathology that drives HIV-SN it is important to consider cell death and axonal degeneration independently. Given this new perspective, studies on axonal degeneration are drawing insight from mechanisms underlying Wallerian degeneration and parallels have been made between Wallerian degeneration and neuropathy induced by the toxic chemotherapeutic agent, Vincristine sulphate (Wang *et al.*, 2000).

Wallerian degeneration is the physiological process of axonal degeneration following separation of the axonal segment from its neuronal cell body, for example after a cut or crush injury. Wallerian degeneration occurs both in the peripheral and central nervous systems although characteristically different (Stoll *et al.*, 2002; Vargas *et al.*, 2007). Macrophages and Schwann cells play an essential accessory role in the process, functioning to clear degenerated debris to create a permissible environment and provide guidance cues for effective neuronal regeneration (Bruck, 1997; Stoll *et al.*, 1999; Camara-Lemarroy *et al.*, 2010).

Classically axonal degeneration was considered to be a passive wasting away phenomenon due to the deprivation of essential components (proteins, nutrients, axonal components and biosynthetic material) at the distal ends of long axons. However introduction of the *Wld^S* (slow Wallerian degeneration) mouse mutant began the suggestion of an active process of self-destruction that is initiated once a threshold of parameters is met. This hypothesis appeared following the observation that the distal portion of *Wld^S* axons remain viable, unlike those from wild-type mice, after transection from and removal of the cell body, furthermore, remaining capable of conducting action potentials for up to 2 weeks (Lunn *et al.*, 1989). Expression of the *Wld^S* gene causes the overexpression of a fusion protein consisting of 1) full-length Nmnat1 (nicotinamide mononucleotide adenylyltransferase: involved in biosynthesis of NAD⁺; an energy source), 2) 18α linker protein and 3) N-terminal portion of an E4 ubiquitin ligase known as Ube4b or UFD2 (involved in multi-ubiquitin chain assembly in order to prepare target proteins for degradation by proteasome) (Mack *et al.*, 2001). Furthermore studies investigating the relative involvement of neurons and Schwann cells in the delayed degenerative phenotype of Ola mice demonstrated that the degenerative properties were intrinsic to the neurons themselves and occurred independently of macrophages or glial cells (Perry *et al.*, 1990; Glass *et al.*, 1993). Together these suggested for the first time that axons possess an intrinsic self-regulated and active process of degeneration as opposed to a passive process of wasting away.

This has had important implications for the mechanisms involved in physiological degeneration, their contribution to neurodegenerative diseases, as well as the potential for therapeutic intervention. As such, the identification of calcium signalling as an important initiator in Wallerian and axonal

degeneration has highlighted commonalities between diseases such as multiple sclerosis, HIV-SN and Alzheimer's previously considered to be independent. In particular the involvement of calpains, ubiquitous cysteine proteases involved in many physiological processes including cell mobility (Potter *et al.*, 1998; Huang *et al.*, 2004), platelet aggregation (Kuchay *et al.*, 2007) and regulation/stabilisation of the neuronal cytoskeleton (Hasselgren, 1999). However calpain involvement in HIV only became highlighted after the initiation of my PhD therefore the implication of calcium regulation, calpain involvement and potential mechanisms linked gp120 signalling and the processes underlying axonal degeneration will be discussed in the General Discussion in section 1.4.1.

1.4. HIV-Associated Sensory Neuropathy and Pain

1.4.1 Basic Pain Pathways

Nociception, the term given to the process of perceiving and processing noxious stimuli, comprises three key events: 1) the transduction of noxious signalling across numerous barriers, from the external original stimulus to internal tissues and across synaptic clefts between neurons, 2) the transmission of both electrical signals along neuronal pathways and chemical transmission from one cell to the next, and 3) the modulation of the intensity of pain perceived through modulatory events occurring at all levels of the process: in the periphery, along primary afferent neurons, centrally in the dorsal horn and all the way in higher brain centres.

All healthy individuals, who process pain 'normally', have the same hard-wired pain processing set-up as each other, the same basic signalling procedures; however at the same time every individual has the possibility to perceive pain differently. This implies that the additional modulatory events within each individual are less hard-wired, which suggests that they are not essential for the perception of pain *per se*. From an evolutionary point of view, with pain being a warning signal to protect the host from potential or further damage, this suggests that modulation of pain signalling events does not drastically affect survivability of the individual; otherwise these components would be more conserved. However research has shown that variation in pain signalling can drastically affect the quality of life of an individual. Modulation that is host-specific may be affected by the genetic make-up of the host, which in turn may affect cytokine profiles, receptor expression and channel excitability (Mogil, 1999; Mogil, 2009). In addition psychological processing, which is altered by previous experiences, may also greatly alter the perception of pain (Eccleston, 2001).

The nociceptive processing begins at the terminals of nociceptive neurons. Noxious signals such as intense heat ($> 48^{\circ}\text{C}$), extreme cold ($< -1^{\circ}\text{C}$), acid, extreme force (mechanical) are transduced to the nerve terminals by selective receptors expressed in a particular pattern on two types of nociceptive neuronal fibres that helps determine their function in pain signalling. These fibres consist of the thinly myelinated A δ -fibres and the unmyelinated C-fibres. C-fibre afferents constitute approximately 65–75% of the afferents into the spinal cord. They are distinguished by their unmyelinated axons and small cell body diameter (10–25 μm) in the DRG, while electrically they are identified by their slower conduction velocity ($< 2\text{ m/s}$) compared to A δ -fibres ($> 2\text{ m/s}$). C-fibres underlie the burning sensation of pain due to the relatively exclusive expression of TRPV1 (transient receptor potential cation subfamily V member 1) channels on these fibres. TRPV1 is activated by noxious stimuli and capsaicin, activation of which the brain perceives as heat, therefore its activation in pain signalling is accompanied by the perception of a burning pain quality and the wide receptive field and slower conduction speed provides the dull, non-discriminative, aching sensation compared to the sharp, distinct, stabbing pain driven by the faster conducting A δ -fibres with their small receptive fields. Both fibre types can transduce chemical signals through the expression of cutaneous nociceptors and mechanoreceptors on their terminal ends. Following cutaneous injury, for example, these nociceptive receptors are exposed to the locally released inflammatory mediators from the injured tissue and infiltrating immune cells, which results in their activation and subsequent sensitisation, as outlined previously.

Sensory afferents may also be defined by their neurochemical responses and be further subdivided into the peptidergic and non-peptidergic. The peptidergic population are characterised by the expression of neuropeptides, such as substance P (SP, calcitonin gene-related peptide (CGRP), somatostatin (SOM) and vasoactive intestinal peptide (VIP) have been identified in DRGs (40%), C-fibres (50%) and A δ -fibres (20%) (McCarthy *et al.*, 1989; Lawson *et al.*, 1996). Non-peptidergic neurons on the other hand are characterised by expressing glycoconjugates that bind the plant lectin IB4. These particular cell markers are not indicative of the target tissue innervated however the proportion of DRG cells specific for each varies between tissue targets with almost all visceral afferents representing a peptidergic population, while cutaneous afferents present approximately half of both populations (Perry *et al.*, 1998). The peptidergic and non-peptidergic populations also express different receptor profiles. This affords them the ability to respond to different stimuli and perform different functional roles. The IB4-positive non-peptidergic neurons also express the purinoceptor, P2X3, responsible for transducing signals mediated by ATP, is primarily expressed (Vulchanova *et al.*, 1998), while, as stipulated previously, the TRPV1 receptor, which mediates

signals of heat and responds to capsaicin and proton binding, is expressed predominantly on peptidergic neuronal populations (Caterina *et al.*, 1997; Michael *et al.*, 1999).

Acute pain is transient and at most lasts several weeks. In the context of inflammation or infection acute pain may persist for the length of time the inflammatory or infectious source is present, which may be up to 6 months (Long *et al.*, 1996). In general acute pain involves the direct transduction of noxious stimuli, such as thermal or mechanical stimuli, into action potentials in nociceptors. In contrast, chronic pain is persistent pain that lasts typically for longer than 3 months and may be subdivided into nociceptive; the physiological but persistent activation of nociceptors, or neuropathic, which has recently been redefined as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system” (Treede *et al.*, 2008). . Unlike acute pain, chronic pain tends to be accompanied by strong immune cell involvement and the indirect amplification of pain signalling by peripheral and central sensitisation. Peripheral sensitization is largely mediated by the local release of pro-inflammatory and excitatory mediators from immune cells (Stein *et al.*, 2009), while central sensitization is the phenomena of neuronal plasticity, brought on by prolonged nociceptor activation, that leads to prolonged but reversible increased excitability and synaptic strength (Woolf CJ, 2011). These will be discussed in more detail in section 1.4.4 and its relevance to painful HIV-SN.

1.4.2 Painful HIV-SN: Prevalence and risk factors

As reviewed earlier HIV-SN is one of the most common complications of HIV and its therapy. Although I have introduced the existence of HIV-SN, an additional point to raise, is the prevalence of pain symptoms that sometimes accompanies HIV-SN. One of the most frequent clinical characteristics of HIV-SN is neuropathic pain. Between 20-90% of patients with HIV-SN report painful symptoms (Letendre *et al.*, 2009; Singer *et al.*, 2010; Phillips TJC *et al.*, 2010; Evans *et al.*, 2011). Increased prevalence of pain correlates with the severity of HIV/ late-stage AIDS (de la Monte *et al.*, 1988), age (Watters *et al.*, 2004; Evans *et al.*, 2011), the use of toxic ARTs (Smyth *et al.*, 2007; Evans *et al.*, 2011), history of diabetes (Evans 2011 AIDS) and the length of time the HIV-SN has been present (Fuller *et al.*, 1993; Marra *et al.*, 1998). As outlined earlier the presence of the TNF- α haplotypes, TNF- α -308 and TNF- α -1021*2, have been recently correlated with an increased risk of developing HIV-SN (Quasney *et al.*, 2001; Cherry *et al.*, 2008; Chew *et al.*, 2011). However, while TNF is known to hold algogenic properties and been implemented in HIV-associated neuropathic pain (Leung *et al.*, 2010; Zheng *et al.*, 2011), the influence of these TNF haplotypes in determining whether an individual with HIV-SN develops associated neuropathic pain has not been dissected.

1.4.3 Painful HIV-SN: Clinical features and aetiology

Pain is a clinical feature accompanying both aetiologies of HIV-SN; HIV-DSP and ATN. On the occurrence of painful-DSPs the onset is gradual, beginning in the feet with sensations predominantly described as burning or aching that is commonly accompanied by the sensation of ‘pins and needles’ and, paradoxically, numbness (Fuller *et al.*, 1993; Verma, 2001; Wadley *et al.*, 2011). Eventually these sensations can also present in the hands however they tend to then remain restricted to these extremities. Diagnosis is revealed using neurological examinations and quantitative sensory testing that identify a reduced or absent ankle tendon reflex in 85–100% of individuals presenting with HIV-SN (So *et al.*, 1988b; Griffin JWCT *et al.*, 1998; Ferrari *et al.*, 2006), reduced vibration sense (Wadley *et al.*, 2011) and loss of thermosensation indicative of the loss of C-fibre nociceptors (Bouhassira *et al.*, 1999; Martin *et al.*, 2003). Painful-DSPs appear to surface earlier in relation to the initiation of particular antiretroviral drugs, than in treatment-naïve patients or those on newer, less toxic compounds (Marra *et al.*, 1998; Smyth *et al.*, 2007; Wadley *et al.*, 2011). In particular, following exposure to d4T, ddC and ddI, which have been largely discontinued in developed countries but still used dominantly in resource-deprived countries (Cherry *et al.*, 2006; Affandi *et al.*, 2008; Wadley *et al.*, 2011). While both forms of HIV-SN are thought to have mechanistically distinct components, the pain associated with either neuropathy is likely to result from a similar source in connection with the axonal degeneration or die-back phenotype present in both. A connection between Wallerian degeneration has been postulated more heavily in the last 10 years, again following studies using the Wld^S mutant mouse, which demonstrated not only a delay in Wallerian degeneration but also attenuated thermal hyperalgesia following a chronic constriction injury of the sciatic nerve (Sommer *et al.*, 1998; Campana *et al.*, 2006). This suggested that peripheral, rather than central, mediators instigate the normal neuropathic-pain like response associated with such an injury and in turn the thermal hyperalgesia was further correlated with the influx of macrophages, a key source of neurotoxic and algogenic mediators, which occurred with the onset of Wallerian degeneration (Sommer *et al.*, 1998). Therefore it is suggested that the pain associated with HIV-SN is likely to be driven by the processes involved or activated by axonal degeneration, a theory supported by a study published in 2002 by Polydefkis *et al.* By comparing the intraepidermal nerve fibre (IENF) density among individuals with HIV-SN they showed that reduction in IENF density, indicative of peripheral axonal degeneration, inversely correlated with the severity of pain experienced, implicating the die-back pathology in the pain syndromes accompanying it (Polydefkis *et al.*, 2002). Furthermore the extent of axonal degeneration has been correlated with the level of macrophage infiltration (Nagano *et al.*, 1996).

A review paper by Uçeyler *et al.* in 2006, succinctly summarised the mechanisms involved in Wallerian degeneration and their association with the development of neuropathic pain (Uçeyler *et al.*, 2006). Therefore our knowledge of what underlies the pain accompanying HIV-SN, and the risk factors or compounds that exacerbate and aid the development of HIV-SN, is expanding; however our knowledge on why only some people develop pain symptoms in association with the HIV-SN while others do not remains elusive.

1.4.4 Role of Immunity in Painful HIV-SN

As elucidated to earlier, cytokines are small regulatory proteins secreted by immune and glial cells that have a diverse array of functions, particularly in response to inflammatory disease and infection. In addition to these established roles, the last decade has seen cytokines robustly implemented in the induction and maintenance of pain (Sommer *et al.*, 2004; Wieseler-Frank *et al.*, 2005; Uceyler *et al.*, 2009). Nerve injury causes the release of a multitude of inflammatory mediators from damaged tissue, mast cells and nerve terminals that include excitatory amino acids, prostaglandins and pro-inflammatory cytokines. These are, commonly referred to as the inflammatory ‘soup’ that promotes inflammation and attracts immune cells. It is a defensive mechanism following the exposure of internal tissue to external pathogens that attempts to avoid further injury or infection as well as providing an environment permissible to tissue recovery. In addition many of the mediators released have algogenic properties responsible for much of the pain experienced during inflammatory responses; functioning as a warning signal to the host of actual or probable tissue damage. To alert the host, evolution has mandated the activation of pain signals in response to tissue injury and inflammation among which, along with the transduction of thermal and mechanical stimuli, the action of cytokines and other molecules are essential mediators. This is a physiological process that can be compartmentalised into two regional mechanisms of sensitisation; one acting peripherally and the other centrally. In addition two terms are used in humans to describe the enhanced sensitivity phenomena that results from activation of these mechanisms; allodynia and hyperalgesia. Allodynia describes pain states initiated by stimuli that would not normally elicit pain, while hyperalgesia depicts an enhanced pain response to normally noxious stimuli.

Peripheral sensitisation, also known as primary sensitisation, is mediated by local cells and macrophages and further amplified by migrating blood cells following tissue injury, through the release of excitatory and pro-inflammatory mediators (Stein *et al.*, 2009). For the purpose of this review I will discuss just the most prominent and heavily researched mediators, which I have

categorised into, 1) small molecules, 2), lipids 3) small peptides, 4) large peptides and 3) hormones and growth factors.

The small molecules include H^+ and adenosine-5'-triphosphate (ATP) both of which are released locally by injured or inflamed tissue and can directly act on nociceptive-specific primary afferents via their relevant receptors (TRPV1 and acid-sensing ion channels (ASICs) for H^+ and P2X receptors for ATP) to sensitise and induce pain.

Lipids involved in pain signalling tend to refer to the eicosanoids that are lipid derivatives of arachidonic acid metabolism, of which only the prostaglandins (PGs) will be discussed here but also include thromboxanes, prostacyclins and leukotrienes. Prostaglandins can act in both an autocrine and paracrine fashion on astrocytes, platelets, endothelial cells and mast cells, and of particular note is their vasodilatory capabilities that underlie the erythema associated with inflammation.

Among the class of small peptides are bradykinin, substance P and the excitatory amine, adrenaline. Bradykinin (BK) is released locally by damaged tissue and mast cells to act directly on BK receptors B1 and B2 expressed on nociceptive afferents. Activation of these receptors leads to neuronal sensitisation. Substance P (SP) binds to neuropeptide 1 receptor (NK1R) and SP-NK1R signalling is critical for the transmission of pain signals into the central nervous system (Zubrzycka M *et al.*, 2000). SP is also released from the peripheral neuronal terminals in the skin joints and muscle where it can aid in the sensitisation neurons by lowering membrane depolarisation thresholds through enhanced Na^+ ion influx (Coutaux *et al.*, 2005), stimulate epidermal cells and immune cells (Kavelaars *et al.*, 1994), lead to vasodilation (Bossaller *et al.*, 1992) and smooth muscle contraction (Couture *et al.*, 1982).

Both PGs and the small peptides listed above have the ability to directly sensitise neurons by acting on their respective G-protein coupled (GPCR) receptors expressed on C-fibre nociceptors (Verri, Jr. *et al.*, 2006). This activation initiates a cascade of intracellular signalling that starts with the binding of the $G\alpha$ subunit to a primary effector e.g. adenylyl cyclase (AC), phospholipase C (PLC) and phospholipase A (PLA). These effectors drive second messenger signalling involving cAMP, diacylglycerol (DAG), inositol triphosphate (IP_3), phosphatidylinositol (PI) and Ca^{2+} , which in turn leads to the release of calcium from intracellular stores and/ or the activation of various enzymes including protein kinase A (PKA) and protein kinase C (PKC). The subsequent enhanced kinase activity leads to the phosphorylation of ion channels, which enhances their sensitivity to subsequent agonist binding subsequently enhancing the sensitivity of the nociceptive neuron, which translates into enhanced pain sensations or hyperalgesia.

The category of large peptide incorporates the diverse array of pro-inflammatory cytokines and chemokines. As reviewed previously these encompass small regulatory and chemotactic proteins produced by many kinds of leukocytes (white blood cells). Intriguingly cytokines display great redundancy in action due to their pleiotropic actions on many and the same cells. In addition they have low binding constants (K_d within the range of 10^{-12} to 10^{-10} M) which means that even at low concentrations, 50% of its selective receptors on a cell are occupied by a cytokine ligand (Sommer *et al.*, 2004; Wieseler-Frank *et al.*, 2005). Furthermore, they induce functional effects at low concentrations (10^{-12} to 10^{-9} g/ml). This makes them potent mediators of inflammation and in turn enhancers of pain.

Nerve growth factor (NGF) is a small secreted growth factor released by a variety of cells including Schwann cells, neurons and fibroblasts. NGF promotes survival and differentiation of sensory neurons during and post-development (cite). More recently it has been demonstrated provide an additional function as an important intracellular signalling molecule in inflammation and tissue repair (Sofroniew *et al.*, 2001). Further research has since revealed NGF-dependent sensitisation of neurons (McMahon, 1996).

Therefore, in the context of peripheral axonal degeneration, as in HIV-SN, infiltrating macrophages release various factors in their role to remove degenerated debris. However, many of these mediators include the algogenic compounds outlined above. In addition to this physiological activation, in the context of HIV, macrophage stimulation and aberrant cytokine signalling mediated by the HIV viral proteins may also occur. Despite this, not all HIV+ individuals, presenting with a sensory neuropathy, experience pain, therefore another level of complexity must exist that modulates the mechanisms involved despite the same driving input. Such factors may include the viral strain involved, with some, namely T-tropic strains proving to be more virulent than their M-tropic partners. Alternatively, or additionally, since the genetic make-up of the host has been shown to manipulate their response to HIV infection, it seems likely that genetics too may affect pain signalling pathways as well as differential immune responses per se determining whether a pain state may occur. Recent examples of this are shown by the identification of TNF- α haplotypes (TNF- α -308 and TNF- α -1021*2), the presence of which have been correlated with an increased risk of developing HIV-SN (Quasney *et al.*, 2001; Cherry *et al.*, 2008; Chew *et al.*, 2011). TNF- α is an important cytokine involved in the early phase of immune activation; functioning as a potent chemoattractant to recruit additional immune cells to the site of injury. Original hypotheses of neuropathic pain commonly ignored the cytokines, considering their only function to relate to inflammation however the last decade has significantly altered this concept. It is now fairly

established that pro-inflammatory cytokines also possess the ability to induce pain signals either directly or indirectly, by sensitising nerve terminals (Thacker *et al.*, 2007; Uceyler *et al.*, 2009). Intrathecal and intraneural injections of TNF- α have been shown to reproduce mechanical hypersensitivity in rats that may equate to the neuropathic pain in humans (Wagner *et al.*, 1996; Reeve *et al.*, 2000; Kwon *et al.*, 2005; Wei *et al.*, 2007). In line with the correlation between increased CSF levels of TNF- α and the neuropathogenesis of HIV (Leung *et al.*, 2010), the correlations between macrophage infiltration and the severity of HIV-SN (McArthur *et al.*, 1989; Griffin JWCT *et al.*, 1998), and between the extent of peripheral nerve die-back and the severity of pain (Polydefkis *et al.*, 2002), it becomes apparent that inflammation, immune response and cytokine signalling are inter-linked and important mediators in the pain that accompanies nerve degeneration.

1.5. HIV-SN and other degenerative disorders: commonalities and differences

HIV-SN is just one among many neurodegenerative disorders that is characterised by degeneration of neurons or neuronal toxicity including diabetic neuropathy, multiple sclerosis and Alzheimer's disease. It is clear that each of these arise from distinct aetiologies; some are the result of infection as with HIV-SN, while others are the outcome of dysfunctional autoimmune signalling. The simple process of aging can be implicated in many of these conditions (Popescu *et al.*, 2009; Cherry *et al.*, 2009; Bertini G *et al.*, 2010; Mevel *et al.*, 2011; Pizza *et al.*, 2011), while the original aetiology of multiple sclerosis is yet to be fully clarified. However, recent advances in the understanding of mechanisms involved in each of these conditions is beginning to unveil interesting commonalities among them, an important point to be aware of as knowledge may be gained from research in different disciplines that may be applied, for example, to my own field of research in HIV-SN. This may be of particular importance for the understanding of multiple sclerosis because if the mechanisms can be fully elucidated, an aetiological basis may be identified.

Therefore, I have reviewed much of the literature; old and new, on these neurodegenerative disorders in order to highlight some common mechanisms. The results have been summarised in Figure 1.5.1. In summary, dysregulation of calcium signalling, mitochondrial toxicity and a malfunctional ubiquitin-proteasome system have been identified in Alzheimer's disease, multiple sclerosis and diabetes and in the context of HIV; all disease states with a neurotoxic component and show evidence of axonal degeneration. Therefore, it is tempting to hypothesise that these pathways may be dominant causes of the resultant pathologies. However, the final step that links these activated mechanisms to the initiation of axonal degeneration remains to be clarified. Furthermore

while the immune phenotypes underlying disease states such as multiple sclerosis and diabetes are quite distinct, those underlying HIV-SN are less well described. A switch from a predominant Th1 to Th2 immuno phenotype has been postulated to underly the progression to AIDS; however the nerve damage that leads to HIV-SN may be driven by a separate phenotype. Understanding the role of the immune system in initiating this nerve damage may highlight how and why the other processes become activated and therefore how they may be controlled. In turn, these insights may be useful in controlling the neurotoxic pathways in the other neurodegenerative diseases.

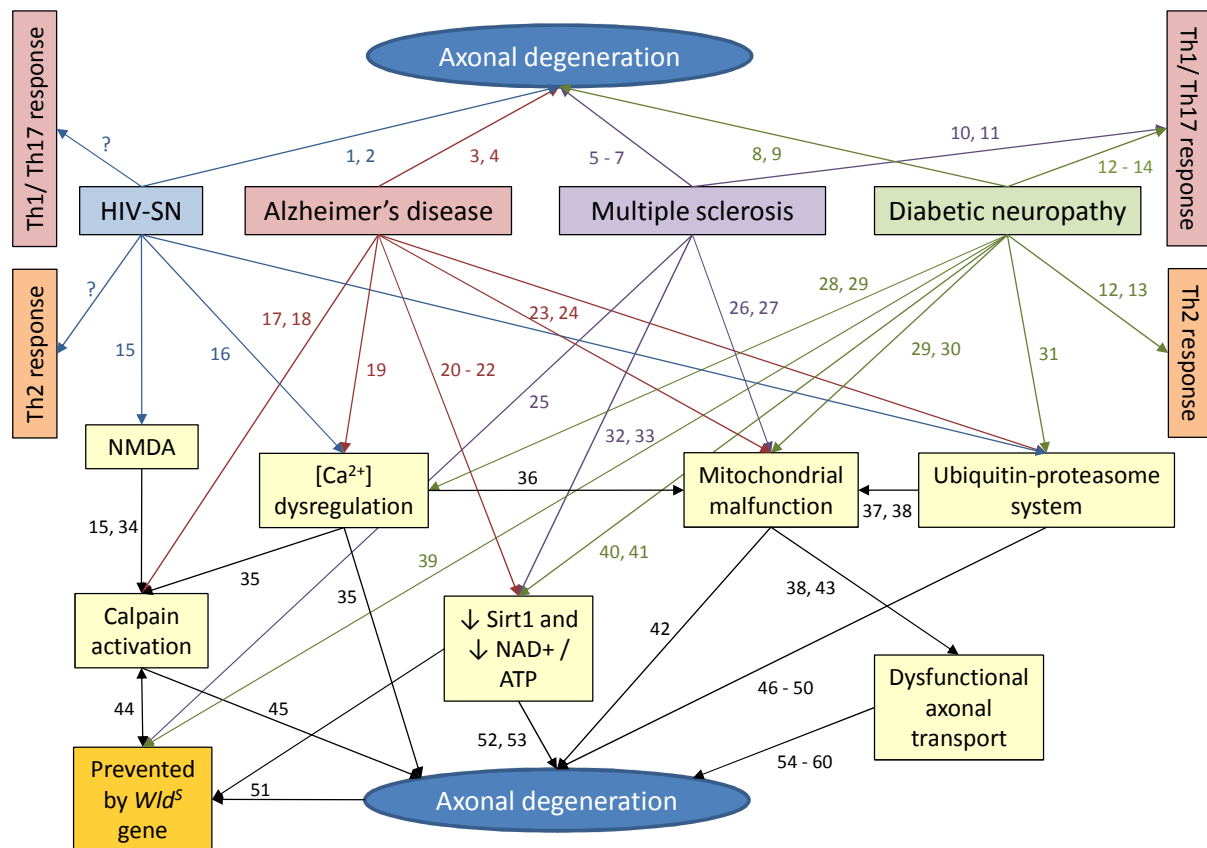


Figure 1.5.1) Diagram depicting the commonalities, and differences, between different neurodegenerative disorders; created by a review of current literature. Citations are select examples and not exhaustive.

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Key of references for Figure 1.5.1

1.6. Aims

Based on the indication that HIV-1 gp120 may play a key role in the mediation of HIV-associated sensory neuropathies I aimed to investigate the mechanisms driven by HIV infection *per se*. My current hypothesis is that gp120 mediates axonal degeneration by two mechanisms, 1) through direct activation of neurons via CCR5 and/or CXCR4, and 2) indirectly as a result of secreted cytokines from gp120-activated Schwann cells and/or macrophages. However, evidence for a direct action on neurons is controversial and mainly limited to embryonic or neonatal studies. Furthermore, the temporal profile of gp120-mediated toxicity, the immune response to gp120 exposure, and the key mediators of axonal degeneration, remain unclear. Therefore the objectives of this project were to:

- 1) Clarify the involvement of neurons in the development of HIV-1 gp120-mediated axonal degeneration.
- 2) Elucidate the temporal profile of HIV-1 gp120-mediated axonal degeneration in the PNS.
- 3) Examine the phenotype of immune response elicited by exposure to HIV-1 gp120.
- 4) Investigate the relative contributions of macrophages and Schwann cells in HIV-1 gp120-mediated axonal degeneration.
- 5) Study the relative involvement of known neurotoxins in the development of axonal degeneration.
- 6) Assess how the *in vitro* findings translate to the *in vivo* environment.

Chapter 2: Direct effects of gp120 on neurite outgrowth *in vitro*

2.1. Introduction

2.1.1 Synopsis

In the general introduction I have outlined the importance of the immune system in HIV infection and eluded to a key role for non-neuronal cells in HIV-associated neurotoxicity. I highlighted the recent research focus on the HIV-1 envelope glycoprotein, gp120, for its neurotoxic capabilities. In this chapter I will explore the role of neuronal cells in gp120-mediated neurotoxicity. Recent evidence for direct interactions between gp120 and neurons will be presented. I will outline my data that explored the relative involvement of neurons in gp120-mediated neurite degeneration and discuss the findings in the context of HIV-associated sensory neuropathy.

2.1.2 Anatomy of Neurons

The structure and morphology of neuronal cells are determined by the cytoskeletal components. The cytoskeleton is required to maintain cell shape and comprises of three filamentous structures: microfilaments, intermediate filaments and microtubules. Microfilaments are found throughout the neuronal cytoskeleton but are particularly abundant in presynaptic terminals, dendritic spines and postsynaptic densities (Ratner *et al.*, 1983; Fifkova *et al.*, 1992). They are produced by the polymerisation of actin, a 43 kDa globular protein, of which two isoforms have been identified in neurons, β and γ actin (Choo *et al.*, 1978). Actin is an ATP-binding protein that requires ATP in order to polymerise and bind with additional monomers. Actin polymers make a double helical formation following the intertwining of actin monomers looking similar to the plaiting of two strings of beads with a diameter of approximately 6nm. Intermediate filaments are fractionally larger with a diameter of about 10 nm formed by the polymerisation of intermediate filament proteins derived from a multigene family of polypeptides (Herrmann *et al.*, 2000). These proteins demonstrate a cell-type expression that alters with its developmental stage. Neurofilament (NF) triplet proteins, of which there are three classes, NF-L (-light), NF-M (-Medium) and NF-H (-heavy), are expressed by differentiating and mature neurons. NF-L and NF-M are expressed at relatively low levels in the majority of neurons at the time of neurite outgrowth. Following maturation of neuronal cells, NF-H is expressed and levels of NF-L and NF-M are upregulated. These proteins form neurofilaments, a class IV type of intermediate filament (IF) that are one of the most abundantly expressed cytoskeletal

elements within large myelinated axons but expression is also evident in smaller diameter neurons. Other examples of intermediate filaments include peripherin (a class III IF), which is selectively expressed in small diameter neurons in the peripheral nervous system, and GFAP (glial fibrillary acidic protein, a class III IF) that acts as a marker for astroglial cells. The third of the cytoskeletal elements, and the largest, are microtubules; hollow fibres with a diameter of approximately 25nm. Microtubules are comprised of a configuration of α - and β -tubulin subunits, which form a globular heterodimer. Numerous tubulin heterodimers polymerise end-to-end to the opposite tubulin subunit (i.e. α -subunit to a β -subunit), forming a 'strand' and the stacking of 13 longitudinal 'strands' forms a protofilament. Multiple protofilaments bundle together to form a hollow cylindrical filament that takes a helical conformation, thereby completing a microtubule. Due to the selective alignment of the α -/ β -tubulin heterodimers one end of the protofilament will expose predominantly α -subunits while the other comprises of mainly β -subunits resulting in a polarised polymer unit with ends referred to as (-) and (+) respectively. Protofilaments bundle in parallel to each other and in the same polarised formation so that the microtubule also consists of one (-) and one (+) end. The assembly and disassembly occurs at the (+) end and, like with microfilaments, the (+) end of microtubules polymerise faster than the (-) end (Howard *et al.*, 2003). Tubulin is a GTP-binding protein and in a heterodimer both the α - and β -tubulin subunits are bound to a molecule of GTP during polymerisation. However, while α - tubulin bound GTP remains stable, β -tubulin bound GTP can rapidly hydrolyse to GDP soon after assembly making it more prone to depolymerisation and GDP-bound β -tubulin units will detach if positioned at the end of the microtubule. Therefore to protect the microtubule from rapid depolymerisation and shortening, a cap of GTP-bound tubulin subunits is maintained at the tip of the microtubule. However if GTP hydrolysis to GDP occurs more rapidly than the addition of new GTP-bound tubulin units, the GTP enriched cap will not be maintained dropping below a critical threshold and the rapid dissociation of tubulin subunits will occur. Depolymerisation can be rescued through the reincorporation of GTP-bound tubulin. It is important to note that microtubules are less dynamic *in vivo* than *in vitro* (Kirschner *et al.*, 1986), which is likely explained by the reduced support network available to cultured neuronal cells and their increased vulnerability. Microtubules are important for the generation and maintenance of neuronal morphology and largely determine the axonal length and neuritic network that extends from the neuronal cell body. In addition the microtubule network act as important passages for the bi-directional shuttling and transport of essential nutrients, organelles and cytoskeletal components between the cell body and the neuritic tips.

There are subtle differences in the dynamics of the cytoskeletal elements. Microfilaments alter their length through the exchange of actin monomers at the ends of the polymer chains. Similar to

microfilaments, microtubules extend and retract through the incorporation and release of tubulin heterodimers at the ends of the protofilament, while intermediate filaments mainly involve the lateral replacement of subunits within the main body of the polymer network (Atkinson SJ *et al.*, 1992). In addition to these core structural proteins many additional proteins associate with the cytoskeleton and are important for the regulation of their structural stability, the dynamics of assembly and disassembly of filaments and their interaction with other cellular organelles. Together these components will determine neuronal outgrowth, morphology and degeneration as well.

2.1.3 Modelling neuronal degeneration *in vitro*

Assessing the ability of compounds and drugs to induce neuronal degeneration *in vivo* is laborious and impractical for high-throughput screening. Cell cultures have become useful tools for assessing neuronal function at a molecular and cellular level with the possibility for high-throughput screening and time-lapse and live-cell imaging. There are numerous advantages to testing the toxicity of compounds in cell cultures rather than *in vivo*, in addition to the major savings gained in time, costs and animal use, (for review see Harry *et al.*, 1998; Costa, 1998). These include allowing for individual neurons and subsets of neurons to be investigated in isolation, which can provide important information on the selectivity of neurotoxic compounds and the mechanisms and receptors involved. The exposure delivery of toxic compounds can also be regulated to be acute, chronic or intermittent and exposure parameters can be strictly controlled, enabling researchers to distinguish between the pharmacological and neurotoxic effects of compounds. Of course the main drawback of working on *in vitro* cultures, particular primary dissociated cell cultures, is that the major cell-to-cell connections and tissue organisations are completely lost. This means there is reduced integration of function, and insight into compensatory mechanisms is lacking. Yet, the loss of the three-dimensional tissue architecture also affords dissociated primary neuronal cultures a great advantage; improved accessibility to the neurons, which can now be directly exposed to treatment compounds. It will always be difficult to confidently translate the results to the *in vivo* setting and to extrapolate the *in vitro* findings to complex end points such as sensory dysfunction or cognitive impairment. However, important targets, mechanisms and understanding of cell type involvement can certainly be highlighted. In particular the quantification of neurite outgrowth *in vitro* has become a frequent tool for assessing neurotoxicity (Harry *et al.*, 1998; Radio *et al.*, 2008) and reductions in neuritic processes *in vitro* are widely accepted to model the process of axonal degeneration in humans (Wang *et al.*, 2000; Zhang F *et al.*, 2009). The extension of axons and dendrites in cultured neuronal cells is a physiological process that has the characteristics of cells maintained *in situ* (Saver MA *et al.*, 1999; Liu *et al.*, 2008a). Neurite outgrowth puts a significant

energetic demand on the cell therefore efficient and productive neurite extension can act as a general indicator of good cellular health. Consequently outgrowth may be affected by the widest possible spectrum of harmful agents, which may deregulate any number of the multiple stages involved in neurite outgrowth. This provides scientists with an opportunity to examine the specific effects of different groups of toxic substances. The mechanisms underlying axonal degeneration remain elusive therefore validation of the relevance of reductions in neurite outgrowth *in vitro* to the clinical setting remains to be performed. However, many aspects of sensory neuronal cultures, are considered to provide a decent replication of the numerous neuronal cell types and their response properties *in vivo* (Malin SA *et al.*, 2007). Furthermore, it is important to highlight that dissociated neuronal cultures represent a better model of regenerating neurons following axotomy, than normal neuronal networks in healthy individuals. Common features between dissociated neurons and regenerating neurons *in vivo* have been identified, as evidenced by the expression of classic markers of regeneration, GAP43 and galanin, on extending neurites *in vitro* and regenerating axons *in vivo* (Woolf *et al.*, 1990). However, providing treatments are applied following extensive outgrowth of neurite networks, the induction and mechanisms underlying neuronal degeneration can thereafter be assessed.

There are many different methods available for the measurement of neurite outgrowth in primary dissociated neuronal cultures, ranging from simple arbitrary methods to complex and accurately quantitative methods. Sholl analysis is a semi-quantitative method that does not require expensive complex computational software (Sholl DA, 1953; Sotrel A *et al.*, 1993; Gutierrez H *et al.*, 2007). This method sees consecutive concentric circles and equi-distance radial lines mapped around a neuronal cell body and the number of intersections on each circle quantified. This provides information on basic neurite length, arborisation, dendritic density and geometry. However, it provides only semi-quantitative information on outgrowth with the accuracy being determined by the number of circle and radial lines inserted and the distances between them. The method relies on neurites radiating out in relatively straight lines and not extending between segments where additional growth would not be quantified if no additional intersections were made. In addition, this method strongly requires isolated neurons for its analysis because it does not account for two neighbouring neurons with intersecting neuronal networks. Alternatively, both basic and complex computational packages now allow for accurate measurements of neurite length to be quantified either manually or automatically by the software program. Parameters analysed can include the longest neurite length, mean neurite outgrowth per neuron, number of neurite extension per cell and information on the complexity of branching and arborisation.

DRG explants have been used in place of dissociated neuronal cells to assess neurite outgrowth due to the maintenance of cellular elements and structural connections. However, while explants main advantage is the presence and maintenance of cell-cell contacts and signalling, it is restrictive as a model because it is highly dependent on the diffusion rate of materials through the exterior perineural membrane, which varies depending on the thickness of this layer, the size of the DRG and physicochemical properties of the compounds in the media (nutrients, oxygen, test compounds). In addition the complexity of the model makes quantification of neurite outgrowth more difficult.

Compartmental cultures, first described by Campenot *et al.* allows for the isolation of pure axonal populations (Campenot RB, 1977). Neuronal cells are plated in the central portion of a three-compartmented chamber; the axons grow beneath the dividing walls, guided by scratches in the base of the dish, into the two side compartments, resulting in their compartmental isolation. The advantage of this system is the ability to assess the differential affects and binding sites of compounds on the axons and cell soma individually. Furthermore, depending on which growth factor is used in the side compartment to attract and guide axons into the side chambers (i.e. NGF for small diameter nociceptors or GDNF for large myelinated cell neurite extension) selective neuronal sub-populations can be investigated.

Strong correlations have been made between the degree of axonal degeneration in HIV-patients with a sensory neuropathy and the intensity of pain that accompanies it (Polydefkis *et al.*, 2002). The increased degree of neuronal degeneration is indicative of increased nerve damage and this nerve damage therefore is likely to instigate and continue to drive peripheral and central changes that lead to increased pain sensations. For this reason investigating gp120-induced neurite degeneration *in vitro* may offer an insight, not only into the scientific mechanisms underlying the degenerative process, but also eventually highlight potential processes involved in HIV-SN neuropathic pain.

2.1.4 Neurons as direct targets for gp120.

Until recently it was believed that the neurotoxic effects of gp120 on sensory neurons occurred solely via interactions with Schwann cells and/ or macrophages. However recent discoveries in the past 10 years have altered this view. In 1988, Brenneman *et al.* were one of the first to demonstrate neurotoxicity following exposure to the HIV envelope glycoprotein, gp120 (Brenneman *et al.*, 1988). Embryonic hippocampal neurons were exposed to gp120 purified from two HIV isolates HIV_{III_B} and HIV_{RF2}, which resulted in nearly identical neuronal loss. This highlighted that the neurotoxicity present in the context of HIV infection may be occurring independently of infection *per se* and may be mediated by gp120 shed from the virus. In 1993, using immunofluorescence microscopy

techniques, Apostolski *et al.* confirmed that HIV-1 gp120_{IIIIB} (a T-/ CXCR4 tropic strain) binds to sensory ganglion neurons, but not fibroblasts or glial cells, in primary mixed neuronal-glial cultures established from embryonic rat DRGs (Apostolski *et al.*, 1993). These studies suggested that the receptors for gp120 were expressed on dorsal root ganglia neurons and in 2001 Oh *et al.* verified, using calcium imaging and immunofluorescence that DRG neurons do express functional CCR5 and CXCR4 receptors (Oh *et al.*, 2001). This highlighted for the first time in particular that small nociceptive neurons express the HIV-1 chemokine coreceptors and, when exposed to the receptor ligands and gp120, excitatory effects were observed combined with the release of substance P, in a neuronal sensory culture system (Oh *et al.*, 2001). While neurons do not express the CD4 receptor, and thus cannot be infected by HIV, a new focus has now been created on the possibility of a direct interaction between gp120 and sensory neurons independent of Schwann cells or macrophages. Chemokine receptors expressed on neurons could potentially mediate the enhanced sensitivity to pain, in inflammatory states or HIV-1 infection, directly. Furthermore evidence for the internalisation and axonal transport of the T-tropic gp120 (gp120_{IIIIB}) in striatal neurons has been recently reported (Bachis 2006 J Neurosci) and could indicate how gp120 contributes to widespread HIV-1-mediated neuronal cell death.

It is now well established that HIV gp120 plays a key role in the pathogenesis of neurological disorders associated with HIV, and is capable of initiating neurotoxic cascades from permissible cells via an interaction with the CXCR4 and/or CCR5. However, it still remains unclear exactly which of these interactions and resulting factors underlie the pathogenesis of HIV-SN. Very little is known about such interactions and minimal research has focussed on the role of the adult peripheral nervous system in the induction of neurotoxicity *in vitro* following HIV exposure despite the majority of HIV infections affecting the adult population.

2.1.5 Aims

The studies highlighted in this chapter aimed to clarify the ability of gp120 to interact directly with adult DRG neurons. As outlined in the introduction, until recently gp120-mediated toxicity was believed to occur solely via activation of non-neuronal cells. However, recent insights have demonstrated the ability of neurons to directly respond to gp120. Subsequent studies that have attempted to investigate this occurrence have done so using embryonic or neonatal culture systems. Therefore the following studies were designed to explore the involvement and importance of gp120-neuronal signalling, in a model of an adult peripheral nervous system, in the development of gp120-mediated axonal degeneration.

2.2. Materials and Methods

2.2.1 Compounds

The compounds used but either referred to or source not described within the Materials and Methods are listed here: Vincristine sulphate (#V8879; Sigma), nocodazole (M1404, Sigma), gp120_{MN} (#1021; ImmunoDiagnostics), biotinylated gp120_{MN} (#B-1021; ImmunoDiagnostics), gp120_{Bal} (generous gift from Prof. Maria Papathanasopoulos, University of the Witwatersrand, Johannesburg), recombinant rat RANTES/CCL5 (#400-13, Peprotech) and recombinant rat SDF-1 α /CXCL12 (#400-32A, Peprotech), and capsaicin (#404-86-4, Fluka).

2.2.2 Establishment of primary mixed adult Dorsal Root Ganglia (DRG) neuronal cultures

Adult mixed dorsal root neuronal-glial cell cultures were obtained from adult female Wistar rats. Spinal dorsal root ganglia were dissected from CO₂ asphyxiated 150-200g female Wistar rats and desheathed from the roots under a dissecting microscope using Vannas micro-dissection scissors (Fine Science Tools). Ganglia were enzymatically dissociated by incubation with 0.125% collagenase XI (Sigma) in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen) supplemented with 0.1 mg/ml DNase II (Sigma) for 1.5h at 37°C. Following incubation ganglia were triturated 10–15 times with a cut 1 ml tip to disrupt the cells. Cell suspensions were centrifuged at 1000 rpm for 5 min, resuspended in warm complete DMEM (C-DMEM; DMEM supplemented with 10% foetal bovine serum (FBS; GIBCO Invitrogen) and penicillin-streptomycin (P/S; 1:100; Sigma) antibiotics) and filtered through a 70 μ m mesh (BD Biosciences). The cell suspension was centrifuged at 1000rpm for 3 min, resuspended in 2 ml C-DMEM. Cell counts were performed using a hemacytometer and the final volume adjusted and supplemented with NGF (50 ng/ml), aphidicolin (10 μ M; Sigma). Cells were plated at between 2000–8000 cells in 500 μ l/well onto poly-L-lysine- (0.1 mg/ml; Sigma) and laminin-coated (10 μ g/ml; Invitrogen) coverslips seeded in 4-well plates. Unless otherwise stated cultures were maintained in 500 μ l C-DMEM at 37°C, in a humidified environment containing 5% CO₂ in air, for 24h before treatment application.

2.2.3 Immunocytochemistry on primary DRG neuronal cultures

For all immunocytochemistry the following protocol was used. DRG neuronal cultures were washed three times with sterile phosphate buffered saline (PBS) then fixed with 4% paraformaldehyde (PFA) in PBS for 10 min at room temperature (R.T.). Cultures were washed, permeabilised for 10 min at

R.T. with 0.1% Triton X-100/PBS followed by incubation with the relevant primary antibody diluted in 10% donkey serum/PBS for 1.5h. Antisera and the working dilutions used are presented in Table 2.2.1. Following incubation with the primary antibody samples were washed three times with PBS then incubated with the relevant fluorescently labelled secondary antibody or streptavidin compound for 1h at R.T. in the dark. Secondary antibodies were used at the working dilutions presented in Table 2.2.1, diluted in 10% donkey serum/PBS. Where stated nuclei were counter-stained, after completion of immunolabelling with secondary, by incubation with Hoescht 32258 diluted 1:10000 in PBS for 10 min at R.T. After PBS washes (3 x 10 min each) cells were rinsed twice briefly with deionised water (dH₂O) then the coverslips were mounted onto glass microscope slides with anti-fade agent AF1 (Citifluor Ltd) and sealed in place with nail varnish. Samples were analysed on a Nikon 80i microscope at 10x magnification and images acquired with a digital Nikon DXM1200F camera controlled with LUCIA G software. Post-acquisition processing was carried out with Adobe Photoshop 7 and neurite analysis performed using CSIRO HCA-vision software as outlined in section 2.2.6. For short-term storage samples were kept at 4°C wrapped in foil to protect them from light exposure, while if long-term storage was required samples were stored at -20°C.

Table 2.2.1) Table of antibodies used for immunocytochemical studies on mixed DRG neuronal-glia cell cultures.

Antibody	Dilution for Cultured DRG	Company	Code
Primary			
Mouse α - β III-tubulin mAb	1:2500	Promega	G7121
Goat α -CCR5 pAb	1:50	SantaCruz Biotech	sc-6129
Goat α -CXCR4 pAb	1:50		sc-6190
Mouse α -S-100 mAb	1:600	Abcam	ab8330
Mouse α -Peripherin	1:500	Chemicon	MAB1527
Secondary			
Cy3 donkey α -mouse	1:1000	Jackson ImmunoResearch Laboratories	715-165-150
FITC donkey α -goat	1:500		711-095-152
Fluorescein (DTAF)-streptavidin	3.6 μ g/ml		016-010-084
Dye			
Hoescht 32258	1:10,000	Gift from Dr. Anita Hall	-
Propidium Iodide	1 μ g/ml	Gift from Dr. Anita Hall	-

2.2.4 Acquisition and analysis of images from mounted slides of immunofluorescently labelled DRG cultures

Following fixation, immunostaining and subsequent mounting, where each treatment was mounted together, in triplicate (three coverslips/ slide), a blinding procedure was implemented. The treatment labels on each slide were covered with a blank label and then randomly labelled A, B, C and so on, by a different participant to the observer. The images were acquired by the experimenter from each slide in alphabetical order completely unaware of which slide related to which treatment sample. To prevent experimenter bias in the selection of images, which could distort the representative population of the sample, selection criteria was established. Standardised positions were determined and outlined in Figure 2.2.1. A minimum of five and maximum of ten images were acquired from each culture coverslip and taken from the same segments across all treatment samples. The minimum five images were taken from position C and N, E, S and W within the orbital region indicated by the red circle. Additional images were acquired within the orbital region of the green circle and included NE, SE, SW and NW. Generally the field of view was selected by visualising the fluorescent light beam on the coverslip on the position of interest however only manual alteration to this procedure was a subtle shift in position, for example, to ensure a particular neuritic network was completely in view.

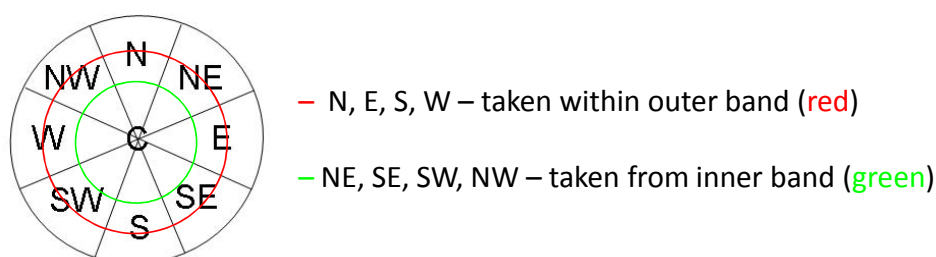


Figure 2.2.1) Example coverslip and markers indicating standardised positions for image acquisition.

Constant exposure times and enhancement settings were applied to all samples within an experiment and where possible across experiments. Positive immunolabelling was determined by quantifying the mean autofluorescence intensity of unlabelled or non-specific fluorescence labelling intensity of cells exposed only to the secondary antibody as a negative control. Immunostaining was considered to be specific and genuine if the mean and maximum fluorescence output intensities were two standard deviations greater than the mean and maximum intensities of the calculated background fluorescence.

2.2.5 Campenot chamber compartmented culture set-up and assessment of biotinylated-gp120_{MN} axonal transport

Campenot-style compartmented chambers were established using a CAMP2 Teflon divider (Tyler Research Corporation) using a method derived from by Pazyra-Murphy (Pazyra-Murphy *et al.*, 2008). 35 mm Ø polystyrene tissue culture-treated dishes (BD falcon) were first prepared by creating a single 2 cm long set of parallel scratches using a 20-pronged pin-rake (CAMP-PR; Tyler Research Corporation) in the central part of the dish. The scratched region only was coated with poly-L-lysine (0.1 mg/ml; Sigma) for 2h prior to set-up, then washed thoroughly with dH₂O and allowed to dry completely. The middle portion of the scratched region (5 mm x 5 mm) was overlaid with 30 µl of mounting solution comprised of 1% methylcellulose (Sigma), 10 µg/ml Laminin (Invitrogen) and 25 ng/ml NGF (Peprotech) diluted in DMEM with P/S antibiotics to prevent complete adherence of teflon divider to culture base and allow space for neurites to maneuver beneath dividing walls into side chambers. All areas of the teflon divider base were greased with a thin (2 mm wide) strip of autoclaved high-vacuum silicon grease (Dow Corning) delivered with precision, and where possible in one continuous flow, using a syringe grease applicator (CAMP-GLSS; Tyler Research Corporation). The scratched dish with mounting solution in place was then carefully flipped and placed on top of the greased side of the Teflon divider so that the scratched region spanned equally across the three chambers. Gentle force was applied in four points of the base of the dish with a pair of tweezers dish to ensure a complete seal was created between the dish and the divider. However, care had to be taken to prevent too much force being applied otherwise direct contact would be made between the dish and divider surface, preventing axons from crossing into the side compartments. These procedures were performed under a stereo-microscope in sterile conditions. Following set-up compartmented dishes were placed at 37°C for 2h to allow the grease to settle after which 200µl DMEM supplemented with P/S Ab were added to each side compartment only, returned to 37°C and left overnight to test for leaks in the chambers. The following day leaky chambers were discarded and remaining sealed compartments were used for experimentation. Chambers were coated with 10 µg/ml Laminin (Invitrogen) for 1.5h at room temperature, then washed three times with pre-warmed DMEM. Primary DRG cultures were established as described in 2.2.1, using the BSA cushion method as described in 1.3.2.3.2.5, plated in the central compartment in 40 µl C-DMEM and left at 37°C for 1.5h to settle and attach to the base. Central compartments were then topped up with 150 µl C-DMEM and the side compartments provided with 300 µl C-DMEM supplemented with 50 ng/ml NGF. Figure 2.2.2 outlines the compartmented cultured set-up following plating of the DRG cells and supplementation with culture media. Media was replaced every 3 days and experiments

performed on culture systems after 7 days *in vitro*, when extensive neurite outgrowth in the side chambers were visible.

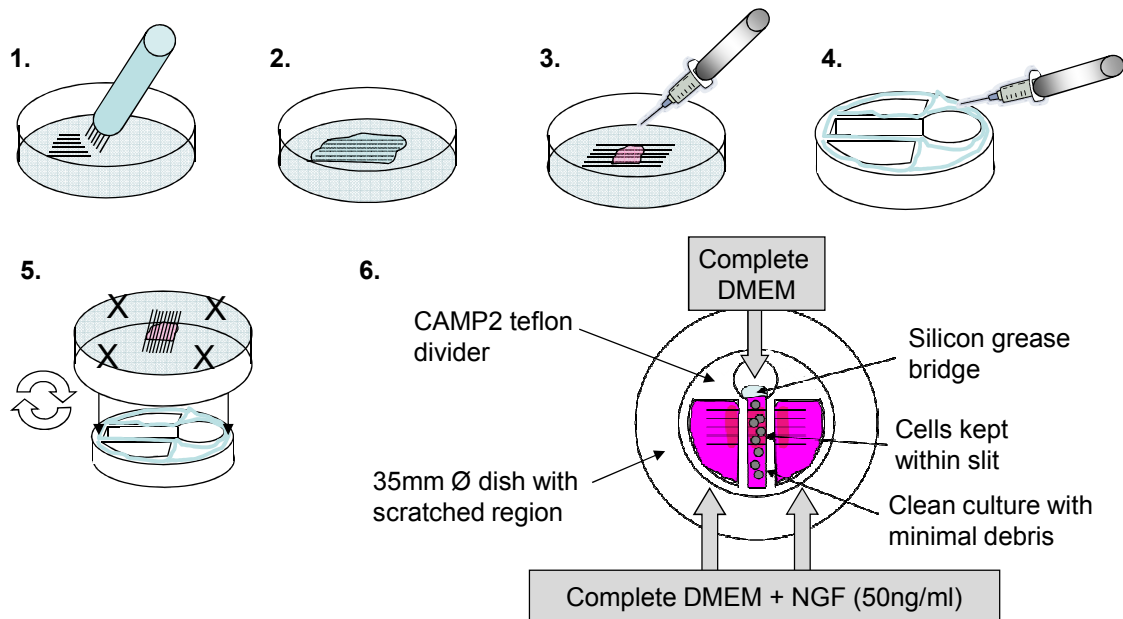


Figure 2.2.2) Diagram depicting set-up of compartmented culture. 1. Make 2 cm long scratch with pin rake; 2. Coat scratched region with 0.1 mg/ml poly-lysine for 1.5h; 3. Add 30 μ l mounting solution onto central portion of scratched region; 4. Apply thin coat of autoclaved grease to one side of sterile teflon divider; 5. Drop scratched dish onto greased divider and press down gently at points marked with X to form seal. Leave for at least 1.5h to allow grease to settle, then fill side chambers with 300 μ l DMEM to check for leaks – leave o/n. Using only non-leaky Campenot chamber, coat chamber surfaces with 10 μ g/ml laminin and perform culture. 6. Final set-up of compartmented culture: Plate 40 μ l DRG cell suspension in the central compartment beyond a silicon grease bridge lying over the scratched region. Maintain DRG cells, in 150 μ l C-DMEM without NGF. Fill side compartments with 300 μ l C-DMEM supplemented with 50 ng/ml NGF to guide and support DRG neurite extension into the side chambers.

The day of the experiment 300 μ l of 2 nM biotinylated-gp120_{MN} (B-gp120_{MN}) was applied to the side compartments in C-DMEM supplemented with 50 ng/ml NGF. Media was renewed in the central compartment with C-DMEM but with no treatment application. Compartmented cultures were treated for 24h at 37°C then washed, fixed and immunolabelled for β III-tubulin and B-gp120 and nuclei counterstained with Hoescht 32258, as outlined in section 2.2.3, with the Teflon divider remaining in place. The teflon Campenot chamber was removed carefully only after immunocytochemistry had been completed. A 25 mm x 25 mm glass coverslip was mounted gently on top of the fixed immunolabelling culture with anti-fade agent AF1 (Citifluor Ltd) and sealed in place with nail varnish. Samples were analysed at 10x magnification on a Nikon 80i upright

microscope and acquired consecutively using a digital Nikon DXM1200F camera and LUCIA G software. Consecutive images were pieced together as a collage in Microsoft Powerpoint software.

2.2.6 Neurite Analysis: HCA-vision

CSIRO HCA-vision software was used for all neurite outgrowth studies (www.hca-vision.com). The software requires the experimenter to set the parameters within three main categories, Neuron Body Detection, Neurite Detection and Neurite Analysis. Neuron Body Detection requested parameters relating to background correction, suppression of neurites, intensity thresholding and fluorescence channel selection (channel 1 to visualise labelling in red wavelength, channel 2 for green labelling and channel 3 to visualise blue labelling). Neurite Detection parameters included pre-processing (Gaussian smoothing), linear feature detection, removal of small objects that are likely debris and gap closing. Details of all parameters and the range of thresholds set throughout all studies presented in this thesis are showed in Table 2.2.2. Images were acquired at 10x magnification with a screen size of 1180 x 920 μm or 1128.7 x 957.9 pixels. This relates to a conversion of 0.95877 $\mu\text{m}/\text{pxl}$, therefore pixel thresholds presented in Table 2.2.2 relate almost 1:1 to μm measurements.

Table 2.2.2) Table presenting parameters available for alteration during CSIRO HCA-vision neurite analysis.
Range set indicates the range of parameter values used during HCA-vision neurite analysis in these studies.

Parameters	Function	Range Set
Neuron Body Detection		
Load input image	Load image of interest	
Select input image channel	Select relevant emission channel	0 = red; 1 = green; 2 = blue
Smoothing (Gaussian Filtering)	Reduces effect of noisy image capture	4-6
Background Correction	Attempts to flatten black part of image. Should be set larger than diameter of largest neuronal cell body	25-30 pixels
Suppression of Neurites	Removes neurite structure to ensure only circular cell bodies detected. Determined by setting a "width" parameter	7-10 pixels
Intensity Thresholding	Sets threshold of emission intensity. Increasing threshold emits fainter labelled neurons but provides more accurate results and fewer false-positives	0.68-0.80
Nucleus channel	Include nucleus emission channel to help in the accurate identification of cell bodies	No nucleus
Object selection	If touching neuron bodies have been merged by the software, here then can be automatically split by setting a maximum neuron body area	Autosplit: 200-300
Neurite Detection		
Load input image	Load image of interest	
Select input image channel	Select relevant emission channel.	0 = red; 1 = green; 2 = blue
Pre-processing (Gaussian Smoothing)	Reduces effect of noisy image capture	4-6
Linear Feature Detection	"Window size" and "intensity" parameters that control how different the brightness of a neurite can be, compared to its background and the size of the window in which the background and foreground are determined for a particular pixel	Window size: 7-11 pixels Intensity: 4.50
Remove Small Objects	Removes small isolated noisy objects picked up following linear feature detection while retaining connected real neurite structure	5 pixels
Gap Closing	Two parameters "Distance" and "Quality" allows for broken neurite segments to be re-joined. This was decreased from default to incorporate only segments identified by the software to be disconnected due to varying emission intensity and not due to structural disconnection, which was my primary outcome measure therefore should not be manipulated here	Distance: 4-9 pixels Quality: 6-10 intensity
Neurite Analysis		
Load input image	Load image of interest	
Debarb Small Neurite Branches	Here any branch in a neurite tree structure with lengths less than this parameter threshold are pruned from the tree	0-7 pixels
Thicken Neuron Bodies	A "width" parameter that thickens detected neuronal cell bodies graphically to ensure detected neurite are touching the neuronal cell body. Increasing too much can result in neurite networks being associated with an incorrect cell body	0-1
Remove Small Trees	"Length" parameter that removes small, spurious neurite trees from the edges of neuron bodies.	4-7 pixels

2.2.7 Viability Assay

To assess the viability of cells after 24h exposure to vehicle or 2 nM B-gp120_{MN}, treated cultures were washed briefly with pre-warmed DMEM then incubated with propidium iodide (PI; 1 µg/ml) for 15 min at R.T. Cells were then washed three times with PBS and fixed as described in Materials & Methods section 2.2.3 with 4% PFA/PBS. PI stained cultures were additionally incubated with FITC (DTAF)-streptavidin to immunolabel B-gp120_{MN} and nuclei counterstained with Hoescht 32258 as outlined in Materials & Methods section 2.2.3.

2.2.8 Calcium imaging of primary DRG cultures

The calcium fluorescent indicator Fluo-4 AM (Molecular probes; Invitrogen) was reconstituted in dimethyl sulfoxide (DMSO) to a final concentration of 4 mM, aliquoted at 1 µl and stored at -20°C. DRG neuronal cultures were established 24h prior to calcium imaging as described in Materials & Methods 2.2.1 and plated onto poly-lysine and laminin-coated 35 mm glass-bottomed culture wells (coverslip 13 mm Ø, no.0). Cultures were washed three times with 1 ml of a balanced salt solution hereby referred to as normal solution (n.s.) (containing (in mM) 140 NaCl, 5 KCl, 2 MgCl₂, 1.8 CaCl₂, 10 Glucose and 10 HEPES, pH 7.4). Cells were then loaded with 1 ml of 4 µM Fluo-4, diluted in n.s., for 30 min at R.T. protected from light exposure. Following three quick washes with n.s., cells were then left in 1 ml n.s. for 30 min for the de-esterification step. Cells were ready for calcium imaging following three final washes in n.s. and left in a final volume of 1 ml n.s. Treatments were applied in 100 µl volumes sequentially. Neurons were imaged on a Leica SP5 inverted confocal microscope and recorded using Leica LAS AF Lite software. Fluo-4 was excited with a 488 nm Argon laser at 15% power and emitted fluorescence was detected in wavelength range 500-570 nm.

Gp120 glycoproteins and chemokines receptor ligands were applied at 12–14x concentrate (diluted in n.s.) in 100 µl volumes, directly to the culture dish to take into account for the increase in final volume following accumulative application of treatments. Treatments were applied accumulatively following 4–6 min baseline recording of the vehicle application in each study. Cells were recorded for 4–6 min following treatment application, with all measurements made at room temperature. 200 µl KCl (60 mM final concentration) was applied following vehicle and treatment application to test cell viability. The number of cells responding to treatments was counted, with a positive response being determined by an increase in fluorescence intensity of greater than 20% (relating to intracellular calcium concentration) from pre-application baseline and accepted providing no responses were observed following vehicle or spontaneously prior to any application.

2.2.9 Statistical analysis

Data is presented throughout as mean $\pm$ standard error mean (SEM) unless stated otherwise. To assess for significant differences between 1) two independent samples testing one variable, unpaired Student's t-tests were performed; 2) two or more independent samples with one variable tested, one-way ANOVAs were performed; and 3) two or more independent samples with two variables tested, two-way ANOVAs were performed. The null hypothesis of no significant difference was rejected if the critical p value was less than or equal to 0.05, unless stated otherwise. Biological replicates refer to a study being performed in an identical manner using an independent source i.e. a new primary culture set-up from a different rat. Treatment replicates derived from one source (i.e. independent culture coverslips derived from one primary culture/ one rat) were classified as technical replicates. Mean and SEM values were all calculated from identical treatment replicates, whether solely, from technical replicates, biological replicates, or technical treatment replicates pooled from across two or more biological replicates. 'n' represents the number of data values that were included in the calculation.

2.3. Results

2.3.1 Cellular localisation of biotinylated gp120_{MN}.

I was interested in further understanding the involvement of gp120 in HIV-associated peripheral neuropathies. Published research to date demonstrates that the HIV coat protein gp120 binds to cells of monocyte lineage, while its potential interactions with other cell types relevant to peripheral neuropathy has been less well studied. CCR5 and CXCR4 chemokine coreceptors for HIV are mainly targeted to lymphocytes and macrophages, however expression has been also identified on neurons and Schwann cells (Oh *et al.*, 2001; Bhangoo *et al.*, 2009; Keswani *et al.*, 2003). Thus a variety of cells are permissive to gp120 binding however the proportions of cells that internalise gp120 have not been clearly elucidated in the adult PNS. Therefore, using primary adult DRG cultures as a model of the adult PNS I investigated the localisation of a dual tropic gp120, gp120_{MN} that targets both CCR5 and CXCR4. Primary dorsal root ganglia (DRG) cultures, containing a mixture of neuronal and non-neuronal cells, were exposed to 2 nM biotinylated gp120_{MN} (B-gp120_{MN}) for 24h then fixed, permeabilised and B-gp120_{MN} labelled with fluorescein (DTAF)-streptavidin immunostained as

outlined in materials and methods (section 2.2.3). It was found that positive B-gp120_{MN} labelling was present at the cell soma in primary mixed DRG-glial cultures (Figure 2.3.1). Two patterns of localisation were observed 1) diffuse only and 2) diffuse with a clustered patch of B-gp120 labelling, which overlay the Hoescht labelling; suggestive of nuclear colocalisation (Figure 2.3.1 A, green + blue channel + inset). If axonal specific staining was present it was undistinguishable from the background fluorescence (data not shown). Control experiments were conducted to verify the authenticity and specificity of the B-gp120_{MN} immunostaining. Naive (untreated) DRG cultures and vehicle-treated cultures, not exposed to B-gp120_{MN} were incubated with fluorescein (DTAF)-streptavidin, the secondary antibody used to label B-gp120_{MN}, to confirm lack of non-specific binding or fluorescence labelling in the absence of B-gp120_{MN}. No distinct nuclear labelling, as normally seen after B-gp120_{MN} treatment, was evident (Figure 2.3.1 B + C). However, there was a high background fluorescence that appeared similar to the diffuse labelling observed in B-gp120_{MN}-treated cultures. Secondly, primary DRG cultures were treated with denatured B-gp120_{MN} (stock aliquot denatured at 90°C for 1h prior to dilution to working concentration and application) then incubated with the fluorescein (DTAF)-streptavidin during the immunostaining protocol. No distinct fluorescent labelling, as seen after B-gp120_{MN} treatment, was evident (Figure 2.3.1 D, green) indicating a lack of binding affinity of denatured B-gp120_{MN} and further verifying the biological validity of the positive immunoreactivity visualised in B-gp120_{MN}-treated cultures. On occasion in these controls, diffuse non-specific staining was noted, however no punctate clustered labelling was evident. These experiments suggested that B-gp120_{MN} immunolabelling resulted from specific and active binding. It should be noted however that the high background that appeared as a consequence of non-specific binding of the secondary antibody DTAF-streptavidin made the diffuse B-gp120_{MN} labelling and the non-specific antibody labelling indistinguishable. Therefore in my future identification of B-gp120_{MN} labelled cells I quantified only those demonstrating the punctate clustered labelling pattern.

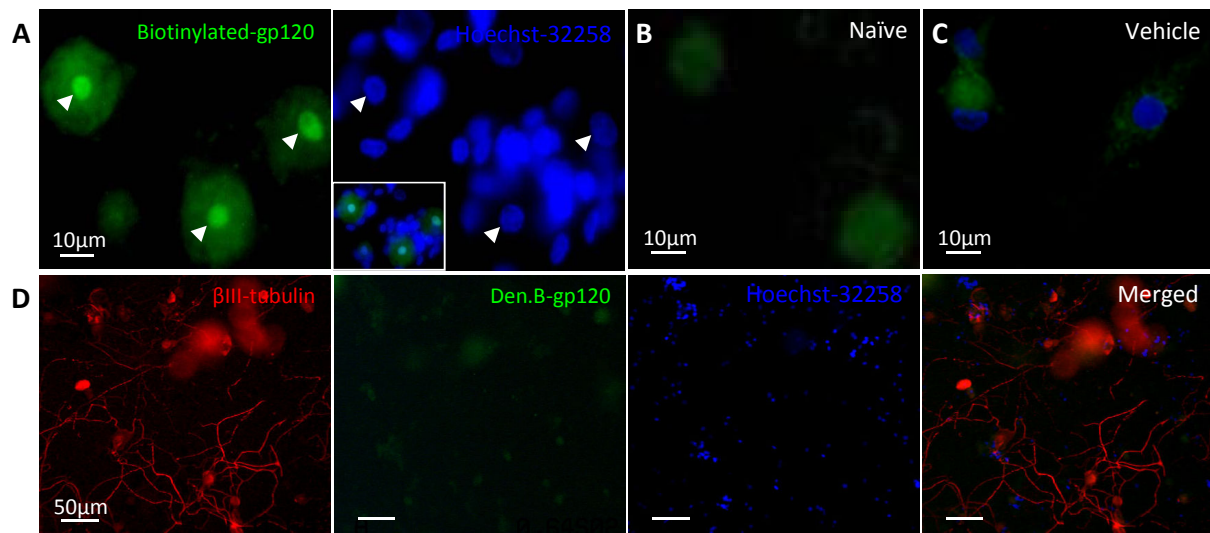


Figure 2.3.1) Biotinylated-gp120_{MN} localisation and immunostaining controls. Neuronal cultures treated with (A) 2 nM biotinylated-gp120_{MN} (B-gp120_{MN}), (B) naïve (untreated) culture, (C) vehicle-treated culture, (D) treated with 2 nM denatured B-gp120_{MN} (Den.B-gp120), all incubated with DTAF (FITC)-streptavidin (green) and nuclei counterstained with Hoechst 32258. Neurons labelled with β III-tubulin (D; red). White arrowheads indicate distinct punctate labelling for B-gp120_{MN} that overlay Hoescht nuclei labelling.

I then aimed to clarify whether B-gp120_{MN} labelling demonstrated membrane-bound or cytoplasmic intracellular localisation and, if internalised, whether this occurred solely via the soma, where it was visualised, or also through axonally-expressed receptors. Using a compartmented Campenot chamber system both the diffuse and clustered patch B-gp120_{MN} labelling was evident on the soma of the neuronal cells in the central chamber following application to the axonal compartment only (Figure 2.3.2). This indicates both that B-gp120_{MN} is actively internalised upon binding and subsequently retrogradely transported to the cell body. These findings suggest that the punctate clustered labelling that overlapped the nuclear labelling represented nuclear localisation of B-gp120_{MN} rather than a singular clustered patch on the membrane.

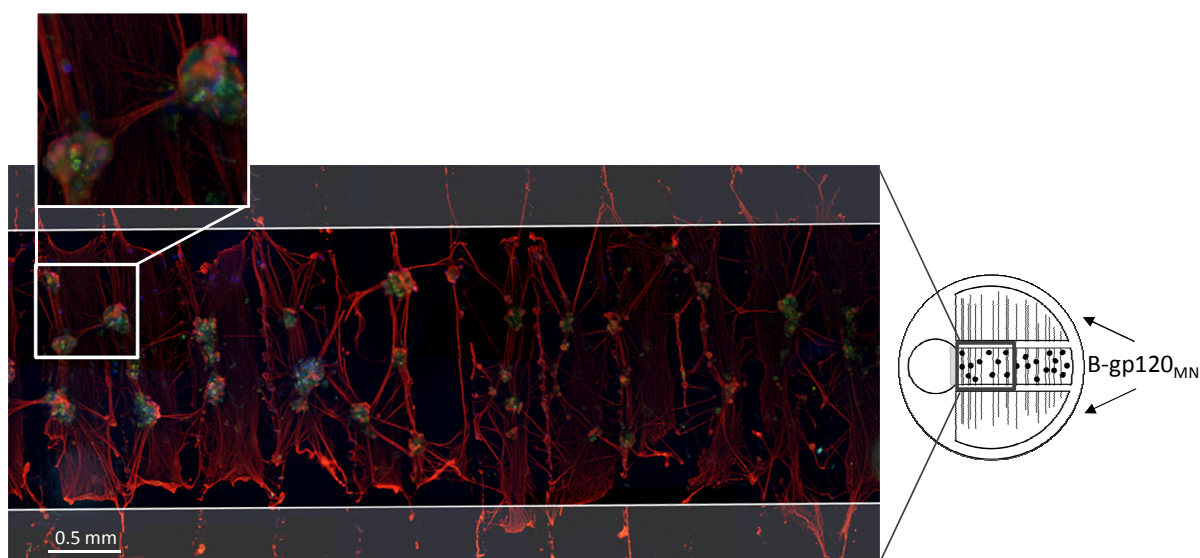


Figure 2.3.2) B-gp120_{MN} labelling shown in the reconstructed image of the central compartment of a Camenot chamber culture system. Primary adult DRG neurons were plated at 10,000 cells into the central compartment of a sealed Teflon divider chamber system and maintained for 7 days to allow neurite extension, beneath the divider walls, into the neighbouring isolated axonal compartments. 2 nM B-gp120_{MN} was applied 7 days post-plating for 24h to the side axonal compartments only, following which neurons were fixed, permeabilised and immunostained after the Teflon divider was removed. Immunolabelling was performed for β III-tubulin (red), B-gp120_{MN} (green) and nuclei counterstained with Hoescht 32258 (blue). Images were acquired, at 10x magnification, in a sequential manner across the culture and then the whole image reconstructed. Transparent grey horizontal bands with white line border represent where the Teflon dividing walls, between the central and side chambers, were positioned. Expanded box represents 1.9x magnification of boxed area.

I also investigated whether the internalisation of B-gp120_{MN} was occurring via an active biological mechanism or as a result of passive diffusion into dying cells. B-gp120_{MN}-treated cultures were incubated with 1 μ g/ml of propidium iodide (PI) for 15 min immediately prior to paraformaldehyde fixation. PI intercalates into double-stranded nucleic acids and can penetrate the disrupted cell membranes of dying or dead cells but is excluded from viable cells with intact membranes. Positive nuclear PI staining was visible in the nucleus of a minority of β III-tubulin immunoreactive neurons (mean $\pm$ SEM: $6.1 \pm 2.0\%$; Figure 2.3.3 A), confirming that the staining protocol was effective and indicating that my culture systems yielded a high proportion of healthy non-PI positive neurons. On quantification of the percentage of neuronal cell bodies immunopositive for B-gp120_{MN}, PI and co-reactive for both it was found that $17.7 \pm 6.9\%$ β III-tubulin-positive neurons demonstrated nuclear localised B-gp120_{MN} staining (Figure 2.3.3) but interestingly only a minority of B-gp120_{MN} positive neurons were PI positive (mean $\pm$ SEM: $3.0 \pm 1.1\%$). However, analysis of the PI stained β III-tubulin-

positive neurons showed a higher proportion immunolabelled for B-gp120_{MN} (mean $\pm$ SEM: 26.7 $\pm$ 5.4%).

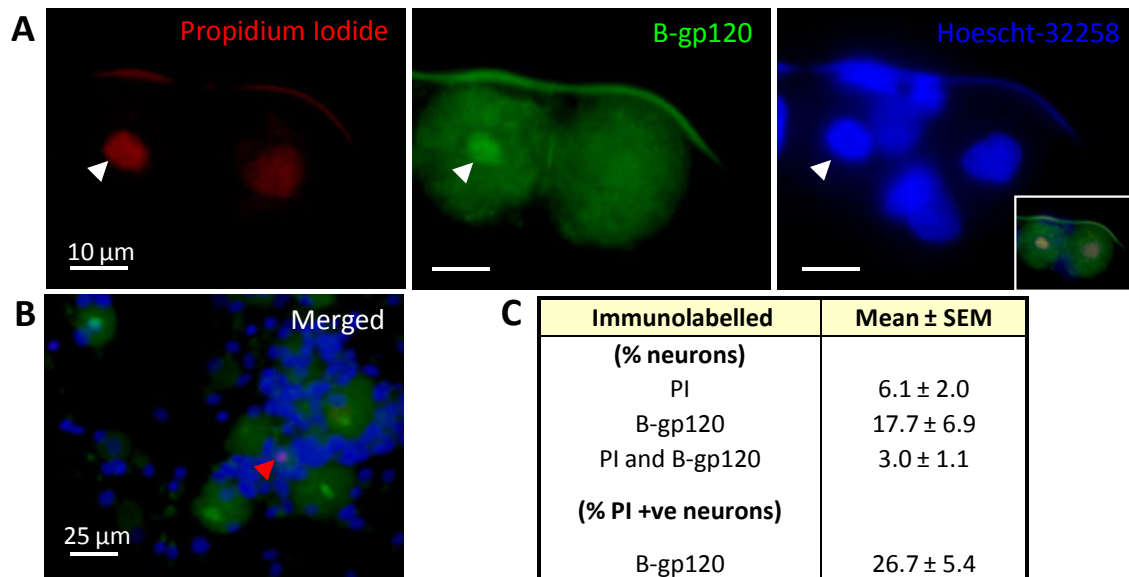


Figure 2.3.3) Representative images and immuno analysis of primary DRG cultures. (A) Split channels for one image demonstrating an example of positive propidium iodide (PI) staining indicated by white arrowhead. (B) Image demonstrating an example of positive for PI staining but negative B-gp120_{MN} immunolabelling indicated by red arrowhead. (C) Quantification of the percentage of neurons immunopositive for B-gp120_{MN}, PI and colocalised with both, averaged across 4 separate but identical studies (5–9 images/study).

On confirmation of the validity of the B-gp120_{MN} immunostaining, colocalisation of B-gp120_{MN} with various markers was investigated to determine the cell types into which B-gp120_{MN} was internalising and the receptor expression profiles of B-gp120_{MN} positive cells (Figure 2.3.4). A greater proportion of β III-tubulin–positive neurons (14.2 $\pm$ 5.4%) showed B-gp120_{MN} localisation compared to S100-positive glial cells (1.1 $\pm$ 0.4%). However, it should be noted that overall there was a greater proportion of S100-positive cells in the culture (41.5 $\pm$ 0.1%) compared to β III-tubulin–positive cells (4.8 $\pm$ 1.5%). Therefore the absolute number of B-gp120_{MN} positive cells was very similar between β III-tubulin–positive cells and S100-positive cells even though proportionally they were different. Negative controls were performed to assess background fluorescence and non-specific binding of the secondary antibodies (Figure 2.3.4 A). Analysis of the cell subsets into which B-gp120_{MN} internalised demonstrated that 1) 13.4 $\pm$ 6.0% were small diameter nociceptive neurons as indicated by labelling for the nociceptive marker, peripherin; 2) 69.2 $\pm$ 2.6% expressed the chemokine receptor CXCR4 and 3) 59.7 $\pm$ 8.8% were positive for the chemokine receptor CCR5 (Figure 2.3.4). Another way of expressing these data is that of peripherin, CXCR4 or CCR5 immunopositive cells 25.9 $\pm$ 13.0%, 48.4 $\pm$ 12.1% and 38.0 $\pm$ 8.6% were B-gp120_{MN} positive, respectively. These data indicate that

B-gp120_{MN} has the ability to bind and internalise into both CXCR4 and CCR5 positive cells, supporting its action as a dual tropic gp120 strain and maintains the ability to interact with both neurons and Schwann cells. The prevalence of CXCR4 and CCR5 co-expression was not assessed in this study because antibodies raised in different hosts were not available. However, data from the literature, suggests that the coexpression of CXCR4 and CCR5 can reach 100% in many cell types (inc. microglia, Schwann cells and neuronal) (Kaul *et al.*, 2007). As stipulated previously, despite coexpression of both coreceptors, gp120 will preferentially bind to just one of these coreceptors depending on the tropism of that strain, determined by the V3 sequence. However as gp120_{MN} is a dual tropic strain it is able to target both coreceptors. Therefore these studies only highlight the prevalence of each coreceptor and not the level of coexpression or which coreceptor gp120 interacted with to internalise.

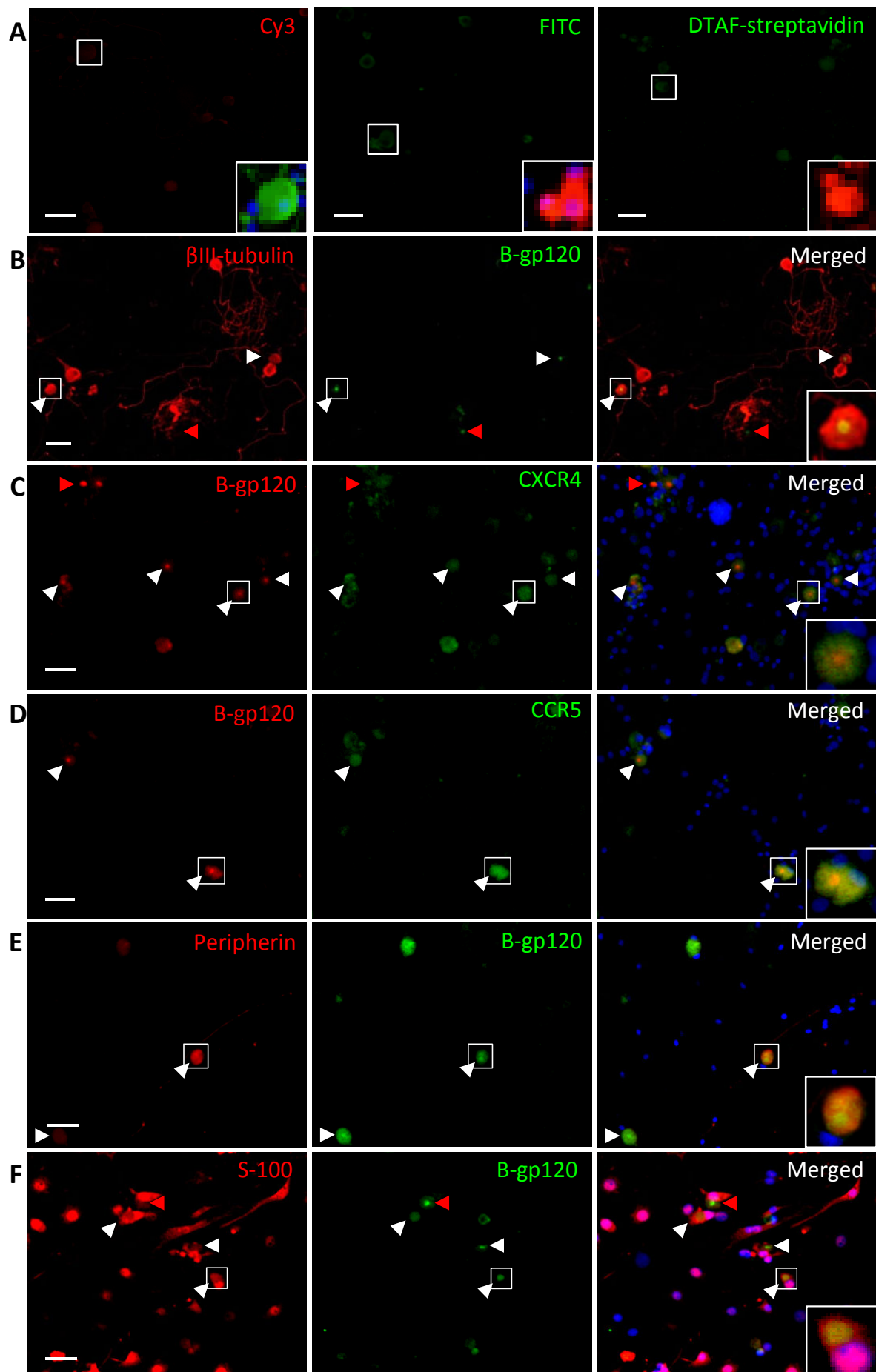


Figure 2.3.4) Biotinylated-gp120_{MN} co-localisation. Representative images from mixed glial-neuronal cell cultures incubated with 2 nM biotinylated-gp120_{MN} (B-gp120_{MN}) for 24h. (A) Negative controls testing non-specific labelling by secondary antibodies. Cultures were incubated without primary antibody, then exposed to Cy3, FITC or DTAF-streptavidin secondaries. Inset depicts magnification of boxed area on negative control merged with channels showing β III-tubulin labelling and Hoescht-32258 staining. B-gp120_{MN} was labelled with either TRITC-streptavidin (C + D: red) or DTAF (FITC)-streptavidin (B, E + F: green) and the culture co-stained for either β III-tubulin (B; Cy3), CXCR4 (C; FITC), CCR5 (D; FITC), peripherin (E; Cy3) or S-100 (F: Cy3). White arrowheads indicate colocalisation between B-gp120_{MN} and target marker immunolabelling. Red arrowheads indicate B-gp120 immunolabelled cells that are negative for the target protein. Blue labelling represents nuclei counterstained with Hoescht 32258. Inset depicts 3x magnification of boxed area. Scale bar = 50 μ m.

Table 2.3.1) Quantitative analysis of B-gp120_{MN} co-localisation depicted in Figure 2.3.4. Data presented as mean percentage $\pm$ SEM and total counts in parentheses. Nuclei labelling using Hoescht 32258 was performed in order to quantify the number of cells present within the images analysed. The sum of labelled-nuclei acted as the normaliser for “% cells +ve for target” in order to perform a comparison across all target proteins.

Target	% cells +ve for target	% target +ve for B-gp120
β III-tubulin	4.8 $\pm$ 1.5 (647/9642)	14.2 $\pm$ 5.4 (71/647)
Peripherin	1.3 $\pm$ 0.6 (49/4424)	25.9 $\pm$ 13.0 (11/49)
CXCR4	1.6 $\pm$ 0.3 (120/7216)	48.4 $\pm$ 12.1 (54/120)
CCR5	4.4 $\pm$ 0.6 (111/2855)	38.0 $\pm$ 8.6 (25/62)
S-100	41.5 $\pm$ 0.1 (3236/7791)	1.1 $\pm$ 0.4 (35/3236)

2.3.2 Establishing a protocol for sensitive analysis of neurite outgrowth.

Neurite outgrowth is a physiological process that acts as a general indicator of cellular health. Consequently it is affected by a wide spectrum of harmful agents and the involvement of multiple stages provides the opportunity to examine the specific effects of different groups of toxic compounds on neurite growth. While the use of neurite outgrowth as an *in vitro* model of assessing neurotoxicity has been previously explored (Abdulla *et al.*, 2006) differences in culture protocol can affect neurite outgrowth analysis between research labs. Therefore a significant effort was made to ascertain a sensitive and robust protocol to obtain a genuine and representative readout of the extent of neuronal neurite outgrowth within my own primary DRG cultures. HCA-vision software was used throughout all neurite analysis studies to determine the extent of neurite outgrowth per

neuron and a description of the steps involved in the set-up of this analysis are depicted in Figure 2.3.5. Details of the protocols for set-up and analysis are outlined in Materials and Methods sections 2.2.4 and 2.2.6. Using the HCA-vision software I obtained information on the number of neuronal cell bodies, the number of cells present as determined by the nuclei count, as well as other analysis parameters including number of branch points and roots.

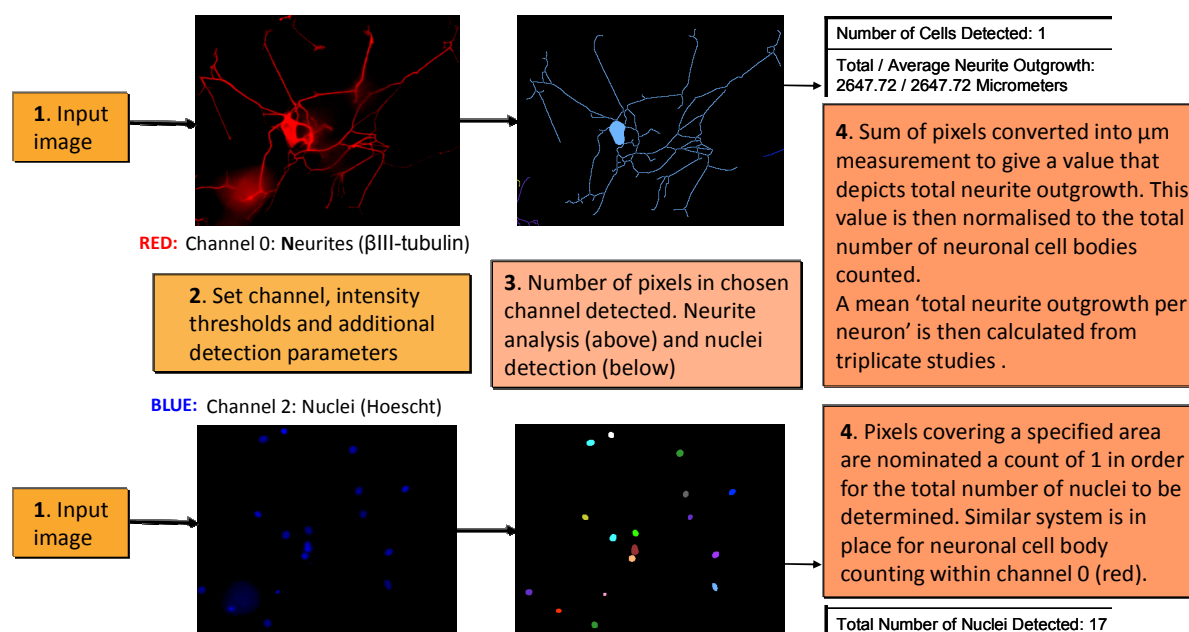


Figure 2.3.5) Flow chart depicting steps for neurite analysis using HCA vision analysis software.

HCA-vision proved to be sensitive and reproducible in its detection of neuronal cell bodies and neurites. However, it was not possible to accurately determine which neurite belonged to which cell body if the cell culture was too densely populated or if neurites from two individual neurons were overlapping. In addition, the software had the tendency to incorrectly detect flattened portions of tubulin networks as a cell body. Therefore it was essential to manually check the results obtained from performing neurite analysis with HCA-vision. Manual validation was performed by checking each image created by HCA following detection of the pixels in the selected channel (Figure 2.3.6 B) and either removing false positives or merging split cells, as outline in Figure 2.3.6 C. To improve the accuracy of the HCA analysis and limit the number of cells requiring manual correction I reduced the plating density from approximately 6000 cells to 2000 cells per 13 mm $\varnothing$ coverslip.

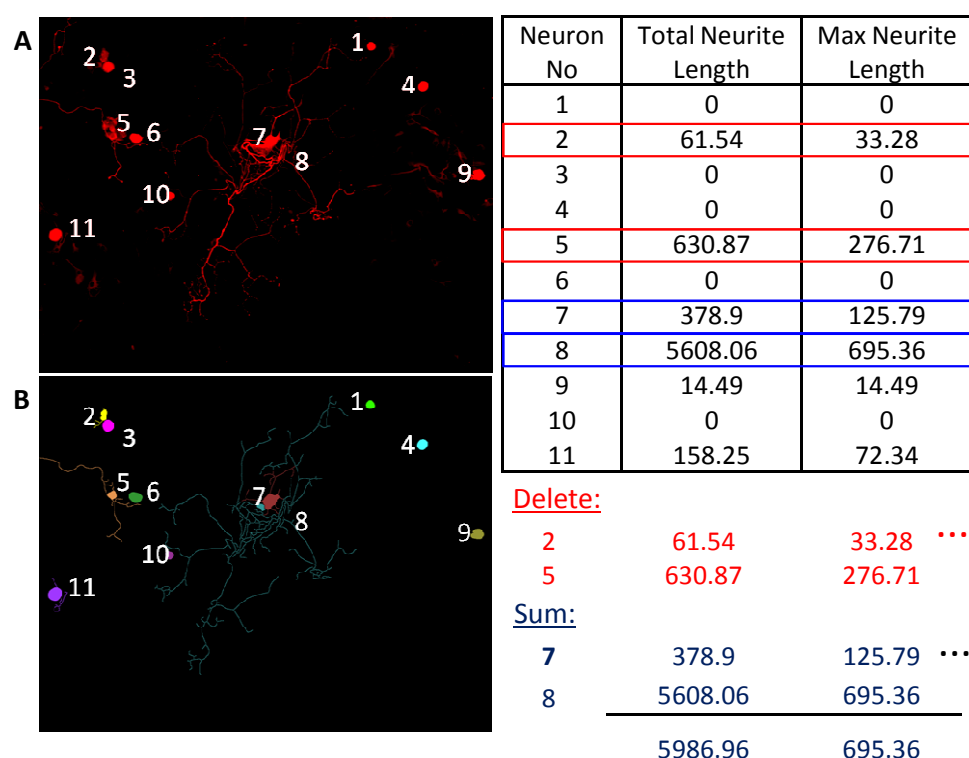


Figure 2.3.6) Example of procedure for manual validation of HCA results and correction strategy. (A) Example image of primary neuronal cells in a mixed DRG-glial culture immunolabelled for β III-tubulin (red). Images are uploaded into HCA-vision and the parameters set as outlined in **Figure 2.3.5** and described in **Materials & Methods section 2.2.6**. (B) HCA-vision-created profile outlining the neurites and cell bodies detected according to the pixels in the selected channel that met parameter criteria. (C) Extract example of neurite analysis data resulting from HCA-vision neurite analysis of image depicted in **A**. White numbers in **A** and **B** correlate to the cell body counts identified by HCA-vision and denoted as Neuron No in **C**. On visual comparison of **A** and **B** it is apparent that cell bodies 2 and 5 have been incorrectly identified as viable neurons by HCA-vision. Therefore the neurite outgrowth data relating to these two cells would be deleted. In contrast neuron no 7 in **A** has been incorrectly identified as two separate cell bodies in **B**. Therefore the neurite data in **C** for these two cells are pooled together and denoted as one cell.

Once the analysis procedure was established, I validated that the culture technique and neurite analysis protocol was capable of identifying reductions in neurite outgrowth by treating DRG cultures with control compounds with known neurotoxic capabilities. The neurotoxic compounds, nocodazole (5 μ M) and vincristine sulphate (55 nM), which both depolymerise microtubules (Jordon *et al.*, 1992), were applied to primary DRG cultures for 24h using the same culture conditions to be used for gp120 studies. Following treatment a significant reduction in the extent of neurite outgrowth per neuron was observed for both nocodazole (mean $\pm$ SEM: 75.4 $\pm$ 14.2 μ m) and vincristine (mean $\pm$ SEM: 32.7 $\pm$ 3.7 μ m) compared to vehicle control (mean $\pm$ SEM: 1356.8 $\pm$ 219.2

µm) (Figure 2.3.7 A), confirming the effectiveness of the treatment and the ability for the protocol to detect alterations in neurite outgrowth.

To limit Schwann cell proliferation aphidicolin or cytosine arabinoside (aka arabinofuranosyl cytidine, AraC) was used throughout maintenance of the cultures, described here and in all subsequent studies. Although both aphidicolin and AraC target proliferating cells and therefore should not affect the health of neuronal cells, which are in a differentiated non proliferating state, some literature on AraC suggests it may have some neurotoxic properties or alter nociceptor sensitivity (Chang *et al.*, 1996; Anand *et al.*, 2008). Therefore the effect the presence of these two compounds had on neurite outgrowth in vehicle-treated cultures was assessed. Cultures maintained in the absence of any inhibitor of proliferating cells showed no significant difference in the extent of neurite outgrowth per neuron (mean ± SEM: 980.1 ± 151.9 µm) compared to when maintained in the presence of aphidicolin (mean ± SEM: 1164.7 ± 165.7 µm) or AraC (mean ± SEM: 929.6 ± 117.4 µm) (Figure 2.3.7 B).

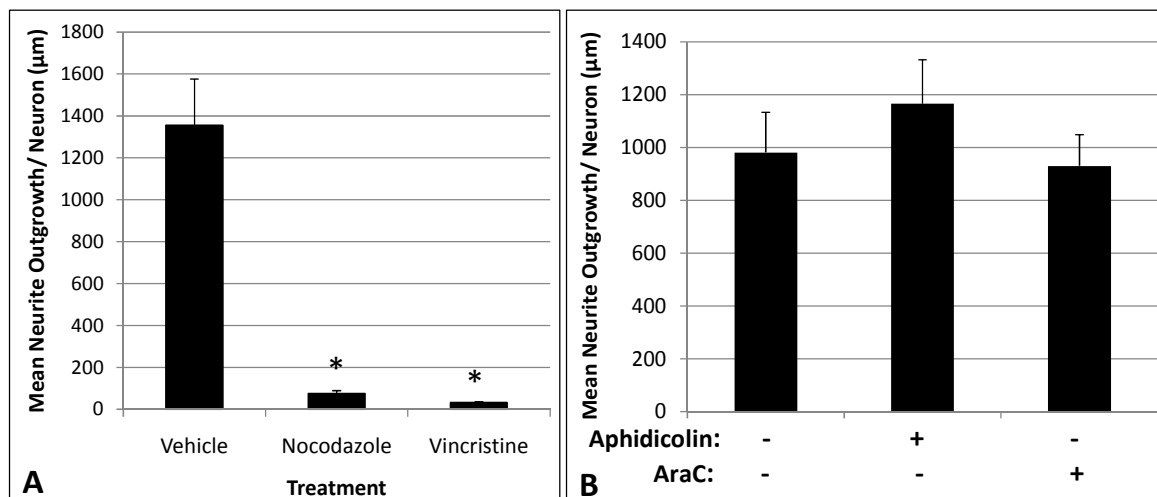


Figure 2.3.7) Effects of neurotoxic compounds and inhibitors of proliferating cells on neurite outgrowth in primary DRG cultures. (A) Neurite analysis following treatment with vehicle, nocodazole (5 µM) or vincristine sulphate (55 nM) verifying sensitivity of culture protocol, HCA software and analysis technique. * p = 0.0003 using single factor ANOVA and Tukey's post-hoc analysis (n=6). (B) Neurite analysis of primary DRG cultures indicating no significant difference in neurite outgrowth between vehicle-treated cultures (0.1% BSA/PBS) maintained either in the absence or presence of aphidicolin (M) or AraC (0.5 µM). p = 0.1517; using single factor ANOVA and Tukey's post-hoc analysis (n=6).

Although I found the primary DRG cultures to be relatively comparable in their extent of neurite outgrowth, for consistency of data and to account for any variations in experimental timing or

culture maintenance, many studies were analysed by comparing the effects of treatments to the internal control. Identical repeats of a study were then pooled by taking the mean variation from the controls for each treatment, presented as percentage of control.

2.3.3 Gp120_{MN} induces reduced neuronal outgrowth in a concentration-dependent manner.

Once the sensitivity of the culture technique and neurite analysis protocol had been confirmed I began my investigations by assessing the effects of different concentrations of recombinant gp120 on neurite outgrowth in primary adult DRG cultures. DRG cultures exposed to 200 pM and 2 nM of gp120_{MN} for 24h showed a significantly reduced mean neurite outgrowth per neuron of $196.4 \pm 48.9 \mu\text{m}$ and $226.8 \pm 49.5 \mu\text{m}$, respectively, compared to naïve untreated cultures ($669.0 \pm 6.2 \mu\text{m}$) or those exposed to only 2 pM gp120_{MN} ($501.8 \pm 83.8 \mu\text{m}$) or 2 pM B-gp120_{MN} ($538.9 \pm 88.9 \mu\text{m}$) respectively (Figure 2.3.8). In addition the biotinylated form of gp120_{MN} (B-gp120_{MN}) induced a similar profile of concentration-dependent neurite reduction indicating that biotinylation does not interfere with its biological activity. Quantification of the mean density of neuron cell bodies per treatment indicated no significant difference between naïve cultures and gp120_{MN}- or B-gp120_{MN}-treated cultures at any of the concentrations tested (data not shown).

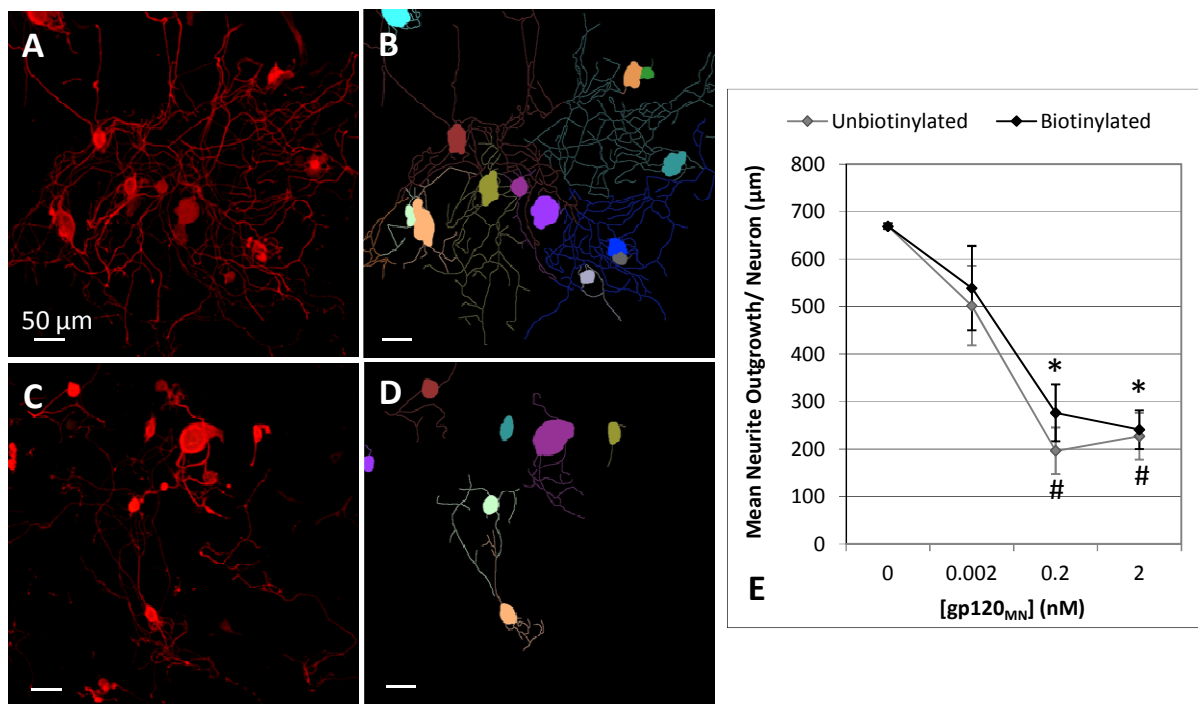


Figure 2.3.8) Representative images and quantification of neurite outgrowth. Neurite analysis of neurite outgrowth following 24h exposure to (A) vehicle solution or (C) 200 pM gp120_{MN}. B and D depict the image created by HCA-vision after inputting images A and C respectively. Cultures were immunostained for β III-tubulin. (E) Mean neurite outgrowth per neuron, following 24h exposure to varying concentrations of either

unbiotinylated gp120_{MN} (gp120_{MN}) or biotinylated gp120-MN (B-gp120_{MN}). Data presented as mean $\pm$ SEM (n=3 technical replicates; 15 images, and an average of 97.4 ± 10 neurons, analysed per coverslip). * = significant at 0.05 level vs. 0 nM gp120_{MN} or B-gp120_{MN} i.e. vehicle control, using two-way ANOVA with repeated measures and Tukey's HSD post-hoc analysis.

2.3.4 Temporal course of gp120_{MN}-associated neurite reduction and its temporal relationship with neuronal B-gp120_{MN} accumulation.

I was interested in the temporal course of the gp120-associated effect on neurite outgrowth to gain a greater understanding of the potential mechanisms involved. Primary DRG cultures were exposed, 41h after plating, to either vehicle solution (0.1% BSA/PBS in 500 μ l DMEM) or 2 nM gp120_{MN} (in vehicle). Untreated naïve cultures were fixed at the time of treatment application (denoted as t=0) to assess the basal level of neurite outgrowth. Vehicle and gp120_{MN} treated cultures were then fixed 2, 4, 8, 16, 24 and 48h post-application. Neurite degeneration was first evident 24h following application of 2 nM gp120_{MN} as shown by a mean neurite length ($580.7 \pm 149.6 \mu\text{m}$; n=6) that was significantly lower than that in vehicle-treated cultures fixed at the same time ($1272.9 \pm 178.0 \mu\text{m}$; n=6). Mean neurite lengths remained significantly different between treatment groups at least until 48h when the study was stopped (naïve: $2594.7 \pm 487.4 \mu\text{m}$ vs. 2 nM gp120_{MN}: $643.0 \pm 548.9 \mu\text{m}$; n=6) (Figure 2.3.9). It is important to note that the term degeneration has been used here rather than the traditional term of 'reduction in neurite outgrowth' because the data demonstrated that the neurite outgrowth per neuron at 24h in gp120_{MN}-treated cultures was significantly lower than mean levels recorded at the 16h time point ($1015.1 \pm 103.2 \mu\text{m}$) eight hours previously. This indicates that the reduced neurite length is likely due to retraction of already established neurites, rather than a lack of neurite outgrowth. In addition neurite outgrowth of gp120_{MN}-treated cultures matched vehicle-treated cultures for the first 16h indicating axonal growth was not slowed or significantly affected until after 16h of gp120_{MN} exposure.

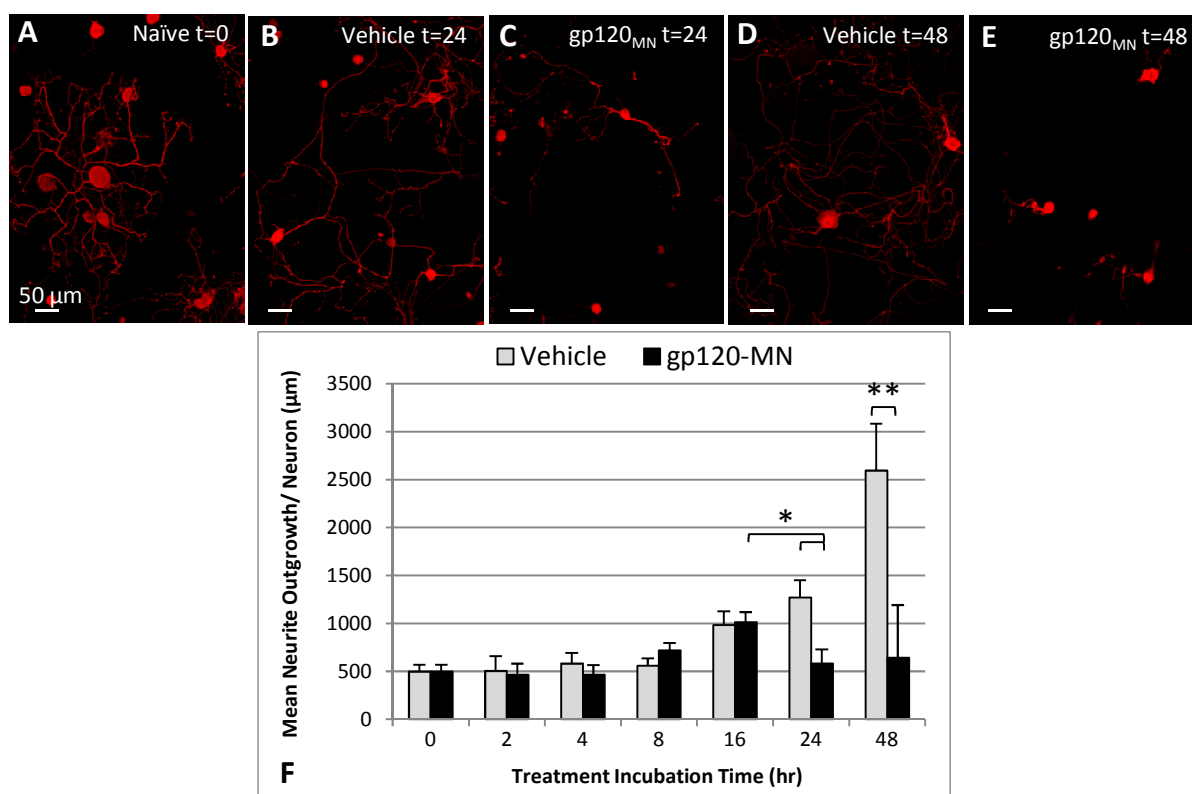
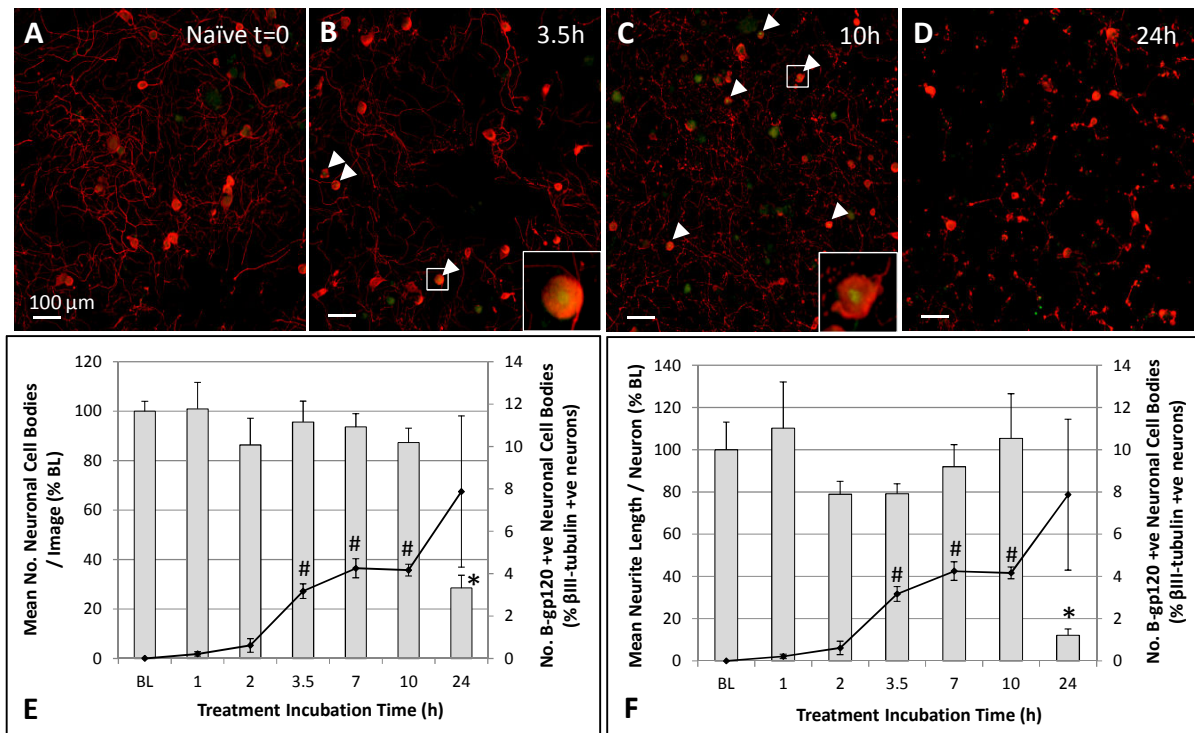


Figure 2.3.9) Representative images and quantification of neurite outgrowth. Representative images of (A) naïve DRG cultures at t=0 and treated DRG cultures after 24h (B + C) or 48h (D + E) exposure to (B + D) vehicle (0.1% BSA/PBS) or (C + E) 2 nM gp120_{MN} (in vehicle solution). Cultures were immunostained for βIII-tubulin. (F) Quantification of the temporal course of neurite outgrowth. Untreated naïve cultures were fixed at time of application to assess the baseline neurite outgrowth (denoted as 0) at time of treatment application. Vehicle and gp120_{MN} treated cultures were then fixed 2, 4, 8, 16, 24 and 48h post-application. Data presented as mean ± SEM (n=6; across two biological replicates, treatments in triplicate per study; 15 images analysed per replicate). * p < 0.05 and ** p < 0.01 vs. vehicle control, using two-factor ANOVA and Tukey's HSD post-hoc analysis.

To further this, the temporal course of neurite degeneration was studied in parallel to the temporal course of biotinylated-gp120_{MN} (B-gp120_{MN}) localisation into neuronal nuclei. Untreated naïve primary DRG cultures were fixed at t=0 (41h post-plating) to determine the basal level of neurite outgrowth at time of treatment application, following which 2 nM B-gp120_{MN} was applied to the remaining cultures. Treated cultures were fixed 1, 2, 3.5, 7, 10 and 24h post-application. It was confirmed that significant reductions in mean neurite length occurred after 24h incubation with B-gp120_{MN} (12.2% of baseline length at time of application) (Figure 2.3.10 E). Interestingly, B-gp120_{MN} was detectable within neuronal cell bodies by 1h post application, significantly earlier than the visible signs of neurite degeneration, albeit in a small proportion of cells (0.2 ± 0.1% neurons; Figure

2.3.10 E). Accumulation of B-gp120_{MN} in neuronally-localised nuclei peaked at 24h ($7.9 \pm 3.6\%$). However there was large variability at this time point, most likely due to the significant loss of neurons from the culture (28.5% of baseline) after 24h. The low percentage of B-gp120_{MN} positive neurons also suggests that a direct, neuronally-mediated mechanism of gp120-induced neurite degeneration, is unlikely to explain the global extent of neurite degeneration observed, and is more consistent with an indirectly mediated toxicity via a non-neuronal cell type.



2.3.5 Non-neuronal cells may act as important mediators of gp120-associated neurite degeneration.

The previous data demonstrated that only a minority of neuronal cells internalised gp120_{MN} and yet neurite reduction affected the global population of neurons. Together with the delayed onset of neurodegeneration these data gave strong indication that the gp120_{MN}-associated neuronal degeneration may be acting via an indirect mechanism in non-neuronal cells. There is some literature supporting direct activation of neurons and calcium influx in small diameter neurons by gp120 in primary neonatal DRG cultures (Oh *et al.*, 2001). However, the data reported by Oh *et al.*, also demonstrated only a low proportion of gp120-responsive neurons. Calcium influx has been attributed to play an important role in neuronal toxicity in the context of many disease states (Siesjo *et al.*, 1999; Medina *et al.*, 1999). Much recent literature indicates an ability of HIV-1 gp120 to initiate calcium influx in neurons *in vitro* however, most of this research has focused on hippocampal and cortical cultures in relation to HIV dementia or DRGs derived from embryonic or neonatal animals. Therefore I aimed to elucidate whether gp120 induces acute alterations to intracellular calcium levels in cultured adult DRG neurons and the relative proportions compared to endogenous chemokine receptor ligands. Further exploration of this phenomenon may provide an insight into whether the neuronal toxicity involves an acute calcium-mediated component.

Changes in calcium influx in cultured adult rat DRG neurons were recorded following sequential applications of treatments. Overall there was no significant difference in the responsiveness of neurons to application of vehicle control ($0.9 \pm 0.7\%$ positive responses) compared to the subsequent application of 100 μ l gp120_{MN} ($1.9 \pm 0.5\%$ positive responses; 2 nM final concentration) (Figure 2.3.11). Meanwhile the application of capsaicin (10 μ M final concentration) following application of vehicle and gp120_{MN} induced calcium influx in a population of DRG neurons ($35.4 \pm 18.5\%$; 88/242 cells). Indicating a similar proportion of neuronal cells are of the nociceptive sensory fibre subset, as was indicated by my immunostaining with anti-peripherin, the marker for small diameter nociceptive fibres.

Due to the low number of responses observed following gp120_{MN} I examined the effects of SDF-1 α and RANTES, the endogenous agonists for CXCR4 and CCR5 respectively, on calcium influx in cultured adult rat DRG neurons to confirm the presence of functional CXCR4 and CCR5 receptors. The proportions of gp120-responsive neurons were equivalent to the neuronal calcium responses following application of SDF and RANTES (50 nM final concentration) (Figure 2.3.11). Initial application of 100 μ l vehicle control solution (0.1% BSA/PBS) to DRG cells, in an initial final volume of 1 ml normal solution, did not alter the $[Ca^{2+}]_i$. In contrast, a minority of cells responded to an

accumulative application of 100 μ l 0.6 μ M SDF-1 α (0.9%; 1/111 cells; 50 nM final conc.) or 100 μ l 0.55 μ M RANTES (2.9%; 3/104 cells; 50 nM final conc.). Further accumulative application of higher concentrations of SDF-1 or RANTES (100 μ l at 2.6 μ M to give 200 nM final conc.) afforded a moderate increase in the number of neurons responding (SDF: 4.5%, 5/111 cells; RANTES: 5.8%, 3/104 cells). Viability of the neurons after application of all treatments was confirmed via final application of 100 μ l 8.4M KCl (to give 60 mM final conc.) to depolarise the cells, which showed that $98.0 \pm 1.2\%$ remained responsive at the end of each experiment (273/277 cells).

Although only a minority of cells responded to gp120 or the chemokine ligands, the mean trace for these responsive cells followed similar temporal outlines to each other. In contrast, the vehicle response times (15.0 ± 2.3 s to first increase) appeared anomalous compared to the chemokine and gp120 response times which showed more latent responses. The time to first increase following gp120_{MN} (170.0 ± 15.0 s) and gp120_{Bal} (138.0 ± 11.0 s) showed no statistical difference compared to 50 nM SDF-1 α (101.5s), 50 nM RANTES (143.5 ± 10.5 s) or 200 nM RANTES (151.2 ± 8.8 s). There was no statistically significant difference between any of the compounds in the time taken to reach peak fluorescence intensity from time of first increase. Although X4/ T-tropic HIV strains are considered more virulent and toxic, the majority of HIV patients present with an R5/ M-tropic strain. Therefore in addition I tested an M-tropic form of HIV-1 gp120, gp120_{Balr}, which preferentially targets and binds to CCR5. Application of gp120_{Bal} increased $[Ca^{2+}]_i$ in $6.1 \pm 1.7\%$ of neurons (n=4/66), both of which also responded to subsequent application of KCl (Figure 2.3.11). Prior addition of vehicle induced no alteration to baseline calcium levels. The temporal profile of the gp120_{Bal}-induced calcium influxes was also comparable to those induced both by gp120_{MN} and the CXCR4 and CCR5 ligands, with the first significant increase in $[Ca^{2+}]_i$ occurring 138.0 ± 11.0 s after gp120_{Bal} application and peaking after 147.0 ± 16.0 s.

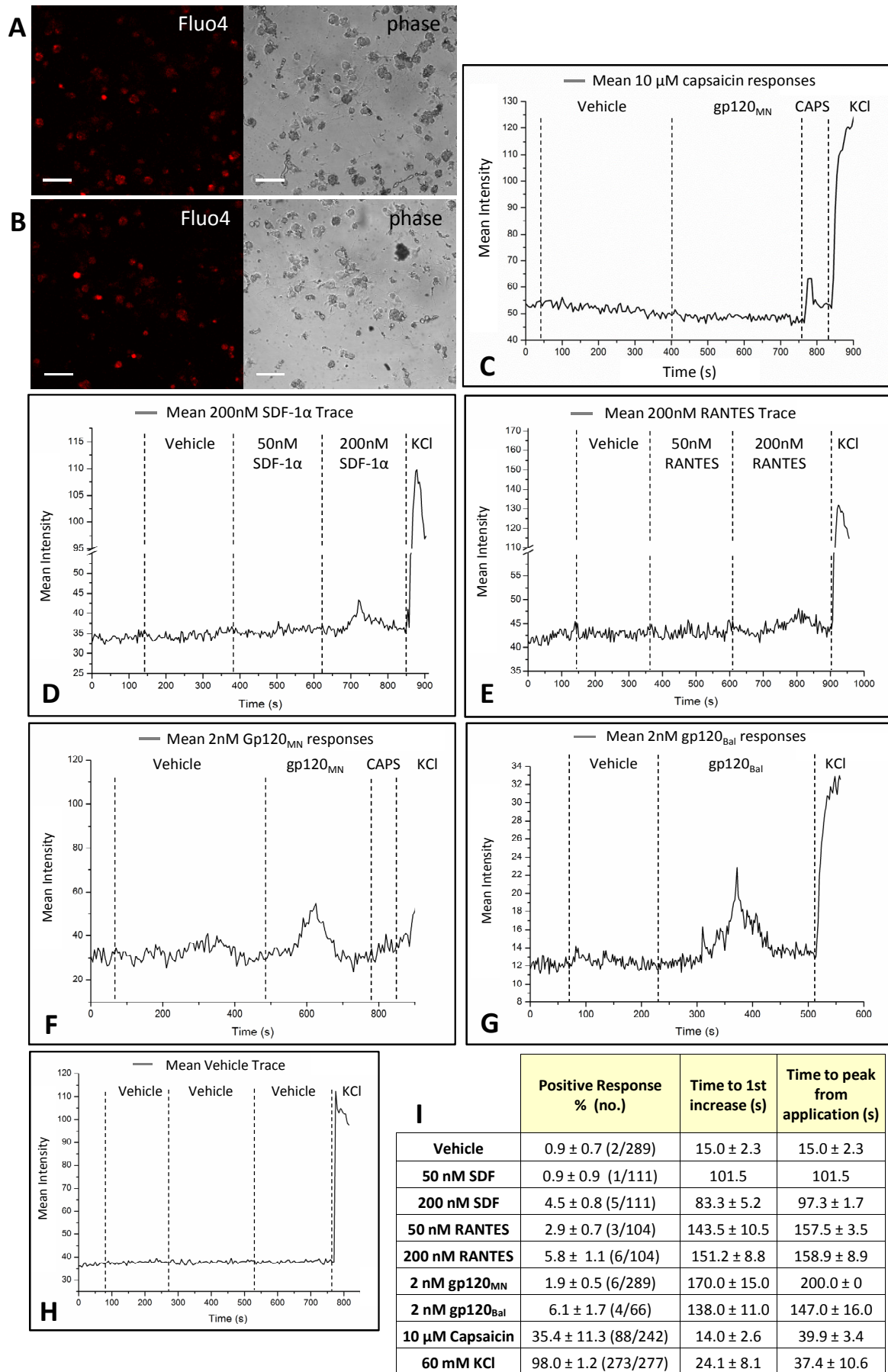


Figure 2.3.11) Assessment of intracellular calcium levels in cultured primary adult DRG neurons following exposure to gp120, RANTES and SDF-1. Channel image for Fluo-4 fluorescence and phase detection acquired prior to application of (A) SDF-1 α and (B) RANTES. Scale bar = 60 μ m. Mean calcium traces from all cells responsive to (C) 10 μ M capsaicin, (D) 200 nM SDF-1 α , (E) 200 nM RANTES, (F) 2 nM gp120_{MN}, (G) 2 nM gp120_{Bal} and (H) consecutive vehicle applications to assess baseline temporal responses of DRG culture. (I) Table displaying quantification of the mean percentage (and pooled number) of responsive cells from 1–2 biological replicates and their temporal response times following application of different chemokines, gp120 proteins and excitatory compounds.

2.4. Discussion

The studies carried out so far have aimed to develop and optimise *in vitro* protocols and experiments, using a mixed sensory neuronal culture, to allow me to investigate more precisely the interaction between HIV-1 gp120 and neuronal cells at a cellular level. I wanted to be able to determine the relative effects of gp120 viral strains and endogenous chemokine coreceptor ligands, and the involvement of neuronal and non-neuronal cells in gp120-mediated neurite degeneration. DRG cultures, derived from embryonic or neonatal animals, have been reported to show reduced neurite outgrowth after exposure to gp120, either directly or after addition of a supernatant from another cell culture exposed to gp120 (Bajetto *et al.*, 2001). Therefore this idea was adopted and a protocol was established in order to test the effects of gp120 in an adult neuronal culture system with neurite outgrowth as my outcome measure. Strict culture, immunostaining, image acquisition and image analysis protocols were established to avoid bias and after this optimisation various additional pilot experiments were carried out in order to start to tease apart the gp120 cellular interactions. It should be noted that for the purpose of this thesis, unless stated otherwise, gp120 has been referred to as a neurotoxin or gp120-induced neurotoxicity has been stated, however, this relates only to its ability to induce neurite degeneration and not its ability to induce cytotoxic effects such as apoptosis, which was not assessed in depth here.

The second purpose of the *in vitro* experiments was to clarify whether direct interactions between gp120 and neuronal cells are necessary for the initiation of gp120-associated neurite degeneration. These were explored *in vitro* at a cellular level, because such investigations would not only be difficult but also costly and time-consuming if performed *in vivo*. Any mechanisms highlighted from these studies will then be evaluated in an *in vivo* model to determine how they function in a whole body system. Each of the studies performed and the results obtained will now be discussed and interpreted in greater detail in this section.

2.4.1. Cellular localisation of biotinylated gp120_{MN}.

Numerous studies across the past decade have explored the biological effects of gp120 interactions, however, relative to this number, only a selection have assessed the cellular localisation of gp120 and correlated this with the functional outcome. I was intrigued to explore this field further however, my main aim was only to verify gp120 binding in my culture systems in order to correctly interpret the results of subsequent studies.

For my localisation studies, initially I used a rabbit anti-gp120 HIV-1 polyclonal antibody (ImmunoDiagnostics #1301) on paraformaldehyde-fixed and permeabilised cell cultures following 24h vehicle or gp120_{MN} exposure. However, I saw no obvious specific staining (data not shown). According to current literature T-tropic gp120_{IIIB} induces calcium influx in only a small proportion (~10%) of small-diameter neurons (Oh *et al.*, 2001) and there is little data elucidating internalisation of gp120 or what proportion of different cell types are sensitive to gp120 binding. In my studies, relatively low concentrations of the gp120 are tested so; joint with the suggestion of low binding probability (~10%) and inadequate antibodies, unconjugated antibodies may result in weak labelling or low numbers of positive labelling. Therefore I decided to test a biotinylated form of the recombinant gp120 strain I wanted to use in my experiments, biotinylated-gp120_{MN} (B-gp120_{MN}), in an effort to amplify the fluorescent signal.

My first results using B-gp120_{MN} showed diffuse immunolabelling at the cell soma, thereby indicating B-gp120_{MN}-binding to cells in my culture. An unexpected finding however, was the patch of clustered B-gp120_{MN}-labelling that appeared to consistently overlay with the Hoescht 32258 nuclear labelling. This apparent colocalisation was suggestive of B-gp120_{MN} internalisation and subsequent nuclear translocation. However, these images were acquired using a conventional wide-field fluorescence microscope at 10x magnification. Therefore from this data I cannot confidently claim that these labelling patterns represent cytoplasmic and nuclear localisation of B-gp120_{MN}, respectively, due to the limited optical resolution of conventional microscopy. Future investigations could clarify this using confocal microscopy; with its ability to increase optical resolution and visualise, accurately, the fluorescence at specific z stack points through the sample thereby providing greater insight into the target location. Few studies have focused on investigating the intracellular localisation of gp120 and to my knowledge nuclear localisation of gp120 has not been previously reported. If the nuclear localisation proved to be accurate this may be a novel finding with interesting proposals. However, the purpose of these experiments was not to confirm internalisation or elucidate accurately the intracellular localisation of gp120 but rather, verify gp120-to-cell binding and to investigate the cell

types sensitive to gp120 and their relative proportions. As a note though, some studies inadvertently partially address these questions and will be discussed in the following sections.

Numerous controls were performed to verify the B-gp120_{MN} staining. No punctate staining was observed under conditions where adult DRG cultures were 1) immunolabelled with the secondary, fluorescein (DTAF)-streptavidin in the absence of B-gp120_{MN}, 2) treated using denatured-B-gp120_{MN}, or 3) immunolabelled with the secondary, fluorescein (DTAF)-streptavidin following treatment with unbiotinylated gp120_{MN}. By using denatured-B-gp120_{MN} I assumed that the denaturation conditions were sufficient to denature the gp120 but I do not know whether biotin would have detached or been denatured itself. Heat denaturation in this manner has been demonstrated to be sufficient to abolish CD4 and coreceptor-mediated activity and gp120 immunogenicity (Grundner *et al.*, 2004). Biotin is known to bind to other biotin molecules thereby resulting in non-specific labelling. If the biotin was undenatured then the lack of punctuate staining provides further verification of the specificity of both the target biotinylated protein and the secondary antibody. In addition the rationale for staining unbiotinylated gp120_{MN}-treated cultures using fluorescein (DTAF)-streptavidin, derived from the idea that cell cultures under conditions of neurotoxicity may present more biotin-derived components than healthy neuronal culture, as in this case induced by gp120, due to depolymerisation of microtubules and restructuring of the cytoskeleton. In these conditions it could be hypothesised that the clustered immunolabeling was a result of increased binding of streptavidin to the increased presence of biotin molecules. No punctate clustered staining was evident under any of these conditions indicating that these hypotheses were unsubstantiated and indicated that the B-gp120_{MN} labelling observed was supportive of gp120-specific binding events.

Due to the somal labelling, and lack of axonal, it may be tempting to speculate that, despite having access to both areas, B-gp120_{MN} interacted only with somal-expressed chemokine receptors rather than with axonally-expressed chemokine receptors. However, a study by Bachis *et al.* strongly indicates that gp120 axonal transport to the cell body is necessary in order for it to exert its neurotoxic activity (Bachis *et al.*, 2006). In some very elegant studies, and one of the few that has accessed the temporal course of gp120 localisation, Bachis exposed neonatal rat cerebellar granule cells (CGCs) cultures to 5 nM T-tropic (X4) gp120_{IIB}, conjugated to colloidal gold particles, and assessed the time-dependent localisation. They show that within 5mins gold particles were localised around the synaptic terminals, after 15–30 mins they were found in microtubules, likely in the process of being axonally transported. By 1h the majority of particles were inside lysosomes. Of course only a general comparison can be made as they use a different gp120 tropic strain that targets CXCR4 receptors, and they mimic a model of an immature CNS as they test neonatal

cerebellar granule cells, while my system is a broad model of the adult PNS; adult DRG neuronal cultures. However, it does provide an indicator of the time course of gp120 interactions.

Campanot chamber culture systems allow for neuronal cell bodies to be compartmented from their axonal extension allowing site-specific application of treatments to be performed. Neuronal cell bodies are plated into the central compartment of a teflon divider and using high concentrations of NGF in the neighbouring side compartments axons are guided beneath the dividing wall, and into the side compartments where they extend across the laminin-coated surface of the dish. Silicon grease beneath the divider provides a seal with the dish base that prevents mixing of media across the compartmented cultures but remains permissive to extending neurites. This compartmented system was used to assess the binding kinetics of B-gp120_{MN} and demonstrated B-gp120_{MN} labelling in the cell somas 24h following B-gp120_{MN} application to the axonal compartment. Prior to cell plating confirmation is gained that the seals between the chambers are not permeable to fluid. Therefore identification of B-gp120_{MN} in the cell bodies is unlikely to be due to passive diffusion of media from the side chamber, containing B-gp120_{MN}, leaking into the central chamber where the cell somas are present. Due to the steps I took to validate the B-gp120_{MN} labelling, I believe this demonstrates that B-gp120_{MN} binds to axonally-expressed receptors, is internalised and then readily transported within 24h to the cell soma, where it was identified with fluorescein (DTAF)-streptavidin labelling. Furthermore these data support my notion that the diffuse and clustered patch of B-gp120_{MN} labelling is indicative of cytoplasmic and possibly nuclear localisation respectively, and not membrane-bound.

Investigations into the viability of the B-gp120_{MN} positive neurons demonstrated that only a minority (approximately $4.1 \pm 2.2\%$) occurred in non-viable cells (i.e. positive for propidium iodide staining; the indicator of non-viable cells). In contrast, normalising against the PI positive cells, showed that $34.0 \pm 12.0\%$ of non-viable PI +ve cells were B-gp120_{MN} labelled. PI can only gain access to the cellular DNA by passing through disrupted cell membranes, which occurs only in cells undergoing cell death. That the B-gp120_{MN} labelling occurs mainly in viable cells adds further support to this being the result of biologically active internalisation of the glycoprotein rather than the result of passive diffusion through disrupted membranes. That the PI positive non-viable neuronal cells show a higher proportion of B-gp120_{MN} labelling could also be interpreted to indicate that being activated by or internalising B-gp120_{MN} has a negative impact on cell survival. Certainly, these data would suggest that the B-gp120_{MN} nuclear-like localisation occurs prior to the cell toxicity that is indicated by PI staining.

Although these data together provides supportive evidence for a nuclear localisation of B-gp120_{MN} it is certainly not conclusive and I remain cautious. However, certainly I demonstrate that the B-gp120_{MN} neuronal binding occurs prior to cellular toxicity, which is suggestive of a causal factor but not confirmed. Here I have not ascertained whether B-gp120_{MN} binding or the subsequent nuclear localisation is involved in neuronal degeneration, however, it is tempting to speculate that the nuclear localisation may well play a key role in gp120-mediated neurotoxicity by modulating transcription factors or activating apoptotic pathways in certain neurons. To the best of my knowledge nuclear localisation has not been previously reported. Gp120 is approximately 120 kDa in size therefore could not diffuse passively into the nucleus via the nuclear pore. Therefore it would require either a nuclear import signal or due to its size, more likely, utilise a 'piggy-back' mechanism involving association with nuclear localisation signal (NLS)-binding proteins and subsequent cotransport into the nucleus, as reported to occur with other proteins (Tsuneoka *et al.*, 1986; Zhao *et al.*, 1988). As this was not the focus of my studies I did not verify this theory however, incubation with wheat-germ albumin (WGA), which inhibits nuclear import, could be a possible way to verify whether gp120 is being actively translocated to the nucleus. Of course an alternative theory for the punctate clustered labelling is that the apparent co-localisation with Hoescht labelled-nuclei is a chance event and in fact this punctate staining may represent accumulation of packaged endocytosed B-gp120_{MN} not in the nucleus but in lysosomal compartments. However, the consistent and robust colocalisation with the Hoescht 32258 nuclear dye does make it tempting to maintain the theory of nuclear localisation. Future investigations would be required to elucidate the intracellular localisation and determine at which point, and by what mechanism, neurite outgrowth becomes inhibited or degenerative and whether gp120 intracellular localisation plays any role in this process or whether the process of binding alone is sufficient or not necessary.

Prior to beginning my assessment of the effects of gp120 on neurite outgrowth I confirmed expression of CCR5 and CXCR4 through which dual-tropic gp120_{MN} would act. In addition I wanted to gain an insight into the proportion of different cell types that internalise gp120 and furthermore whether a specific cell type showed increased or reduced gp120 labelling. HIV-sensory neuropathies present with axonal degeneration of the longest neurons that effects typically and intriguingly the small diameter neurons that are the main transmitters of pain signals; the nociceptive sensory neurons. Peripherin is a class III intermediate filament subunit that forms part of the neuronal cytoskeleton, is found selectively in small diameter neurons (Parysek *et al.*, 1988; Rhrich-Haddout *et al.*, 1997), both in the peripheral and central nervous system, and has therefore become a uniform marker of the nociceptive neuron population. Unfortunately anti-peripherin showed poor labelling

in the axons therefore I was unable to perform selective neurite analysis on peripherin positive neurons and neurite networks (data not shown).

I assessed B-gp120_{MN} binding in neuronal cells (β III-tubulin), small diameter nociceptors (peripherin), Schwann cells (S-100) and cells expressing the gp120 coreceptors; CXCR4 and CCR5, independently. I found a high proportion of CXCR4- and CCR5- expressing cells showed binding with B-gp120_{MN}. Peripherin and S-100 were both raised in mouse and thus could not be co-labelled for β III-tubulin, which was also of mouse origin; therefore, additional labelling for neuronal cells with anti- β III-tubulin was not possible in these studies. Only Hoechst 32258 nuclear staining was available therefore it was not possible to determine whether the CXCR4 or CCR5 positive cells are of neuronal or non-neuronal origin. That the B-gp120_{MN} positive cells showed greater than 50% for both CXCR4 and CCR5 suggests that some of these cells would have expressed both coreceptors but in that case I cannot determine which receptor was responsible for the binding. Studies with CXCR4 and CCR5 antagonists would be useful here to elucidate the involvement of each of these receptors in gp120 binding and whether this varies with cell type.

It should be noted that the proportion of different cell types recorded in Figure 2.3.4 does not amount to 100% and no single cell marker showed a prevalence of >50% in the cell culture. This is most likely due to an underestimation of positive cells due to the strict criteria used to distinguish positive immunolabelling from the non-specific background fluorescence. I calculated the mean maximum fluorescence intensity detectable in the control immunofluorescence images (secondary in the absence of a primary) and then, using the identical acquisition settings, accepted labelling as genuine when the fluorescence intensity of a labelled cell was greater than 2 standard deviations from this mean maximum value. However, these strict conditions may have resulted in many neurons and Schwann cells being excluded in the analysis, due to inadequate fluorescence levels, which may explain the lower than expected staining proportions. In addition other non-neuronal cell types, including fibroblasts and monocytes, have not been accounted for here but may also make up a proportion of the culture. It should be noted that fibroblasts are not thought to be targets for gp120 binding (Apostolski *et al.*, 1993).

2.4.2. Establishing a protocol for sensitive analysis of neurite outgrowth.

In vitro models have become useful tools for investigating the toxicity of different compounds, their effects on neurite outgrowth and elucidating the mechanisms underlying axonal degeneration. I previously reviewed how *in vitro* models of neurite outgrowth translate to axonal degeneration and clinical neuropathies *in vivo*, therefore here I will discuss the techniques I used in my own model and

analysis protocol and review it compared to other methods available and what improvements could be made.

My rationale for investigating gp120-mediated neurotoxicity in cultured primary adult DRG cultures was driven by the fact that differences in the functional role and phenotypes of neurons from different regions can result in varying neurotoxic effects, including in neurite outgrowth. Just as exemplified by two studies, assessing gp120-induced neurotoxicity; *in vitro* exposure to gp120 induced cell death in mouse hippocampal neuronal cultures (Brenneman *et al.*, 1988); Savio T 1993 J Neurosci Res), while cultures of fetal rat cerebral cortex (Bernton *et al.*, 1992) or embryonic-derived cultures of neurons and oligodendrocytes (Pulliam *et al.*, 1991; Pulliam *et al.*, 1993) showed no neuronal death in response to gp120 (1 pM to 4 nM) but did cause alterations to astrocytes. Similarly it was shown by Giulian *et al.* that HIV-1-infected human myeloid cell lines released toxic factors that were detrimental to rat neuronal cultures, while the same did not occur in infected lymphoid cell lines (Guilian *et al.*, 1990). Most of the work exploring gp120 has been performed to date on embryonic and neonatal cultures. Given the differences between embryonic, neonatal and adult neuronal cultures in terms of their trophic requirements (Goldberg, 2003);(Patterson PH, 2000), I aimed to elucidate gp120-mediated effects on adult DRG neuronal cultures, especially considering that 92.5% HIV patients are adults, as shown by the WHO 2009 global report (World Health Organization (WHO), 2009).

As outlined in the Introduction to this chapter, section 2.1.3, dissociated primary neuronal cultures have become one of the most common *in vitro* systems for assessing neurotoxicity of various compounds. The loss of the three-dimensional tissue architecture affords dissociated primary neuronal cultures another advantage; greater accessibility to the neurons, which can now be directly exposed to test compounds. For this reason I chose dissociated primary cultures for my experiments rather than an alternative method that uses DRG explants. While explants main advantage is the presence and maintenance of cell-cell contacts and signalling, it is restrictive as a model because it is highly dependent on the diffusion rate of materials through the exterior perineural membrane, which varies depending on the thickness of this layer, the size of the DRG and physicochemical properties of the compounds in the media (nutrients, oxygen, test compounds).

The main aims of these investigations were to establish a method of assessing neurite outgrowth that provided the most accurate, reproducible and representative data and minimised, at every step, the chance of predictability or bias.

Culture Methodology

The first thing I will discuss is the plating density of the DRG neurons because this was found to be one of the most critical parameters in the success of this model. By reviewing prior *in vitro* studies that assessed neuronal outgrowth, I found that a typical plating density ranged from 10,000 to 13,000 cells per 13 mm Ø coverslip. I began my studies plating at a density of approximately 6000 cells per 13 mm Ø coverslip however, I found that the neurite growth was extremely rapid and just 24h after plating, a complex network of neurites was established due the strong trophic support and growth cues between each other. For the analysis of neurite growth I used imaging software called HCA-vision developed by CSIRO (Wang *et al.*, 2010a). HCA-vision detects the pixels for a given fluorescent probe within the fluorescent channel of choice, and using complex algorithms accurately quantifies neurite outgrowth and other parameters. Therefore, it is possible to assess the total amount of neurites present in the culture. However, the ability of the software to accurately trace one neurite back to its correct cell body is completely lost when such a dense network of neurites exists. It may not be necessary to gain knowledge about the specific neurite lengths of each neuron because when using a neurotoxic compound, such as vincristine or nocodazole, which act on the microtubules, indiscriminately of neuronal subset, so that an obvious loss of neurites is visible even if only the total neurite outgrowth was analysed. In this case, providing the total neurite outgrowth value is normalised against the total number of neuronal cell bodies present, to account for the slight variation in the number of neurons contributing to the neurite outgrowth, this method can act as acceptable tool to assess neurotoxicity. However, where more subtle effects are expected or where maybe only one neuronal subset may be affected, this technique may lead to a masking of the neurotoxic effect by the remaining insensitive neurons that continue to extend neurites.

Another aspect that was considered was the manner in which the neurons were plated and randomisation of the treatments. I understood that the plating density significantly affected the resultant extent of neurite outgrowth. Therefore if care and consistency was not maintained when plating the neurons this may have lead to large culture variations, which could have potentially masked subtle differences induced by the treatments. To ensure consistency and equality of culture plating I distributed the dissociated cells in a method derived from the ABCBC alternating treatment design. Effectively, as treatments were to be treated in triplicate I did not want to plate the cells in blocks for each treatment because, it is possible that the first part of the dissociated cell suspension contained more cells, despite thorough mixing, and is plated for all the vehicles, and then the last portion is plated for the toxic treatment but contained many fewer cells. Despite normalising to cell number, this inconsistency in plating would still influence the neurite outgrowth patterns due to

different trophic support and neuron-to-neuron interactions. Therefore, before plating, I planned in advance which culture well would receive which treatment; also randomised across all the wells in an ABCBC alternative treatment-like design, see Methods for more details. Then I plated the cells into one well per treatment sequentially and alternated the sequence of plating across the treatments. This guaranteed that each treatment was tested on a culture that was of similar density and derived from similar portions of the suspension as all the other treatments.

The DRG cultures used as my system for assessing neurotoxicity were not pure neuronal cultures. The proliferating, non-neuronal cell populations were restricted through constant exposure to anti-mitotic agents, such as aphidicolin or cytosine arabinoside (AraC). However, I did not remove the non-neuronal cell population completely because I felt their presence would correlate most accurately to the *in vivo* setting. It is widely known that Schwann cells, the predominant non-neuronal cell type present in DRG neuronal cultures, are essential for providing trophic factors to neurons (Purves *et al.*, 1988; Franke *et al.*, 2000; Pellitteri *et al.*, 2001; Bhatheja *et al.*, 2006; Hu *et al.*, 2011).

The typical time course of studies assessing gp120-mediated effects on primary DRG neuronal cultures, tests cells 24h post-plating and expose them to treatment for between 16-48 h. I adopted a similar time course with a treatment exposure time of 24h, because according to the literature this would provide sufficient time to observe gp120-induced neurite effects, while being short enough to prevent significant overlapping of neuronal processes and maintaining distinction between individual neurites. The implications of these time points will be discussed later on.

Analysis

I used a semi-automated quantitative method of analysis whereby the selection of images was manually acquired by the observed, the analysis was automated using HCA computational software but then completed by manual validation to ensure accuracy. This method was chosen above the simpler Sholl analysis method, which I considered to be insufficiently sensitive, as it is only semi-quantitative being only accurate to the distances separating each circle. As should be the case with any experiment, considerable effort should be made to limit observer bias and to ensure random sampling of the data and consistency of protocol between treatments. This is never truer than with neurite analysis because neurite outgrowth is highly dependent on the position of the neuron in the culture well and the surrounding cell environment, therefore when manual image acquisition is used, neurite analysis becomes highly vulnerable to selection bias.

Therefore strict selection criteria were in place before analysis began and this was maintained consistently throughout all the experiments performed. Although the culture wells were plated and tested following an alternate treatment ABCBC experimental design, at the point of testing the observer (myself) was aware of which dish received which treatment. At this step, this was acceptable as that knowledge would not have influenced the culture outgrowth however, following fixation, immunostaining and subsequent mounting, as described in Materials and Methods (see section 2.2.3), a blinding procedure was implemented. This enabled me to acquire the images completely unaware of which slide contained which treatment thereby limiting bias. An additional selection procedure was implemented that not only limited further selection bias, by pre-determining where the image was acquired from (i.e. one from each octant sector), but also ensuring a selection of images was acquired that would be most representative of the effects to the whole culture. I was guided by the work of previous groups, in particular Keswani *et al.* who investigated gp120-mediated neuronal toxicity in a mixed embryonic DRG-Schwann cell co-culture system. However, I adapted my own protocol because of concerns over their image acquisition methods (Keswani *et al.*, 2003). In Keswani's study, and commonly in other papers, no explanation was provided on how images were acquired, how the images were selected or how the immunocytochemistry was quantified or normalised. It was noted that while 13,000 cells were plated, they analysed only around 10 neurons per treatment, which I propose is extremely under-representative of the global culture effects. Primary neuronal cultures exhibit considerable variability in neurite outgrowth, within cultures, from one neuron to the next, therefore there is great importance in measuring a considerable proportion of the cells in order to reach, not only statistical significance, but biological relevance also.

I used the CSIRO HCA-vision software, which offers automated image analysis and provides extensive information on neurite length, arborisation, number of branch points, number of roots and much more (Vallotton *et al.*, 2007; Wang *et al.*, 2010a). There are many advantages to using an automated system such as this. Firstly it standardises the assessment across all treatment images, guaranteeing that every image is analysed using identical criteria, without any variation caused by human error. It delivers a huge quantity of data with minimal time requirements from the observer and finally its detection capabilities are far more sensitive than the limited optics, from this respect, of the human eye that affords automated systems a level of accuracy that is unattainable by manual analysis. Using this system I was provided with vast amounts of information describing neurite analysis on many different levels, e.g. total neurite outgrowth, mean neurite outgrowth, neurite area, primary neurites, secondary neurite levels, number of roots, branching points and so on. The most common outcome measures reported, in studies assessing neuronal outgrowth *in vitro*, are 1) mean total

neurite outgrowth, 2) mean total neurite outgrowth/ neuron, or 3) mean longest neurite per neuron. In my studies I chose to express my outcome measure as the mean total neurite outgrowth per neuron. In some of my earlier studies I found that the plating densities remained too high so that following 48h *in vitro*, adult DRG neuronal cultures had extended neuritic processes that were fully intertwined with neighbouring neurite extensions, making it impossible to trace neurites back to their cell body. In these studies I used the total neurite outgrowth (that is, the total number of pixels detected by HCA-vision and converted into μm according to the set conversion size taken from the dimensions of the image acquired) and normalised this value against the total number of neuronal cells visible in the analysed images. It is important to note that this would include neurons that were bald of neurites (outgrowth $<20\ \mu\text{m}$) and therefore did not contribute to the total outgrowth value. In these cases it was not possible to detect specific effects on individual neurons, to look at the distribution of the different neurite outgrowths per neuron or to assess the selective effects of specific neuronal subsets. However, this method would still sensitively detect global reductions in neurite outgrowth affecting the majority of neurons. Once I had optimised the plating density (~ 2000 cells/ $13\ \text{mm}\ \varnothing$ coverslip) I performed the analysis on an individual neuron basis. HCA-vision was able to, in most cases; correctly identify which neurite processes belonged to which neuronal cell body. At this point I had to introduce a final manual validation step because this procedure was not faultless, for example; 1) if two cell bodies were too closely positioned, the software commonly categorised them as one body, 2) if a neuron had an extensive network of neuritic processes that spanned a large proportion of the image, any neurites that the network overlapped that were derived from a different cell body, were often merged so that some cells were allocated the wrong neurites and others lost theirs. The manual validation step, as describe in materials and methods, ensured that the correct number of cell bodies were counted and, in the later studies, that neurite networks were matched to the correct cell body. Ensuring that the correct number of cell bodies was recorded was probably the most important parameter factor in this analysis. Whereas any errors in detecting neurite outgrowth would have occurred consistently across all experiments, cell body detection errors could vary from one image to the next depending on how the cell bodies were positioned. This could lead to profound inaccuracies in the data because this critical cell number was my normalising number, therefore an over-calculated cell number would give a lower mean neurite outgrowth per neuron value thereby depicting a reduction in neurite outgrowth when in fact this is an artefact of incorrect cell counting. The reliability of any automated system is highly dependent on setting biologically relevant thresholds for each parameter such as neuronal cell body detection criteria, background adjustment and removing artefacts. A great deal of effort was spent on ensuring that the thresholds selected resulted in the minimal number of false identification errors or missed

detection points, however, I maintained the manual validation step following this automated analysis to maintain high analytical standards and to increase confidence in my interpretation of the data. Therefore it was considered that 'mean total neurite outgrowth per neurons' with manual validation represented the most accurate and representative method for neurite analysis.

To test face validity of this technique and culture protocol I treated primary adult DRG neuronal cultures, established following are described protocol, with nocodazole and vincristine. Using the neurite analysis protocol and technique I confirmed that my *in vitro* system correctly demonstrated the neurotoxic effects of nocodazole and vincristine, which are both well-known neurotoxic compounds. Of course these are both potent anti-tubulin agents that directly depolymerise microtubules, resulting in their disassembly, disruption of fast axonal transport and break-down of the axonal structure, as depicted by reduction in neurite outgrowth. Such drastic effects would be easily noticeable whether I recorded total neurite outgrowth normalised to cell number or measured every neurite per cell individually and obtained the mean neurite length per cell. Thus, while it demonstrates that the culture system can detect dramatic reductions in neurite outgrowth, it offers only partial face validity because this does not inform whether it would be sensitive enough to significantly detect subtle changes. However, I obtained robust cultures and, across multiple studies, using the same culture systems, obtained a mean neurite length per neuron of approximately $1098.5 \pm 92.9 \mu\text{m}$ for all vehicle-treated cultures (n=14, across five biological replicates). This level of consistency across a very large time range and across multiple studies indicates that it should be sensitive enough to detect as little as a 10% reduction in neurite outgrowth from baseline.

While there may be limitations and drawbacks with the culture system, as with any *in vitro* model, I felt I established a system that was accurate, sensitive and representative. For future investigations it would be interesting to pursue these studies further using a Campenot chamber system or microfluidic chambers. This sees the compartmentalisation of neuronal cell bodies away from their axonal extensions so that the selective affects on cell soma and axons can be investigated separately. This is also a fantastic system to access axonal transport and furthermore it is an excellent method to test neuronal axons in the complete absence of contaminating non-neuronal cells, such as Schwann cells, thereby allowing direct affects on neurons to be investigated.

2.4.3. Gp120_{MN} induces reduced neuronal outgrowth in a concentration-dependent manner.

While many hypotheses underlying HIV-mediated neurotoxicity are now fairly established, including the involvement of non-neuronally-mediated axonal injury as a result of aberrant cytokine release, the complete picture of HIV-associated neurological toxicity remains poorly elucidated. Until recently it was believed that the neurotoxic effects of gp120 on sensory neurons occurred solely via interactions with glia and macrophages. While neurons do not express the CD4 receptor and thus cannot be infected by HIV, my data supports that of previous studies (Oh *et al.*, 2001; Bhargoo *et al.*, 2009), demonstrating that DRG neurons express CCR5 and CXCR4 making them a target for gp120 binding. A study by Oh *et al.* demonstrated for the first time that small nociceptive neurons express the HIV-1 chemokine coreceptors and, when exposed to the receptor ligands and gp120, excitatory effects were observed combined with the release of substance P, in a neuronal sensory culture system (Oh *et al.*, 2001). Therefore new focus has been put on the possibility of a directly-mediated toxicity on sensory neurons, by gp120, independent of Schwann cells or macrophages. Furthermore evidence for the internalisation and axonal transport of the T-tropic gp120 (gp120_{IIIB}) in striatal neurons has been recently reported (Bachis *et al.*, 2006) and could indicate how gp120 contributes to widespread HIV-1-mediated neuronal cell death.

Having established a robust culture system and neurite analysis procedure, and confirmed gp120 neuronal binding in my own cultures, I tested the functional effects of gp120_{MN} on adult DRG neuronal cultures. My studies demonstrate that gp120_{MN} induced a significant reduction in the total neurite outgrowth in a concentration-dependent manner. A similar profile was observed using the same biotinylated gp120_{MN} that I used for my immunocytochemistry studies, confirming that the biotin conjugate did not alter the biological activity of the glycoprotein. In turn, this may allow us the assumption that unbiotinylated gp120 exhibits similar cellular localisation to B-gp120_{MN}.

This experiment clearly demonstrated that gp120 appears to cause significant effects to neurite outgrowth however; from this study alone, I cannot deduce whether this was a result of early inhibition of neurite outgrowth or active degeneration of established neuronal processes and whether gp120 acted directly on the neurons or indirectly via non-neuronal cells, or both. Additionally it is noted that a plateau appears in the concentration-response curve. This may be an artefact of testing too few different concentrations, or not testing for long enough however, this may also be indicative of a capping on the neurotoxic effect or saturation of the available chemokine receptors.

One current theory for the onset of axonal degeneration is disruption to axonal transport and diminished replenishment of essential nutrients, proteins and cytoskeletal components (Campenot *et al.*, 1996; Chevalier-Larsen *et al.*, 2006). Previous studies have reported a capping of the degenerative effects once neurites had retracted to approximately 100–200 µm in length (Keswani *et al.*, 2004), which correlates with the perceived capping in my concentration-response curve. This may simply be due to the concentrations selected however, in view of the concept of nutrient deprivation it may be conceivably hypothesised that once neurites have degenerated within close proximity of the cell body the issue of a limited energy supply or dysfunctional axonal transport may be overcome as they are close enough to the cell body to be replenished by diffusion or utilising the same or alternative supply mechanism. This new close proximity may offer the neurites resistance from continuous insult as they now have the resources to, perhaps not recover when gp120 is still present, but prevent further degeneration. It would be interesting to investigate the effects of higher concentrations of gp120 on the rate and degree of neurite reduction.

It should also be noted that I was testing very early on in the life of the culture; the neurons have just been axotomised (24h) and are currently in a process of recovery and rapidly regenerating their neuritic processes, at the time of treatment application. Therefore this may have considerable impact on the mechanisms I am interfering with and may differ from gp120-mediated effects in an uninjured state i.e. a healthy HIV patient. On the other hand this model may be utilised to clarify our understanding of the synergistic or unmasking effects of gp120 in the setting of a pre-existing injury, or vice versa with NRTI HIV therapy unmasking an underlying sub-clinical gp120-induced toxicity, as proposed by numerous studies (Keswani *et al.*, 2002; Wallace *et al.*, 2007b). Furthermore at the time of treatment application extensive neurite networks have been established therefore, I make the assumption that the degeneration that follows utilises a mechanism similar to that *in vivo*.

2.4.4. Temporal course of gp120_{MN}-associated neurite reduction and its temporal relationship with neuronal B-gp120_{MN} accumulation.

The temporal course of the gp120-mediated reduction in neurite outgrowth was investigated in order to address the question of whether this was due to an inhibition of ongoing neurite outgrowth or a result of active degeneration of already established axons. I show that up till 16h post gp120, neurite outgrowth in gp120-treated cultures matched that of vehicle-treated cultures, indicating that at least till this time point, no inhibitory or degenerative effects on neuronal axons were evident. Significant loss of neurite outgrowth was confirmed by 24h of gp120 exposure but most

importantly, this reduced the neurite outgrowth to a level that was below that seen 8h earlier at the 16h time point, while the vehicle-treated cultures showed continued growth from its earlier time point. This strongly supports the theory that gp120 induces neurite degeneration, as reported by *in vivo* studies (Toggas *et al.*, 1994; Wallace *et al.*, 2007b) and in HIV patients, therefore the culture system is accurately portraying biologically relevant effects of gp120 and likely utilising similar mechanisms. No further degeneration was noted when the gp120-treated cultures were exposed for 48h, while again the vehicle-treated cultures showed significant continued growth. At the same time the 48h gp120-treated cultures showed no evidence of regeneration. Expression of β III-tubulin has been identified during olfactory neurogenesis in embryonic and adult rat (Roskams *et al.*, 1998) and during axonal regeneration of rat DRGs *in vivo* (Moskowitz *et al.*, 1995), therefore immunolabelling for β III-tubulin was a suitable marker for measuring neurite length during potential axonal regeneration. It would be interesting to repeat these experiments with later time points to determine whether the axons are permanently degenerated, even once the gp120-mediated input is removed, or whether they regain their regenerative ability.

The temporal course of B-gp120_{MN} binding, assessed in parallel with the temporal course of neuronal neurite outgrowth, highlighted that B-gp120_{MN} interacted with neuronal cell bodies within 1h of exposure, correlating with similar early internalisation shown by Bachis *et al* using gp120-conjugated to gold particles (Bachis *et al.*, 2006). B-gp120_{MN} accumulation peaked at 24h however, with large variability in the proportion of cells positively labelled across the treatment triplicate. This may be explained by the significant drop in the 'number of neurons visible per image' that was also observed at 24h. This indicates a reduction in neuronal density likely due to cell death and loss, therefore the total number of neuronal cell bodies analysed was overall lower, which increased the proportion of B-gp120_{MN} positive cells and in turn the variance due to variable degrees of cell loss.

An interesting point that may be interpreted from this data is that, if the early gp120-binding is one of the instigating factors for neurite degeneration, it demonstrates a much delayed onset of the gp120-mediated effects on neurite outgrowth after early activation. This delay may be further indicative of the activation of multiple complex pathways, initially in non-neuronal cells that eventually converges on a neuronal-based component, such as neuronal intracellular calcium modulation (Lipton, 1991a; Lipton, 1991b). This therefore activates a common pathway, synergising and eventually leading to the process of axonal degeneration, which interestingly has been associated with deregulated calcium levels (Glass *et al.*, 1994; LoPachin *et al.*, 1997; reviewed by Coleman, 2005). The potential mechanisms involved will be reviewed in greater detail in the General Discussion (see section 1.4.1), where guidance is taken from studies on Wallerian degeneration,

which many believe, acts via the same, or similar mechanisms (Wang *et al.*, 2000; Finn *et al.*, 2000). The time course also parallels that of Wallerian degeneration both *in vitro* (Wang 2000 J Neuropathol) and *in vivo* (Beirowski *et al.*, 2005), with its delayed onset (16–48h) but then rapid degeneration (~50% reduction in 8h in my data), further indicating the potential similarities and the plausible existence of an active regulated mechanism (Raff *et al.*, 2002; Coleman, 2005). However, it is important to highlight that despite this indication of early gp120 binding, the maximum proportion of neurons demonstrating positive gp120 binding was only $7.9 \pm 3.6\%$ after 24h, and yet reduction in neurite outgrowth effected the global population of neurons. This low percentage of B-gp120_{MN} positive neurons joint with the delayed onset of axonal degeneration suggest that a direct toxicity of B-gp120_{MN} in neuronal cells is unlikely to fully explain the global extent of neurite degeneration observed, if mediated solely by a neuronally-based mechanisms, and allows me to speculate that, at least, additional mechanisms via non-neuronal cells are required. Hence, it could be hypothesised that the involvement of indirect activation via non-neuronal cells and the subsequent release of excitotoxic intermediaries mediates degeneration in the gp120-insensitive (and sensitive) neurons. This could explain why degenerative effects were evident in the majority of neurons and also be fitting with this delayed onset due to the multiple cells and pathways involved.

It should be noted here that some assumptions are made and alternative or additional interpretations may be possible. I have interpreted the B-gp120_{MN} labeling as being indicative of gp120-binding and a marker of gp120-sensitive neurons. In addition I am associating these gp120 interactions with activation of the neurons however, from these studies alone, this is speculative. I cannot confirm that the gp120-binding I have visualised is involved in the reduction in neurite outgrowth observed or gp120-labelled neurons are the only candidates. Other molecules of gp120 may have bound and been released, are therefore were not labeled however that may have been sufficient to activate the cell and yet is unaccounted for.

To directly implicate the gp120-binding quantified, with the axonal degeneration, antagonists for the CXCR4 and CCR5 receptors should be used both prior to treatment and at different time points during the treatment to elucidate the time course of the initiation of axonal degeneration and the temporal involvement of these receptors. This would be interesting to determine whether single activation of these cells is sufficient to instigate the pathways leading to axonal degeneration, or whether maintained activation is essential for these deleterious effects to occur.

I must, however, consider how translatable the data, and that of other research, are that report neuronally-mediated effects *in vitro*. I demonstrated that my culture was modeling axonal degeneration rather than inhibition of neurite outgrowth and is therefore likely to utilise similar

mechanisms. However, the neurons remain present in a pathological state, fully exposed to the action of excitatory mediators and neurotoxins, having lost the fundamental protective shielding from the ensheathing Schwann cells *in vivo*. Furthermore, I must draw attention to the concentrations of gp120 utilised in these experiments. Such concentrations were determined from previous work that reported negative effects to neuronal survival and axonal outgrowth at concentrations tested from 1 pM to 1 μ M, but it should be discussed how biologically relevant these concentrations may be. At the lower concentrations, which are more biologically relevant, gp120-mediated effects are controversial, while at the higher concentrations, more consistency in the negative effects are reported however whether such high concentrations are reached in the plasma of HIV patients, the *in vivo* equivalent of my culture media, is questionable. The two most commonly cited papers that address this issue, detected gp120 plasma levels in AIDS patients in the range of 0.1–0.8 nM (Oh *et al.*, 1992) and 2–20 pM (Gilbert *et al.*, 2003). These demonstrate quite different ranges and the accuracy of these recordings were reviewed by Klasse drawing attention to the limitations of the capture enzyme-immunoassay techniques used and underestimation due to the interference by plasma antibodies (Klasse *et al.*, 2004). However, both ranges are between two and four orders of magnitude greater than those predicted by Klasse who extrapolated gp120 concentrations from more accurate recordings of plasma viral load, determined by measuring p24 antigen levels (the core protein in HIV virions), which tend to be 40–60-fold greater than gp120 levels (Layne *et al.*, 1992; Chertova *et al.*, 2002) giving a considerable lower range of 0.03–0.07 pM. This is under the assumption that HIV virions are the only source of gp120; however concentrations may be increased by the production and secretion of gp120 from infected host cells (Chertova *et al.*, 2002). In light of this, the concentrations I have used in my studies are significantly higher (2nM). Higher concentrations were considered necessary based on affinity binding studies that report the K_d for gp120-CD4 binding is 1–10 nM (Ashkenazi *et al.*, 1990; Moore *et al.*, 1990; Ivey-Hoyle *et al.*, 1991; Moebius *et al.*, 1992) and in the absence of CD4, as on neuronal cells, the K_d for gp120 binding to CXCR4 and CCR5 is much more controversial at 70–200 nM (Hesselgesser *et al.*, 1997; Babcock *et al.*, 2001) and 4 nM, respectively. Conversely, other reports identify no CXCR4 binding at gp120 concentrations as high as 0.5 μ M (Mondor *et al.*, 1998; Doranz *et al.*, 1999) and only minimal binding with CCR5 (Trkola *et al.*, 1996; Wu *et al.*, 1996), in the absence of CD4. Therefore in light of this it may be concluded that at biologically relevant levels, relating to the concentrations discussed, gp120 would exhibit little or no binding to neuronal cells. Yet, neurodegenerative effects have been demonstrated *in vivo* following low level acute gp120 exposure (Toggas *et al.*, 1994). It is important to note that these estimates of plasma [gp120] relate to the circulating gp120 levels that are highly diluted and distinct from the sites of injury. At the sites of productive infection such as lymph node

and at sites of injury containing many infiltrated macrophages, more concentrated levels of gp120 may be found (Schacker *et al.*, 2001; Santosuosso *et al.*, 2011). Further investigations are required to elucidate the gp120 concentrations in different compartments and localized at specific tissue sites, which in turn would aid in the biological relevance of my data.

Enhanced binding may be achieved through association with alternative binding partners such as C-type lectin receptors (Geijtenbeek *et al.*, 2002; Turville *et al.*, 2002) and GAG moieties of proteoglycans (Ugolini *et al.*, 1999; Moulard *et al.*, 2000) as previously reported, which may permit gp120-mediated effects at lower concentrations. Since I clearly demonstrate neurotoxicity induced by exposing mixed DRG-glia culture to 2 nM gp120 and importantly B-gp120_{MN} labeling in neuronal cells that do not express CD4 this suggests that additional receptors or moieties may have enhanced the ability of gp120 to bind neuronal cells at this concentration that would be considered to be far below the K_d for CXCR4 or CCR5 binding. Indeed recent investigations aiming to address this demonstrated similar effects in CD4+ T cells in which signaling was activated following concentrations as low as 10 pM virion-bound gp120 (Melar *et al.*, 2007).

2.4.5. Non-neuronal cells may act as important mediators of gp120-associated neurotoxicity.

I have demonstrated that my culture system is accurate, representative and biologically relevant. From my previous data it was demonstrated that only a minority of neuronal cells internalised gp120_{MN} and yet the neurite reduction was observed in the global population of neurons. In concert with the delayed onset of neurodegeneration these data gave strong indication that the gp120_{MN}-associated neuronal degeneration may be acting, at least in part, via an indirect mechanism on non-neuronal cells. However, studies have reported evidence for direct activation of neurons and calcium influx in small diameter neurons by gp120 (Oh *et al.*, 2001). With the knowledge that a mechanism must be activated within neuronal cells to initiate the degenerative process, it may be plausible that a direct gp120-initiated neuronal response remains an important component in the initiation of gp120-associated axonal degeneration. Calcium dysregulation has been frequently implemented in other neurodegenerative diseases (Verkhatsky *et al.*, 2008; Bezprozvanny *et al.*, 2008) as well as in HIV-associated pathologies (Dreyer *et al.*, 1990) and peripheral neuropathies in general (Ferryhough *et al.*, 2010). One study has shown evidence for gp120-induced calcium influx and activation of peripheral neurons in neonatal and adult cultures (Oh *et al.*, 2001) however to my knowledge few others studied these effects in primary adult DRG cultures. Using calcium imaging I demonstrate that, while present, only a very low proportion of cultured DRG neurons (~5%) expressed functional

CXCR4 and CCR5 receptors. I feel that a sufficient number of neurons were analysed although a greater number of repeats would have been beneficial to be more conclusive. Despite the low number of gp120- or chemokine-responsive neurons, I am confident that these figures are representative of the responsiveness of adult DRG neurons *in vitro* to these compounds because they corroborate the findings of Oh *et al.* who recorded 14% (10/74 cells) and 6% (10/154 cells) of cultured primary adult DRG neurons responding, with increased $[Ca^{2+}]_i$, to RANTES/ CCR5 and SDF-1/ CXCR4 respectively, (Oh *et al.*, 2001). Gp120 exposure induced calcium modulations in a similar proportion of cells as those reported by Oh and the temporal profiles of these traces were shown to correlate with the temporal profile of responses to RANTES and SDF-1. I did not confirm whether the gp120-responsive neurons also expressed the relevant functional CXCR4 or CCR5 receptors, but use of CXCR4 and CCR5 antagonists would be useful to determine which receptor are required to mediate these effects. Interestingly calcium imaging data reported Melar *et al.* is in contrast to the temporal profiles I recorded. It was found that, RANTES and SDF-1 induced rapid mobilisation of intracellular Ca^{2+} levels at similar concentrations (30 nM), while monomeric gp120 induced a delayed (3.72 ± 0.9 min to peak after application) and sustained plateau of increased $[Ca^{2+}]_i$ (Melar *et al.*, 2007), which is more similar to my own data for both gp120 and RANTES/ SDF (overall mean: 2.5 ± 0.1 min). These differences may be explained by the cell types tested; primary T cells in the studies by Melar *et al.*, which has significantly higher expression levels of CCR5 and CXCR4 than primary neuronal cultures, which were used in these studies. To further control for the possibility that my responses were simply a temporal event that occurred, regardless of gp120 exposure, I applied only vehicle control solutions sequentially to the cultures following the same time course used in gp120 treatment experiments and observed no significant responses. In addition the temporal profile of the gp120 responses correlated closely to those acquired for both RANTES and SDF-1 therefore strongly supporting the interpretation that these highlight specific responses to gp120 exposure. Of course the decision to apply and investigate treatments sequentially in this manner raises the issue that an acumulative effect may occur with the secondary and tertiary compounds applied that may not be biologically specific to that compound. Additional studies testing the compounds separately on individual neuronal cultures would be beneficial to elucidate and validate the selective effects of individual compounds. However, this technique was necessary so that each study has a negative and positive control correlating to it.

Such low levels of functional CXCR4 and CCR5 receptors may offer a reasonable explanation to the variability of the gp120 *in vitro* model and may also provide further indication that the gp120-induced neurite degeneration is mediated predominantly via non-neuronally mediated pathways. Immune cells, such as macrophages and microglia, express the CD4 receptor, while neurons and

Schwann cells do not. Physiologically this is the first and essential point of contact between the HIV virus and CD4⁺ cells and, as reviewed earlier, this gp120-CD4 binding induces a conformational change within the gp120 protein structure that exposes the CXCR4 and CCR5 binding sites, allowing it to bind to these coreceptors with greater efficiency (Wu *et al.*, 1996; Trkola *et al.*, 1996). It is conceivable that this enhanced efficiency overcomes the low expression levels. Furthermore Schwann cells and macrophages functional role is to amplify signals both in an autocrine- and paracrine-signaling fashion (Jones *et al.*, 2001; Tofaris *et al.*, 2002; Mirsky *et al.*, 2002). This may explain the delayed onset of the axonal degeneration as multiple signals and pathways were required prior to their eventual activation of neuronally-based mechanisms. Furthermore synergy of various mechanisms may occur and converge onto a common pathway in neuronal cells that subsequently leads to degeneration of their neurites.

These data inform us that certainly functional CXCR4 and CCR5 receptors are present in cultured adult DRG neurons albeit at a low level, and that gp120, like the endogenous coreceptor ligands, can also induce modulation to intracellular calcium levels in a small proportion of cells. However I cannot implicate these calcium responses directly to the axonal degeneration. It demonstrates that a calcium modulatory pathway is activated during the cause of gp120-mediated mechanisms that may be occurring neuronally or non-neuronally however, whether its presence lends itself to the synergistic neurotoxic pathways later would require further investigation. Many of the effects to neurite outgrowth were latent and internalisation occurred in a small minority therefore I speculate that calcium pathways were unlikely to be directly or acutely related to the toxic effect. However it is plausible that a slow gradual increase in calcium may have occurred but beyond the time frame in which I recorded for these studies, assessed using fast time-lapse imaging. This may be elucidated by performing the calcium imaging experiments as described, in the absence of KCl application, maintain the cultures post-imaging and then, 24h later, compare the neurite outgrowth of neurons that demonstrated calcium responses to those that did not.

While the culture protocol and analysis procedure *per se* were robust it should be noted that the gp120 *in vitro* model was less robust. The gp120-mediated effects were variable and appeared susceptible to some unknown variation in the culture condition. When a gp120-mediated effect was observed it was robust and significant however these effects were not always reproducible. As stipulated previously, primary neuronal cultures, and their neurite outgrowth, are dependent on the microenvironment around them, and are strongly influenced by the trophic support offered to them by non-neuronal cells, in particular Schwann cells, present in the culture. In the uninjured nerves

Schwann cells provide axonal myelination or ensheathment and paracrine trophic support to neurons (Campana, 2007). However, following nerve injury, or axotomisation (as in the establishment of a primary dissociated neuronal culture), Schwann cells undergo a phenotypic switch allowing them the capacity to proliferate and secrete factors that control both Wallerian degeneration and nerve regeneration (Hammarberg *et al.*, 1998; Hoke, 2006). This involves both the release of pro-inflammatory cytokines that can sensitise neurons, and the production of factors that opposes these mediators, such as erythropoietin (Epo) and affords protection to the neurons (Campana *et al.*, 2003; Keswani *et al.*, 2004). With the ability to offer such opposing influences on the culture I hypothesised that despite a possible potent neurotoxic pathway induced via these cells by gp120, their own ability to release NGF and other factors that promote neurite outgrowth and are neuroprotective may explain the variability with this model. This was clearly demonstrated by Melli *et al.* who showed that Schwann cells exhibit “spatially distinct and functionally independent mechanisms” in gp120-mediated neurodegeneration after exposure to a dual-tropic HIV-1 gp120 (Melli *et al.*, 2006). Using a compartmented neuronal culture system, they demonstrated that exposure of embryonic neuronal cell bodies to dual-tropic gp120_{MN} induced neuronal apoptosis and axonal degeneration independently of neurons, instead requiring the presence of Schwann cells. In contrast direct gp120-mediated activation on neuronal axons induced a local toxicity that was independent of both the neuronal cell bodies and Schwann cells, likely mediated through gp120 binding to axonally expressed chemokine receptors. Furthermore the presence of Schwann cells in the axonal compartment offered some protection from this direct axonal toxicity. Therefore using the compartmented culture Melli *et al.* was able to dissect apart the opposing functions of Schwann cells however my un compartmented culture will have these multiple pathways converging on each other and may underpin some of the variability I observed.

Therefore I suggest that the variability of the *in vitro* gp120 neuronal model may be highlighting the involvement of multiple pathways and cell types in gp120-mediated neurotoxicity. In fact these results are more fitting with the clinical situation that reports that HIV-SN develops in treatment-free HIV+ individuals predominantly towards the latter stage of infection and on progression to AIDS and that HIV-ATN may unmask a neuropathy earlier that was previously subclinical but mediated by HIV *per se*. This suggests that on acute exposure gp120 may not immediately induce neurotoxicity. Instead, either neuronal responses to gp120 slowly accumulate over time, for which chronic stimulation is required. Alternatively the effects may be counteracted or prevented by neighbouring neuroprotective cells either through downregulation of the CCR5/ CXCR4 receptors or trophic and nutrient support. Therefore, in the setting of a prior injury a pro-inflammatory or sensitisation state is induced or the neuroprotective stimuli removed, which may allow gp120 to be able to activate

neurons more chronically and induce axonal degeneration due to chemokine receptor expression changes and enhanced cell responses. Understanding this network of signalling however may offer an explanation as to what determines the development of HIV-SN in 35% of HIV individuals and the absence of neuropathy in the remainder.

2.5. Summary

In summary these data inform us that 1) gp120 interacts with both neuronal and non-neuronal cells, 2) gp120 appears to interact with only a small minority of neuronal cells in primary adult sensory neurons, 3) gp120 interactions occur soon after gp120 application and yet gp120-mediated neurite degeneration is delayed in onset implying multiple complex mechanisms are involved, 4) the observed neuronal interactions do not explain the global effect to neurite outgrowth, 5) non-neuronal cells may be important instigators of the axonal degeneration and 6) gp120 induces transient calcium modulation only a small proportion neurons on first exposure.

Taken together these data suggest that direct interactions between gp120 and neuronal cells do occur. Furthermore, gp120-neuron interactions occur to a similar degree in *in vitro* models of the adult nervous system as in culture models of an embryonic or neonatal system. However, the relevance of the gp120-neuronal binding in gp120-mediated axonal degeneration is still to be elucidated. If the binding of gp120 does activate important pathways in neurons relevant to neurite degeneration, as indicated by observation of calcium modulation, this may only occur in a small proportion of susceptible neurons. Therefore, although potentially present, direct neuronally-mediated mechanisms are insufficient to explain the global reduction in neurite outgrowth observed in these studies. Therefore, non-neuronally-mediated mechanisms may act as the predominant instigator of neurite degeneration. However, it would be interesting to explore whether the non-neuronal responses alter subsequent responses of neurons to gp120 responses. Upregulation of chemokine receptor expression (including CXCR4) has been demonstrated following nerve injury (Bhangoo *et al.*, 2009) therefore it is tempting to speculate that activation or injury induced by non-neuronally secreted compounds may sensitise neurons making them more vulnerable to direct gp120-binding or activation.

Chapter 3: Indirect effects of gp120 on neurite outgrowth *in vitro*

3.1. Introduction

3.1.1 Synopsis

The distribution, physiological roles and involvement of macrophages and Schwann cells in HIV infection will be discussed in the introduction of this chapter. In the general introduction I have highlighted the overall involvement of immunity in HIV infection (see section 1.2). The aims of this chapter were to profile the immune response to gp120 exposure and investigate the responses of non-neuronal cells to gp120. I will present and discuss results focusing on the relative involvement of key non-neuronal cells in HIV-1 gp120-mediated toxicity and highlight potential connections with HIV-SN and associated neuropathic pain.

3.1.2 Macrophages

a. Distribution and functional roles

As previously outlined macrophages are derived from a lineage of monocytic cells produced in bone marrow. As their name suggests they play a key role in the phagocytosis and endocytosis of foreign microorganisms and the elimination of degenerated or necrotic/ apoptotic debris and are an essential first line of defence in immunity. Macrophages are distributed widely throughout the entire peripheral nervous system, with a considerable number residing within the endoneurium of peripheral nerve (Glass *et al.*, 1993) and dorsal root ganglia (Moalem *et al.*, 2006). These resident neural macrophages express typical macrophage/microglial cell markers, similar to microglial populations in the brain, including ED2, and CD68 in humans or the alternate F4/80 antigen in mice. In addition they express MHC I and II antigens, the CD4 glycoprotein and complement receptors in humans, mice and rats (Stevens *et al.*, 1993; Bonetti *et al.*, 1993). The remaining macrophages are hematogenous meaning they originate in and are circulated by the blood. Resident macrophages are rapidly replaced by blood-derived macrophages (Vass *et al.*, 1993), which infiltrate through the more permeable blood-nerve barrier, unlike microglial equivalents in the brain, which demonstrate minimal turnover (Hickey *et al.*, 1992) and are less easily replaced due to the tighter blood-brain barrier.

Macrophages have evolved with the ability to target and eliminate debris and foreign microorganisms from the system by phagocytosis, stimulate lymphocytes and other immune cells to respond to pathogens, contribute to tissue repair, as well as being capable of extracellular killing of infected and altered self target cells. This occurs following their activation, which affords them the ability to respond to cytokines and other stimulators or cues in the microenvironment around them. Activated macrophages are highly metabolically active and contain organelles such as lysosomes enriched with hydrolytic enzymes primed for phagocytosis. The response of macrophages to their environmental cues can include the up- or down-regulation of membrane expressed receptors or antigens and chemotactic movement. Chemotaxis is a process where the direction of movement of a cell is determined by a gradient of chemical substances in the microenvironment surrounding it. Locomotion towards higher concentration of a substance is referred to as positive chemotaxis, however chemotaxis can also be negative whereby cells are deterred by a substance and migrate away from it, towards areas of lower concentration.

An important response of macrophages is the presentation of antigens in response to phagocytosis of foreign pathogens or following stimulation by important cues. These antigens include major histocompatibility complexes (MHCs) I and II and intracellular adhesion molecule-1 (ICAM-1) is an important requirement for antigen presentation. MHCs are important in the process of alerting the immune system of the presence of a foreign pathogen inside a cell. Following phagocytosis and digestion of pathogens, macrophages will constitutively present MHC ("self") complexes with fragmented pieces or "antigens" from the ingested pathogen. This complex will stimulate additional immune cells, in particular T cells and natural killer (NK) cells to initiate pathogen surveillance. If the MHC:peptide complex is recognised from the immune cells "memory", an adaptive immune response will propagate with the production of antibodies specific to the antigen by B cells, to target and bind with the pathogen. In addition to antigen presentation, macrophages release a complex array of cytokines that act as cues to attract other immune cells to the site of their release and stimulate cells to promote an immune response. Binding of antibodies to the pathogen enhances its targeting and phagocytosis by further recruited immune cells.

In addition to the targeting of foreign pathogens, macrophages also have cytotoxic properties. A particularly fitting example is the destruction of tumor cells and somatic cells infected with fungi or parasites. The aberrant cells are identified by T cell lymphocytes, which release chemical mediators or lymphokines. In turn these activate macrophages into a particularly aggressive state. These activated macrophages can then engulf and digest cells more efficiently however, unlike adaptive

immunity, this response is not directed against a specific antigen therefore all cells present local to the activated macrophages can be affected.

b. Macrophage polarisation

Mirroring the Th1/Th2 nomenclature of T cells, macrophage activation states have also been broadly grouped into equivalent M1/ M2 phenotypes. These functional phenotypes have been incorporated into a conceptual framework that describes the plasticity of macrophage function and is known as macrophage polarisation. Macrophage polarisation is determined by particular tissues and immunological microenvironment surrounding macrophages, inducing distinct morphological and functional changes. The macrophages in an M1 phenotype are referred to as classically-activated macrophages and are induced by interferon- γ (IFN- γ) alone or combination of microbial stimuli (e.g. lipopolysaccharide, LPS) and cytokines (e.g. TNF- α and GM-CSF). They are distinguished typically by an IL-12^{high}, IL-23^{high}, IL-10^{low} phenotype and their efficient production and release of specific mediators (reactive oxygen and nitrogen intermediates) and pro-inflammatory cytokines (IL-1, TNF- α , IL-6), highlighting their role as potent immune activators (Mantovani *et al.*, 2004). All other phenotypic states achieved by macrophages that are not M1 have been categorised into the broad M2 group of 'alternatively-activated' macrophages, a group rapidly proving to be more complex and diverse than originally thought. The M2 phenotype can be further sub-divided into three distinct groups: M2a (induced by IL-4 or IL-13), M2b (induced by immune complexes) and M2c (induced by IL-10 and glucocorticoid hormones) (Mantovani *et al.*, 2004). These discoveries also emphasized the importance of mediator combinations because while LPS and TNF- α , jointly, lead to an M1 phenotype, LPS in combination with immune complexes lead to an M2b (or type II) phenotype which are characterised, in complete contrast to M1, by an IL-10^{high} and IL-12^{low} phenotype. Therefore different combinations of immune complexes, macromolecules and cytokines lead to distinct activation states of macrophages each with varying functional roles. A summary of these macrophage properties is shown in Figure 3.1.1.

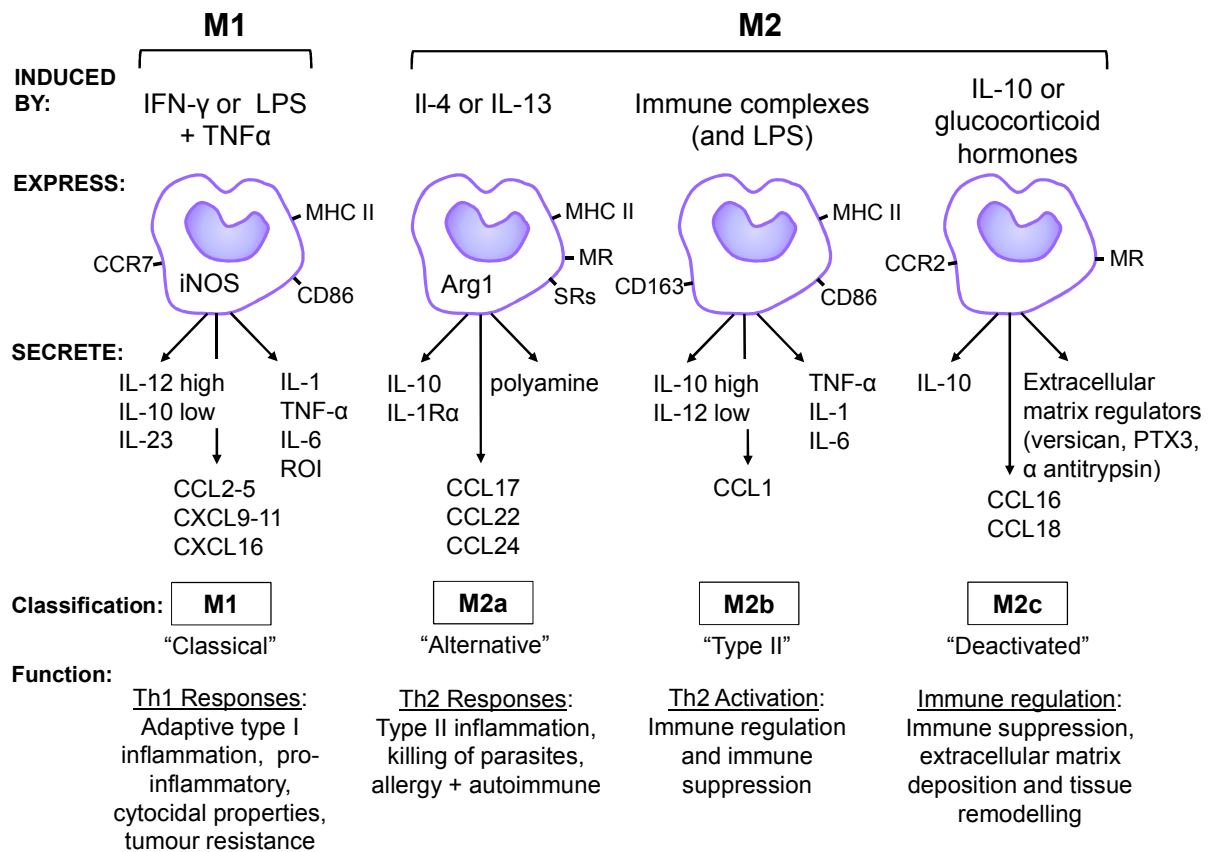


Figure 3.1.1) Summary of macrophage activation and polarisation (adapted from Mantovani *et al.*, 2004). In response to different environmental cues macrophages polarise and acquire differential cellular and functional properties. "Classically activated" M1 macrophages are induced by IFN- γ or LPS and TNF- α and are associated with pro-inflammatory and cytotoxic properties, while M2a and M2b macrophages, induced by IL-4 and immune complexes respectively exert immunoregulatory functions. In contrast M2c, activated by IL-10 or on exposure to glucocorticoid hormones is associated with wound healing and tissue repair. Abbreviations: iNOS, inducible nitric oxide synthase; Arg1, arginase 1; MR, mannose receptor; SR, scavenger receptors; ROI, reactive oxygen intermediates; PTX3, the long pentraxin PTX3.

c. Involvement of macrophages in HIV neurotoxicity

A resident population of macrophages exists in peripheral nerves and dorsal root ganglia (Moalem *et al.*, 2006). However, in times of nerve injury or inflammation the resident macrophages are assisted by haematogenous macrophages that infiltrated into peripheral nerves during clearance of cellular debris (Bendszus *et al.*, 2003); a requirement if peripheral nerve regeneration is to occur (Perry *et al.*, 1987; Correale *et al.*, 2004). Expression of CCR5 and CXCR4 receptors on the macrophage cell surface, however, also makes them a key target for the HIV gp120 protein and thus can be infected thereby acting as a reservoir of ongoing virus replication disseminated throughout the body. Macrophages contain an assortment of powerful excitatory and toxic agents that allow them to

eliminate a targeted pathogen but under HIV infection there appears to be a dysregulation of macrophage function that causes the inappropriate release of these agents and may contribute to local inflammation and injury as well as further recruitment of additional inflammatory and/or target cells. It is noteworthy that macrophages also invade the dorsal root ganglia following peripheral nerve lesions (Hu, 2011) and may contribute to neuropathic pain by release of excitatory agents that generate ectopic activity in sensory neurons. Moreover, soluble gp120 shed from viruses can result in the activation of macrophage chemokine receptors in the absence of infection. It has been shown that increased numbers of macrophages are present in sciatic nerve tissue treated with gp120 and this correlated temporally with the presence of pain behaviour (Wallace *et al.*, 2007a). Additionally it has been demonstrated that macrophages produce cytokines and chemokines and other soluble mediators after exposure to gp120 (Lee *et al.*, 2003). Testing of recombinant gp120_{JRFL} and gp120_{IIIB} on primary human monocyte-derived macrophages induced diverse signalling events including K⁺, Cl⁻ and nonselective cation currents and intracellular Ca²⁺ increases. The increased chemokine gene expression was demonstrated to be the result of mitogen-activated protein kinase (MAPK) activation and included chemokines such as monocyte chemoattractant protein-1 (MCP-1, also known as CCL2), macrophage inflammatory protein-1 β (MIP-1 β , also known as CCL4) and TNF- α . In line with this, significant secretion of CCL2 was demonstrated in primary human MDM cultures following exposure to a recombinant R5 gp120 and was shown to be mediated independently of CD4 and driven by NF- κ B signalling (Fantuzzi *et al.*, 2008). Enhanced CCL2 expression in the DRG of rats following nerve injury model has been correlated with the development of neuropathic pain (Tanaka *et al.*, 2004). Gp120 therefore can potently stimulate and recruit macrophages and infiltration of macrophages has been positively correlated with the severity of HIV-associated neurotoxicity (Griffin JWCT *et al.*, 1998). Therefore parallels may be drawn between gp120-mediated macrophage activation, immune cell recruitment, cytokine production (CCL2, TNF- α) and the induction of enhanced pain signalling.

3.1.3 Schwann cells

Schwann cells represent the 'glia' or 'supporting cells' of the peripheral nervous system and act to make up the myelin sheath around myelinated axons, as well as enveloping small bundles of unmyelinated axons (Remak bundles). This myelination has long been believed to be Schwann cells primary role in that it provides an insulating sheath to the axons aiding in the conduction and preservation of action potentials, and also functions as guidance for regenerating axons re-growing back to their targets after injury (Bolin *et al.*, 1995). However, recent research has highlighted that the role of Schwann cells may not be just passive and supporting but could well be involved strongly

with immune function since it has been shown that they interact with the immune system in T-cell-mediated immune responses (Bergsteinsdóttir *et al.*, 1992).

Schwann cells are known to produce neuroactive substances in response to stimulation including MCP-1 (Karanth *et al.*, 2006), TNF- α (Wagner *et al.*, 1996), IL-1 (Bergsteinsdóttir *et al.*, 1991) and IL-6 (Bolin *et al.*, 1995). In line with this, gp120 interacts with Schwann cells expressing CXCR4 and CCR5 *in vitro*. An experiment by Keswani *et al.* provides evidence for the involvement of Schwann cells in gp120-associated neuropathy (Keswani *et al.*, 2003). The supernatant fluids from a pure Schwann cell culture exposed to dual-tropic gp120_{MN} (1 pg/ml–1 μ g/ml) when transferred to a mixed DRG culture instigated a similar degree of neuronal toxicity to that when gp120 was added direct to a coculture preparation (dorsal root ganglion sensory neuron cultures plated onto a Schwann cell monolayer). Exposure to gp120 also caused a significantly increased level of RANTES, which can be released by Schwann cells, therefore the authors hypothesised that the neurotoxicity was mediated via gp120 binding to Schwann cells that resulted in the release of RANTES. They suggested in turn that secreted RANTES stimulate neurons via CCR5, resulting in neuronally-released TNF- α and TNF- α mediated neuronal apoptosis. To further this, a more recent study by Melli *et al.* has reported ‘spatially distinct and functionally independent mechanisms of axonal degeneration’ after exposure to a dual-tropic HIV-1 gp120 (Melli *et al.*, 2006). By using a compartmentalised neuronal culture system, Melli *et al.* was able to demonstrate that dual-tropic gp120_{MN} caused neuronal apoptosis and subsequent axonal degeneration as an indirect effect of gp120, requiring the presence of Schwann cells. However, in addition to this there was a clear direct local toxicity of gp120 to the neuronal cell bodies that was independent of both cell bodies and Schwann cells and was likely mediated through gp120 binding to axonally expressed chemokine receptors.

These findings underpin a key role for Schwann cells in the mechanisms behind gp120 toxicity however also unveil a potential but important Schwann cell-independent mechanism. Despite this, it is still not clear whether one, both or either of these direct or indirect mechanisms revealed *in vitro* are the same as the mechanisms *in vivo* and ultimately whether they cause the HIV-associated painful neuropathies.

3.1.4 *In vivo* models of gp120-mediated neurotoxicity

Ultimately *in vivo* models of HIV-associated sensory neuropathy represent the closest system to the clinical setting while still allowing researchers the ability to manipulate specific parameters.

As outlined earlier in the Thesis Introduction 1.3.2, some of the earliest indications of gp120-mediated toxicity came from *in vivo* studies where systemic and intracerebral injection in adult rats resulted in morphological damage to neurons and evidence of retardation from complex behaviour testing (Kimes *et al.*, 1991; Glowa *et al.*, 1992; Hill *et al.*, 1993). Nerve toxicity and associated hypersensitivity behaviour has been shown in gp120 transgenic mouse models (Toggas *et al.*, 1994) and following acute delivery mechanisms including intradermal injection (Oh *et al.*, 2001), perineural application (Herzberg *et al.*, 2001; Wallace *et al.*, 2007b) or intrathecal injection (Milligan *et al.*, 2001) in rat models. Therefore the mechanisms highlighted in primary culture systems clearly represent a genuine phenomenon occurring *in vivo*. The main drawback with regard to current *in vivo* models is the delivery mechanism by which animals are exposed to gp120 and the concentrations delivered. The biological relevance of and evidence gained from current *in vivo* models of HIV-1 gp120-associated neuropathy has been recently reviewed (Kamerman *et al.*, 2011, manuscript submitted). The report makes important references to the obvious differences between the acute onset of the toxicity and hypersensitivity in these animal models compared to the slower progression apparent clinically in HIV-infected individuals. Accurate temporal characterisation of the concentration of gp120 in specific tissue compartments remains to be established. However the slow progression of toxicity in HIV-infected individuals is considered to be due to the latent phase in lentiviral HIV infection in which little viral replication is occurring and thus exposure to gp120 is minimal. The main other disadvantage in the collection of *in vivo* studies performed to date is the difficulty in interpreting and translating the data to different experimental models due to the potentially confounding effects of species differences that may relate to co-receptor expression, immune responses and receptor tropism. However regardless of this, key commonalities between *in vitro* results, *in vivo* animal models and data from HIV-phenotyping or post-mortem tissue such as axonal degeneration, mechanical hypersensitivity and microglial activation and up-regulation of specific cytokines. This has given enough precedence to allow neurotoxicity and hypersensitivity following gp120 exposure to becoming widely accepted. The key steps to move forwards will be to identify the mediators or cytokines and chemokines involved in these effects *in vivo*, which still remain unknown.

3.1.5 Aims

The following chapter focuses on identifying and investigating indirect mechanisms underlying gp120-mediated neurite degeneration. I aimed to explore the relative contributions of macrophages and Schwann cells in gp120-mediated axonal degeneration, as well as profiling and comparing the gene expression changes bone-marrow derived macrophages and Schwann cells in response to gp120 exposure. An attempt is made to identify potential neurotoxins that are released from gp120-activated cells that may play a key role in the initiation of neurite degeneration. In addition I investigated the biological validity and relevance of my *in vitro* studies by assessing how the gene expression changes translate *in vivo*.

3.2. Materials and Methods

3.2.1. Compounds

The compounds used but either referred to or source not described within the Materials and Methods are listed here: Maraviroc (#11580, NIH AIDS Reagent Program), RANTES/CCL5 (#400-13, Peprotech), LPS (#L2630, Sigma), gp120_{MN} (#1021, ImmunoDiagnostics), gp120_{CN54} (#7749, NIH AIDS Reagent Program) and gp120_{Bal} (generous gift from Prof. Maria Papathanasopoulos, University of the Witwatersrand, Johannesburg), TNF- α (#PHC3016, GIBCO) and quinolinic acid (#0225, Tocris).

3.2.2. Primary Macrophage Culture

Bone-marrow cells were obtained from female Wistar rats (150–250 g) using a protocol derived from Zhang *et al.* (Zhang *et al.*, 2008). Briefly, female Wistar rats were euthanised by CO₂ asphyxiation, the femur and tibiae removed bilaterally and the bone marrow flushed from the bones using a 25G needle attached to a 20 ml syringe filled with ice-cold sterile wash medium (Dulbecco's phosphate buffered saline (D-PBS) without calcium and magnesium; Invitrogen). Flushed bone marrow was collected in a sterile 50 ml Falcun tube, then passed through a 70 μ m filter (BD Biosciences) to remove excess tissue or bone fragments and collected in new sterile 50 ml tube. Filtered suspension was centrifuged at 1500 rpm for 5 min at room temperature (R.T.) then resuspended in 50 ml macrophage complete medium (C-IMDM) comprising Iscove's Modified Dulbecco's Medium (IMDM; Invitrogen), 10% fetal bovine serum (FBS; GIBCO Invitrogen) and Penicillin/Streptomycin antibiotics (P/S Ab; 1:100; Sigma) and further supplemented with macrophage colony stimulating factor (M-

CSF, 5ng/ml; #CYT-308, ProSpec) to differentiate monocytes into macrophages. Cell suspension was plated 10 ml each onto 5 x 100 mmØ Petri dishes and incubated in a 95% air/5% CO₂ humidified incubator. Dishes were washed once after 1 day *in vitro* (DIV) and 3 DIV with pre-warmed C-IMDM then media replaced with 10 ml C-IMDM supplemented with 5 ng/ml M-CSF. After 7 DIV supplementation with M-CSF was halted, media was removed and discarded, remaining adherent cells were washed with 5 ml pre-warmed D-PBS then 3 ml pre-warmed non-enzymatic cell dissociation solution (#C5914; Sigma Aldrich) was applied to each dish and incubated at 37°C for 5 min. Cells were gently detached by pipetting media on top, dislodged cells were pooled into a 50 ml centrifuge tube and centrifuged at 1500 rpm for 10 min at 4°C. The pellet was recovered by careful removal of supernatant and resuspension of pellet in 3 ml pre-warmed C-IMDM. Cell count was quantified using a hemacytometer and the volume of media adjusted in order for differentiated macrophages to be plated at 3×10^5 cells/ well in 6-well tissue culture plates or 35 mm Ø culture dishes in 1 ml C-IMDM. Cells were allowed to settle at 37°C for 24h then media was replaced with 1 ml C-IMDM unless, when stated, macrophages were serum-deprived and maintained in 1 ml IMDM (plus P/S Ab) only, for 24h. Where IL-4 priming conditions are stated, macrophage cultures were supplemented with recombinant rat IL-4 (5 ng/ml; #400-04, Peprotech) at the same time as serum-depriving (24h prior to experimentation) in IMDM + P/S Ab. Macrophage cultures were tested at 9 DIV (2 days post-re-plating) by removal of media and replacement with treatment solution diluted in 1 ml C-IMDM or IMDM + P/S Ab when in serum-free conditions.

3.2.3. Primary Schwann Cell Culture

A fast and efficient protocol for the establishment of pure rat adult Schwann cell cultures was derived from a method described previously by Haastert *et al.* (Haastert *et al.*, 2007). Briefly, the enrichment technique that allows for enhanced yield of Schwann cells is achieved through a process of degeneration of extracted sciatic nerves. Sciatic nerves from 150-250 g female Wistar rats, euthanised by CO₂ asphyxiation, were extracted bilaterally. The epineurium was stripped off using fine forceps and Vannas microdissection scissors under a dissecting stereo microscope in sterile conditions. Epineurium-free nerve tissue was placed in a 35 mm Ø polystyrene culture dish containing DMEM and P/S Ab (1:100) and transferred to a tissue culture hood for preparation. Tissue sections were washed with sterile DMEM, chopped into 1 cm segments and split across two 35mm Ø polystyrene culture dishes. Tissue medium was supplemented with C-DMEM and incubated at 37°C in a 95% air/5% CO₂ humidified incubator with medium replaced every 3 days for 9 DIV. Pre-degenerated peripheral nerve tissue was dissociated by replacing culture media with 2 ml 0.125% Collagenase XI (Sigma) and 0.1 mg/ml DNase II (Sigma) in DMEM for 2h at 37°C then enzymatic

action was reduced by addition of 2 ml pre-warmed C-DMEM. Tissue sections were pooled and transferred to a 50 ml Falcun tube and dissociated by triturating with a cut 1ml tip approximately 15 times until a cloudy cell suspension was achieved. Samples were centrifuged at 1200 rpm for 5 min at R.T., supernatant discarded and pellet resuspended in 2 ml C-DMEM. Cell suspension was passed through a 70 µm filter (BD Biosciences) and flow through topped up to 5 ml volume with C-DMEM, supplemented with 1% BSA, and plated five x 1 ml onto five x poly-L-Lysine (0.1 mg/ml; Sigma) and laminin (10 µg/ml; Invitrogen) coated 35 mm Ø polystyrene culture dishes at a density of $\sim 10^6$ cells/dish (Post-plating day 0; PPD 0). Addition of 1% BSA for 24 h enhances the seeding efficiency of primary Schwann cells. To obtain a pure Schwann cell culture 24h after plating, media was replaced with 1 ml pre-warmed C-DMEM in the absence of BSA but supplemented with 100 µM cytosine arabinoside (AraC) for 30h. AraC is a chemotherapeutic agent and anti-mietabolic compound that damages DNA during the S phase of the cell cycle resulting in premature termination of DNA synthesis and cell death. Fibroblasts proliferate more rapidly than Schwann cells in the first 48 h therefore the application of AraC will predominantly kill fibroblastic cells, with only minimal affect on Schwann cells in the first 24h. Following AraC application cells (PPD 2) were washed three times with pre-warmed C-DMEM to remove residual AraC and maintained in C-DMEM supplemented with 2 µg/ml forskolin for 48h to enhance Schwann cell proliferation. Prior to experimentation (PPD4) Schwann cells were serum-deprived for 24h maintained in DMEM plus P/S Ab. Experiments were performed on pure Schwann cell cultures on PPD 5 (14 DIV).

3.2.4. Primary Mixed Dorsal Root Ganglia (DRG) Neuronal Culture

Primary adult mixed DRG neuronal-glial cell cultures were established exactly as described in Materials and Methods section 2.2.1

3.2.5. BSA cushion method of non-neuronal cell removal during DRG culture set-up

Where stated, BSA cushions were used during the establishment of primary DRG cultures to reduce the number of contaminating non-neuronal cells present in the culture on plating. As directed in the protocol for DRG establishment 2.2.1, following filtration through a 70 µm mesh, each 1 ml of cell suspension was gently loaded on top of a 2 ml BSA (PAA) cushion, in a 15 ml falcun tube, comprised of 0.7 ml 30% BSA and 1.3 ml DMEM, thoroughly mixed, to give a final concentration of 10.5% BSA. Loaded BSA cushions were centrifuged at 800 rpm for 6 min. All supernatant debris was removed and discarded starting from the BSA-DMEM interphase. DRG cells pellet at the base were recovered by resuspending in pre-warmed C-DMEM to wash and remove residual BSA, pooled together and

centrifuged one final time at 1000 rpm for 3 min. Pellet was resuspended in final volume of pre-warmed C-DMEM and plated as previously described 2.2.1.

3.2.6. Immunocytochemistry on primary cell cultures

Immunocytochemistry on primary macrophage, Schwann cell and DR neuronal were performed following the same protocol outlined in section 2.2.3. Antisera and the working dilutions used are presented in Table 3.2.1.

Table 3.2.1) Table of antibodies used for immunocytochemical studies on primary cell cultures.

Antibody	Dilution for <i>in vitro</i> culture	Company	Code
Primary			
Mouse α -rat ED1 (CD68) mAb	1:500	Millipore	MAB1435
Mouse α - β III-tubulin mAb	1:2500	Promega	G7121
Goat α -CCR5 pAb	1:50	SantaCruz Biotech	sc-6129
Goat α -CXCR4 pAb	1:50		sc-6190
Secondary			
Cy3 donkey α -mouse	1:1000	Jackson ImmunoResearch Laboratories (Stratech Scientific UK)	715-165-150
FITC donkey α -mouse	1:500		715-096-151
FITC donkey α -goat	1:500		711-095-152
Fluorescein (DTAF)-streptavidin	3.6 μ g/ml		016-010-084
Dye			
Hoescht 32258	1:10,000	Gift from Dr. Anita Hall	-

3.2.7. RNA extraction and cDNA synthesis

On completion of treatment exposure primary cell cultures were removed from 37°C incubation At time and total RNA was extracted using the peqGOLD MicroSpin Total RNA Kit (PeqLab) following PeqLab manufacturers instructions for 'RNA Isolation from Animal Tissue or Cell Culture'. All procedures were performed in RNase-free conditions, using auto-claved and RNase-free equipment (Ambion). In brief, cells were washed with sterile PBS at R.T., the wash discarded and 300 μ l RNA Lysis Buffer applied on top for approximately 5 min. Cells were dislodged from culture surface using a cell scraper and transferred into auto-claved RNase-free 1.5 ml eppendorf tubes. Cells were homogenised thoroughly by triturating with a syringe and 25G needle for 15 repetitions, loaded onto a DNA-Removing Column and spun at 10,000 x g for 1 min. DNA binds to the column while RNA

passes through, therefore the flow through was collected and the RNA precipitated by addition of 200 μ l of 100% isopropanol and mixed thoroughly by pipetting. The solution was then transferred onto a PerfectBind RNA Column held within a 2 ml collection tube. The PerfectBind RNA Columns were spun at 10,000 x g for 1 min and the flow through discarded. Columns were transferred to a clear 2 ml collection tube. First wash was performed by addition of 500 μ l with RNA Wash Buffer I and spun at 10,000 x g for 1 min. This was followed by wash applications with 500 μ l Wash Buffer II, spun at 10,000 x g for 1 min. Flow through was discarded carefully and washed with Wash Buffer II and the spin repeated. The columns were spun again at 10,000 x g for 2 min to dry the PerfectBind matrix and ensure removal of any residual wash buffer. The column was then placed into a sterile RNase-free 1.5 ml eppendorf tube and RNA eluted by application of 25 μ l of RNase-free water, left at R.T. for 3 min and spun at 6,000 x g for 1 min. Approximately 22 μ l eluted RNA was recovered and each was aliquoted into 2 x 11 μ l aliquots and stored immediately at -80°C. cDNA synthesis was performed, within 48h of RNA extraction. All steps were performed in RNAase-free conditions and using autoclaved RNase-free Eppendorf tubes and pipette tips (Ambion). RNA samples were defrosted on ice and concentrations of nucleic acids were established using a spectrophotometry (Nanodrop, Ambion). DNase treatment was performed on samples prior to reverse transcription to digest and remove any contaminating DNA in the RNA sample. 0.5 μ l DNase I (1:20; 2.5-18.8 units/ μ l) was combined with 1 μ l 10x DNase I buffer and 8.5 μ l (200-400 ng) RNA sample and incubated at 37°C for 10 min. Following incubation, 1 μ l of the chelating agent EDTA (50 mM), was added to samples in order to prevent RNA from hydrolysing with divalent cations during subsequent heating. Samples were quick-spun (8s) and incubated for 10 min at 75°C to deactivate DNase I. Reverse transcription was performed using the First-Strand SuperScript® III First-Strand System (#18080-051; Invitrogen) following a protocol based on the manufacturer's instructions (SuperScript® III; Invitrogen). 8 μ l of DNase I treated sample was mixed with 1 μ l random hexamers (50 ng/reaction) and 1 μ l dNTP mix (10 mM) and incubated at 65°C for 5 mins. Samples were placed on ice to bring to R.T. and quick-spun before next step. A 20 μ l reaction for reverse transcription was performed using the 10 μ l RNA/hexamer mixture from the previous step in mixed with 2 μ l 10x reverse transcriptase (RT) buffer 4 μ l 25 mM MgCl₂, 2 μ l 0.1M DTT, 1 μ l RNase OUT™ (40 U/ μ l) and 1 μ l SuperScript® III Reverse Transcriptase (200 U/ μ l). RT reaction was performed at 25°C for 10 min followed by 50 min at 50°C. The reaction was terminated at 85°C for 5 min then chilled on ice and quick-spun (8s) to recollect evaporated sample. RNase H (1 μ l) was added to each sample and incubated for 20 min at 37°C to digest and remove RNA strands. Synthesised cDNA was aliquoted at -20°C until next use, at which point it was defrosted slowly on ice just prior to use.

3.2.8. Quantitative real-time polymerase chain reaction (qPCR)

Expression levels of target mRNA in primary cultured adult rat bone-marrow-derived macrophages and Schwann cells were examined by quantitative real-time PCR (qPCR) on a 96-well StepOnePlus™ Real-Time PCR instrument, using the comparative Ct method, normalised to the endogenous house-keeping genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or YWHAZ (14-3-3 protein zeta/delta) and assessed relative to the calibrator (untreated/ naive or vehicle-treated cultures). All PCR reactions were performed either in duplicate or triplicate per sample.

PCR reactions (20 µl) consisted of 1 µl target gene or house-keeping gene forward and reverse (F+R) primer mix (0.1–0.5 µM), 1 µl cDNA sample template (10–20 ng), 10 µl Fast SYBR green master mix (Applied Biosystems) and 8 µl RNase-free water (peqLab). Primer sequences are presented in Table 3.2.2.

Table 3.2.2) Characteristics of primers for selected targets and house-keeping genes

Gene Symbol	Forward Primer	Reverse Primer	Reaction Conc. (µM)	Amplicon Size (bp)	Cit. ⁿ
TNF-α	ATCCGAGATGTGGAAGTGGCA	CCGGACTCCGTGATGTCTAAGT	0.2	659	¹
CCL2	ATGCAGTTAATGCCCCACTC	ATGCAGTTAATGCCCCACTC	0.5	167	²
iNOS	CACCTTGGAGTTCACCCAGT	ACCACTCGTACTTGGGATGC	0.5	167	³
Arg1	TATCGGAGCGCCTTTCTCTA	ACAGACCGTGGGTTCTTCAC	0.5	189	
IL-6	TCCTACCCCAACTTCCAATGCTC	TGGATGGTCTTGGTCTTAGCC	0.4	79	⁴
IL-1β	CACCTCTCAAGCAGAGCACAG	GGGTTCATGGTGAAGTCAAC	0.4	79	⁴
EMR1	CCTTGCCTGCTTCTTCTGGATG	AGCATCTTGATGTTGCGAGAC	0.5	104	⁵
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	0.5	87	
YWHAZ	GCTACTTGGCTGAGGTTGCT	TGCTGTGACTGGTCCACAAT	0.5	61	⁶

Citations (Cit.ⁿ): ¹ Ronaldson, P.T. and Bendayan, R. (2006) *Mol Pharm* 70:1087-1098; ² Thacker, M.A. *et al.* (2009) *Eur J Pain* 13:263-272; ³ Invitrogen: <http://tools.invitrogen.com/content.cfm?pageid=9716>; ⁴ Peinnequin, A. *et al.* (2004) *BMC Immunol* 5:3; ⁵ Haitina, T. *et al.* (2008) *BMC Neurosci* 9:43; ⁶ Sousa, C.T. *et al.* (2011) *Braz J Med Biol Res* 44(6):562-572.

Quantitative PCR (qPCR) amplification consisted of 45 cycles of denaturing at 95°C for 3s, annealing at 58°C for 15s and extending at 72°C for 15s. Specificity of primer products and lack of primer dimers was assessed by running a melt analysis at the end of each qPCR starting at 58°C and raising to 95°C in +0.6°C increments.

Due to the shift in melting temperature of the iNOS primer products a further validation step to confirm specificity was performed by agarose gel electrophoresis. Briefly, the melt curve step was omitted from the iNOS qPCR run and primer products were collected immediately after cycling completion and frozen at -20°C. 15 µl undiluted primer product, mixed with 5x DNA sample buffer, was run on a 2% agarose gel containing ethidium bromide at a constant voltage until the bands of interest were separated. DNA bands were visualised under UV light and the image captured.

The relative fold changes are given by $2^{-\Delta\Delta C_t}$ where $\Delta\Delta C_t = \Delta C_{t_{\text{target}}} - \Delta C_{t_{\text{calibrator}}}$ and ΔC_t is the C_t of the target gene subtracted from the C_t of the housekeeping gene. C_t (cycle threshold) is the number of PCR cycles required before a target is amplified to sufficient quantities to allow the fluorescence signal (SYBR green) associated with it to exceed the threshold (background levels). C_t s < 28 are indicative of abundant target nucleic acid, C_t s of 30–37 are indicative of moderate amounts of target nucleic acid in the sample while C_t s of 38–40 are weak reactions indicative of minimal amounts of target nucleic acid. C_t s > 40 are considered to be false amplification readings represented by formation of primer dimers or environmental contamination.

3.2.9. Comparative ΔC_t Validation Experiment for TNF- α Primer

To validate that the target TNF- α gene primers and normalising GAPDH gene primers had equal amplification efficiencies a validation experiment was performed following the method outlined in the Applied Biosystems “Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR”. In brief, a qPCR was run with TNF- α (0.2 µM) and GAPDH primers (0.5 µM) set at a constant concentration but the amount of input cDNA (derived from one sample) spanned 3 logs across the multiple singleplex reactions run in the same qPCR. For the validation experiment to be accepted the resultant ΔC_t ($C_{t_{\text{target}}} - C_{t_{\text{housekeeping}}}$) at each quantity of the sample cDNA should be almost equal. Equality was determined by plotting the ΔC_t against the log of the input cDNA and measuring the slope of the linear trendline which should be ≤ 0.1 to be accepted.

3.2.10. Cytokine Array

BMDM cultures were established as outlined previously. Two biological replicates were performed in duplicate per treatment. Concentrations of nucleic acid were established using spectrophotometry (Nanodrop, Ambion) and RNA integrity confirmed using the Eukaryote Total RNA Nano 6000 assay run with an Agilent 2100 Bioanalyser (Agilent). Reverse transcription was performed as previously described, using 200 ng RNA, using random hexamers (50 ng/reaction) according to manufacturer's

instructions (SuperScript III, Invitrogen). All cDNA samples were run in a qPCR reaction for TNF- α to validate that the alterations observed in previous experiments were present; to confirm consistency of the experiment before processing on a cytokine array. cDNA samples were loaded in duplicate, in a randomised order, onto three Applied Biosystems 384-well TaqMan[®] microfluidic array cards custom made by John Dawes (King's College London, used for published data in Dawes *et al.* 2011 Pain; accepted manuscript) using the Applied Biosystems website (www.appliedbiosystems.com) and run as per manufacturer's instructions. cDNA samples were mixed with DNase-free water to a final volume of 100 μ l then mixed 1:1 with 2x TaqMan[®] PCR Master mix to give a final cDNA concentration of 1 ng/ μ l (Applied Biosystems). Samples were centrifuged briefly then carefully loaded into the individual reservoirs at the top of each microfluidic low-density card. The plates were then centrifuged, fixed in a 96-well card plate holder, at 1200 rpm for 2 x 1 min, in order for the cDNA samples in the reservoirs to spin down and fill the 384-wells available that were pre-loaded with 96 TaqMan gene expression primers. The microfluidic wells were then isolated from the reservoirs by using a dedicated apparatus with precision stylus carriage to seal the main fluid distribution channels from the array. Using scissors the isolated reservoirs were trimmed from the TaqMan array. The array cards were then run individually in a 7900HT Fast Real-Time PCR system (Applied Biosystems) by Dr. John Dawes (Neurorestoration Group, King's College London) using automated run protocols and software supplied with the array cards that consisted of 40 run cycles. Cards that were prepared but waiting to be run were kept at 4°C until running. The relative fold changes are given by $2^{-\Delta\Delta Ct}$ where $\Delta\Delta Ct$ was normalised to the geometric mean of four endogenous house-keeping genes; GAPDH (ID: Gapdh.Rn99999916_s1), β -actin (ID: Actb.Rn00667869_m1), Hprt1 (hypoxanthine phosphoribosyl transferase 1, ID: Hprt1.Rn01527840_m1) and X18S (Eukaryotic 18S rRNA; ID: X18S.Hs99999901_s1), and assessed relative to the vehicle-treated cultures. Where expression was undetermined in less than 50% of the sample replicates, the mean Ct value was calculated from the remaining data values and undetected sample was excluded. Where expression was undetermined in more than 50% of sample replicates, the sample was given a default Ct of 38, unless this occurred in both vehicle-control or treatment-treated in which case no fold change was calculated and denoted NA (not analysed).

Statistical analysis on gene expression array data was performed by Dr. John Dawes using a two-sided, Welch's test on the ΔCt values against an adjusted p value to limit false positives, using custom-made software designed by Jim Perkins (Bioinformatics Research Group, University College London).

3.2.11. Cell-Conditioned Media Study Conditions and Protocols

i Macrophage-conditioned media-treated DRG cultures

Primary BMDM cultures were established as outlined previously 3.2.1 and at ~200,000 cells/ dish in 35mm Ø culture dishes maintained in 1.5 ml C-IMDM + 5 ng/ml M-CSF. 48h prior to Experimental Day differentiated BMDM cells were isolated from non-adhered and undifferentiated cells and maintain in 1.5 ml IMDM + P/S Ab. 24h prior to Experimental Day a primary adult DRG culture was established as described in Materials & Methods 2.2.1 with DRG cells plated at 2000 cells/ well in a 12-well plate on poly-L-lysine and laminin-coated glass coverslips. DRG cultures were maintained in serum-free conditions in 500 µl DMEM media supplemented 1:1 with F-12 Nutrient Mixture (Invitrogen) plus P/S Ab and 50 ng/ml NGF. Differentiated BMDM (8 DIV) were serum-deprived 24h prior to Experimental Day in 1.5 ml IMDM + P/S Ab. On treatment day BMDM culture media was replaced with 1.5 ml treatment solutions; vehicle (0.1% BSA/PBS), 2 nM denatured-gp120_{Bal} (den.gp120_{Bal}; stock aliquot (10 µl 100 ng/µl gp120_{Bal}) denatured at 90°C for 1h), or 2 nM gp120_{Bal} in 1.5 ml IMDM + P/S Ab. Treatments were applied for 4h, in duplicate. 1 ml supernatant from each culture duplicate was collected, pooled to 2 ml and spun at 1500 rpm for 5 min to pellet cells and debris. 1.5 ml cell-free supernatant was carefully collected and diluted 1:1 with 1.5 ml 2x DRG media (DMEM, 2x NGF (100 ng/ml) and 2x aphidicolin). DRG culture media was removed and replaced with 500 µl of macrophage-conditioned media (MCM)/DRG media mix. Treatments were applied in triplicate in an alternating treatment design (i.e. ABCBCA) across the DRG culture wells and exposed for 24h before fixation, permeabilisation and immunostaining for βIII-tubulin and counterstaining with Hoescht 32258 exactly as outlined in Materials & Methods 2.2.3. Neurite analysis was investigated using CSIRO HCA-vision software as described in Materials & Methods 2.2.6.

i Schwann cell-conditioned media-treated DRG cultures

Primary Schwann cell cultures established from pre-degenerated adult rat sciatic nerves were established as outline previously 3.2.3 and re-plated onto 35 mm Ø culture dishes maintained in 1.5 ml C-DMEM then serum-deprived 24h prior to Experimental Day in 1.5 ml DMEM + P/S Ab. 24h prior to Experimental Day a primary adult DRG culture was established as described in Materials & Methods 2.2.1 with DRG cells plated at 2000 cells/ well in 4-well plates on poly-L-lysine and laminin-coated glass coverslips. On treatment day Schwann cell culture media was replaced with 1.5 ml treatment solutions; vehicle (1.8 µl 0.1% BSA/PBS), 0.5 nM den.gp120_{Bal} (stock aliquot (10 µl 100 ng/µl gp120_{Bal}) denatured at 90°C for 1h), 0.5 nM gp120_{Bal} in 1.5 ml DMEM supplemented with P/S Ab. Treatments were applied for 24h, in duplicate. 1 ml supernatant from each culture duplicate was

collected, pooled to 2 ml and spun at 1500 rpm 5 min to pellet cells and debris. 1.5 ml cell-free supernatant was carefully collected and diluted 1:1 with 1.5 ml 2x DRG media (DMEM, 2x NGF (100 ng/ml) and 2x aphidicolin). Treatments were applied in triplicate in an alternating treatment design (i.e. ABCBCA) across the DRG culture wells and exposed for 24h before fixation, permeabilisation and immunostaining for β III-tubulin and counterstaining with Hoescht 32258 exactly as outlined in Materials & Methods 2.2.3. Neurite analysis was investigated using CSIRO HCA-vision software as described in Materials & Methods 2.2.6.

3.2.12. Neurite Analysis

Neurite analysis following application of macrophage- or Schwann-cell conditioned media or direct exposure to TNF- α (0.25–2 nM final conc.), quinolinic acid (0.7–2 μ M final conc.) or gp120_{MN} (2 nM final conc.), was performed exactly as outlined previously in Materials and Methods section 2.2.6.

3.2.13. Calcium imaging of primary DRG cultures

Calcium imaging on primary DRG cultures was performed following the same protocol as described in Materials and Methods section 2.2.8.

Gp120 glycoproteins and chemokines receptor ligands were applied in 100 μ l volumes diluted in n.s. directly to the culture dish consecutively after 4–6 min vehicle application within each study. Cells were recorded for 4–6 min following treatment application, with all measurements made at room temperature. 200 μ l KCl (60 mM final conc.) was applied following vehicle and treatment application to test cell viability. The number of cells responding to treatments was counted, with a positive response being determined by an increase in fluorescence intensity of greater than 20% (relating to intracellular calcium concentration) from pre-application baseline and accepted providing no responses were observed following vehicle or spontaneously prior to any application.

3.2.14. Intraplantar Injection *in vivo* and gene expression changes

Intradermal injection of 20 μ l control treatment, 400 ng denatured-gp120_{Bal} (10 μ l 100 ng/ μ l stock aliquot denatured at 90°C for 1h) or 400 ng gp120_{Bal} to the plantar surface of the left hindpaw of adult female Wistar rats was performed as outlined in Oh *et al.* (Oh *et al.*, 2001). In brief, female Wistar rats (150 g) were anaesthetised under 2% isoflurane in 1% O₂ and 1% N₂O. In aseptic surgical conditions, the plantar surface of the left hindpaw was sterilised with an isopropanol swab and the area dried. A 25 μ l Hamilton syringe was used to deliver 400 ng of either denatured-gp120_{Bal} or

gp120_{Bal}, in 20 µl volume, intradermally within the sterilised area. The needle point was kept in place for 1 min following injection to prevent back-flow of the injected material. Rats were allowed to recover for 5h then the animals were euthanased with CO₂ asphyxiation and a 5 mm x 5 mm section of tissue, centred by the injection sight, was removed down to the epidermal layer, before subcutaneous fat. Total RNA extraction, cDNA synthesis and quantitative PCR were performed as outlined in sections 3.2.7 and 3.2.8, respectively.

3.2.15. Statistical Analysis

Data is presented throughout as mean ± standard error mean (SEM) unless stated otherwise. To assess for significant differences between 1) two independent samples testing one variable, unpaired student's t-tests were performed; 2) two or more independent samples with one variable tested, one-way ANOVAs were performed; and 3) two or more independent samples with two variables tested, two-way ANOVAs were performed. The null hypothesis of no significant difference was rejected if the critical p value was less than or equal to 0.05, unless stated otherwise. Biological replicates refer to a study being performed in an identical manner using an independent source i.e. another primary culture set-up from a different rat. Treatment replicates derived from one source (i.e. independent culture coverslips derived from one primary culture/ one rat) were classified as technical replicates. Mean and SEM values were all calculated from identical treatment replicates, whether solely, from technical replicates, biological replicates, or technical treatment replicates pooled from across two or more biological replicates. 'n' represents the number of data values that were included in the calculation.

3.3. Results

3.3.1. HIV-1 gp120 induces expression of cytokines in primary bone-marrow derived macrophages and the release of compounds capable of initiating neurite degeneration.

Macrophages originate from monocytes and play a key role in both innate and cell-mediated immunity. Expression of CCR5/CXCR4 receptors on the macrophage cell surface also makes them a key target for the HIV-1 and its gp120 envelope protein, allowing them to be infected and later act as a reservoir of ongoing virus replication throughout the body. Macrophages contain an assortment of powerful excitatory and toxic agents that allow them to eliminate a targeted pathogen. However in the setting of HIV infection there appears to be a dysregulation of macrophage function that

causes the inappropriate release of these agents and may contribute to local inflammation and injury as well as further recruitment of additional inflammatory and/or target cells. It has been demonstrated that macrophages produce cytokines and chemokines and other soluble mediators after exposure to gp120 (JRFL, IIB strains) (Lee *et al.*, 2003). Research has explored the responses of macrophages, derived from a variety of tissues, in terms of their ability to be productively infected by different HIV viral strains, and their responses to gp120 in terms of specific cytokine release. However, few have attempted to correlate gp120-stimulated non-neuronal cell responses with gp120-induced neurite degeneration. Furthermore, the key mediators released from activated macrophages that are involved in initiating neurite degeneration remain to be identified. Therefore I aimed to investigate the responses of bone marrow-derived macrophages (BMDMs) to gp120, assess their involvement in gp120-mediated neurite degeneration and perform a detailed cytokine profile of BMDMs in response to gp120 to attempt to identify the key compounds involved.

My first aim was to clarify the responses of BMDMs to different HIV-1 gp120 tropic strains. I characterised BMDMs in terms of their expression levels of the two chemokine receptors targeted by gp120, CCR5 and CXCR4. Primary bone-marrow derived monocytes of adult rats were differentiated to give cultures containing $96.0 \pm 1.7\%$ cells ($n=3$) immunoreactive for the macrophage marker, ED1 (Figure 3.3.1). Positive immunolabelling was determined by quantifying the mean autofluorescence intensity of unlabelled or non-specific fluorescence labelling intensity of cells exposed only to the secondary antibody as a negative control. Immunostaining was considered to be specific and genuine if the mean and maximum fluorescence intensities were 2 standard deviations greater than the mean and maximum intensities of the calculated background fluorescence. ED1 immunoreactive macrophage-like cells demonstrated immunolabelling for both chemokine receptors however a significantly greater proportion of CXCR4 co-immunoreactive ED1+ cells ($76.6 \pm 0.1\%$; $n=6$) were present than CCR5 co-immunoreactive ED1+ cells ($43.9 \pm 3.3\%$; $n=6$) (Figure 3.3.2). Of the CXCR4- and CCR5-immunoreactive cells $100.0 \pm 0.0\%$ of both populations were identified as macrophages through positive co-labelling with ED1. This indicated that while the population of cells present within the culture did not reach 100% purity, the only cells, apparently expressing CCR5 or CXCR4 were ED1+ macrophages and therefore, to my knowledge, would be the only cells susceptible to gp120 binding.

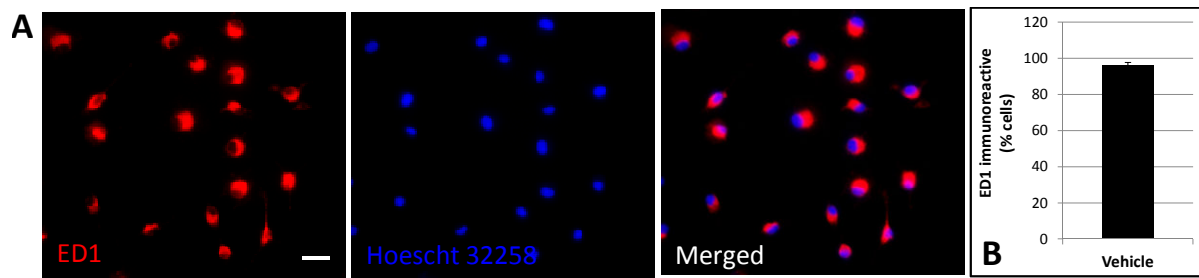


Figure 3.3.1) Macrophage purity in primary cultured bone marrow-derived macrophage cultures. (A)

Representative image of a BMDM culture immunostained for the macrophage-specific marker, ED1 (red) and nuclei counterstained with Hoescht 32258 (blue). Scale bar = 25 µm. (B) Quantification of the percentage of nuclei showing positive colocalisation with ED1 in vehicle-treated BMDM. Data presented as mean ± SEM (n=3 technical replicates).

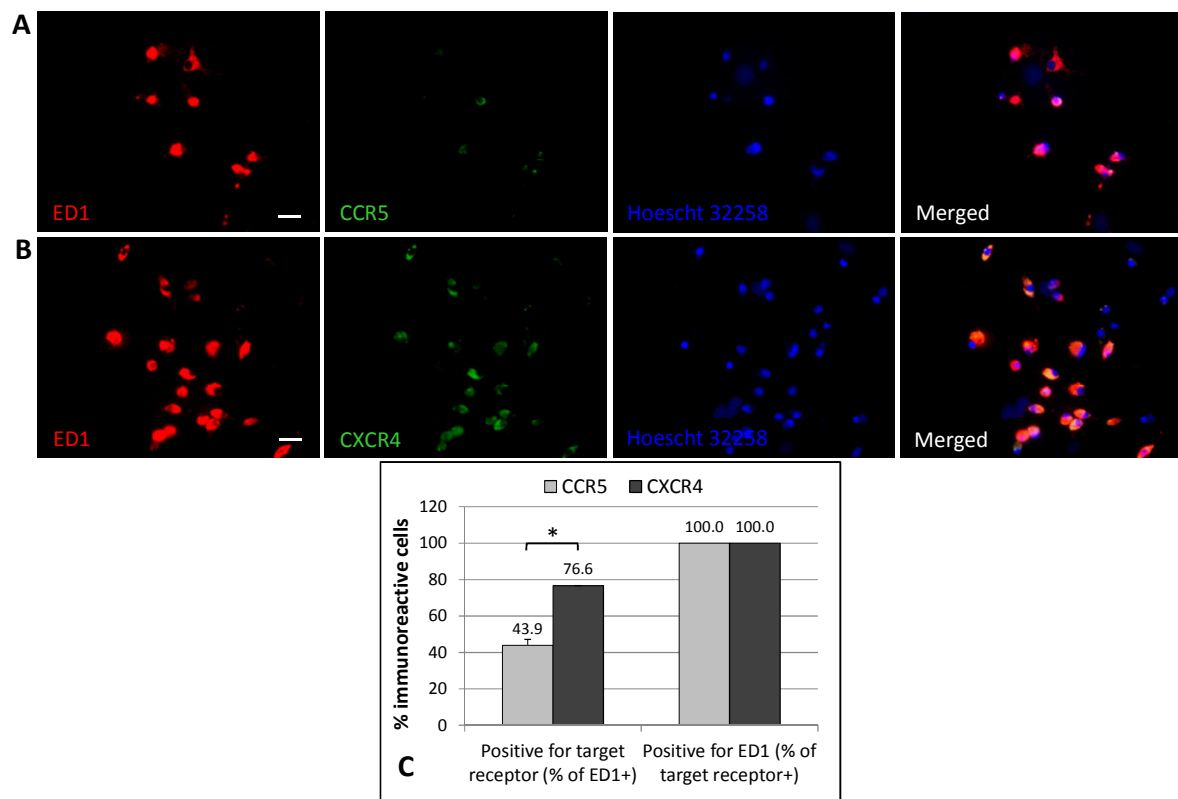


Figure 3.3.2) CXCR4 and CCR5 immunoreactivity in primary cultured bone marrow-derived macrophage cultures. (A)

Representative images of BMDM cultures immunostained for the macrophage-specific marker, ED1 (red) and either (A) CCR5 (green) or (B) CXCR4 (green). Nuclei were counterstained with Hoescht 32258 (blue). Scale bar = 25 µm. (C) Quantification of the percentage of ED1 immunoreactive cells that showed positive colocalisation with CCR5 or CXCR4 and the relative proportion of cells immunoreactive for CCR5 or CXCR4 that were immunopositive for ED1, i.e. macrophages. Data presented as mean ± SEM (n=6, data from two biological replicates). * p<0.01 using unpaired two-sample t-test.

I aimed to explore the changes in expression levels of important cytokines and chemokines within BMDM cells following acute gp120 application using qPCR as a high-throughput but sensitive assay. My initial readout was induction of TNF- α expression as this is one of the most commonly induced genes following macrophage activation and therefore acts as a useful initial assay for activation and to assess the relative effects of different compounds on BMDM activation.

In order for the $\Delta\Delta C_T$ calculations, obtained using the comparative C_T method, to be valid without performing a standard curve for each gene target, the target primer and reference primer amplification efficiencies must be approximately equal. The technique verifies that when qPCR is performed on a range of dilutions of the sample cDNA, both gene target and calibrating gene target will act with comparable amplification efficiencies, shown by minimal variation in the calculated ΔC_T . When the log cDNA quantity is plotted against the resultant ΔC_T the minimal variability is depicted by the slope of the linear trendline. No variability would give a slope value of 0 and primer efficiency should be rejected if the slope of ΔC_T vs. log input is > 0.1 . I performed a validation experiment for the primers to TNF- α and the house-keeping gene, GAPDH, which gave a slope of 0.0055 (Figure 3.3.3), thereby confirming comparable primer efficiencies. In addition, from this data I determined my C_T cut-off value to be 40.0, by taking the C_T reading for the lowest cDNA input. All following studies will be limited to this C_T cut-off threshold, where lower C_T values would be considered genuine and the data analysed, while anything greater would be classified as undetected and given the maximum value of 40.0.

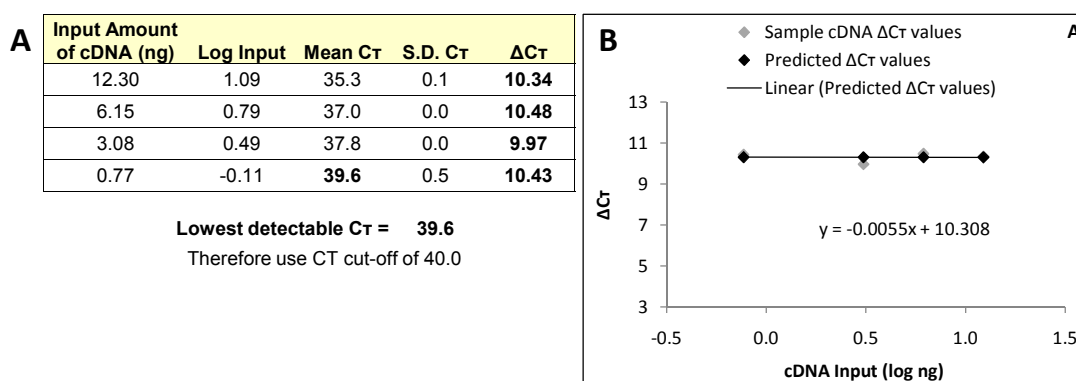


Figure 3.3.3) Assessment of TNF- α primer qPCR efficiencies. The amplification PCR efficiencies of TNF- α and GAPDH were assessed by comparing how the ΔC_T (C_T target – C_T reference) varied with template dilution. Input amount of cDNA was diluted and all run in the same qPCR reaction with primers for TNF- α or GAPDH. (A) ΔC_T calculations for the validation experiment and (B) validation plot of ΔC_T vs. log input amount of cDNA (ng). With 0.77 ng cDNA input the TNF- α primers maintained equivocal detection and amplification efficiencies as with higher cDNA amounts giving a ΔC_T of 39.6 therefore as the efficiencies, at least with this input amount was maintained, a cut-off threshold <40.0 was determined and ΔC_T values >40.0 were considered genuine and accurate.

On completion of these essential validation steps I aimed to explore the relative changes in TNF- α expression in BMDMs following exposure to different gp120 strains and the endogenous CCR5 ligand, RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted). Induction of TNF- α gene expression has been considered a good indicator of macrophage activation (Gifford *et al.*, 1987; Haddad *et al.*, 1997) and therefore was used here to assay gp120-activation.

I will also note that at the time of these experiments, which was towards the very end of my PhD, I received a new gp120 compound, gp120_{Bal}, as a generous gift from Prof. Maria Papathanasopoulos (University of the Witwatersrand, Johannesburg). Unlike the previous gp120 strains I had obtained (gp120_{MN} from ImmunoDiagnostics and gp120_{CN54} from NIH AIDS Reagents program), which were produced in insect cells using a baculovirus expression system, gp120_{Bal} had been produced in HEK293T cells using a mammalian expression system. The significance of this will be discussed in the discussion, section 1.2.13.5.1.

Primary adult BMDMs were incubated for 4h with either vehicle (0.1% BSA/PBS), 20 nM RANTES, or 2 nM gp120_{MN}, gp120_{CN54} or gp120_{Bal} in 1 ml IMDM. Of the gp120 strains tested, only the fully glycosylated M-tropic strain, gp120_{Bal}, induced significant upregulation of TNF- α expression (107.3 ± 44.1 mean fold change; 88.7–138.9 standard range) (Figure 3.3.4). LPS is known to strongly induce cytokine expression in macrophages, therefore LPS (5 ng/ml), was used here as a positive control for responsiveness of the BMDMs, inducing TNF- α expression by 333.5 ± 193.2 fold relative to vehicle, with a standard range of 243.0–459.2. Intriguingly the endogenous CCR5 receptor ligand, RANTES (or CCL5), did not induce a significant increase in TNF- α expression at the concentration tested (1.5 ± 0.3 mean fold change; 1.25–1.84 standard range), nor did two other gp120 strains, the dual-tropic (X4/R5) gp120_{MN} (0.3 ± 0.18 mean fold change; 0.3–0.5 standard range) or the M-tropic (R5) gp120_{CN54} (1.3 mean fold change; 0.65–2.55 standard range). Melt curves were performed at the end of each run to verify specificity of the primer for the target (data not shown but for example melt curves from additional qPCRs see Figure 3.3.15).

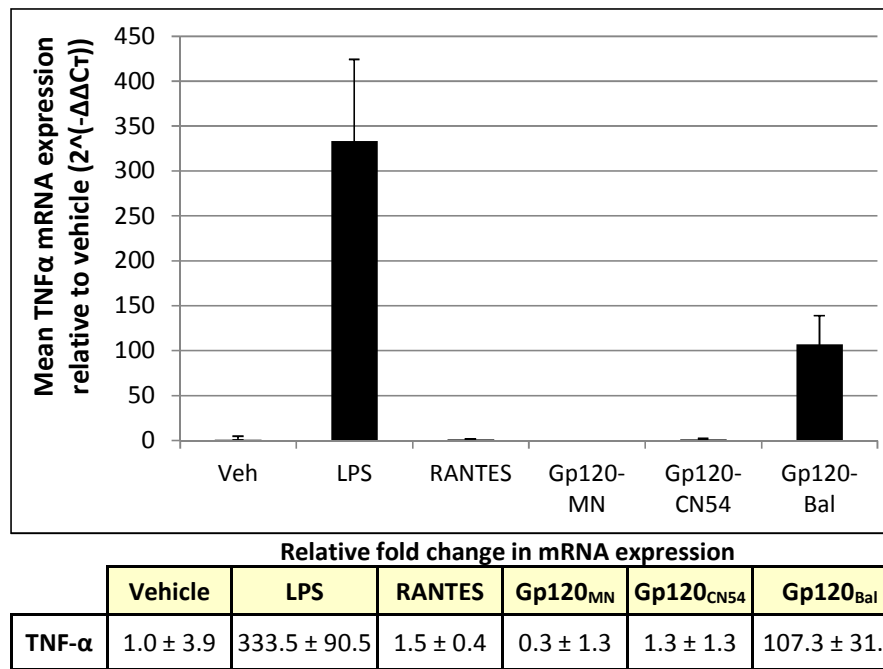


Figure 3.3.4) Changes in TNF-α mRNA expression in bone-marrow derived macrophages (BMDMs) following 4h treatment exposure. Primary BMDM cells were stimulated for 4h with vehicle (0.1%), 5 µg/ml LPS, 20 nM RANTES, 2 nM gp120_{MN}, 2 nM gp120_{CN54} or 2 nM gp120_{Bal} in 1 ml IMDM. Changes in TNF-α mRNA gene expression were assessed, relative to levels in vehicle-treated macrophages. Data presented as mean $2^{-\Delta\Delta C_t} \pm$ SEM (n=2 technical replicates, PCR reactions performed in duplicate per sample).

As gp120_{Bal} induced the strongest response in BMDMs I further explored the pharmacology underlying this response. Gp120_{Bal} induced TNF-α expression in BMDM cells in a concentration-dependent manner that was significant at gp120_{Bal} concentrations of 200 pM (64.9 ± 3.8 fold increase), 1 nM (415.3 ± 77.7 fold increase) and 2 nM (627.5 ± 180.5 fold increase) relative to cultures treated with vehicle (1.0 ± 0.0 fold increase) and denatured-gp120_{Bal} (27.1 ± 7.9 fold increase) (Figure 3.3.5 A). In addition the induction in TNF-α expression by 2 nM gp120_{Bal} was significantly greater than that induced by both 2 pM and 200 pM gp120_{Bal}.

Next, I aimed to verify whether these effects were mediated through the CCR5 chemokine receptor, which gp120_{Bal} is postulated to target primarily due to its classification as a macrophage R5-tropic gp120 strain. Primary cultured BMDMs were pre-incubated either in the absence or presence of the CCR5 antagonist, Maraviroc, at concentrations of 1 nM, 10 nM or 100 nM for 1h prior to treatment. Culture media was then supplemented with media containing the same pre-treatment compounds in addition to treatment comprising vehicle (0.1% BSA/PBS in 1 ml IMDM), 1 nM denatured gp120_{Bal} (in vehicle) or 1 nM gp120_{Bal} (in vehicle). Pre-incubation and continued presence of 1 nM Maraviroc was sufficient to significantly attenuate the induction of TNF-α expression by 1 nM gp120_{Bal} (Figure

3.3.5 B). Application of higher concentrations of Maraviroc maintained this attenuation however with no additional significant reduction, indicating a plateau of inhibition had been reached. Expression levels induced by 1 nM gp120_{Bal} in the presence of 10 nM and 100 nM Maraviroc matched those induced by 1 nM denatured gp120_{Bal} in the presence and absence of Maraviroc and application of 10 nM Maraviroc in the absence of gp120 induced no induction of TNF- α expression by itself.

Therefore these studies confirmed that gp120_{Bal} activation of BMDMs and induction of subsequent cytokine expression was mediated via binding to CCR5 receptors expressed on the BMDMs cell surface.

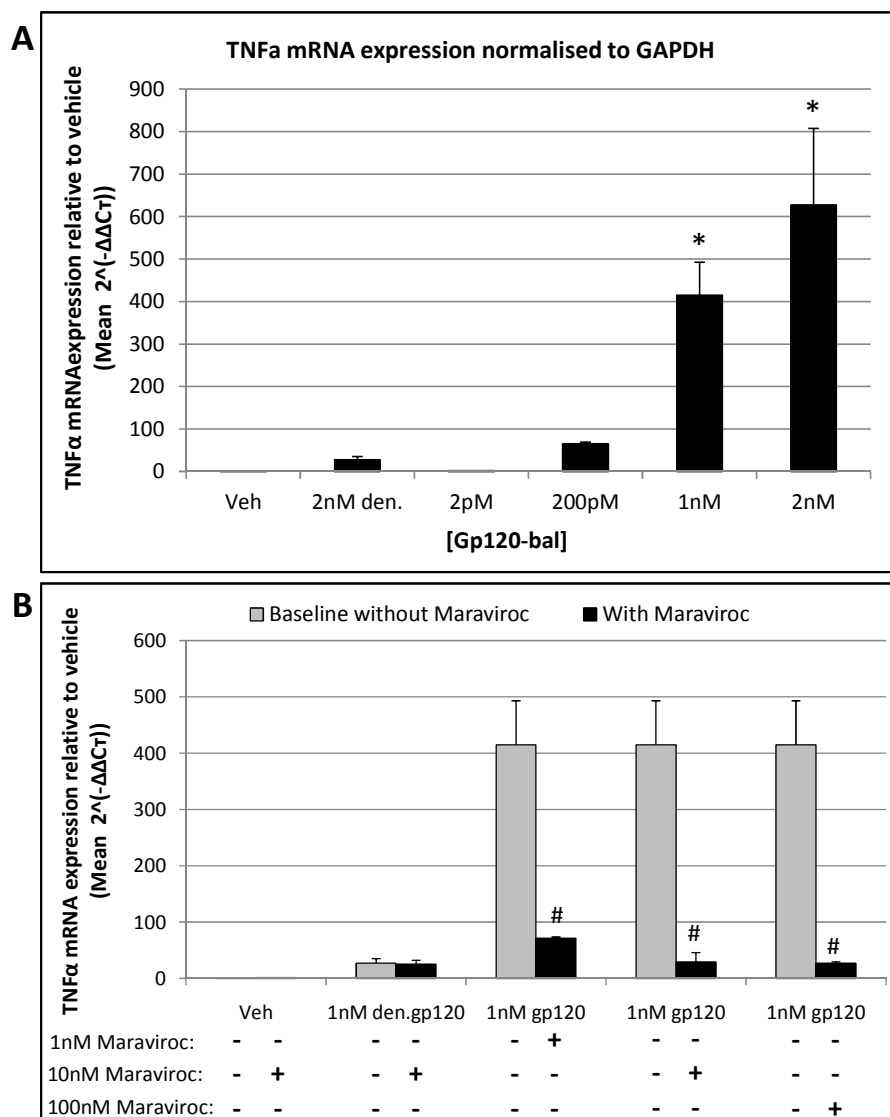


Figure 3.3.5) Relative expression changes in TNF- α mRNA in BMDMs. (A) Gp120_{Bal} induced a concentration dependent increase in BMDM TNF- α expression following 4h stimulation. Primary BMDM cells were stimulated for 4h with vehicle (0.1% BSA), 2 nM denatured gp120_{Bal} in 1 ml IMDM or 2 pM, 200 pM, 1 nM or 2 nM gp120_{Bal} (stocks of lower concentrations were obtained through serial dilution of highest concentration).

* $p < 0.05$ vs. vehicle- and den.gp120-treated control levels using one factor ANOVA and Tukey's post-hoc analysis, $n=4$. (B) Gp120-induced TNF- α expression could be inhibited by the CCR5-specific antagonist Maraviroc. Primary BMDM cells were incubated in the absence or presence of 10 nM Maraviroc for 1hr prior to treatment for 4h with vehicle (0.1% BSA/ PBS), 1 nM denatured gp120_{Bal} or 1 nM gp120_{Bal} in the absence (1 ml IMDM only) or presence of 1 nM, 10 nM or 100 nM Maraviroc, in 1 ml IMDM. Changes in TNF- α mRNA gene expression were assessed relative to levels in vehicle-treated BMDMs. Data presented as mean $2^{-\Delta\Delta Ct}$ fold change $\pm$ SEM ($n=4$; across two biological studies, treatments performed in duplicate per study, PCR reactions performed in duplicate). # $p < 0.05$ vs. 1 nM gp120-treated baseline values, using two factor ANOVA and Tukey's post hoc analysis,.

To this point BMDMs have been maintained and treated in the presence of 10% FBS, however it was noted that many studies induce a quiescent state in macrophages through 24h serum deprivation, in order to ensure homogeneity of their activation state. Therefore I explored the effects of serum-deprivation on the BMDM responses. BMDM cultures were maintained in either serum-free media (IMDM, 0% FBS) or media containing serum (IMDM + 10% FBS) 24h prior to treatment. At time of testing, IMDM media was replaced, either with or without serum, and supplemented with 1 ml of the relevant treatment solution: vehicle (0.1% BSA/PBS), 20 nM RANTES (in vehicle) or 2 nM gp120_{Bal} (in vehicle). Cultures were exposed to treatments for 4h then RNA was immediately extracted and synthesised into cDNA as described in materials and methods (3.2.3.2.7).

Quantification of the changes in TNF- α expression levels in each condition, using the Comparative C_T Method, identified a significant difference in the magnitude of induction elicited by 2nM gp120_{Bal} in serum-free conditions (792.7 ± 181.5 fold increase) compared to the same treatment in the presence of 10% FBS (137.9 ± 55.0 fold increase) (Figure 3.3.6 D). No increase in TNF- α expression was observed following treatment with 20 nM RANTES, whether in the presence or absence of 10% FBS. In addition the purity of the macrophage cultures appeared unaltered when maintained in serum-free ($84.4 \pm 2.4\%$ macrophages) conditions compared to with serum ($93.0 \pm 4.0\%$ macrophages) (Figure 3.3.6). Therefore to ensure I observed maximal effects of gp120 I used serum-free conditions in all subsequent experiments.

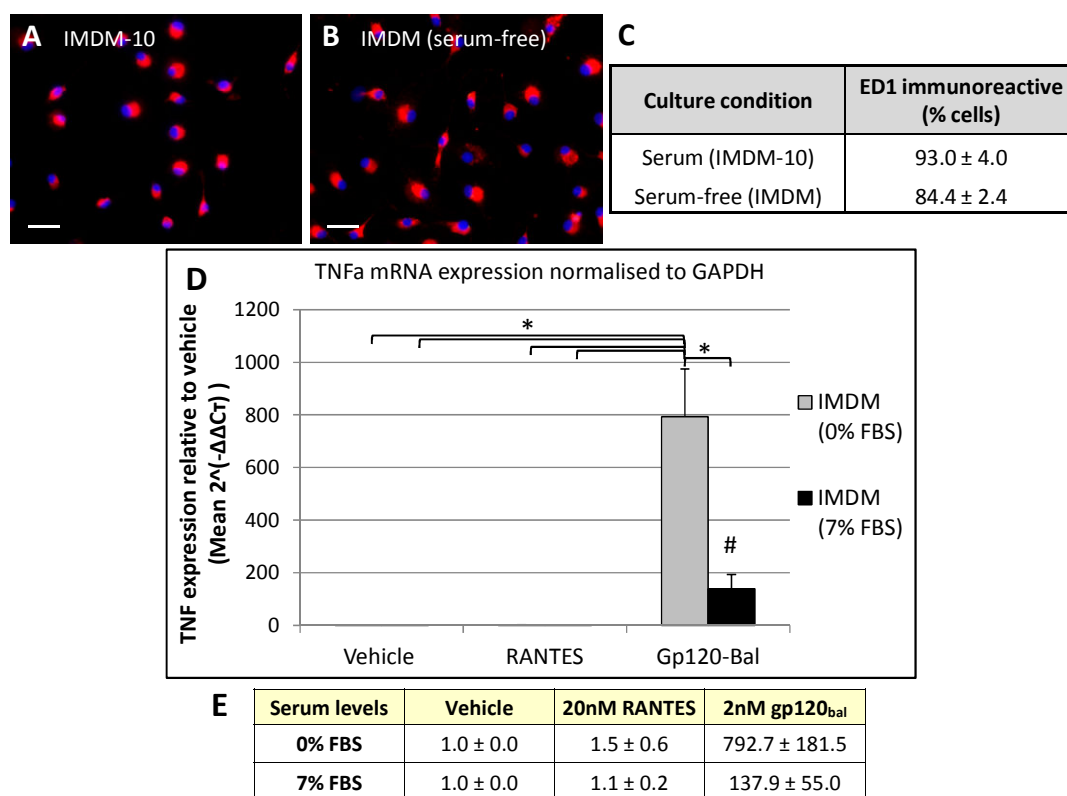


Figure 3.3.6) Effect of serum-deprivation on the purity and gp120-induced TNF- α expression changes in bone-marrow derived macrophages. (A) Representative images of BMDMs immunostained for the macrophage-marker, ED1 (red) with nuclei counterstained with Hoescht 32258 (blue). BMDMs were cultured in IMDM either in the presence or absence (serum-free) of 10% FBS and treated with vehicle or 2 nM gp120_{Bal}. (B) Assessment of the effects of FBS on the purity of macrophage cultures through quantification of the percentage of ED1 positive cells as a percentage of the number of nuclei present. Scale bar = 20 μ m. (C) Quantification of the percentage of ED1 positive cells as a percentage of the number of nuclei present in macrophage cultures maintained in serum +ve (IMDM + 10% FBS) or serum-free conditions (IMDM + 0% FBS). Data presented as mean $\pm$ SEM (n=4; across two biological studies with treatments tested in duplicate per study). (D) Comparison of gp120-induced TNF- α gene expression, relative to vehicle control, in serum and serum-free conditions. (E) Tabulation of the data displayed in D. Data expressed as mean $2^{-\Delta\Delta C_t}$ fold change $\pm$ SEM (n=6; across two biological studies with treatments tested in triplicate per study).

In summary of this section, I have confirmed that primary BMDMs *in vitro* express both CCR5 and CXCR4 chemokine receptors. BMDMs can be dramatically activated by both LPS and gp120_{Bal} following an acute 4h incubation and subsequently induce significant upregulation of TNF- α gene expression. Interestingly the dual-tropic gp120_{MN} and M-tropic gp120_{CN54} (produced in an insect expression system) were unable to induce TNF- α gene expression. The gp120-induced TNF- α gene upregulation is most likely mediated through binding of gp120_{Bal} to the CCR5 receptors expressed on the surface of BMDMs as TNF- α upregulation was almost completely ablated by pre-incubation with the CCR5 antagonist maraviroc.

3.3.2. Effects of compounds released from primary gp120-treated bone-marrow derived macrophages on DRG neurite outgrowth.

I have demonstrated that gp120_{Bal} activates BMDMs *in vitro* by inducing TNF- α expression in primary BMDM cultures. I then attempted to correlate the gp120-activated BMDM responses with gp120-mediated neurite degeneration. This was achieved by assessing whether gp120-treated BMDMs released compounds had the ability to initiate neurite degeneration in primary adult DRG neurons in a mixed adult neuronal-glial culture.

The primary neuronal cultures, although established from primary DRGs, were not pure neuronal cultures as non-neuronal cells commonly contaminate cultures produced in the manner outlined in 2.2.2. Therefore an attempt was made first to establish purer neuronal cultures in order to minimise secondary effects of the supernatant compounds on neurons released by non-neuronal cells present in the culture. Use of a 10% bovine serum albumin (BSA) cushion, as outlined in 3.2.3.2.5, through which I centrifuged dissociated cells before culture set-up, significantly reduced the mean density of non-neuronal cells (nuclei count minus neuronal cell body count; mean 121.7 ± 19.5 per image) that contaminated the DRG cultures compared to cultures established without the use of a BSA cushion (396.2 ± 46.5 per image) (Figure 3.3.7). Importantly this appeared not to affect the mean neurite outgrowth per neuron of vehicle-treated DRG cultures as no significant difference was observed between cultures established without a BSA cushion (1375.5 ± 206.8 μm per neuron; n=6) or using a BSA cushion (994.9 ± 171.2 μm per neuron; n=6). In addition, there was no significant difference in the overall plating density of neuronal cells between either of the culture methods (BSA: 15.2 ± 2.9 neurons/ image; no BSA: 21.0 ± 1.9 neurons/ image) indicating the plating protocol was consistent and comparable. Therefore use of a BSA cushion was employed and the same culture conditions maintained in subsequent studies in order to minimise secondary effects of non-neuronal cells and explore the specific effects of treatments on neuronal cells.

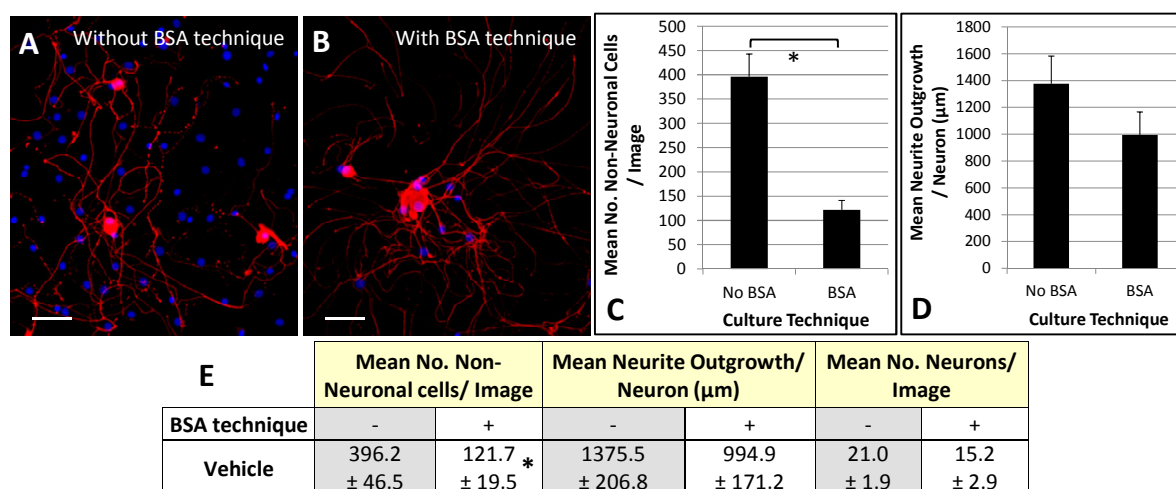


Figure 3.3.7) Assessing the effectiveness of the BSA cushion technique during DRG culture set-up. Representative images of a DRG neuronal culture following normal culture set-up without use of a BSA cushion (A) and with centrifugation through a BSA cushion (B). Scale bar = 50 μm. (C) Comparison of the mean number of nuclei detected per image (C) or the mean neurite outgrowth per neuron (D) between cultures established with or without the BSA technique, across 6 replicate studies. (E) Data values for results indicating significant drop in proportion of non-neuronal cells after BSA cushion but no significant change to neurite outgrowth or the number of neuronal cells plated. Data presented as mean ± SEM (n=6; two biological studies with treatments tested in triplicate per study). * p < 0.001 (p=0.0003) using student's unpaired t-test.

Primary BMDM cultures or naive cell-free media were incubated for 4h with either vehicle (0.1% BSA/PBS in 1 ml IMDM), gp120_{Bal} (2 nM in vehicle) or denatured-gp120_{Bal} (2 nM; gp120_{Bal} stock aliquot denatured at 90°C for 1h then diluted in vehicle). Adult DRG cultures were established 24h prior to testing, in serum-free conditions (DMEM/F12). To limit the possibility of gp120 being carried over from the BMDM media to the neuronal culture and mediating further effects, BMDM supernatant was passed through a 50 kDa Vivaspinn column that only permits particles with molecular weights lower than 50 kDa to pass through. Gp120 has a molecular weight of 120 kDa while cytokines range from 6 to 50 kDa. Therefore this technique should prevent any carryover of gp120 and allow the effects of the supernatant to be attributed to the BMDM-secreted compounds alone. At time of testing DRG media was replaced in a ratio of 1:1 with DRG culture media (DMEM/F12) and culture media (IMDM) from 4h treated BMDM cultures. After 24h treatment exposure there was no significant difference in neurite outgrowth between vehicle-treated DRG cultures and those treated with the culture media from BMDM cultures incubated with 2 nM denatured-gp120_{Bal}. In contrast, application of 2 nM gp120_{Bal}-treated BMDM culture media resulted in a subtle but significant reduction in neurite outgrowth of 18.9 ± 5.3% (Figure 3.3.8). There were minimal differences in the nuclei density (mean number of nuclei per image) or in the neuronal

plating density (number of neuronal cell bodies per image between any of the treatments indicating firstly that if any cells were carried over from the macrophage culture media it was constant across the conditions. Secondly it informs that the DRG plating protocol was reliable and constant. Serum-deprivation of DRG cultures appeared to cause no significant changes to mean neurite outgrowth per neurons ($1239.1 \pm 78.9 \mu\text{m}$) compared to the mean neurite outgrowth per neuron in studies maintained in serum conditions ($1146.2 \pm 73.5 \mu\text{m}$) (Figure 3.3.8 F).

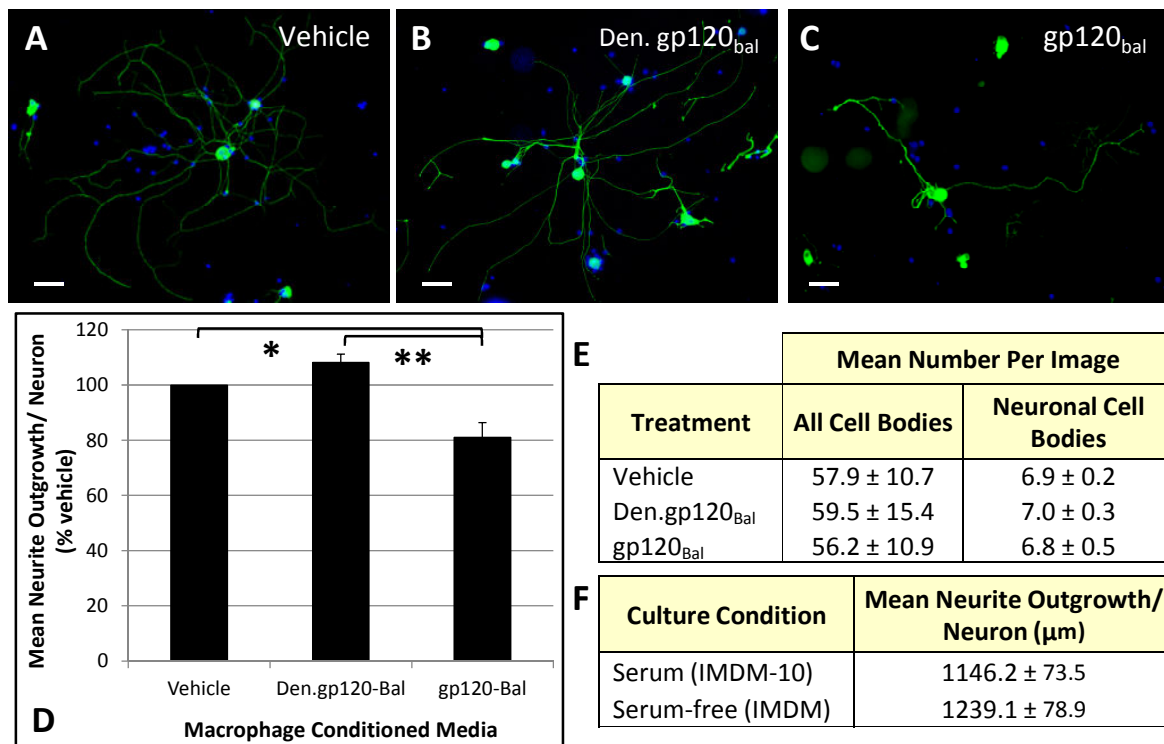


Figure 3.3.8) Neurite analysis following exposure to conditioned BMDM cell media. (A-C) Representative images of primary mixed DRG-glia cultures immunostained for β III-tubulin (green) and nuclei counterstained with Hoescht 32258 (blue). 1 day old DRG cultures were treated for 24h with DMEM/F-12 supplemented 1:1 with the conditioned culture media from bone marrow-derived macrophage stimulated for 4h prior with (A) vehicle (0.1% BSA/PBS in 1 ml IMDM), (B) 2 nM denatured gp120_{bal} (in vehicle), or (C) 2 nM gp120_{bal} (in vehicle). Scale bar = 50 μm . DRG cultures treated with culture media from gp120_{bal}-treated BMDMs showed a significant reduction in mean neurite outgrowth per neuron, as a percentage of vehicle-treated cultures (D). Conditioned media from denatured-gp120_{bal}-treated BMDMs induced no change in neurite outgrowth relative to vehicle control. No significant difference in the density of non-neuronal or neuronal cells plated (E) existed between any of the treatment groups. (F) There was also no difference in the mean neurite outgrowth per neuron when DRG cultures were tested in the presence (serum +ve) or absence (serum-free) of 10% FBS. * $p < 0.05$ vs. vehicle-treated cultures; # $p < 0.05$ vs. gp120_{MN}-treated culture media in the absence of cells, using single factor ANOVA and Tukey's post hoc analysis ($n=5-6$; five-six technical replicates across two biological studies).

3.3.3. Non-neuronally released neurotoxins may play an indirect role in gp120-mediated axonal degeneration although synergistic pathways may be needed.

Following from the data obtained so far there was strong indication that compounds released from gp120-activated macrophages were able to initiate neurite degeneration in primary DRG cultures within 24h. I also demonstrate that BMDMs extensively upregulate TNF- α gene expression on stimulation by gp120_{Bal}, which may be indicative of TNF- α cytokine release. Other mediators have also been identified to be upregulated in different *in vitro* macrophage models after gp120 treatment, including pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6), excitatory mediators (quinolinic acid, glutamate), lipid mediators (arachidonate metabolites) and free radicals (nitric oxide). CSF concentrations of quinolinic acid (QUIN) have been significantly correlated to the severity of the neuropsychological deficits (Heyes *et al.*, 1991). QUIN is an “excitotoxic” metabolite, released from activated microglia and macrophages, and an agonist of the NMDA receptor. Specific TNF- α haplotypes have been identified to be associated with increased risk of neuropathy in the setting of HIV (Affandi *et al.*, 2008; Chew *et al.*, 2011) and with increased TNF- α levels in other diseases (Park *et al.*, 2006). Furthermore TNF- α levels are increased in the CSF of patients with HIV (Castagna *et al.*, 1990; Grimaldi *et al.*, 1991) and stimulation of macrophages release substantial amounts of TNF- α (Lee *et al.*, 2005). QUIN and TNF- α both have been identified in HIV patient sera and CSF and/or shown, from my data, to be induced at the transcriptional level, in Schwann cells and BMDM by gp120. Therefore I aimed to investigate the relative involvement of TNF- α and quinolinic acid in the initiation of neurite degeneration and whether synergism between multiple compounds is involved.

I performed a concentration-response analysis for the effects of increasing concentrations of TNF- α and quinolinic acid on neurite outgrowth. Primary DRG cultures were exposed for 24h to vehicle (PBS in DMEM), 0.25–2 nM TNF- α or 0.7–2 μ M QUIN, concentrations based on the CSF and plasma levels found in HIV patients exhibiting a sensory neuropathy. TNF- α and QUIN both demonstrated concentration-dependent reduction of mean neurite outgrowth per neuron that were significant with 2nM TNF- α ($74.3 \pm 5.9\%$ of vehicle) and all concentrations of QUIN tested (0.7 μ M = $79.2 \pm 6.3\%$; 1 μ M = $72.5 \pm 1.7\%$ of vehicle, 2 μ M = $61.0 \pm 2.4\%$ of vehicle) (Figure 3.3.9). Concentrations below 2 nM TNF- α (0.25 nM, 0.5 nM and 1 nM TNF- α) showed a trend towards increased reduction in mean neurite outgrowth per neuron (82.0 ± 8.0 , 87.0 ± 5.1 and $81.1 \pm 8.4\%$ of vehicle respectively), however these values were not significantly different to mean neurite outgrowth per neuron in vehicle control.

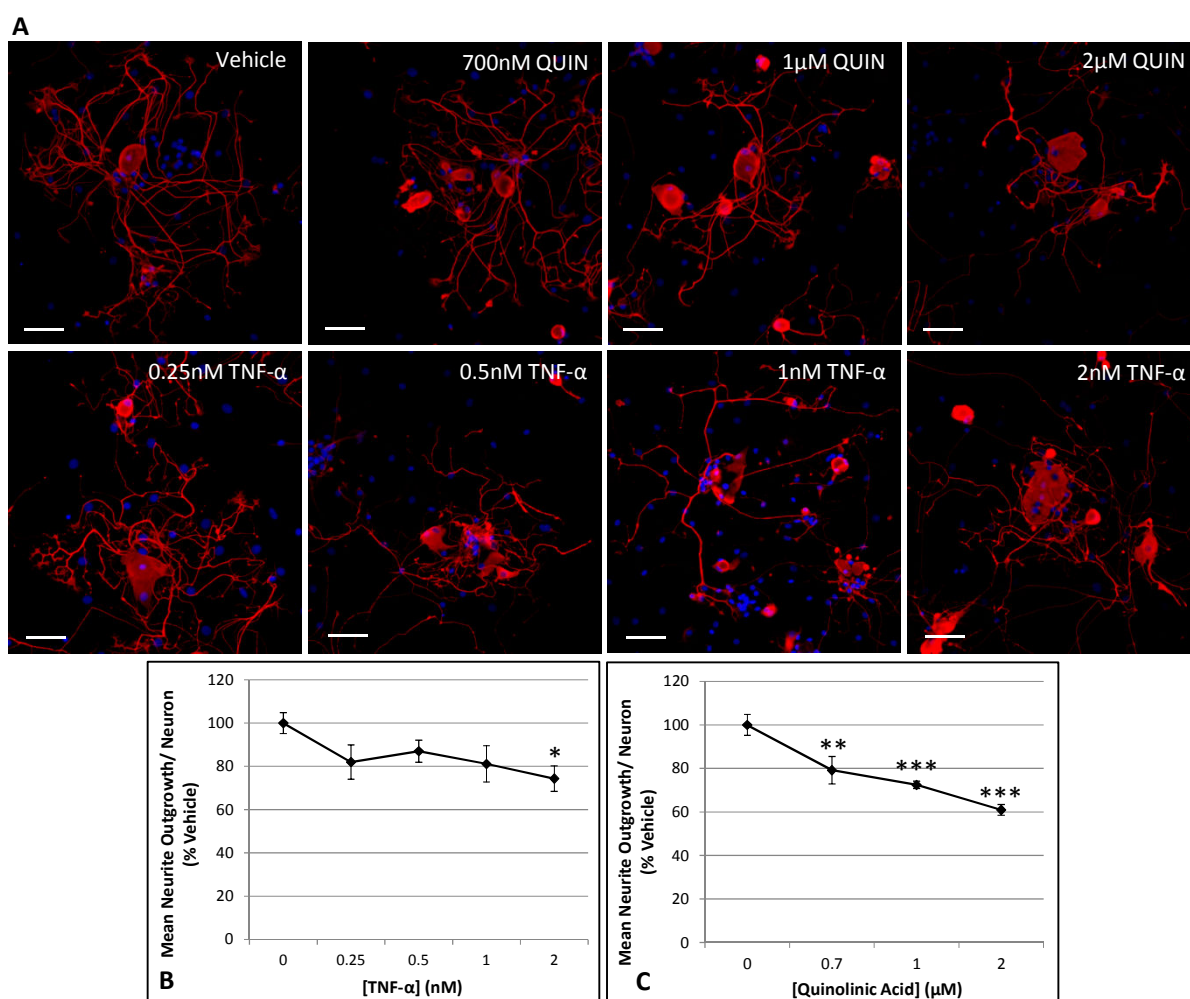


Figure 3.3.9) Concentration-dependent effects of TNF- α and quinolinic acid on neurite outgrowth in primary adult DRG cultures. (A) Representative images of adult primary DRG cultures treated for 24h with vehicle (0.1% BSA/PBS), 0.25 nM, 0.5 nM, 1 nM or 2 nM TNF- α) or 0.7 μ M, 1 μ M or 2 μ M quinolinic acid (QUIN) in 500 μ l C.DMEM. Scale bar = 50 μ m. Addition of TNF- α (B) and quinolinic acid (C) induced reduction in neurite outgrowth in a concentration-dependent manner that was significant at concentrations above and respectively. Data presented as mean neurite outgrowth/neuron, following 24h treatment, normalised to (percentage of) the mean neurite outgrowth/neuron of vehicle-treated cultures (n=3–12; technical replicates across one to four biological studies). * p<0.05, ** p<0.01, *** p<0.0025 using single-factor ANOVA and Tukey's post-hoc analysis.

Synergy between neurotoxic compounds has been previously reported in the context of neuronal apoptosis and axonal degeneration (Chao *et al.*, 1995; Turrin *et al.*, 2000). Given that multiple mediators are released following gp120-stimulation I aimed to elucidate whether specific released factors from non-neuronal cells act synergistically with gp120 to enhance the neurotoxic effect on neurite outgrowth. Primary DRG cultures, purified using a BSA cushion, were tested either individually or in combination with recombinant TNF- α , quinolinic acid and gp120_{MN}, while vehicle

(0.1% BSA/PBS) acted as a negative control. TNF- α and QUIN both individually caused significant reduction in mean neurite outgrowth per neuron relative to vehicle-treated cultures (81.1 ± 8.4 , 79.2 ± 6.3 , % vehicle respectively) (Figure 3.3.10). However, surprisingly when these treatments were combined together with 2 nM gp120_{MN}, there appeared to be no synergistic effect with a mean neurite outgrowth per neuron of $77.0 \pm 5.5\%$ vehicle. Of note, TNF- α signalling is commonly linked with activation of apoptotic pathways, therefore I also assessed cell survivability by quantifying the proportion of neurons that exhibited signs of apoptosis, namely condensed and fragmented Hoescht-labelled nuclei. Against expectations, no significant difference was observed between vehicle-treated cultures ($45.0 \pm 3.2\%$ “apoptotic” neurons) and those exposed to TNF ($47.6 \pm 0.9\%$ “apoptotic” neurons) or QUIN ($44.4 \pm 4.0\%$ “apoptotic” neurons). Conversely, combination of these compounds with gp120_{MN} resulted in significantly reduced number of apoptotic neurons ($25.3 \pm 1.0\%$ “apoptotic” neurons).

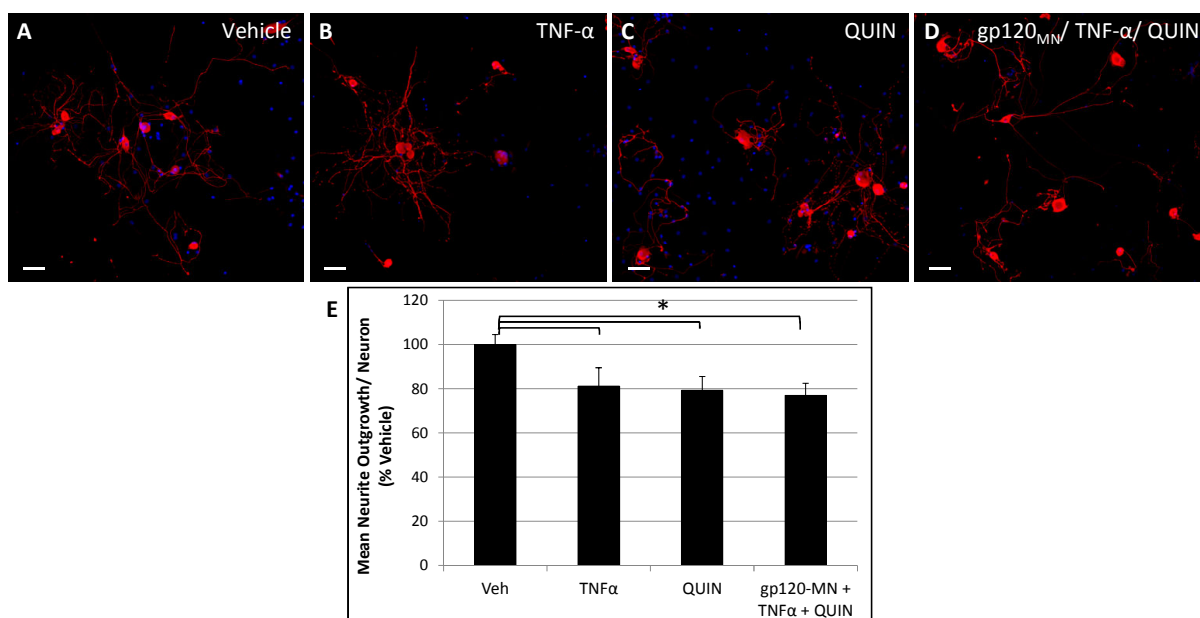


Figure 3.3.10) Single and combined effects of neurotoxins on primary DRG neurite outgrowth *in vitro*. (A) Representative images of adult primary DRG cultures treated for 24h with (A) vehicle (0.1% BSA/PBS), (B) 1 nM TNF- α , (C) 0.7 μ M quinolinic acid (QUIN) or (D) 1 nM TNF- α and 0.7 μ M QUIN in combination with 2 nM gp120_{MN}, in 500 μ l C.DMEM. Scale bar = 50 μ m. (E) Quantification of the mean neurite outgrowth/ neuron following 24h treatment exposure. Data presented as mean $\pm$ SEM (n=9; technical replicates across three biological studies). * p<0.05 using single factor ANOVA and Tukey’s post-hoc analysis.

Dysregulation of calcium homeostasis has become a common feature associated with HIV-associated neurotoxicity and apoptosis, and is frequently implicated in other neurodegenerative diseases. I have shown that both TNF- α and QUIN were able to induce neurite degeneration. In addition, QUIN

is able to induce calcium excitotoxicity through overstimulation of the NMDA receptor (Braidy *et al.*, 2009). However, it is suggested that TNF neurotoxicity and in particular TNF-induced apoptosis occurs independently of calcium modulation. Research suggests that the mechanisms underlying apoptosis are distinct from those determining axonal degeneration (Finn *et al.*, 2000) but this raises speculation that two different pathways may be involved and that synergism between these, and potentially more, may determine the development of peripheral neuropathy. I have highlighted TNF- α due to its suggested involvement in either, or both, the degeneration of sensory neurons and the pain associated with it, in the context of HIV. Furthermore under aberrant macrophage activation, QUIN may act as a key mediator of the HIV-associated calcium toxicity observed. Therefore I aimed to elucidate the effect of TNF- α and QUIN on $[Ca^{2+}]_i$ levels in primary adult DRG neuronal cells to determine 1) whether acute calcium mobilisation is involved in the TNF- α - and QUIN-mediated neurodegeneration I observed and 2) the relative involvement of these compounds in calcium dysregulation.

The effects of TNF- α and QUIN on calcium mobilisation were investigated in primary DRG cultures. No acute modulation of intracellular calcium levels was observed in cultured DRG neurons following application of TNF- α or QUIN up to concentrations of 1 nM or 2 μ M, respectively (data not shown); concentrations comparable to those that induced neuronal degeneration. Increases in intracellular calcium levels were observed at appreciably higher concentrations of TNF- α (3 nM) and QUIN (25 μ M) however only a minority of cells responded (1.9% and 5.4% responsive cells, respectively) (Figure 3.3.11). However concentrations of this magnitude are only evident in HIV-positive patients with extensive neurological defects notably after prior nerve damage and lower TNF- α and QUIN CSF levels, which raises questions about the biological significance of this data. Of note, 200 μ M QUIN proved to act potently on DRG neuronal cells, activating $[Ca^{2+}]_i$ increases in 89.6% of cells analysed. This indicated that functional NMDA receptors, through which QUIN acts, are likely to be expressed on the majority of neuronal cells present in the primary adult DRG culture and that QUIN has the ability to induce acute calcium influx, unlike TNF- α . However the activation was in fact so efficacious that it resulted in excitotoxic cell death in the majority of activated cells as noted by the condensed morphology seen 660s phase contrast image compared to that at 0s (Figure 3.3.11 F) and the reduced percentage of cells responding to KCl (39.2% responsive cells) and reduced amplitude in the fluorescence trace following KCl given by those that did respond.

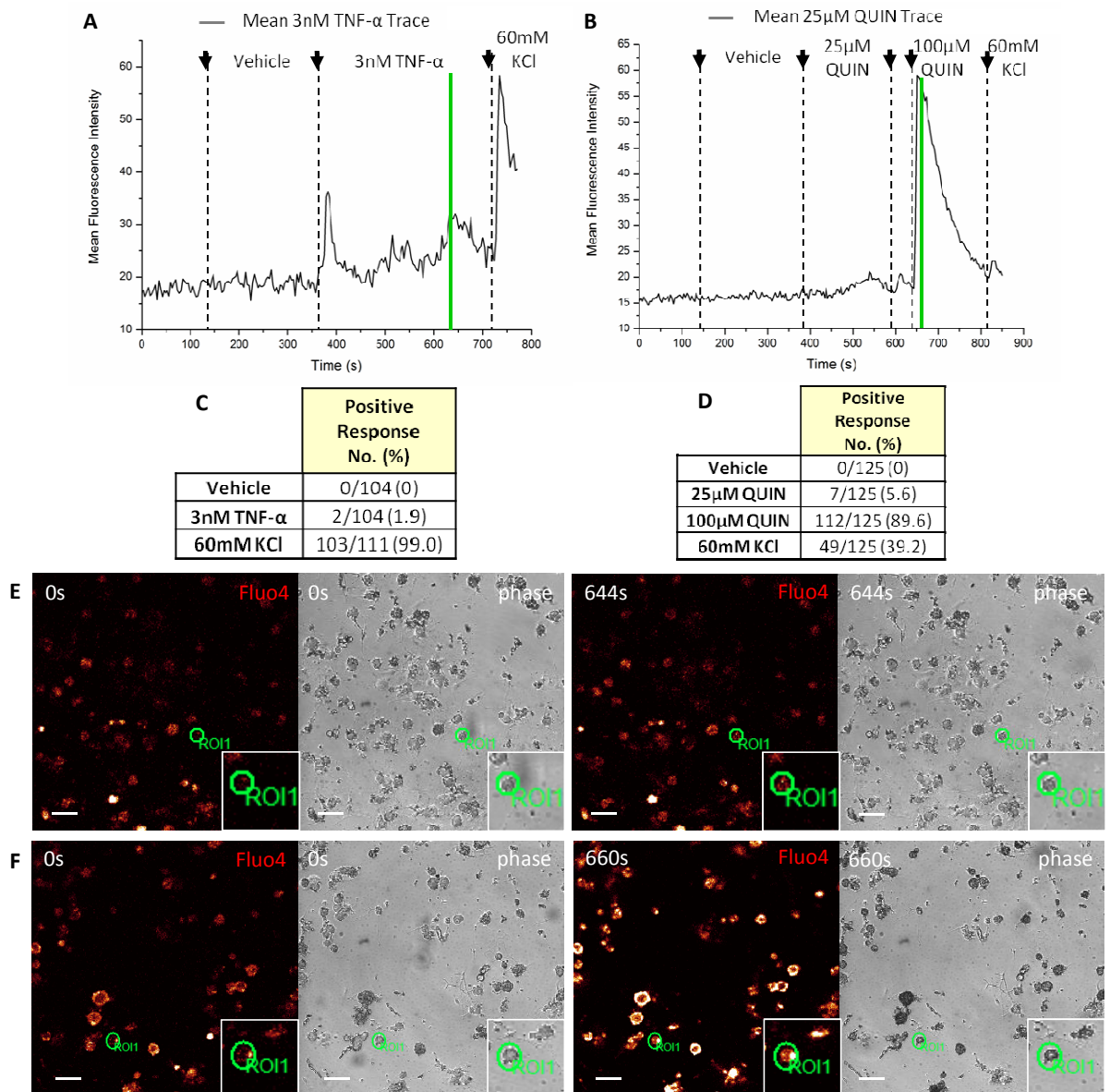


Figure 3.3.11) Fluo-4 based calcium imaging of a primary adult DRG culture tested with high concentrations of TNF- α and quinolinic acid. Pooled fluorescence intensity trace from all cells that responded to 3 nM TNF- α (A) or 25 μ M quinolinic acid (QUIN; B) subsequent to application of vehicle control and followed by a final application of KCl to assess cell viability. (C and D) The number of cells responding to TNF- α (C) and QUIN (D) were quantified. The percentage of cells responding from the population visualised is indicated in the parentheses. Positive responses were recorded when the mean fluorescence showed a significant increase from baseline prior to application. (E and F) Channel images acquired at 20x magnification, showing the Fluo4 fluorescence and phase channel prior to experiment and at the peak of mean fluorescence intensity for TNF- α (E) and QUIN (F). Scale bar = 50 μ m.

TNF- α and QUIN were selected for their current highlighted presence in numerous neurodegenerative diseases and the identification that TNF haplotypes, which result in increased

TNF production, and high TNF- α and QUIN sera and CSF levels have been correlated with enhanced HIV pathogenesis. Both TNF- α and QUIN induced significant neurite reduction in neurite outgrowth in a concentration dependent manner however no synergism was observed when these compounds were combined together with gp120. Furthermore, the reductions observed were more subtle than reductions observed in previous studies where gp120 was applied directly in isolation. This signifies that, although these compounds may be involved in gp120-mediated neurite degeneration they are not sufficiently affective in isolation and therefore alternative cytokines or excitotoxic mediators may be required. Both TNF- α and QUIN also induced calcium mobilisation however only at the highest concentrations tested that are far above the levels likely to be experienced in the clinical setting. Therefore it suggests that, at least acutely, these compounds do not activate acute calcium responses. However, this does not rule out that alternative pathways were activated that may later lead to increased calcium levels.

3.3.4. Cytokine Array: high-throughput assessment of gene expression changes in BMDM cells after gp120 exposure.

It has been proposed that host products from HIV-1-infected and gp120-stimulated macrophages, may alter neuronal physiology and induce apoptosis through the release of neurotoxic compounds, including TNF- α , IL-1 β , quinolinic acid and arachidonic acid metabolites, which are among the most frequently referenced as highlighted in Introduction 3.1. Despite this insight, specific mechanisms, and the critical intermediaries involved in gp120-mediated neurotoxicity, remain unclear. Macrophages have the ability to express, synthesise and release over 100 different cytokines, chemokines and metabolites, many of which have cytotoxic potential. Therefore there remains many unexplored cytokines and chemokines that may play a currently unknown but crucial role in gp120-mediated pathogenesis. In addition the different gp120 strains used and various macrophage populations tested in research allow for individual and condition-specific results to be obtained that do not necessarily translate from one experimental condition to the next. I have shown previously that, while TNF- α and QUIN have become dominant candidates for underlying gp120-mediated neurite degeneration, the application of these compounds acutely to DRG neuronal cultures had only subtle negative effects on neurite outgrowth. Longer incubation or further optimisation may be required however it may also highlight that these compounds are not the only factors involved. Activation of other pathways by other cytokines or excitotoxic compounds may be required. Therefore I aimed to explore the changes in expression of 96 of the most commonly implemented genes in inflammation, disease states and neurotoxicity (outlined in Table 3.3.1) within BMDM cells following acute gp120 application, using qPCR as a high-throughput but sensitive assay.

Primary cultured BMDMs were maintained in serum-free media for 24h prior to treatment with either vehicle (0.1% BSA/PBS in IMDM without serum), 2 nM denatured gp120_{Bal} (in vehicle) or 2 nM gp120_{Bal} (in vehicle) (n=4, from 2 biological replicates with 2 technical replicates per study). Cultures were exposed to treatments for 4h then RNA was immediately extracted. Prior to cDNA synthesis, the RNA integrity of all samples was assessed using an Agilent RNA Nano 6000 electrophoresis chip run on the Agilent 2100 Bioanalyzer machine. Assessment of the RNA Integrity Number (RIN) has become a reliable tool to compare integrity of RNA samples, determined through a complex algorithm based on a Bayesian learning technique that combines ribosomal ratio comparison with other features determined during the running of the Agilent nano chip. RIN values range from 10 (intact) to 1 (totally degraded) with a usual cut-off RIN value of 7. All 12 of the RNA samples were allocated RIN values >7 therefore indicating minimal or no RNA degradation (Figure 3.3.12).

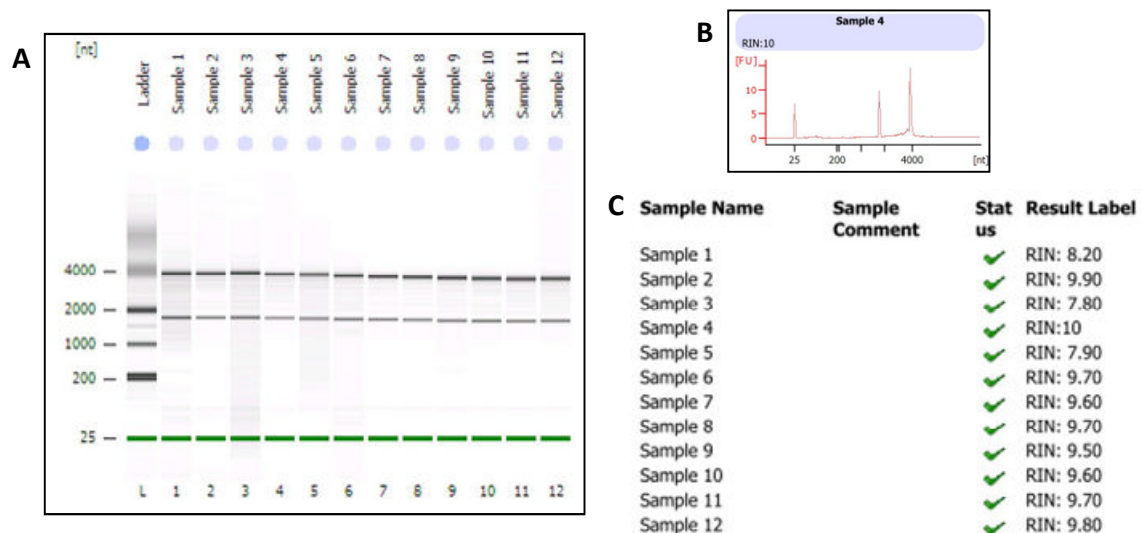


Figure 3.3.12) RNA integrity validation using Agilent RNA Nano 6000 kit. In total, 12 RNA samples (4 biological replicates for each treatment condition) were loaded onto the Nano 6000 chip and run on an Agilent Bioanalyzer to assess ribosomal ratios (A), total RNA quantity and RNA integrity (C) for all the samples. (A) Gel trace depicting the RNA samples run on the microcapillary electrophoresis chip with visible 28S and 18S ribosomal bands. (B) Example electropherogram trace for sample 4 determined from laser-induced fluorescence (LIF) detection and assessment of the miniaturised gel depicted in A. (C) RNA integrity numbers (RIN) allocated to each sample by the Agilent Bioanalyzer algorithm. A sample with RIN value >7.0 is classified as having good RNA integrity.

Following validation of RNA integrity all samples were synthesised into cDNA as described in materials and methods (3.2.3.2.7). Four cDNA samples per treatment (100 ng) were loaded in duplicate, in a randomised order, onto three Applied Biosystems TaqMan cytokine array cards and run as per manufacturers' instructions.

Table 3.3.1) (p140-141) List of all gene targets assessed, their Applied Bioscience ID number and the expression changes relative to vehicle control in denatured-gp120_{Bal}⁻ and gp120_{Bal}-treated BMDM cultures. P values refer to the fold changes in respective treatment groups relative to vehicle-treated.

ID	Target	Denatured gp120 _{Bal}				gp120 _{Bal}			
		2 ^{-ΔΔCt}	(min - max)		p value	2 ^{-ΔΔCt}	(min - max)		p value
Actb.Rn00667869_m1	B-actin	1.0	0.9	1.1	0.974	1.1	1.1	1.1	0.069
Aif1.Rn00567906_g1	Aif1 (Iba1)	0.7	0.5	1.0	0.880	0.8	0.6	1.1	0.709
Alox15.Rn00696151_m1	Alox15 (Arachidonate 15-lipoxygenase)	0.8	0.3	2.0	0.923	0.8	0.4	1.8	NA
Alox5.Rn00563172_m1	Alox5 (Arachidonate 5-lipoxygenase)	0.8	0.5	1.2	NA	NA	NA	NA	NA
Areg.Rn00567471_m1	Areg (amphiregulin)	NA	NA	NA	NA	NA	NA	NA	NA
Artn.Rn01761472_g1	Artn (artemin)	0.3	0.2	0.4	0.801	0.3	0.2	0.4	NA
Bdnf.Rn01484928_m1	BDNF	NA	NA	NA	NA	NA	NA	NA	NA
Btc.Rn00673281_m1	Btc (betacellulin)	NA	NA	NA	NA	NA	NA	NA	NA
C3.Rn00566466_m1	C3 (Complement component 3)	2.1	1.2	3.8	0.903	1.9	1.0	3.6	0.543
C5.Rn01436156_m1	C5 (Complement component 5)	NA	NA	NA	NA	NA	NA	NA	NA
Ccl1.Rn01752376_m1	CCL1	1.5	0.7	3.1	0.801	2.0	0.6	6.0	NA
Ccl11.Rn00569995_m1	CCL11	NA	NA	NA	NA	NA	NA	NA	NA
Ccl12.Rn01464638_m1	CCL12	12.4	0.4	374.5	0.801	1167.2	1005.0	1355.0	0.020
Ccl17.Rn00589290_m1	CCL17	+	NA	NA	NA	NA	NA	NA	NA
Ccl19.Rn01439563_m1	CCL19	0.8	0.6	1.1	0.801	14.0	8.7	22.5	NA
Ccl2.Rn00580555_m1	CCL2	2.1	1.0	4.4	0.821	134.8	107.9	168.4	0.001
Ccl20.Rn00570287_m1	CCL20	1.7	0.7	3.8	NA	276.6	133.9	571.6	0.0005
Ccl21b.Rn01764651_g1	CCL21β	NA	NA	NA	NA	NA	NA	NA	NA
Ccl22.Rn01536591_m1	CCL22	0.7	0.2	2.2	NA	1.1	0.4	3.2	NA
Ccl24.Rn01481451_m1	CCL24	NA	NA	NA	NA	NA	NA	NA	NA
Ccl25.Rn01403351_m1	CCL25	NA	NA	NA	NA	NA	NA	NA	NA
Ccl26.Rn01481484_m1	CCL26	NA	NA	NA	NA	NA	NA	NA	NA
Ccl27.Rn01437037_m1	CCL27	NA	NA	NA	NA	NA	NA	NA	NA
Ccl28.Rn00586715_m1	CCL28	NA	NA	NA	NA	NA	NA	NA	NA
Ccl3.Rn00564660_m1	CCL3	1.4	0.2	9.0	0.974	127.1	53.9	299.9	0.005
Ccl4.Rn00587826_m1	CCL4	2.5	1.0	6.2	0.821	306.3	205.7	456.1	0.004
Ccl5.Rn00579590_m1	CCL5	0.9	0.2	3.3	0.983	474.5	342.2	657.9	0.008
Ccl6.Rn01456402_g1	CCL6	1.1	0.6	1.9	0.974	1.3	0.5	3.2	0.792
Ccl7.Rn01467286_m1	CCL7	1.4	0.8	2.5	0.821	93.4	81.8	106.6	2.00E-07
Ccl9.Rn01471276_m1	CCL9	1.0	0.4	2.2	0.974	7.5	4.3	13.0	0.030
Csf1.Rn00576849_m1	M-CSF1 (macrophage colony stimulating factor 1)	1.2	0.9	1.6	0.958	2.9	1.9	4.4	0.030
Csf2.Rn01456851_m1	Csf2	NA	NA	NA	NA	+	NA	NA	NA
Csf3.Rn00567344_m1	Csf3	NA	NA	NA	NA	+	NA	NA	NA
Cx3cl1.Rn00593186_m1	CX3CL1	1.6	0.9	2.8	0.880	41.4	22.8	75.1	0.001
Cxcl1.Rn00578225_m1	CXCL1	3.9	2.2	6.9	0.166	248.0	198.5	309.9	2.00E-05
Cxcl10.Rn00594648_m1	CXCL10	5.1	2.2	11.4	0.252	939.9	737.5	1198.0	0.0003
Cxcl11.Rn00788262_g1	CXCL11	5.9	1.6	22.5	0.801	3205.5	2241.9	4583.4	3.00E-05
Cxcl12.Rn00573260_m1	CXCL12	1.0	0.6	1.6	0.974	0.9	0.5	1.8	0.884
Cxcl13.Rn01450028_m1	CXCL13	0.9	0.5	1.7	NA	3.2	1.2	8.2	NA
Cxcl14.Rn01441840_m1	CXCL14	1.2	1.0	1.5	0.821	1.0	0.7	1.4	0.969
Cxcl16.Rn01496393_m1	CXCL16	1.1	0.7	1.7	0.974	2.3	1.8	2.8	0.035
Cxcl17.Rn01764053_m1	CXCL17	NA	NA	NA	NA	+	NA	NA	NA
Cxcl2.Rn00586403_m1	CXCL2	10.0	3.2	31.4	0.821	2060.5	1186.4	3578.6	0.052
Cxcl3.Rn00593435_m1	CXCL3	NA	NA	NA	NA	NA	NA	NA	NA
Cxcl6.Rn00573587_g1	CXCL6	2.0	0.8	4.7	0.880	20.6	5.2	80.9	0.040
Cxcl9.Rn00595504_m1	CXCL9	2.1	0.9	5.2	0.801	2082.9	1525.0	2844.0	3.00E-05
Ebi3.Rn01527778_m1	Ebi3 (Epstein-Barr virus induced 3)	0.9	0.7	1.2	0.974	1.7	1.3	2.3	0.045
Edn1.Rn00561129_m1	Edn1 (Endothelin1)	0.9	0.5	1.4	0.974	3.1	2.0	4.9	0.078
Ereg.Rn00572454_m1	Ereg (epiregulin)	1.3	0.5	3.3	0.923	6.5	3.6	11.7	0.212
Fgf7.Rn00573319_m1	Fgf7 (Fibroblast growth factor 7)	3.9	1.9	7.9	0.974	1.3	0.6	3.0	0.296

ID	Target	Denatured gp120 _{Bal}				gp120 _{Bal}			
		2 ^{-ΔΔCt}	(min - max)		p value	2 ^{-ΔΔCt}	(min - max)		p value
Gapdh.Rn99999916_s1	GAPDH	1.0	0.8	1.3	0.974	0.9	0.8	1.1	0.758
Hbegf.Rn00564075_m1	Hbegf (Heparin-binding EGF-like growth factor)	0.9	0.3	3.0	0.974	1.8	1.3	2.3	0.445
Hprt1.Rn01527840_m1	Hprt1 (hypoxanthine phosphoribosyl transferase 1)	3.6	0.2	69.3	0.903	0.8	0.6	1.0	0.334
Ifng.Rn00594078_m1	IFN γ	NA	NA	NA	NA	+	NA	NA	NA
Il10.Rn01644839_m1	IL-10	1.0	0.7	1.3	0.983	2.3	0.6	9.2	NA
Il11.Rn00591721_m1	IL-11	0.0	NA	NA	NA	NA	NA	NA	NA
Il12a.Rn00584538_m1	IL-12 α	NA	NA	NA	NA	4.6	1.1	19.5	0.297
Il12b.Rn00575112_m1	IL-12 β	NA	NA	NA	NA	+	NA	NA	NA
Il13.Rn00587615_m1	IL-13	NA	NA	NA	NA	NA	NA	NA	NA
Il15.Rn00689964_m1	IL-15	1.4	0.7	2.7	0.801	43.1	30.1	61.7	0.003
Il16.Rn01477722_m1	IL-16	0.0	NA	NA	NA	-	NA	NA	NA
Il18.Rn01422083_m1	IL-18	1.3	0.6	2.6	0.974	2.9	1.9	4.3	0.145
Il19.Rn01490483_m1	IL-19	NA	NA	NA	NA	NA	NA	NA	NA
Il1a.Rn00566700_m1	IL-1 α	8.9	3.2	25.2	0.503	2016.3	1336.0	3043.0	0.0004
Il1b.Rn00580432_m1	IL-1 β	8.5	5.4	13.6	0.155	948.3	798.4	1126.0	0.001
Il2.Rn99999181_m1	IL-2	NA	NA	NA	NA	NA	NA	NA	NA
Il20.Rn01483978_m1	IL-20	NA	NA	NA	NA	-	NA	NA	NA
Il21.Rn01755623_m1	IL-21	NA	NA	NA	NA	NA	NA	NA	NA
Il23a.Rn00590334_g1	IL-23 α	NA	NA	NA	NA	+	NA	NA	NA
Il24.Rn00591159_m1	IL-24	NA	NA	NA	NA	NA	NA	NA	NA
Il27.Rn01510484_m1	IL-27	6.5	3.7	11.2	0.821	2446.0	1857.3	3221.5	0.026
Il3.Rn00580435_m1	IL-3	NA	NA	NA	NA	+	NA	NA	NA
Il33.Rn01759837_m1	IL-33	0.5	0.3	1.0	0.821	1.2	1.0	1.4	0.792
Il4.Rn01456866_m1	IL-4	NA	NA	NA	NA	+	NA	NA	NA
Il5.Rn01459975_m1	IL-5	NA	NA	NA	NA	+	NA	NA	NA
Il6.Rn00561420_m1	IL-6	1.3	0.1	23.5	0.983	18.6	1.3	266.0	NA
Il7.Rn00681900_m1	IL-7	1.2	0.5	3.2	0.923	0.5	0.3	0.8	NA
Il9.Rn01448718_m1	IL-9	NA	NA	NA	NA	NA	NA	NA	NA
Kitlg.Rn00442977_m1	Kitlg (KIT ligand, MCF, SCF stem cell factor)	0.8	0.2	2.9	0.974	0.2	0.1	0.5	0.073
Lif.Rn00573491_g1	LIF	1.4	0.3	5.9	0.923	23.0	21.6	24.5	0.095
LOC301289.Rn01757168_m1	LOC301289 (similar to IL-17 precursor (CTLA-8))	NA	NA	NA	NA	+	NA	NA	NA
Lta4h.Rn01503878_m1	Lta4h (Leukotriene A4 hydrolase)	1.2	0.9	1.6	0.821	1.0	0.8	1.4	0.884
Mif.Rn00821234_g1	MIF (macrophage migration inhibitory factor)	1.0	0.9	1.1	0.974	0.7	0.6	0.8	0.138
Ngf.Rn01533872_m1	NGF	1.1	0.7	1.6	0.983	2.0	0.6	7.0	NA
Nos2.Rn00561646_m1	iNOS	2.0	0.7	5.7	0.801	2244.0	1846.0	2728.0	0.0002
Nrg1.Rn00580917_m1	NRG1 (neuregulin 1)	0.7	0.2	2.0	0.923	0.2	0.2	0.4	NA
Nrg1.Rn01482165_m1	NRG1 (neuregulin 1)	0.0	NA	NA	NA	0.0	0.0	0.0	NA
Pf4.Rn01768298_g1	Pf4 (CXCL4, platelet factor 4)	1.0	0.8	1.3	0.974	0.8	0.6	1.1	0.553
Ppbbp.Rn00596603_g1	Ppbbp (CXCL7, pro-platelet basic protein)	NA	NA	NA	NA	-	NA	NA	NA
Prok2.Rn01421308_m1	Prok2 (Prokineticin)	NA	NA	NA	NA	-	NA	NA	NA
Ptges.Rn00572047_m1	Ptges (prostaglandin E synthase)	1.1	0.2	6.4	0.983	46.8	15.4	142.2	0.020
Ptgs2.Rn01483828_m1	Ptgs2 (COX2, prostaglandin-endoperoxide synthase 2)	0.4	0.2	1.2	0.923	57.2	43.1	76.0	0.307
Tnf.Rn99999017_m1	TNF- α	2.4	0.7	8.6	0.821	313.7	199.1	494.3	2.00E-05
Txlna.Rn01516136_m1	Txlna (α -Taxilin)	0.9	0.7	1.0	0.821	0.5	0.3	0.9	0.145
X18S.Hs99999901_s1	X18S (Eukaryotic 18S rRNA)	1.0	0.9	1.2	0.974	1.0	0.9	1.1	0.803
Xcl1.Rn00592605_m1	XCL1 (Lymphotactin)	1.9	0.4	8.9	0.801	5.2	0.6	43.2	0.351

The enormity and range of differences in gene expression between denatured-gp120_{Bal}-treated BMDM samples and gp120_{Bal}-treated BMDM samples is revealed in Figure 3.3.13. The largest alteration in mRNA expression in denatured-gp120_{Bal}-treated BMDM samples remains below 15 fold change relative to vehicle, and none of these change reach statistical significance. As shown in Table 3.3.2, in contrast, 2 nM gp120_{Bal} induced significant fold increases in the expression of 25 genes relative to vehicle-control, including iNOS (inducible nitric oxide synthase; 2240.0 fold change), IL-1 α (2016.3 fold change), IL- β (948.3 fold change), TNF- α (313.7 fold change), CCL2 (134.8 fold change), COX2 (57.2 fold change) and PGES (prostaglandin E synthase; 46.8 fold change). A further 11 genes showed increased expression but these did not reach statistical significance due to missing data values for one or two samples in the analysis, including IL-6 (18.6). Two genes were downregulated, KIT ligand (Kitlg, also known as stem cell factor; 0.2 fold change) and (Artn; 0.3 fold change) however only KIT ligand downregulation reached significance using the normal unadjusted p value statistical paradigm (p=0.037) but not when using the adjusted p value statistical paradigm (p=0.073). In addition Artn was downregulated to the same extent in both gp120_{Bal}⁻ and denatured gp120_{Bal}⁻ treated macrophages however rather than a drop in expression it was a drop in presence i.e. the samples went from 2/4 vehicle-treated samples showed detectable Artn mRNA to 0/4 in denatured gp120_{Bal}⁻ and gp120_{Bal}-treated samples. For this reason it was not possible to perform the same statistical test that was applied to the other genes in the array and therefore is given the term NA, Not Analysed, within (Table 3.3.2). In gp120_{Bal}-treated BMDM samples, 23/96 gene targets statistically showed no significant difference in expression, while 56/96 gene targets were unchanged in denatured-gp120_{Bal}-treated BMDM samples, both compared to vehicle control (Table 3.3.2). Interestingly among these unaltered gene targets were CCL11 (a Th1/M1 pro-inflammatory cytokine); IL-10, CCL1, CCL22, CCL24 and MIF (macrophage migration inhibitory factor) (Th2/ M2 anti-inflammatory cytokines); and CXCL12 (SDF-1, the endogenous ligand for HIV CXCR4 coreceptor, which demonstrates a predominant increase in pro-inflammatory cytokines and relatively unchanged anti-inflammatory cytokine expression. 28/96 gene targets in denatured-gp120_{Bal}-treated BMDM samples and 35/96 gene targets in gp120_{Bal}-treated BMDM samples were unable to be analysed statistically either due to missing sample replicate data points or complete lack of expression. Of the 35 targets that showed undetectable expression in some gp120-treated BMDM samples, 4 showed a trend towards upregulation through the detectable expression in 2/4 samples compared to 0/4 in vehicle- and denatured-gp120_{Bal}-treated BMDM samples. These 4 genes were IL-12 β (M1 macrophage released cytokine), CSF2 (granulocyte-macrophage colony stimulating factor, which controls the production, differentiation, and function of granulocytes and macrophage), IL-23 α (M1 macrophage released cytokine and the cytokine recently distinguished to

induce the distinct Th17 inflammatory phenotype in CD4+ T cells) and CCL17 (M2a macrophage released cytokine). Due to lack of expression in more than 1 out of 4 of the samples for each gene these targets was not processed using the statistical test employed for the array data. However use of the Mann-Whitney statistical test determined this detection of expression (2/4) to be not statistically significant from the complete lack of expression in vehicle-treated samples (0/4) likely due to low n number (statistics not shown). To support the suggestion that the gp120-mediated gene expression changes occurred via a biologically relevant and gp120-specific mechanism, it was found that 2 nM denatured-gp120_{Bal} induced no significant increase or decrease in the expression levels of any of the gene targets investigated compared to vehicle-treated BMDM samples.

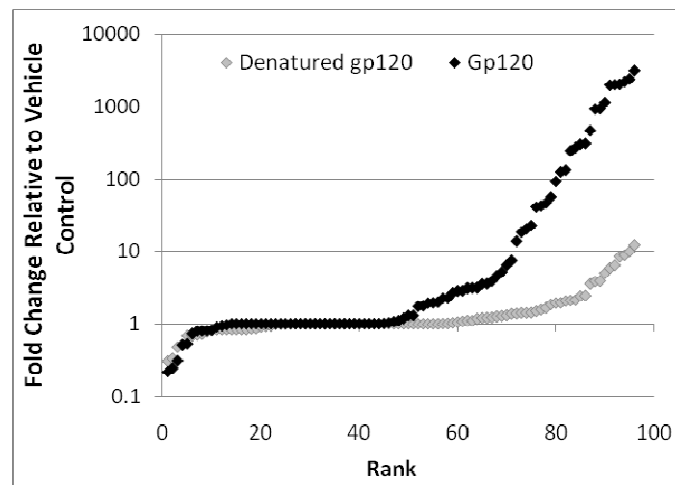


Figure 3.3.13) Fold changes (log scale) in mRNA expression in denatured-gp120- and gp120-treated BMDMs presented in rank order from smallest to largest. There was a much greater effect on gene expression after gp120 compared with denatured-gp120 where the highest fold change was <14.

Table 3.3.2) Cytokine array data: Mean $2^{-\Delta\Delta Ct}$ fold change (and $2^{-\Delta\Delta Ct}$ standard range) of all gene expression profiles affected by gp120_{Bal} exposure (n=4; two biological repeats with two technical replicates in each).

Target	Fold difference in den.gp120 relative to vehicle	Fold difference in gp120 relative to vehicle	p value
UPREGULATED			
CXCL11	5.9 (1.6 - 22.5)	3205.5 (2241.2 - 4583.4)	***
IL-27	6.5 (3.8 - 11.3)	2446.1 (1857.3 - 3221.5)	*
iNOS	2.0 (0.7 - 5.7)	2244.0 (1846 - 2728)	***
CXCL9	2.2 (0.9 - 5.2)	2082.9 (1525 - 2844)	***
CXCL2	10.0 (3.2 - 31.4)	2060.5 (1186.4 - 3578.6)	*
IL-1 α	8.9 (3.2 - 25.2)	2016.3 (1336 - 3043)	***
CCL12	12.4 (0.4 - 374.5)	1167.2 (1005 - 1355)	*
IL-1 β	8.6 (5.4 - 13.6)	948.3 (798.4 - 1126)	***
CXCL10	5.1 (2.2 - 11.4)	939.9 (737.5 - 1198)	***
CCL5/ RANTES	0.8 (0.2 - 3.1)	474.5 (342.2 - 657.9)	**
TNF α	2.4 (0.7 - 8.6)	313.7 (199.1 - 494.3)	***
CCL4	2.5 (1.0 - 6.2)	306.3 (205.7 - 456.1)	**
CCL20	1.7 (0.7 - 3.8)	276.6 (133.9 - 571.6)	***
CXCL1	3.9 (2.2 - 6.9)	248.0 (198.5 - 309.9)	***
CCL2	2.1 (1.0 - 4.4)	134.8 (107.9 - 168.4)	***
CCL3	1.4 (0.2 - 9.0)	127.1 (53.9 - 299.9)	**
CCL7	1.4 (0.8 - 2.5)	93.4 (81.8 - 106.6)	***
COX2	0.3 (0.1 - 0.9)	57.2 (43.1 - 76.0)	
PGES	1.1 (0.2 - 6.4)	46.8 (15.4 - 142.2)	*
IL-15	1.4 (0.7 - 2.8)	43.1 (30.1 - 61.7)	**
CX3CL1	1.6 (0.9 - 2.8)	41.4 (22.8 - 75.1)	***
LIF	1.4 (0.3 - 5.9)	23.0 (21.6 - 24.5)	
CXCL6	2.0 (0.8 - 4.7)	20.6 (5.3 - 80.9)	*
IL-6	1.3 (0.07 - 23.5)	18.6 (1.3 - 266.0)	
CCL19	0.8 (0.6 - 1.1)	14.0 (8.7 - 22.5)	**
CCL9	1.08 (0.4 - 2.2)	7.5 (4.3 - 13.0)	*
Ereg	1.3 (0.5 - 3.3)	6.5 (3.6 - 11.7)	
IL-12 α	no change	4.6 (1.1 - 19.5)	
CXCL13	0.9 (0.5 - 1.7)	3.2 (1.2 - 8.2)	
Edn1	0.9 (0.5 - 1.5)	3.1 (2.0 - 4.9)	
M-CSF1	1.2 (0.9 - 1.6)	2.9 (1.9 - 4.4)	*
IL-18	1.3 (0.6 - 2.6)	2.9 (1.9 - 4.3)	
CXCL16	1.1 (0.7 - 1.7)	2.3 (1.8 - 2.9)	*
IL-12 β	no change (0/4 detected)	increase (2/4: $\mu = 33.6 \pm 0.1$)	Δ
Csf2	no change (0/4 detected)	increase (2/4: $\mu = 33.8 \pm 0.1$)	Δ
IL-23 α	no change (0/4 detected)	increase (2/4: $\mu = 33.8 \pm 0.5$)	Δ
CCL17	increase (1/4: 34.91)	increase (2/4: $\mu = 34.7 \pm 0.6$)	Δ
DOWNREGULATED			
Kitlg	0.8 (0.2 - 2.9)	0.2 (0.1 - 0.5)	
Artn	0.3 (0.2 - 0.4)	0.3 (0.2 - 0.4)	

Data from 2 nM denatured gp120_{Bal} or 2 nM gp120_{Bal}-treated BMDMs after 4h compared to those tested with vehicle control (0.1% BSA/PBS) for 4h, all in serum-free conditions. Values were normalized to the mean of four housekeeping genes. * = level of significance and refer to gp120-treated fold changes only. *p<0.05; **p<0.01; ***p<0.001,. No fold changes for denatured-gp120 were statistically significant. Δ denotes cases

where expression was detected in more than 1/4 but less than 4/4 samples. This detectable expression was different than in samples from vehicle- and denatured gp120_{Bal}-treated BMDMs that were generally undetectable, however due to the reduction in sample number the same statistical test could not be performed. Instead a chi squared test can be performed however this finds that only if 3 or more of the 4 samples becomes detectable from 0/4 in vehicle-treated conditions, will this increase be considered statistically different at $p < 0.05$ level.

Some of the gene expression changes detected by the cytokine array, deemed to be of particular interest to my investigations into macrophage-associated neurotoxicity, were verified using individual quantitative PCR. Quantitative PCR was performed on all samples from the same cDNA with primer targets for iNOS, IL-1 β , TNF- α or COX-2 (Figure 3.3.14). Melt curves following qPCR cycling verified primer specificity to the target by recognition of a single melting point (Figure 3.3.15), detected by the loss of SYBR green fluorescence at the temperature that causes dissociation of all double-stranded PCR products and resultant unbinding of SYBR green. Interestingly, application of gp120_{Bal} but not the denatured form, or vehicle control, caused a rightward shift in the iNOS qPCR melt curve indicating an increase in the melting temperature of the iNOS primer product. This has never been reported before following gp120 exposure, however I found the same effect in primary cultured Schwann cells (Figure 3.3.19) and in *in vivo* plantar tissue (Figure 3.3.22), indicating that gp120 may activate common pathways in BMDMs and in Schwann cells, both *in vitro* and *in vivo*, which leads to modulation of iNOS gene splicing. iNOS PCR products were further validated on an electrophoresis gel, thereby additionally confirming specificity of the PCR product and induction in expression mediated specifically by gp120_{Bal} (Figure 3.3.16). All targets assessed verified the data obtained from the cytokine array and in most cases to a similar degree highlighting the robustness and validity of the technique and data respectively.

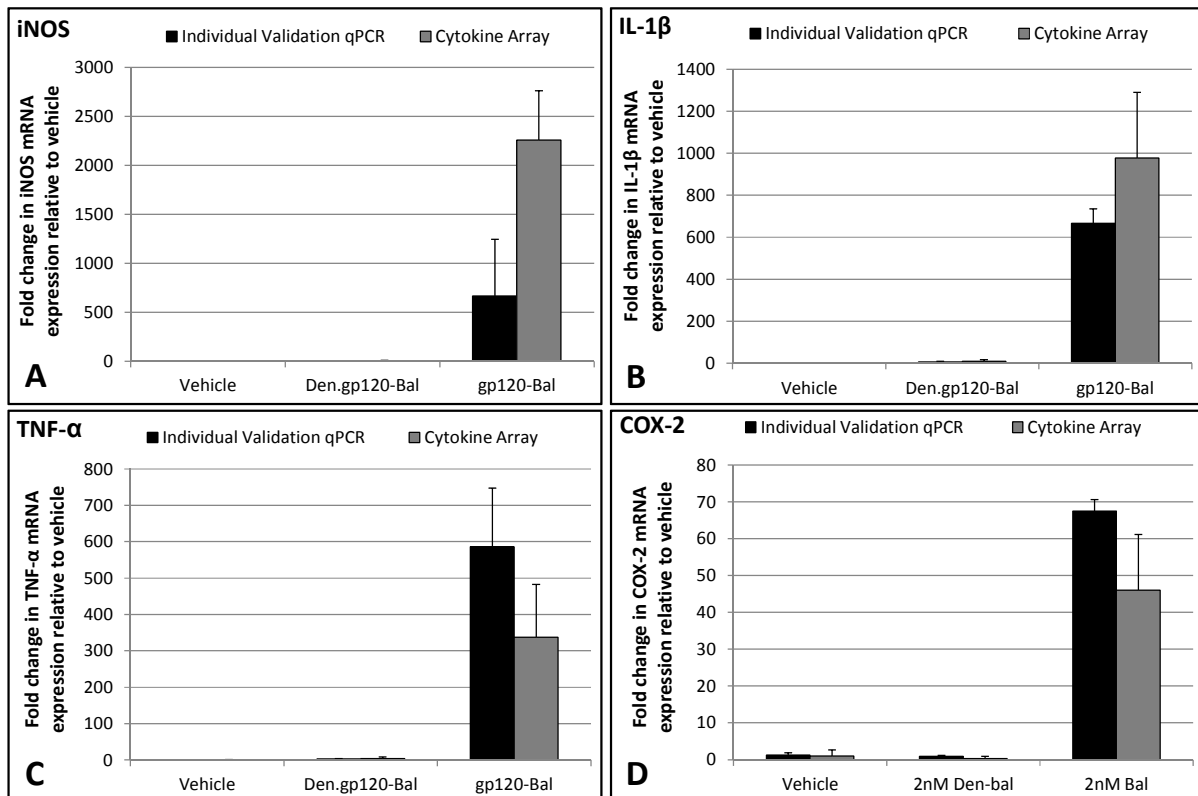


Figure 3.3.14) Validation qPCR on BMDM-derived cDNA. Primary BMDM cells were serum-deprived for 24h prior to stimulation for 4h with vehicle (0.1% BSA/PBS), 2 nM denatured gp120_{Bal}, or 2 nM gp120_{Bal} in 1 ml IMDM. Changes in mRNA gene expression relative to levels in vehicle-treated macrophages was assessed for (A) iNOS, (B) IL-1 β , (C) TNF- α and (D) COX-2 to confirm expression changes identified by the cytokine array. All qPCRs confirmed the effects of gp120 exposure identified by the cytokine array. In addition c-Jun gene expression was unaltered by gp120 exposure. Data presented as mean $2^{-\Delta\Delta Ct}$ fold change $\pm$ SEM (n=2, data from two samples of the four technical replicates used in the cytokine microarray).

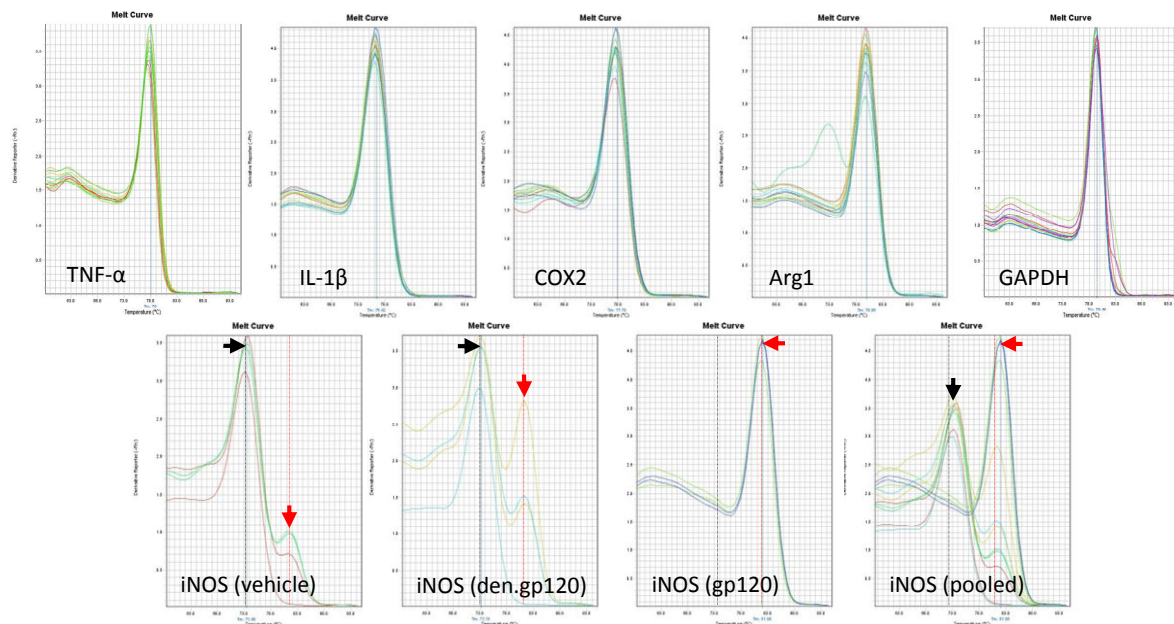


Figure 3.3.15) Representative melt curves following qPCR on BMDM-derived cDNA confirming specificity of the PCR products. All melt curves highlight specificity of all PCR products. The melt curves of iNOS PCR products following treatment, with either vehicle or denatured gp120_{Bal}, displayed no observable differences. Black arrowheads indicate the melting point recorded in unprimed vehicle- or denatured gp120_{Bal}-treated BMDM cells. In contrast gp120_{Bal} induced a highly specific increase in melting temperature of the iNOS PCR product (indicated by red arrowhead).

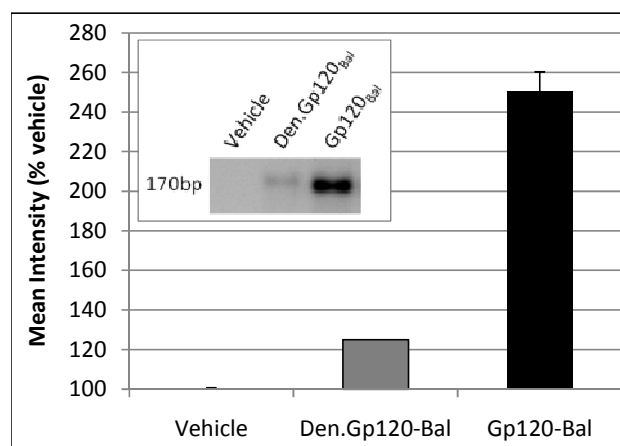


Figure 3.3.16) Gel electrophoresis validation of iNOS PCR product specificity and increase. Histogram displaying the mean intensity of ethidium bromide gel band, displayed in inset image, after running qPCR iNOS products on a 2.5% agarose gel by electrophoresis.

3.3.5. Differential responses of M1- and M2-type macrophages to gp120 exposure.

Macrophages are multi-functional, ever-changing, heterogeneous populations of cells that adapt quickly and drastically in response to the microenvironment around them. Macrophages have been broadly classified into two phenotypes; the 'classically activated' M1 and the 'alternatively activated' M2. In these phenotypes, macrophages express different receptors, produce and secrete different compounds and play very different functional roles. As macrophages have been highlighted to play a fundamental role in the pathogenesis of gp120-mediated neurotoxicity I aimed to investigate whether macrophage phenotype influenced the neurotoxic response to gp120.

Macrophage phenotypes can be broadly distinguished by the ratio of expression levels of inducible nitric oxide synthase (iNOS) and arginase-1 (Arg1). iNOS is an enzyme produced almost exclusively by monocytes and macrophages that facilitates nitric oxide production. In contrast, arginase 1 (Arg1), plays the antagonistic role to iNOS by enzymatically converting L-arginine into L-ornithine leading to collagen synthesis and reduced NO production. These enzymes are mutually exclusive in that if the expression levels of one are increased, the levels of the other are decreased. M1 macrophages produce high levels of iNOS and low levels of Arg1, while for M2 macrophages the opposite is true; they reduce iNOS levels and upregulate Arg1, hinting to their roles as neurotoxic/phagocytic and neuroprotective/wound-healing macrophages, respectively. Therefore an increase in iNOS expression levels, as in M1 macrophages, will be identified by qPCR, as a lower C_T value, and in turn will produce an increase in the normalised ΔC_T iNOS:Arg1, while a reduced iNOS:Arg1 ratio would be indicative of a M2 phenotype resulting from increased Arg1 and reduced iNOS. This ratio therefore can act as a useful marker for phenotypic switch. Macrophages switch to a particular phenotype in response to their microenvironment and particularly to the cytokines and signals present. M1 phenotypes can be induced by IFN- γ and LPS or TNF- α , in contrast IL-4, IL-10 or IL-13 are capable of inducing an M2 phenotype (Adams D.O. *et al.*, 1984; Somers *et al.*, 1989; Mantovani *et al.*, 2004). In these different phenotypes, macrophages alter receptor expression and up- or down-regulate specific chemokines and cytokine levels, both at a transcriptional and translational level, enabling them to take on often functionally opposing roles (Mills *et al.*, 2000; for review see Mantovani *et al.*, 2004). It is noted however that the M1 and M2 phenotypes are likely to represent just the two extremes of a wide spectrum of functional states. Therefore I aimed to determine how the polarization state of primary BMDMs affected their gp120-mediated responses and whether inducing an M2 phenotype, known for its neuroprotective abilities (Kigerl *et al.*, 2009), could attenuate this response. I assessed the relative expression levels of iNOS and Arg1 as markers for an M1 or M2 phenotype, respectively, in unprimed and 24h IL-4-primed macrophages, subsequently

stimulated for 4h with vehicle (0.1% BSA/PBS), 2 nM denatured gp120_{Bal} or 2 nM gp120_{Bal} in 1 ml IMDM (Figure 3.3.17). Denatured gp120_{Bal} did not alter the expression levels of either iNOS or Arg1, in either priming group, relative to vehicle-treated macrophages. In the unprimed macrophages, 2nM gp120_{Bal} induced a significant increase in iNOS expression (fold change: 7.0 ± 0.7) compared to vehicle-treated macrophages or those treated with denatured gp120_{Bal} (fold change: 0.2 ± 0.1). In contrast, the gp120_{Bal}-induced expression of iNOS, seen in unprimed macrophages, was significantly reduced in macrophages primed for 24h prior with IL-4 (fold change: 1.3 ± 1.0), almost to control levels. Furthermore significant increases in the mean basal gene expression of Arg1 (fold change: 38.8 ± 5.2) across all treatment groups of IL-4-primed macrophages compared to their unprimed macrophage equivalents (fold change: 1.0 ± 0.1). For clarity, these values were depicted as ΔC_T iNOS:Arg1 ratios and normalised to vehicle control in unprimed macrophages (Figure 3.3.17 C). This portrays the significantly increased ΔC_T iNOS:Arg1 ratios in IL-4 primed conditions, relative to unprimed, resulting from increased Arg1 expression in all cultures. In addition it illustrates the drop in ratio due to increased iNOS expression following gp120_{Bal} exposure in unprimed cultures, a drop that was lost in IL-4 primed cultures, to a significant level. This confirms that IL-4 priming was successful at inducing an M2 phenotype prior to treatment application and that exposure to gp120 was no longer able to switch macrophages to an M1 phenotype, although a subtle shift may be occurring. As previously shown application of gp120_{Bal} (Figure 3.3.17 D) and in addition IL-4 priming (Figure 3.3.17 E) alone caused a rightward shift in the melting temperature of the iNOS primer product indicating a possible splice variant.

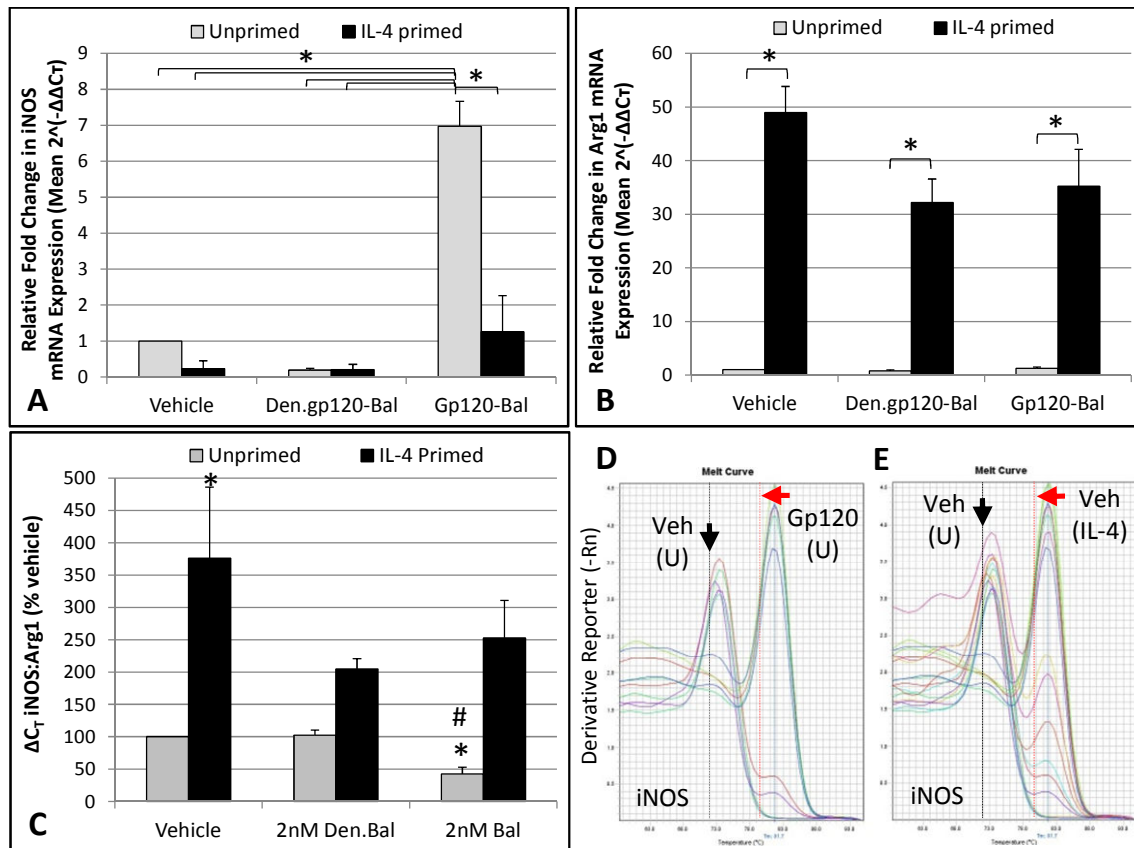


Figure 3.3.17) Relative expression levels of iNOS and Arginase1 in unprimed and IL-4 primed cultured bone-marrow derived macrophages. Primary BMDM cells were serum-deprived either in the absence or presence of IL-4 (5 ng/ml) for 24h prior to stimulation for 4h with vehicle (0.1% BSA/PBS), 2 nM denatured gp120_{Bal}, or 2 nM gp120_{Bal} in vehicle. iNOS (A) and Arg1 (arginase 1; B) mRNA expression changes in unprimed and IL-4-primed BMDM following treatment relative to the expression levels in unprimed vehicle-treated BMDMs (n=4). (C) The ΔC_T iNOS:Arg1 ratio was quantified, for each treatment in both unprimed and IL-4-primed BMDMs, from their relative qPCR ΔC_T values and presented as a percentage of the ΔC_T iNOS:Arg1 ratio in unprimed vehicle-treated BMDMs (n=4). Melt curves for iNOS primer products obtained following qPCR cycling with cDNA from vehicle- and gp120-treated unprimed BMDMs (D) and vehicle-treated IL-4-primed BMDMs only (E). Data presented as mean $2^{-\Delta\Delta Ct}$ fold change $\pm$ SEM or as a percentage of vehicle mean $2^{-\Delta\Delta Ct}$ (n=4 biological replicates, qPCRs performed in duplicate for each sample). Significance depicted by * relative to vehicle (unless stated otherwise) and # relative to denatured gp120_{Bal} at p<0.01 using two-factor ANOVA and Tukey's post-hoc analysis.

I then assessed how IL-4 priming affected the gp120-induced TNF- α expression seen previously in unprimed macrophages. I assessed the relative expression levels of TNF- α in unprimed and IL-4-primed (24h) macrophages following 4h treatment with vehicle (0.1% BSA/PBS in 1 ml IMDM), 2nM denatured gp120_{Bal} (in vehicle), or 2 nM gp120_{Bal} (in vehicle) (Figure 3.3.18). As previously seen, in unprimed macrophages, 2 nM gp120_{Bal} induced a significant and substantial increase in TNF- α

expression compared to vehicle-treated macrophages or those treated with denatured gp120_{Bal}. Incredibly, this potent response was drastically and significantly reduced in the IL-4-primed BMDMs. In fact, the response seen in gp120-treated IL-4 primed macrophages was no longer significantly different to that seen following treatment with vehicle control or denatured gp120_{Bal}. To ensure that the IL-4 priming condition itself was not altering the basal regulation of TNF- α transcription, the expression levels of vehicle-treated macrophages in unprimed and IL-4 primed conditions were compared. No significant difference was found (1.0 and 1.7 fold increase relative to unprimed, respectively; Figure 3.3.18 B) indicating that IL-4 priming did not alter the regulation or basal level of TNF- α transcription.

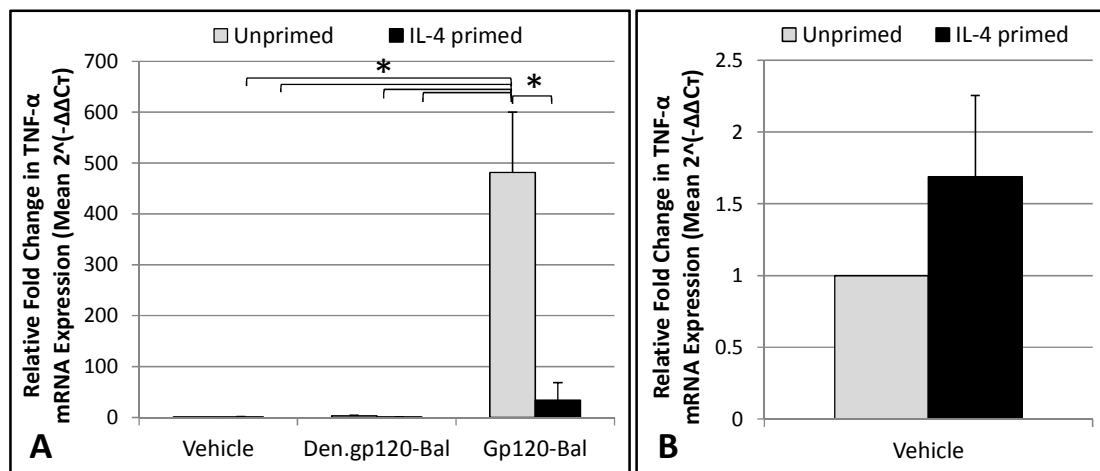


Figure 3.3.18) Relative expression levels of TNF- α mRNA expression in unprimed and IL-4-primed primary cultured bone marrow-derived macrophages (BMDM). Primary BMDM cells were serum-deprived either in the absence or presence of IL-4 (5 ng/ml) for 24h prior to stimulation for 4h with vehicle (0.1% BSA in 1 ml IMDM), 2 nM denatured gp120_{Bal}, or 2 nM gp120_{Bal} in 1 ml vehicle. (A) TNF- α mRNA expression changes in unprimed and IL-4-primed BMDM following treatment relative to the expression levels in vehicle-treated macrophages from the same priming conditions. * $p < 0.0025$ using two-way ANOVA and Tukey's post hoc analysis, $n = 3$ /treatment/priming condition. (B) TNF- α mRNA expression changes in unprimed and IL-4-primed vehicle-treated BMDM conditions relative to the expression levels in unprimed vehicle-treated macrophages. Data presented as mean $2^{-\Delta\Delta Ct}$ fold change $\pm$ SEM ($n = 3$ biological replicates, qPCRs performed in duplicate for each sample). $p = 0.174$ using a one-tailed unpaired student's t-test..

3.3.6. HIV-1 gp120 induces expression of cytokines in Schwann cells and the subsequent release of neurotoxic compounds.

Schwann cells are known to produce neuroactive substances in response to stimulation including MCP-1 (Karanth *et al.*, 2006), TNF- α (Wagner *et al.*, 1996), IL-1 (Bergsteinsdóttir *et al.*, 1991) and IL-6

(Bolin *et al.*, 1995). In line with this, gp120 interacts with Schwann cells expressing CXCR4 and CCR5 *in vitro* to cause the release of cytokines and chemokines including RANTES/CCL5, which can in turn affect neuronal function and survival (Keswani *et al.*, 2003; Melli *et al.*, 2006). My experiments on neuronal DRG cultures treated directly with gp120 indicated a large non-neuronal cell component may be important in mediating a global toxicity to the culture, including those that may be gp120-insensitive. I have shown that one non-neuronal cell type, macrophages, demonstrate potent gene modulation following exposure to gp120 therefore I was interested to perform a comparative study to determine whether primary cultured Schwann cells, the other key non-neuronal cell potentially involved in neurotoxicity, shows similar changes in gene expression. Unfortunately there was not enough time remaining to explore Schwann cell responses to the same depth as for macrophages therefore I focused on some of the key cytokines implicated in inflammation and shown to be upregulated in my BMDM cultures.

Primary adult Schwann cell cultures were established from *in vitro*-degenerated adult rat sciatic nerves and serum-deprived 24h prior to 4h treatment application with vehicle, 50 nM SDF-1 α , 5 ng/ml LPS, 2 nM denatured gp120_{MN} or 2 nM gp120_{MN} in serum-free conditions. LPS was used as a positive control for responsiveness of Schwann cells. LPS and gp120_{Bal} both significantly upregulated the gene expression levels of IL-1 β , TNF- α , and iNOS compared to all other treatments (Table 3.3.3). In addition LPS induced significant upregulation of IL-6 but changes to IL-6 following gp120_{Bal} did not reach significance. No significant upregulation was evident following incubation with denatured gp120_{Bal}, indicating that that gp120_{Bal} induced changes were biologically valid. Melt curves were performed immediately following qPCR cycling to verify primer specificity for its target (Figure 3.3.19). Intriguingly, and as observed in the BMDM cultures studies, application of gp120_{Bal}, but not the denatured form or vehicle control, caused a rightward shift in the iNOS melt curve indicating an increase in the melting temperature of the iNOS primer product. This has never been reported before but may indicate that gp120 directly or indirectly modulates splicing of the iNOS gene in primary cultured Schwann cells.

Table 3.3.3) Fold change (with standard errors), relative to vehicle-control, of gene expression profiles in primary Schwann cell cultures.

	Vehicle	50nM SDF	5ng/ml LPS	2nM MN	2nM den.Bal	2nM Bal
IL-1β	1.0 (0.98 - 1.02)	0.8 (0.4 - 1.7)	14792 ** (13111 - 16688)	3.5 (0.8 - 14.9)	1.3 (0.3 - 6.4)	1129 * (447 - 2851)
TNFα	1.0 (0.2 - 4.3)	0.9 (0.2 - 3.5)	503 ** (423 - 600)	1.00 (0.19 - 5.26)	0.73 (0.15 - 3.55)	23.7 ** (6.9 - 81.3)
IL-6	1.0 (0.6 - 1.6)	1.2 (0.6 - 2.4)	91.5 ** (80.9 - 103.4)	1.2 (0.6 - 2.4)	1.1 (0.6 - 2.1)	4.4 (0.9 - 22.1)
iNOS	1.0 (0.7 - 1.5)	0.3 (0.2 - 0.5)	892 ** (835 - 953)	0.5 (0.52 - 0.53)	0.5 (0.48 - 0.53)	24.4 * (4.0 - 148.6)

Primary Schwann cell cultures were incubated for 4h with vehicle (0.1% BSA/PBS in 1 ml DMEM), 50 nM SDF, 5 ng/ml LPS, 2 nM gp120_{MN} (MN), 2 nM denatured-gp120_{Bal} (den.Bal); 2 nM gp120_{Bal} (Bal). All treatments diluted in vehicle solution. Gene expression changes were assessed for IL-1 β , TNF- α , IL-6 and iNOS. Data presented as fold change increase relative to vehicle control with standard range reported in parentheses. Data presented as mean $2^{-\Delta\Delta C_t}$ fold change ($2^{-\Delta\Delta C_t}$ standard range) (n=4, technical replicates across two biological studies, qPCRs performed in duplicate for each sample). * denotes level of significance: *p<0.05, **p<0.01 using single factor ANOVA and Tukey's post-hoc analysis to assess significant changes within each primer target;

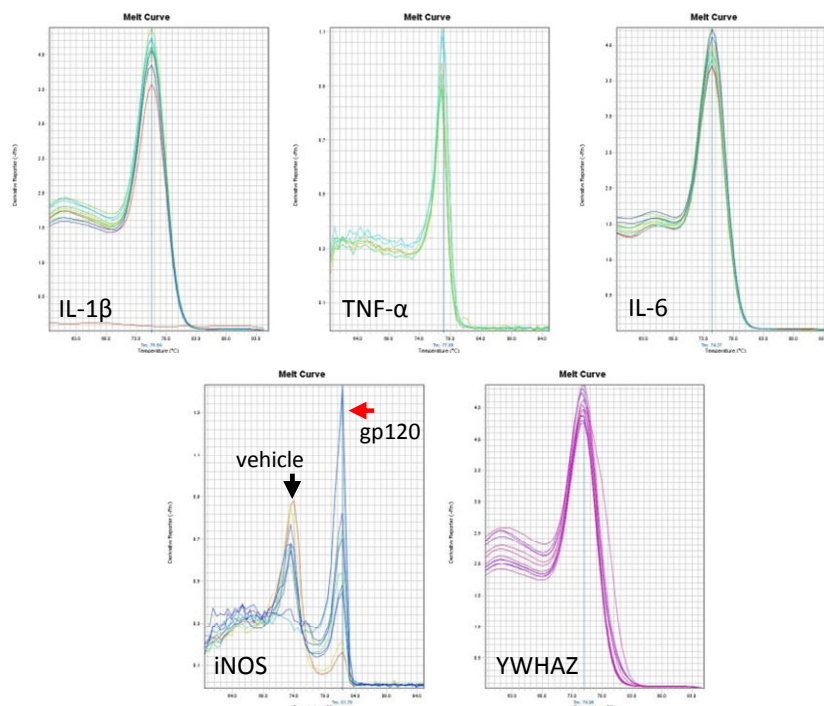


Figure 3.3.19) Representative melt curves following qPCR on Schwann cell culture-derived cDNA confirming specificity of the PCR products. Melt curves highlight specificity of all the PCR products. The melt curves of iNOS PCR products following treatment, with either vehicle or denatured gp120_{Bal}, displayed no observable differences. Black arrowheads indicate the melting point recorded for iNOS from unprimed vehicle- or

denatured gp120_{Bal}-treated BMDM cells. In contrast gp120_{Bal} induced a highly specific increase in melting temperature of the iNOS PCR product (indicated by red arrowhead).

These gene expression profiles in Schwann cells were obtained at the end of my thesis and unfortunately no additional time was available to pursue the relevance of these proteins in gp120-neurotoxicity or to extensively investigate the involvement of Schwann cells in gp120-mediated neurodegeneration. However at an earlier time point during my PhD, during the time I performed the studies presented in Chapter 2, I also performed some experiments to assess whether gp120_{MN} stimulates Schwann cells to release compounds that are toxic to primary DRG cultures. If more time was available I would have repeated these studies and instead of gp120_{MN} used gp120_{Bal}, which has since demonstrated to be more potent and more robust than gp120_{MN}.

To my knowledge at the time, all studies investigating the effects of gp120-stimulated Schwann cells on neurite degeneration had been performed on embryonic DRG cultures, which exhibit different receptor expression and neurophysiological properties to adult DRG cultures. As the majority of HIV patients are in the adult population I wanted to investigate the role of Schwann cells in this setting. Neurotoxicity was assessed in terms of the effects on neurite outgrowth. Primary Schwann cell cultures, were supplemented for 24h with either vehicle, 1 nM denatured gp120_{MN} or 1 nM gp120_{MN}. To identify how pre-incubation at 37°C affects gp120 biological activity, I incubated gp120_{MN} in a dish of DMEM media, in the absence of cells, for 24h in parallel with treated Schwann cell cultures. Adult DRG cultures were established 24h prior to testing and treated for 24h either directly with vehicle or 1 nM gp120_{MN} or indirectly through 1:1 media supplementation with the cell-free supernatant from 24h-treated Schwann cell cultures or DMEM-gp120 test media. There was no significant difference in neurite outgrowth between vehicle-treated cultures and those treated with the supernatant from denatured gp120_{MN}-treated Schwann cell cultures (Figure 3.3.20). In contrast direct application of 2 nM gp120_{MN} or treatment with gp120_{MN}-treated Schwann cell culture media both induced a significant reduction in the extent of neurite outgrowth compared to vehicle-treated control cultures. Application of media that had contained gp120_{MN} but in the absence of cells induced no changes in neurite growth. This implies that, following 24h at 37°C, gp120, being an unstable compound, becomes sufficiently denatured to lose its ability to bind and activate chemokines receptors, at least in a way that induces neurotoxicity. There was no difference in the density of non-neuronal cells present between vehicle-treated cultures and those treated with gp120_{MN} or Schwann cell conditioned media (Figure 3.3.20 F). However addition of gp120_{MN} cell-free media to DRG cultures resulted in a significantly lower density of non-neuronal cells compared to those

supplemented with denatured gp120_{MN}-treated Schwann cell-conditioned media, most likely due to the possibility of carryover of free-floating cells in the Schwann cell culture media.

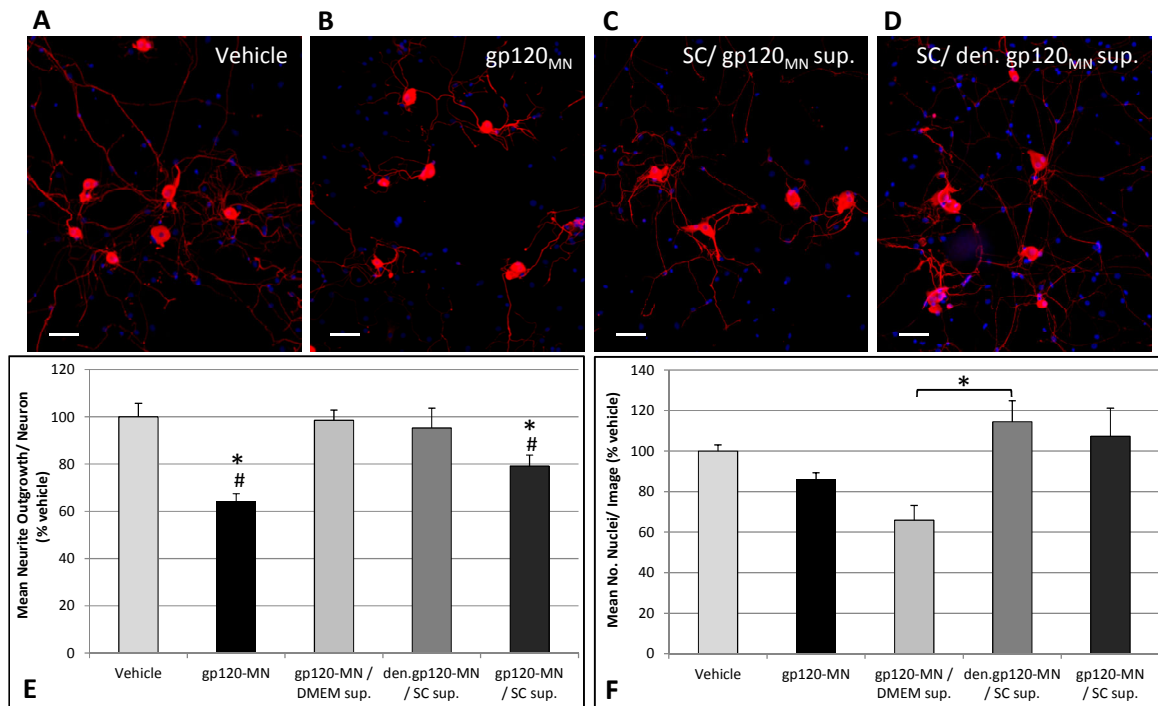


Figure 3.3.20) Neurite analysis following exposure to conditioned Schwann cell media. (A-D) Representative images of primary mixed DRG-glia cultures immunostained for β III-tubulin (red) and nuclei counterstained with Hoescht 32258. 1 day old DRG cultures were treated for 24h either directly with (A) vehicle (0.1% BSA/PBS in 500 μ l DMEM) or (B) gp120_{MN} (2 nM in vehicle) or indirectly either with cell-free culture media containing gp120_{MN} or with the supernatant (sup) from Schwann cell cultures treated for 24h prior with (C) gp120_{MN} (1 nM in vehicle; SC/gp120_{MN} sup.) or (D) denatured gp120_{MN} (1 nM in vehicle; SC/den.gp120_{MN} sup.). Scale bar = 50 μ m. (E) DRG cultures treated directly with gp120_{MN} or indirectly with culture media supernatant from gp120_{MN}-treated Schwann cells showed a significant reduction in mean neurite outgrowth per neuron, as a percentage of vehicle-treated cultures. (F) A significant difference in the density of nuclei occurred only between cultures supplemented with gp120_{MN}-cell free media and those treated with denatured gp120_{MN}-treated Schwann cell supernatant. * $p < 0.05$ vs. vehicle-treated cultures; # $p < 0.05$ vs. gp120_{MN}-treated culture media in the absence of cells, using single factor ANOVA and Tukey's post hoc analysis; $n = 4-6$ (data from 4-6 technical replicates across two biological studies).

3.3.7. Gp120 induces cytokine expression changes *in vivo* following intraplantar injection.

Chronic pain is a common phenotype that accompanies HIV patients with peripheral neuropathy. Numerous studies now provide consistent data demonstrating the induction of hypersensitivity

behaviour in animals exposed to HIV-1 gp120. A frequently cited model sees intrathecal administration of gp120 induce potent microglial and astrocyte activation and consistent pain-like behaviour in rodents that is associated with release of TNF- α , IL-1 β and IL-6 (Ledebor *et al.*, 2005; Schoeniger-Skinner *et al.*, 2007; Loram *et al.*, 2010). Furthermore peripheral application of gp120 delivered either by intraplantar injection (Oh *et al.*, 2001) or perineurally (Herzberg *et al.*, 2001; Wallace *et al.*, 2007a) induced acute and persistent mechanical hypersensitivity, respectively, in the ipsilateral paws of gp120-treated rats. Perineural application has also been associated with axonal swelling, increased TNF- α production within the nerve trunk and intense spinal pathology including astro- and microgliosis (Herzberg *et al.*, 2001; Wallace *et al.*, 2007a). Hypersensitivity following intradermal injection of gp120_{IIIIB} was demonstrated to appear precipitously reaching greater than 50% reduction in paw withdrawal threshold within 10 minutes of injection. The small amounts injected make it unlikely that gp120 was able to reach and activate spinally located cells therefore this is suggestive of a local mechanism of action. My data so far has demonstrated that gp120_{Bal} can intensely activate bone-marrow derived macrophages and result in the upregulation of a vast array of pro-inflammatory cytokines. Many of the upregulated pro-inflammatory cytokines identified in gp120-treated BMDMs have been demonstrated to have allogenic abilities. Therefore to validate that the results I found *in vitro* and the culture models I tested were biologically relevant and to implicate the cytokines in the gp120-mediated hypersensitivity, I examined cytokine expression at the site of injection following intraplantar injection of gp120_{Bal}.

Melt curves were performed immediately following qPCR cycling to verify primer specificity for its target (Figure 3.3.22). Intriguingly, and as also observed in the BMDM and Schwann cell cultures studies, injection of gp120_{Bal}, caused a rightward shift in the iNOS melt curve indicating an increase in the melting temperature of the iNOS primer product. The melt curves of iNOS PCR products in naive untreated tissue samples and those from the contralateral hindpaw of treated animals gave an iNOS T_m melting temperature of 73.6°C similar to that observed in vehicle-treated BMDM and Schwann cell culture studies. However interestingly in these *in vivo* studies, two melting points peaks were also evident following iNOS qPCR on denatured-gp120_{Bal} derived tissue samples, which was less prominent in the *in vitro* cultures. Gp120_{Bal} iNOS T_m peaks showed just one distinguished melting point however 2 samples did not demonstrate the rightward shift, which may be suggestive of poor delivery of, or activation by, gp120.

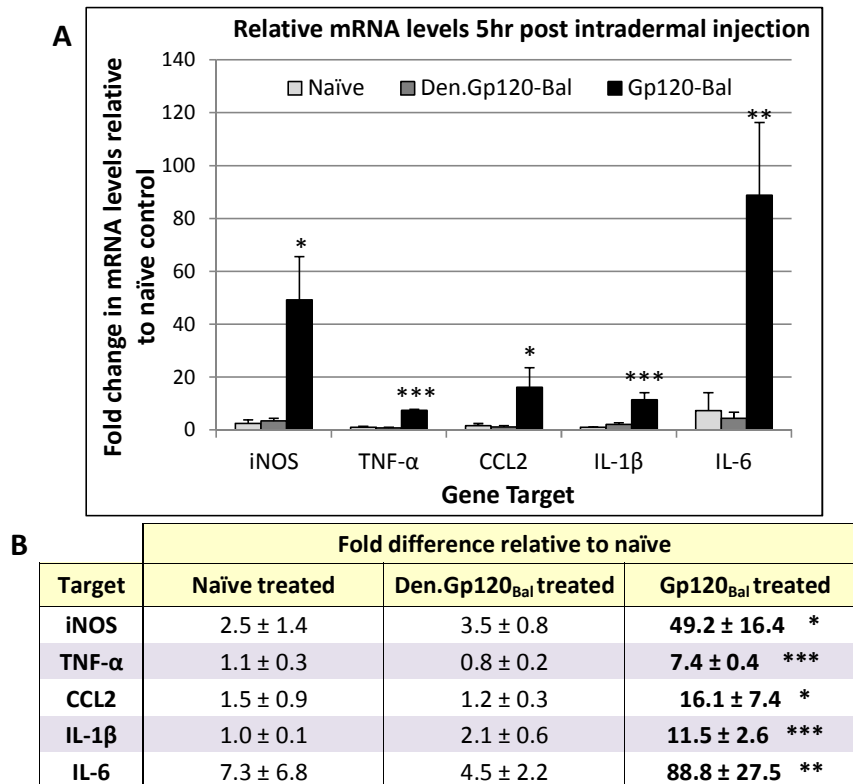


Figure 3.3.21) Fold change (mean ± SEM) of key pro-inflammatory cytokines in ipsilateral epidermal plantar tissue 4h following intradermal injection. Ipsilateral plantar paws were intradermally injected with 20 µl containing either 400 ng denatured-gp120_{Bal} (Sham) or 400 ng gp120_{Bal}. * p<0.05, ** p<0.01, ***< 0.0025 determined using one-way ANOVA with Tukey's post-hoc analysis (n=4; cDNA from four animals, qPCR performed in duplicate for each sample).

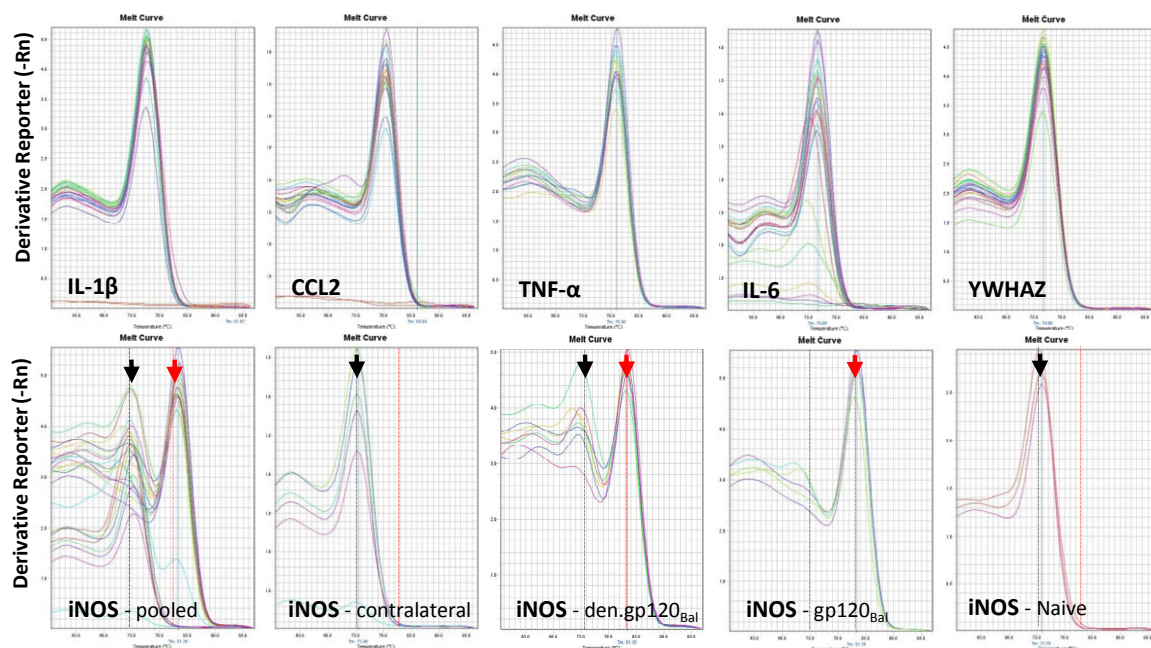


Figure 3.3.22) Representative melt curves following qPCR on ipsilateral intraplantar tissue samples confirming specificity of the PCR products. Melt curves highlight specificity of all the PCR products. Black

arrowheads indicate the melting point recorded for iNOS from unprimed vehicle- or denatured gp120_{Bal}-treated BMDM cells. In contrast both the denatured-gp120_{Bal} and gp120_{Bal} treated tissue samples induced a highly specific increase in melting temperature of the iNOS PCR product (indicated by red arrowhead), demonstrated as a single peak in gp120_{Bal} samples and two peaks (one normal and one shifted) in denatured-gp120_{Bal} samples.

3.4. Discussion

3.5.1. HIV-1 gp120 induces expression of pro-inflammatory cytokines in primary bone-marrow derived macrophages.

I found previously that application of gp120_{MN} resulted in a reduction of the mean neurite outgrowth per neuron in a mixed DRG neuronal-glial culture within 24h. However, only a minority of neuronal cells demonstrated gp120 binding, raising the question of how a global reduction in outgrowth, affecting the whole neuronal population, transpired. These data indicated that a direct mechanism of action of gp120 on neuronal cell is unlikely to be the leading pathway involved and that non-neuronal cells may be required. HIV-1, along with many other lentiviruses, infect cells of monocyte/macrophage lineage (Clements *et al.*, 1996; Miller *et al.*, 2000; Clapham *et al.*, 2002), only evolving to additionally infect CD4⁺ T-cells most likely as a result of host adaptation (Kuroda, 2010). The different HIV strains (M-tropic or R5 vs. T-tropic or X4) specifically target and infect certain cell types via the selectivity of gp120 to one or both of the CCR5 or CXCR4 chemokine coreceptors. Macrophages are the primary target for M-tropic CCR5-targeting HIV strains via the CCR5 receptor and, unlike other infectable cells, appear resistant to HIV-mediated apoptosis (Geleziunas *et al.*, 2001; Pinti *et al.*, 2003; Swingle *et al.*, 2007), thus they can act as a maintained reservoir and continuous producer of HIV virions. Macrophages are known for their phagocytic properties and ability to release a diversity of pro-inflammatory mediators (Mosser *et al.*, 2008), therefore, joint with their role as a target for and producer of HIV and their ability to cross the blood brain barrier (BBB) and deseminates the virus, they became one of the first cells deemed responsible for HIV pathogenesis and associated pain (Herbein *et al.*, 2010). Supportive of this, Liu *et al.* were able to correlate the alleviation of thermal hyperalgesia with macrophage depletion in injured nerves (Liu *et al.*, 2006). Soluble gp120 can be shed from viruses allowing monomeric free-floating gp120 to have access to macrophages present in the blood. As the damage occurring to neurons is local and if mediated by non-neuronal cells then this would include tissue-resident macrophages. However, despite the large number of studies committed to this topic, a specific mechanism and the relative involvement of macrophages in HIV-1 gp120-mediated axonal degeneration and development of HIV painful peripheral neuropathies still

remains elusive. Some studies report a neuroprotective effect of monocytes in HIV infection (Tong *et al.*, 2000; Gras *et al.*, 2003) while others a deleterious role (Lee B *et al.*, 1999; reviewed by Herbein *et al.*, 2010). This is most likely due to the heterogeneity of macrophages, which can differ between tissue locations and vary depending on the cues and compounds they are exposed to in their microenvironment. Use of different experimental models, different HIV viral strains and a lack of characterisation of the macrophage phenotype and population tested, makes interpretation of contradictory data difficult. Therefore it still remains to be determined which role (neurotoxic or neuroprotective) is predominant in the context of HIV and, if both, when, why and how does this switch in function occur.

Therefore, I used rat bone marrow-derived macrophages (BMDMs) as an *in vitro* model of resident macrophages that is considered to closely mimic the clinical setting. I decided against using peritoneal-derived macrophages because their functionality *in vivo* is to retain their host defences while simultaneously promoting the absence of inflammation to prevent oversensitisation in their typical intestinal environment, enriched with presence of intestinal flora (Morland *et al.*, 1984; Nicaise *et al.*, 1993). I therefore considered that the responses of peritoneal macrophages may be misleading and non-representative of the general immune response in HIV patients.

Thus, using BMDMs, I aimed to clarify the responses to gp120 exposure, the phenotypes involved and the relative contribution to gp120-associated neurite degeneration. I used the monoclonal mouse antibody against rat CD68 glycoprotein, also known as the ED1 clone (Millipore, #MAB1435), to identify the macrophage population. The ED1 clone antibody recognises a single chain glycoprotein of 90–100 kDa that is expressed on the lysosomal membrane of myeloid cells (Dijkstra *et al.*, 1985; Damoiseaux *et al.*, 1994) although weak cell surface expression can also occur. Some studies have reported that CD68 expression may not be restricted to cells of macrophage lineage as expression has also been identified by flow cytometry, RT-PCR and immunohistochemistry in fibroblasts derived from normal skin and breast cancer tissue (Kunz-Schughart *et al.*, 2003). However, further studies have clarified that while other cell types are only seldomly labelled (Damoiseaux *et al.*, 1994) all macrophages are targeted allowing it to be now widely used as a macrophage specific marker. High levels of ED-1 expression have been associated with macrophage identification and activated microglia while low level expression indicates quiescent microglia (Graeber *et al.*, 1990; Slepko *et al.*, 1996; Kingham *et al.*, 1999). Therefore, given that I was utilising primary cultured BMDMs, which gave considerably homogeneity in cell-type, I concluded that the ED-1 antibody would be appropriate to quantify the macrophage population.

Cultures obtained were approximately 96% ED1+ve macrophages indicating a relatively pure culture. The identity of the ED1 negative population was not explored immunologically although, morphological identification of a faint flattened spindle-shaped cell body using phase contrast microscopy, suggested they comprised mainly of fibroblasts. Of importance though, the non-macrophage population showed no CCR5 or CXCR4 expression, which was restricted only to the macrophage ED1 +ve cells, therefore I hypothesised that, to my knowledge, the ED1 –ve cells would not contribute to the gp120-mediated responses assessed in terms of modulation of cytokine expression. This is supported by studies that report recombinant gp120 does not bind to rat fibroblasts (Apostolski *et al.*, 1993).

I then aimed to explore the changes in gene expression levels of important cytokines and chemokines within BMDMs following acute gp120 application using qPCR as a high-throughput but sensitive assay. My initial readout was the induction of TNF- α gene expression because this is frequently induced in association with macrophage activation. Due to the simplicity and sensitivity of the qPCR technique this was considered a useful assay for comparing the relative effects of different gp120 tropic strains and chemokine receptor ligands on BMDMs. I showed that gp120_{Bal} induced significant upregulation of TNF- α expression after 4h exposure. In contrast, the two other gp120 strains tested, gp120_{MN} and gp120_{CN54}, induced no significant changes to TNF- α gene expression. I chose an incubation time of 4h based on data from previous studies that demonstrated LPS-induced TNF- α expression in cultured macrophages peaked within 3–6h (Sariban *et al.*, 1988; Zuckerman *et al.*, 1991; Lee *et al.*, 2005; Wang *et al.*, 2010b). As shown by the responses following LPS and gp120_{Bal} stimulation, this time point was more than sufficient to induce TNF- α gene expression. I also investigated TNF- α mRNA levels following 24h incubation with 2 nM gp120_{Bal} and 5 μ g/ml LPS as a positive control, however at this later time point expression levels were in fact lower than control levels (LPS-induced mean fold change: 1.0, 0.8–1.2 standard range; gp120-induced mean fold change: 0.2, 0.1–0.6 standard range) indicating only an acute and transient response to acute gp120 exposure (data not shown). The lack of effect after 4h with gp120_{MN} and gp120_{CN54} are therefore unlikely to be a result of insufficient incubation time. Gp120_{MN} is a dual-tropic strain targeting both CXCR4 and CCR5. In fact I show that, in the BMDM cultures, the predominate coreceptor expression was for CXCR4, with $76.7 \pm 0.1\%$ of macrophages showing positive immunostaining, while there was significantly less for CCR5 ($43.9 \pm 3.3\%$ CCR5+ macrophages). This raises the question as to why no gene expression changes were seen; if both coreceptors are present and especially considering that gp120_{MN} induced significant affects to neurite outgrowth in cultured DRG neurons, potentially via a non-neuronally-mediated pathway. One possible explanation may be offered by the different expression systems used to produce these glycoproteins. As, previously stipulated, gp120 is one of

the most heavily glycosylated proteins in nature (Geyer *et al.*, 1988; Mizuochi *et al.*, 1988). As glycosylation complexity has evolved through the kingdoms of the biological system (Drickamer *et al.*, 1998; Dell *et al.*, 2010), it is important to remember that the expression system used to produce proteins will result in glycosylation variation and may significantly impact its biological activity (Raju, 2003; Kong *et al.*, 2010). Gp120_{MN} and gp120_{CN54} were both produced in a baculovirus expression system, which tends to produce glycoproteins with low-mannose core oligosaccharides due to partial trimming of mannose receptors as it is exported (Dirckx *et al.*, 1990). In contrast, gp120_{Bal} was produced using a HEK293T mammalian expression system allowing the addition of N-linked glycans of the complex-type and the remainder being of the high-mannose or hybrid types (Geyer *et al.*, 1988; Mizuochi *et al.*, 1988; Raska *et al.*, 2010). This will have fundamental effects on their relative abilities to bind to CD4 and consequently their coreceptors (Li *et al.*, 1993; Kong *et al.*, 2010), which relies heavily on correct protein folding and is determined by the selectivity of the V3-4 variable loop of gp120 (Wyatt *et al.*, 1995; Cardozo *et al.*, 2007); an area flanked by important, conserved N-linked glycosylation sites (Borbe *et al.*, 1995; Kock M *et al.*, 2003; Sirios *et al.*, 2007). The apparent differential responses between neurons and macrophages to gp120_{MN} may also be explained by the differences in the receptors utilised. Neurons do not express the CD4 receptor therefore gp120-mediated responses act via CXCR4 or CCR5 independently of the CD4 receptor (Hesselgesser *et al.*, 1997). In contrast, macrophages, like other immune cells, express CD4 to which gp120 will bind in the first instance followed by subsequent binding to the chemokine coreceptors following induction of a conformational change in gp120 protein structure. Therefore I have speculated whether differences in glycosylation may affect the more complex binding of gp120 on immune cells, while having little or less influence on neuronal binding.

Alternatively, another explanation may simply be offered by the comparative timings of the experiments. The DRG culture neurite outgrowth studies with gp120_{MN} were completed a couple of years before I obtained gp120_{Bal} and began acquiring this qPCR data. The instability of gp120 is frequently reported and its biological activity has been demonstrated to diminish over time (Papandreou *et al.*, 1996), even when stored at -80°C, therefore this lack of effect may simply be a consequence of the loss of biological activity of the glycoprotein over time. At the time of these experiments, gp120_{Bal} had been recently received while gp120_{MN} and gp120_{CN54} had been stored at -80°C for more than 9 months. Furthermore, I demonstrated that the biological effects of gp120_{MN} appeared to be lost after incubation at 37°C for 24h (Results 3.3.6). The activity of newly purchased gp120_{MN} and gp120_{CN54} would need to be compared against the newly acquired gp120_{Bal} to ensure these effects were not due to biological inactivation.

In my analysis of the qPCR runs, I use the comparative C_T method, also known as the $\Delta\Delta C_T$ method, which uses an arithmetic formula to determine relative changes in mRNA expression changes. This was as a substitute for the traditional relative standard curve method that determines cDNA expression levels through interpolating a sample value from a standard curve obtained through dilution of a calibrating control target. Unlike the standard curve method, the $\Delta\Delta C_T$ method allows for a variety of different gene targets to be assessed at the same time and requires smaller quantities of cDNA template, making it more of an efficient high-throughput method. The only prerequisite for this method to be valid is that the PCR efficiencies of the primers tested are approximately equal to that for the house-keeping gene. This relates to the main disadvantage of the $\Delta\Delta C_T$ method in that any differences in PCR efficiency between primers will decrease the accuracy of the calculated fold changes, which can greatly affect the interpretation of results when only small fold changes are observed (i.e. 2-fold, 5-fold). However, where large fold changes are observed (i.e. 100-fold, 1000-fold), as was the case in the majority of my experiments, variation in primer efficiency is unlikely to greatly affect the interpretation of the results. As many of my validation experiments and expression comparisons depended on investigating expression changes in TNF- α mRNA, I considered it to be critical to confirm that my TNF- α primer had a similar amplification efficiency to the house-keeping gene, GAPDH, against which it was normalised. This validation experiment confirmed approximately equal efficiencies for these two primers, therefore confirming the validity of my qPCR experiments and supporting the interpretation of subsequent experiments. I did not perform validation experiments for the other cytokine and chemokine targets assessed because, given that the expression changes observed were commonly greater than 100-fold, I concluded that any dissimilarities in primer efficiency were unlikely to be the main cause of these observed results. I therefore interpreted them to be representative of a specific consequence of the treatment applied.

These studies tested M-tropic gp120_{Bal}, which is reported to target and activate cells via the G-protein coupled chemokine receptor, CCR5 (Weissman *et al.*, 1997). I show that gp120_{Bal}-induced graded responses in TNF- α expression over a broad range of concentrations. It has been previously reported that gp120 stimulation of macrophages via CCR5 results in Pyk2 phosphorylation and activation of JNK/SAPK and p38 MAPK pathways through a $G\alpha_i$ (G inhibitory protein)-independent pathway (Lee *et al.*, 2003). However, further studies have indicated that CCR5 can also be coupled with $G\alpha_{q/11}$ (G other protein), $G\alpha_s$ (G stimulatory protein) and $G\alpha_i$ pathways (Arai *et al.*, 1996; Kuang *et al.*, 1996; Lin *et al.*, 2006; Harmon *et al.*, 2008). Activation of CCR5, via $G\alpha_{q/11}$ coupling, leads to increased $[Ca^{2+}]_i$ levels through Ca^{2+} channel activation or PIP2 activation, IP3 and DAG production and subsequent Ca^{2+} release from intracellular stores. In contrast, CCR5 $G\alpha_i$ signalling is involved in

MAPK activation and ERK signalling (Yang *et al.*, 1999). Signalling downstream of Gα_i recruitment has been involved in the regulation of HIV-1 infectivity at various levels and may be important for amplifying viral replication post-entry (Lin *et al.*, 2006). The graded concentration-response observed therefore may result either from increased binding to currently free CCR5 receptors or enhanced recruitment or activation within these intracellular signalling pathways.

To confirm whether gp120_{Bal} induced gene expression changes in BMDMs via the CCR5 receptor I investigated whether these changes could be inhibited using the CCR5 antagonist, Maraviroc. Maraviroc has been demonstrated to act as a specific and potent antagonist at the CCR5 receptor (Yost *et al.*, 2009) with the ability to inhibit 90% of viral growth at a mean IC₉₀ of 2 nM (Dorr *et al.*, 2005). Even at the lowest concentration I tested (1 nM) Maraviroc almost completely ablated the gp120-induced gene expression of TNF-α. This supports the hypothesis that gp120_{Bal} acts specifically and primarily via binding with CCR5. Obviously this does not determine whether secondary binding with alternative coreceptors is required, however CCR5 binding is clearly an essential step. Expression levels induced by 1 nM gp120_{Bal} in the presence of 10 nM and 100 nM Maraviroc matched those induced by 1 nM denatured gp120_{Bal} in the presence and absence of Maraviroc. Application of 10 nM Maraviroc, in the absence of gp120, induced no changes to TNF-α gene expression. Taken together this suggests that the subtle increase in expression after application of denatured gp120_{Bal} is mediated independently of CCR5 and may represent an antigenic response of macrophages to the denatured but highly glycosylated foreign glycoprotein.

Confirmation of CCR5 signalling does raise the question of why no induction in TNF-α expression was initiated following treatment with the endogenous CCR5 ligand, RANTES. To my knowledge the effects of RANTES on gene expression in BMDMs has not been previously investigated therefore these results may indicate functional differences in CCR5 activation induced by RANTES and gp120_{Bal}. However studies comparing the alternative functional affects of RANTES with those induced by CCR5-using gp120 and HIV, have observed similar functional effects via CCR5 signaling in different measurable outcomes. In neonatal and adult DRG cultures, R5-tropic gp120 (*in vitro*: 200 pM; *in vivo*: 250 g) and CCL5 (*in vitro*: 50 nM; *in vivo*: 250 g) both activated calcium signalling pathways (Bolin *et al.*, 1998; Oh *et al.*, 2001) and induced excitatory responses in neurons (Oh *et al.*, 2001). These effects were also observed *in vivo* as enhanced mechanical sensitivity following intradermal injection of gp120 (Oh *et al.*, 2001). I used a rat specific recombinant chemokine therefore the lack of effect was unlikely to be due to lack of receptor specificity. There are numerous explanations for this outcome, firstly, it may be that insufficiently high concentrations were used and that higher levels were necessary to bind or activate the receptor. I based the selection of the chemokine final

concentrations (50 nM) on those used, shown to be effective, by Oh *et al.*, Kaul *et al.* and Keswani *et al.*, in activating intracellular signalling and inducing calcium influxes. In my own studies assessing calcium mobilisation, admittedly I did not see significant increases in $[Ca^{2+}]_i$ until much higher concentrations (200 nM), similar to concentrations used by Signoret *et al.* However an important difference is that my studies tested adult-derived cultures while those cited previously were embryonic or neonatal. Therefore different expression levels of CCR5 may explain these differences and higher concentrations of RANTES on adult cultures may be required to induce the same responses observed in embryonic or neonatal cultures.

Despite studies showing similar signalling pathways between CCR5 ligands and gp120, more evidence in fact supports differential signalling of RANTES and gp120, such as the work of Kaul *et al.* who found that CCL5 provided neuroprotection against gp120-induced neurotoxicity (Kaul *et al.*, 2007). Therefore the lack of cytokine gene expression by RANTES in my study may, in fact, be an indication of how RANTES may bind and activate the CCR5 receptor but induce signalling pathways that do not lead to the same, and potentially neurotoxic, effects induced by gp120. It remains unclear exactly how gp120 recruits such different intracellular signalling via the same receptor however it is hypothesised that the CCR5-binding site of gp120 and RANTES overlap but are not identical (Wu *et al.*, 1997). The differential binding, afforded by the glycosylation of gp120, may allow for different G proteins to be recruited and activated, allowing different pathways to be established. In addition it is noted that while CCR5 ligands act on CCR5 receptors without binding to an additional receptor; gp120, on the other hand, first binds to the CD4 receptor. This induces the necessary conformational change in gp120 that exposes the coreceptor binding sites allowing it to bind with higher affinity to the chemokine coreceptors. This creates a ligand-receptor tricomplex compared to the dual-component complex of RANTES with CCR5, thus potentially allowing a different combination of signals to be activated, including those activated by CD4. In the absence of CD4 receptors, as on neuronal cells, gp120 has shown capacity to bind independently to CXCR4 and CCR5 (Hesselgesser *et al.*, 1997). Whether, in this case, similar or differential signalling patterns are activated remains controversial. Either way, modulation through differential extracellular binding, prior to internalisation, is the most likely explanation. Potential for differential modulation of intracellular signalling, post-internalisation, is limited by a strict pathway that endocytoses the glycoprotein and packages it for degradation in lysosomal compartments without causing toxicity (Kozak *et al.*, 1999; Lassot *et al.*, 2007; Trujillo *et al.*, 2007).

Serum-deprivation is commonly performed by researchers to induce what is often referred to as a 'quiescent' state in microglia and macrophages. Quiescent cells are often used as they are

considered a more representative model of the *in vivo* setting. I found that 24h serum-deprivation prior to 4h treatment incubation significantly enhanced the degree of gp120-induced TNF- α expression, compared to when serum was present. The importance of serum factors for the maintenance of primary cells has been well established (Honn *et al.*, 1975), as too has the influence of serum deprivation on the cell cycle of proliferating cells (Gos *et al.*, 2005) and the survival of maturely differentiated cells. However few studies, using serum-deprivation, comment on how this may have influenced the functionality of the quiescent cell culture in their experiment. One study that attempted to address this, reported an inhibitory effect of serum factors on macrophage activation when maintained in 10% fetal bovine serum (Yamamoto *et al.*, 1993). This is further support by a study by Chen *et al.* (Chen *et al.*, 1994) who shows that, while FBS is well known to directly potentiate the LPS-induced TNF- α production in macrophage cell lines and primary macrophages through receptor binding (Kirikae *et al.*, 1993; Hailman *et al.*, 1994), serum factors can in fact inhibit macrophage activation in response to other stimulatory compounds through a currently unknown but likely indirect mechanism (Yamamoto *et al.*, 1993). Both studies report that, despite these differential modulatory effects, no substantial differences in cell death or morphology were observed. This supports my own findings that demonstrate no observable differences in macrophage ED1+ staining and its observable morphology when maintained either in 10% FBS or serum-free conditions. In a lung cancer cell line, serum withdrawal induced phosphorylation of Akt, I κ B and p38 MAP kinase (Liu *et al.*, 2001; Uemura *et al.*, 2004). These results may suggest that serum deprivation, also in macrophage cells, primes cells to a state that enables them to respond rapidly on stimulation with gp120, through NF- κ B activation or MAP kinase signaling.

3.5.2. Effects of gp120-induced neurotoxin release from primary bone-marrow derived macrophages on DRG neuronal outgrowth.

Circulating monocytes rapidly infiltrate into sites of nerve injury or infection, becoming activated infiltrated macrophages. Infiltrated macrophages are a common hallmark in the nerves and DRGs of HIV patients with peripheral neuropathies. Many pro-inflammatory cytokines and neurotoxic mediators, as reviewed previously, have been implicated in axonal degeneration so given the clear M1/ pro-inflammatory associated profile of the gp120_{Bal}-stimulated BMDM I wanted to correlate this response with axonal degeneration. I assessed whether the gene modulation correlated with macrophage-release of compounds that affected neurite outgrowth in primary adult DRG neuronal-glial cultures. I demonstrate that compounds released, into the culture media, by BMDMs treated

with gp120_{Bal}, are neurotoxic to adult cultured DRG neurons as seen by a subtle but significant reduction in neurite outgrowth of $18.9 \pm 5.3\%$ from vehicle control. BMDMs treated with denatured-gp120_{Bal} did not secrete compounds that affected neurite outgrowth indicating this effect was specifically driven by gp120_{Bal} however these effects were more subtle than expected.

As described previously, BMDM supernatant was passed through a 50 kDa Vivaspin column to limit the possibility of gp120 being carried over from the BMDM media to the neuronal culture. This should ensure that observations made on the DRG culture and neurite outgrowth should be attributed to compounds released by BMDMs into the culture media collected and not gp120-mediated.

Despite these controls, this study is still vulnerable to various drawbacks, for example it may be claimed that adequate time was not given for maximal protein secretion. I incubated the BMDMs for 4-6h, based on the 4h time point used for the qPCR cytokine array data, which indicated potent activation of gene expression by this time point. Gene expression is tightly regulated by post-transcriptional regulation so that once the inducible signal is stopped mRNA is degraded, with typical half-lives, especially those relating to pro-inflammatory cytokines, of around 60 min or less (Seko *et al.*, 2006; Newbury, 2006). The integrity analysis I performed on the extracted RNA, showed that all 12 samples collected were of high integrity indicating that intracellular signalling mechanisms were in place to maintain mRNA stability and were likely to have been sustained for sufficient time for translation of new protein. However, importantly, it can be argued that the strong gene expression changes did not relate to protein expression. It may be that none, or only some, of the induced genes led to protein synthesis and even then many, such as the TNF- α membrane-bound precursor, are synthesised first as a prohormone that then require additional processing before being secreted (Akira *et al.*, 1990; Kim *et al.*, 1993). Unfortunately I was unable to confirm cytokine release, or their identity; however the observation that gp120-stimulated macrophage media induced significant effects to neurite outgrowth while that from denatured-gp120_{Bal}-treated macrophages did not, strongly suggests neurotoxins were secreted as a result of this activation. Other studies have highlighted that TNF- α , NO, IL-1 and CCL5, are commonly released from gp120-stimulated macrophage cultures (Pietraforte *et al.*, 1994; Lee *et al.*, 2005). My own qPCR data would suggest that high levels of NO particles, TNF- α and IL-1 β may be among some of the neurotoxins secreted in this study, that mediated the neurotoxicity observed.

On the other hand, another explanation for the subtle effect may be due to the quantity of culture media I applied. Unfortunately I had insufficient time to determine the identity or concentrations of the compounds present in the media. A dilution of 1:1 (macrophage-conditioned media:neuronal

culture media) was decided however if the initial concentrations were low this further dilution may have resulted in concentrations that had little biological activity. From this respect, the subtle effect on neurite outgrowth may imply minimal insult to neuronal cells due to the low level presence of macrophage-secreted compounds.

As stipulated previously, the neurite outgrowth studies are performed in the presence of non-neuronal cells, including Schwann cells and fibroblasts. Therefore just as direct application of gp120 to the neuronal culture is not evidence for a direct action on neurons, neither is the application of conditioned media evidence for neurotoxins acting directly on neurons. It is more than plausible that BMDM-secreted compounds present in the media interacted with non-neuronally expressed receptors, inducing them to respond either in a proinflammatory (amplifying) manner or in a neuroprotective manner, through the release of trophic factors that promote axonal growth. Therefore, combined with the drawbacks I have highlighted, these data may represent a battle between two opposing pathways, one that is neurotoxic due to activation of an immune response and one that is neuroprotective, driven by Schwann cells through the release of NGF that promotes axonal growth (Bosch *et al.*, 1988; Pellitteri *et al.*, 2001).

Once again I highlight how greater understanding of the relative contributions of different cell types and different pro-inflammatory mediators in axonal degeneration will come about when these effects are investigated *in vivo* where the complete biological environment is in place.

3.5.3. Non-neuronally released neurotoxins may play an indirect role in gp120-mediated axonal degeneration although synergistic pathways may be needed.

Many pro-inflammatory cytokines and neurotoxic mediators, as reviewed previously, have been implicated in axonal degeneration. I have shown gp120_{Bal}-stimulated BMDM rapidly induce gene expression of an array of pro-inflammatory cytokines and chemokines and additionally release compounds that negatively affect neurite outgrowth. A similar pattern was found via Schwann cells therefore suggesting that, independent of the initial source, specific or a combination of pro-inflammatory molecules acting synergistically, may act as the mediators of axonal degeneration.

I assessed the relative effects of two of the most highly cited compounds relating to HIV pathogenesis and neuronal damage; TNF- α and QUIN. Keswani *et al.* suggested that the gp120-associated axonal degeneration they observed in their studies occurred via TNF- α signalling (Keswani *et al.*, 2003). I found that both TNF- α and QUIN induced a reduction in neurite outgrowth in a

concentration-dependent manner. This effect appeared more robust with quinolinic acid. The concentrations tested reached considerably high levels yet a powerful effect on neurite outgrowth i.e. >50% reduction was not observed. Many studies have reported a synergistic effect between non-neuronally-released neurotoxins and gp120 while individual effects are less robust. However I find it intriguing in the study where 1 nM TNF- α , 0.7 μ M quinolinic acid (both of which had induced approx. 20% reduction in neurite outgrowth) showed no additional significant synergistic effect on neurite outgrowth when combined together with gp120_{MN} although a trend is observed. I find it difficult to fully explain this seemingly contradictory effect. One plausible explanation may relate again to the strong non-neuronal cell presence in the DRG culture. As previously discussed Schwann cells, through the release of trophic factors (Bosch *et al.*, 1988; Pellitteri *et al.*, 2001), and essential nutrients present within the media, may offer protection to the neurites. Thereby counteracting and masking any effects induced by the excitatory neurotoxins that require activation of multiple complex pathways in order to be effective. It is important to note that the lack of dramatic neurite reduction should not be considered to be indicative of healthy neurons. It is very likely that dramatic intracellular signalling was activated by both of these compounds and may be simply establishing a subclinical condition. This may become unmasked following one or more direct insult to the neurons or following a more maintained chronic exposure where the system eventually becomes fatigued and is unable to sustain the neuroprotection. Groups focusing specifically on the instigation of axonal degeneration have highlighted disruption to energetic and nutrient support to potentially play a role (Campenot *et al.*, 1996; Chevalier-Larsen *et al.*, 2006). As mentioned previously peripheral sensory neuropathies show a characteristic degeneration of the longest axons, degenerating from the most distal point and developing proximally, the points that would be first effect by disruption in nutrient supply.

Alternatively, as these studies were performed a significant time after the first neuronal outgrowth studies the same problem reported previously regarding the reduced stability and biological activity of recombinant gp120 glycoproteins after long storage periods, may have been present. In this situation gp120 may be a redundant inactive glycoprotein in this study. However, it does seem slightly surprising that the combination of TNF- α and quinolinic acid did not show synergistic effects. Of course there are multiple cytokines and neurotoxins that could be tested and in multiple different combinations. I chose TNF- α and quinolinic acid for their different mechanisms of action and their cited involvement in HIV neurotoxicity however these results either indicate 1) that despite initiating mechanisms via separate pathways, perhaps they activate pathways that ultimately counter-act each other or 2) the activated pathways converge on the same common mechanism that directly

regulates axonal degeneration however a rate-limiting step may be present that prevents further insult.

TNF- α signalling is commonly linked with activation of apoptotic pathways therefore I also assessed the levels of neuronal cell death as indicated by fragmented condensed nuclei. No significant difference was observed between vehicle-treated cultures and those exposed to TNF or QUIN. Conversely, and against my expectations, combination of these compounds with gp120_{MN} resulted in a significantly reduced number of neurons with fragmented nuclei. It should be noted however that counting fragmented nuclei is only an indicator of the final stages of apoptosis and does not identify cells that have activated apoptotic pathways and are currently at an earlier stage in the process. Therefore this data may be underrepresentative of the cytotoxic effects. In addition, the significantly lower percentage of cells with fragmented nuclei in cultures treated with TNF, QUIN and gp120 may have resulted due to the loss of apoptotic cells during immunofluorescence, which would signify apoptosis was induced earlier or occurred more quickly and may be taken as a more cytotoxic effect. Although research reporting TNF-signalling predominantly focuses on its neurotoxic abilities more recent reports are highlighting differential signalling of TNF- α via TNFR1 and TNFR2 that in fact acts opposing roles; TNFR1 inducing classical neurotoxic effects while neuroprotection may be offered through activation of TNFR2. A study in 1997 by Shen *et al.*, demonstrated, for the first time, an indication of an active neuroprotective effect of TNFR2 signalling as opposed to the previously considered passive non-neurotoxic effect using antisense targeted against oligonucleotides p75 TNFR (TNFR2) (Shen *et al.*, 1997). Further support for this theory was provided by Fontaine *et al.* who compared the effects of glutamate excitotoxicity in TNFR1- and TNFR2-deficient mice (Fontaine *et al.*, 2002). Interestingly it was found that direct TNF action on primary murine neurons induced glutamate excitotoxicity in TNFR2-deficient mice but not TNFR1-deficient mice. TNFR1 is rapidly bound and activated by soluble TNF- α with high affinity (K_d of 19 pM) (Grell *et al.*, 1998). In contrast, the affinity of soluble TNF- α is considerably lower for TNFR2 (K_d of 0.42 nM) and binding is less stable (Grell *et al.*, 1995). However, in retrospect, these studies tested a considerably high concentration of soluble TNF- α (1–2 nM). Therefore it is conceivable to consider that, since expression of both TNFR1 and TNFR2 was likely present in my DRG cultures, as reported by others (Pollock *et al.*, 2002), and a high concentration of TNF- α was tested, which was greater than the K_d for TNFR2, this activated both receptor signalling pathways that resulting in a clash between activation of neurotoxic signalling and neuroprotective signalling or conversely that neuroprotection was achieved via signalling through both TNFR1 and TNFR2. In support of this, another study also testing 2 nM TNF- α observed neuroprotection against β -Amyloid-associated toxicity in relation to Alzheimer's disease (Viel *et al.*, 2001) that occurred via an NF- κ B dependent mechanism. Since the effects following

quinolinic acid appeared more robust than TNF- α exposure, and in light of my discussion I consider TNFR-signalling to have been underlying the opposing effects against toxicity induced by quinolinic acid and potentially gp120 as well.

Finally I assessed the ability of both TNF- α and QUIN to induce acute increases in intracellular calcium levels. No acute calcium responses were observed at concentrations equivalent to the CSF and plasma concentrations identified in HIV patients with extensive neuropathology. Calcium mobilisation was evident at extremely high concentrations however while these concentrations may be biologically irrelevant they confirm the activity of the compounds and the presence of functional TNF and NMDA receptors. Interestingly, 500 nM QUIN, which induced no acute changes in $[Ca^{2+}]_i$ levels within the 15 min time frame recorded, caused significant neurite reduction after 24h exposure. This suggests either that QUIN-mediated neurotoxicity acts via a calcium-independent pathway such as the proposed release of free-radicals (Behan *et al.*, 1999; Behan *et al.*, 2002; Braidy *et al.*, 2009). Alternatively maintained exposure and activation of neurons by QUIN may be required to induce low level but consistent activation and calcium mobilisation that reach excitotoxic levels over time. In addition to demonstrating functional receptors, the extreme concentration of QUIN was also potentially cytotoxic in fact causing instant cytolysis. Therefore maintained levels at lower concentration can be envisaged to slowly activate neurons, causing gradual calcium mobilisation that may eventually run a positive feedback loop that eventually leads to toxicity.

Taken together, this section highlights that the ‘traditional’ concept of a “one mediates all” mechanism is an unrealistic ideal and no longer fitting modern research. Therefore research should turn towards a systems approach, to gain a greater understanding of the interplay, synergy and temporal profile of the immune response.

3.5.4. Cytokine Array

Since the first reported large scale gene expression studies following HIV or accessory protein exposure (Geiss *et al.*, 2000), many more studies have utilised the technique to gain a greater understanding, on a high-throughput scale, of the functional correlates between diverse gene modulation and HIV-1 pathogenesis. However surprising few have accessed changes to genes relating to inflammation or immune response on a large scale. The immune response of BMDM has been highly implemented in HIV pathogenesis therefore it is deemed critical that all potential mediators are highlighted.

Primary macrophage cultures were stimulated for 4h with gp120_{Bal} and the gene expression changes assessed using a custom-designed TaqMan Gene Cytokine Array, targeting pro-inflammatory cytokines, chemokines and mediators commonly associated with inflammatory disease and pain. Stringent procedures were employed to ensure the most reproducible, accurate and biological relevant data was obtained. To avoid negative-positive findings four separate biological samples were obtained for each treatment condition. In addition, throughout the study randomisation was asserted at all stages, during RNA extraction, cDNA synthesis and loading onto the cytokine array, to ensure all samples were treated identically and exposed equally to possible human error or temporal variability.

In this next section, I briefly review the functional relevance of a few of the key genes shown to be significantly induced following 4h gp120_{Bal} exposure in BMDMs. Some of these genes reviewed were expected changes and highlight and support my hypothesis regarding M1 phenotypic activation of macrophages following gp120 exposure. Other genes highlighted offer novel targets that although generally well studied within a specific context, their relevance to HIV pathogenesis has not been adequately explored and may offer greater understanding in the underlying network of mechanisms involved.

CXCL11 (fold change: 3205.5), CXCL9 (fold change: 2082.9) and CXCL10 (fold change: 939.9)

From the cytokine array, the most highly induced gene identified was, CXCL11, a C-X-C chemokine, which has potent effects on Th1 activated lymphocyte trafficking (Bonecchi *et al.*, 1998). CXCL11 acts via the CXCR3 receptor (Cole *et al.*, 1998) which is almost exclusively expressed on activated T-cells (Tensen *et al.*, 1999). CXCL11 also goes by the name of I-TAC (Interferon-inducible T-cell alpha chemoattractant) (Zlotnik *et al.*, 2000). There are three known ligands for CXCR3: CXCL11/ I-TAC, CXCL9/ mig and CXCL10/ IP-10 and interestingly, genes for these latter two were also among the most highly induced genes in gp120_{Bal}-stimulated macrophages. Induction of CXCL10 and CXCL11 are also considered key biomarkers of an M1 macrophage phenotype (Cassol *et al.*, 2009). Other studies have shown similar expression responses where all three of these chemokine genes are induced in response to a single common signal (McColl *et al.*, 2004). They postulated that this apparent redundancy indicated potential differences in CXCR3 binding sites and/ or their binding abilities, giving possible control over activation when cells display different receptor conformations as a result of different activation states. In addition, not only do CXCL9, CXCL10 and CXCL11 attract Th1 cells via CXCR3, it has also now been shown that they concomitantly act as antagonists on the CCR3 receptors found on Th2 cells thereby blocking Th2 migration and enhancing the polarization to a Th1 phenotype (Loetscher *et al.*, 2001).

Together the relevance of CXCL9, CXCL10 and CXCL11 gene expression in response to gp120_{Bal} stimulation in macrophages suggests induction of an M1-Th1 immune response and potent chemotaxis of activated T-cells. This may allow for further recruitment of additional immune cells and amplification of the Th1, phagocytic response.

IL-27 (fold change: 2446.1)

The IL-27 protein is a member of the IL-6/IL-12 cytokine family, comprised of two subunits, namely; Epstein-Barr virus-induced gene-3 protein and IL-27 p38 protein (Pflanz *et al.*, 2002). IL-27 is one of a group of cytokines (along with IL-18, IL-21 and IL-32) recently identified as ‘novel cytokine targets’ due to their reported role in HIV-associated pathogenesis (Clerici, 2010). IL-27 gene expression showed the second highest increase in gp120_{Bal}-stimulated macrophages and IL-18 expression was also induced albeit to a much lesser degree (mean fold change: 2.9). IL-27 is reported to play both anti- and pro-inflammatory roles, with its early actions driving a Th1 phenotype in naive CD4 T cells and naive B cells. However, most importantly, IL-27 has been highlighted as a potent inhibitor of HIV-1 replication using a similar pathway to IFN-mediated HIV inhibition through the Jak/STAT pathway (Fakruddin *et al.*, 2007) and induction of antiviral genes in monocyte-derived macrophages (Imamichi *et al.*, 2008). The anti-inflammatory role of IL-27 is similar to that of IL-18 where a negative feedback program is activated that limits and impairs T-cell and NK-T cell function (Clerici, 2010). IL-18 production has been reported to be augmented in HIV infection (Sailer *et al.*, 2007; Iannello *et al.*, 2009). One study found elevated plasma levels of IL-18, CXCL10 and CCL2 in HIV/Hepatitis B (HBV) co-infected patients compared with controls, which were associated with immune reconstitution inflammatory syndrome (IRIS) in response to initiation of HBV/ antiretroviral treatment (Crane *et al.*, 2009). Therefore increased IL-18 levels and the resultant impairment on NK cells may contribute significantly to the debilitating effects of HIV on the immune system.

Taken together this highlights firstly the complexity and interplay that occurs in the immune response between pro- and anti-inflammatory responses. However, given that the increase in IL-27 gene expression is far greater than IL-18, it suggests the BMDMs’ first response to gp120 drives an M1/Th1 phenotype that aims to limit viral replication and to attract Th1 lymphocytes, however there are components that suggest a possible switching of function in the context of a currently unknown factor that would in turn lead to immune suppression.

iNOS (fold change: 2244.0)

Nitric oxide synthases (NOS) are enzymes that convert L-arginine into L-hydroxyarginine and eventually to the production of nitric oxide. There are three forms of nitric oxide synthase; neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS), each with specific tissue locations but all enzymes that catalyse the production of nitric oxide (NO); a simple molecule with diverse biological functions. Macrophages are the predominant producers of iNOS, although expression has been recently identified in other cell types including endothelial cells, fibroblasts, cardiac myocytes and dendritic cells (Rao, 2000). Unlike the other isoforms, iNOS is absent in resting cells and from my data, vehicle-treated BMDMs showed no detectable expression of iNOS supporting the theory that serum-deprivation induced a quiescent or resting state in these cells. However on activation, this gene can be rapidly induced in response to pro-inflammatory stimuli such as cytokines (Bredt, 1999) and, as shown by my data (>2000 fold increase) and supported by others (Pietraforte *et al.*, 1994), induced by exposure to HIV-1 gp120 protein.

iNOS has been highlighted as a mediator of HIV pathogenesis and in particular with HIV-associated dementia. iNOS is not normally detected in healthy brains however, evidence from human postmortem studies and experimental models, have correlated elevated iNOS levels with the severity and progression of HIV dementia (Dawson *et al.*, 1993; Adamson DC *et al.*, 1999; Rostasy K *et al.*, 1999). As previously stipulated, increased iNOS expression acts as a key indicator of the M1 macrophage phenotype. Therefore given the indication of an M1 phenotype from CXCL11 and IL-27 expression, an M1 phenotype is further supported by my findings with iNOS expression. Due to the direct role of iNOS in NO production this large induction in gene expression makes it tempting to speculate that the BMDMs in this study were also producing significant quantities of NO, which, in the clinical setting, if chronic, would cause deleterious effects on neighbouring cells and is commonly identified in neurodegenerative diseases (Tan *et al.*, 1998; Boje, 2004).

It is important to note that production and release of NO is not always associated with pathogenesis. In fact, at physiological basal levels, NO plays important roles providing neuroprotection against NMDA-excitotoxicity (Guix 2005 Prog Neurobiol), vasodilation in the prevention of vascular disease (Lundequist *et al.*, 2010; Weerateerangkul *et al.*, 2011) and in the enhancement of memory formation and neuroplasticity (Susswein *et al.*, 2004). Furthermore inhibition of NO has been shown to induce apoptosis via caspase 3 activation (Contestabile *et al.*, 2004; Calabrese *et al.*, 2007). It has since been clarified that NO, via cGMP and activation of protein kinase G, stimulates the kinase Akt and transcription factor CREB survival pathways (Contestabile *et al.*, 2004; Riccio *et al.*, 2006), two mediators important in neurotrophin-mediated cell survival.

However there is no longer debate over the neurotoxic potential of NO when produced in excess, as is evident in the majority of neurodegenerative diseases (Dawson VL *et al.*, 1998), including Alzheimer's disease (Hölscher, 1998; Domek-Lopacinska *et al.*, 2010), Parkinson's disease (Surendran *et al.*, 2010) and HIV infection (Lipton *et al.*, 1994). In excess, NO has been shown to activate isoforms of cyclooxygenases, which release free radicals and induce the formation of prostaglandins during its catalytic cycle (Mancuso *et al.*, 2007), thereby indirectly inducing neurotoxicity. Furthermore excessive NO causes fundamental disruption to ATP, energetic and glycolytic homeostasis through reversible or irreversible inhibition of GAPDH (glyceraldehydes 3-phosphate dehydrogenase) enzymatic activity (Chuang *et al.*, 2005) leading to mitochondrial stress and eventual apoptosis and neurotoxicity (Sultana *et al.*, 2006).

In the setting of this study it is tempting to speculate that the dramatic induction of iNOS expression correlates with iNOS synthesis and would in turn lead to the production and secretion of large quantities of NO. Following an acute stimulation this may be manageable and reversible, however it is easy to visualise chronic excess of NO in the setting of HIV infection and the initiation of a multitude of neurodegenerative pathways.

Of potential interest is the rightward shift of the T_m melt point observed in all studies investigating the gene expression levels of iNOS by qPCR in primary BMDM cultures, primary Schwann cells and plantar in vivo. As the melting point following qPCR correlates with the size of the amplicon, a significant and robust rightward shift in the melt point would be indicative of a larger amplicon product as more energy is required to denature the double strand. This led me to speculate that the presence of two robust peaks in the melt curve represented two splice variants of iNOS. The cDNA samples from the treatment samples had the higher T_m and were also associated with significant upregulation of iNOS expression. Therefore this phenomenon suggests that a spliced iNOS variant exists in normal naïve or untreated conditions and the rightward shift would indicate newly expressed iNOS mRNA exists in a state prior to splicing, which gives it the larger size. Other occurrences of alternative splicing have been recently described (Eissa 1998; Tiscornia 2004) some of which were associated with interference of iNOS dimerisation and decreased cell viability. Confirmation of this hypothesis and subsequent sequence identification may lead to a novel splice variant whose functional activity may offer an insight into gp120-specific functional effects.

IL-1 α (fold change: 2016.3) and IL-1 β (fold change: 948.3)

There are two members of the IL-1 cytokine family, IL-1 α and IL-1 β , both of which were found to be significantly induced at the transcriptional level in BMDMs following exposure to gp120_{Bal}. IL-1 induction was not surprising due to their heavily cited roles in inflammation, neurotoxicity and function as a neuromodulator (Vitkovic *et al.*, 2000). IL-1 α or IL-1 β are not considered to be directly toxic to neurons and IL-1 injection in healthy brains (Lawrence *et al.*, 1998) or direct application *in vitro* to pure neurons in culture (Simi *et al.*, 2007) does not lead to tissue damage or cell death. However their expression has been associated with neurotoxicity via microglia and macrophages, and have been strongly implicated in many neuropathological diseases (Allan *et al.*, 2001). Furthermore, neuronal activation (Desson *et al.*, 2003; Viviani B *et al.*, 2003), sensitisation and hyperalgesia have been reported as a result of both IL-1 α and IL-1 β exposure (Ferreira *et al.*, 1988; Watkins *et al.*, 1994; Watkins *et al.*, 1995; Reeve *et al.*, 2000). In the context of additional cytokine modulation, synergy of functions may lead to ultimately different outcomes. It has been shown, in fact, that a synergistic effect exists between TNF- α and IL-1 (both IL-1 α and IL-1 β) (Chao *et al.*, 1995; Caldwell *et al.*, 1995; Turrin *et al.*, 2000) that leads to the production of NO and activation of NMDA receptors (Chao *et al.*, 1995).

Of course, as always when interpreting qPCR gene expression changes, it should be remembered that I am not assessing changes at the protein level and I do not know how basal levels of expression compare from one gene to the next. Relative fold changes do not necessarily equate to higher expression levels relative to another or even mean that they are of biological relevance.

However, if I was to assume that these gene expression changes relate to increases in protein expression it becomes possible to visualise how, in the setting of HIV, neurotoxicity may occur. Infiltrating macrophages that are activated, either through HIV-infection or by shed viral proteins, release these potent IL-1 cytokines. These may amplify the signal via other immune cells, act synergistically with other cytokines and directly sensitise neurons, via an alternative signalling mechanism, involving NMDA-R phosphorylation and enhanced calcium entry, making them vulnerable to secondary insults (Viviani *et al.*, 2003).

CCL5/ RANTES (fold change: 474.5)

CCL5/ RANTES is the endogenous ligand for CCR5. The function of this chemokine in the setting of HIV has been controversial. One study suggests it plays a direct role in the neurotoxicity of HIV-1 gp120. Keswani *et al.* demonstrated that gp120 negatively impacts neurite outgrowth through CXCR4-mediated activation of Schwann cells *in vitro* to cause the release of CCL5 which in turn affects neuronal function and survival via neuronally expressed CCR5 receptors (Keswani *et al.*,

2003). They claim CCL5-mediated CCR5-activation induces neuronal release of TNF- α that induces apoptosis and neurotoxicity, in an autocrine/paracrine fashion, via neuronally-expressed TNFR1. However in contrast, numerous other studies have reported a neuroprotective role of RANTES secretion in gp120-mediated neurotoxicity through pharmacological antagonism at the CCR5 receptor (Simmons *et al.*, 1997; Simmons *et al.*, 2000) without CCL5-induced TNF- α release. Furthermore one study found no significant difference in serum concentrations of CCL5 between HIV-1 infected patients and AIDS patients, suggesting that CCL5 expression is not a key mediator underlying, at least progression, to AIDS (McKenzie *et al.*, 1996). It is difficult to conclude what the role of BMDM CCR5 gene increase may be in the setting of this study. However it could be suggested that, as it was stimulated likely via the CCR5 receptor, subsequent release of CCL5 could be seen as a mechanism to antagonise further gp120_{Bal} binding without causing a cycling of the same effects, as has been reported in astrocytes (Avdoshina *et al.*, 2010). Numerous studies suggest that M-tropic (R5) gp120 glycoproteins, despite acting via the same receptor (Davis *et al.*, 1997), induce different downstream signalling pathways to the endogenous ligands, as previously discussed. Further understanding of the interaction between CCR5 and HIV-1 gp120, in comparison with the endogenous ligands, could elucidate these effects and their relevance in HIV infection.

TNF- α (fold change: 313.7)

TNF- α induces the expression of numerous cellular genes through activation of the cellular transcription factor NF- κ B (Aggarwal *et al.*, 2003) including stimulation of HIV-1 replication in infected cell (Duh *et al.*, 1989; Folks *et al.*, 1989; Poli *et al.*, 1990). TNF- α -mediated neurotoxicity has been well described in the literature, as previously outlined. Gp120-induced TNF- α gene expression was used in my assay for relative macrophage activation prior to the cytokine array so its reference here is to highlight the confirmation and validity of my previous studies and to take the opportunity to review some of the key functions of TNF- α relevant to HIV infection. TNF- α is among one of the most studied cytokines in the pathogenesis of HIV. Some reports have even suggested that HIV disease should be considered a TNF- α -driven disease (Herbein *et al.*, 2008) with full involvement by the whole virus, certainly with some strong evidence. Early in infection the actions of viral proteins, expressed early in the viral cycle namely Nef, Tat and Vpr, attempt to mimic the biological effects of TNF- α (Geleziunas *et al.*, 2001). This occurs by promoting HIV replication through Nef- and Tat-mediated NF- κ B activation (Varin *et al.*, 2003; Brady *et al.*, 2005), Vpr-mediated transcriptional enhancement of HIV (Kino *et al.*, 2002) and Nef-inhibition of TNFR-mediated apoptosis via the Bcl complexes (Geleziunas *et al.*, 2001). These effects function to sustain viral replication and to inhibit death of the infected cell so as to further prolong its own survival and dissemination. However, this TNF mimicry appears to occur in the setting of low levels of TNF- α and other proinflammatory cytokine production and shows little neurotoxicity. Later in the stage of HIV disease and at the onset

of AIDS, high plasma and CSF levels of TNF- α have been identified and correlate with dramatic cell death (de Oliveira Pinto *et al.*, 2002), immune suppression (Wig *et al.*, 2005) and the pathogenesis and neurotoxicity associated with HIV. There is a dramatic increase in the protein expression of TNF- α and CC- chemokines due to chronic immune activation (Fantuzzi *et al.*, 2003). In this setting viral proteins switch away from modulating TNFR signalling to enhancing TNF- α -mediated apoptosis resulting in accelerated immune suppression through CD4+ T cell death (Herbein *et al.*, 2008). As I have demonstrated that gp120 potently induces TNF- α gene expression in BMDMs on acute exposure, this process may underlie the sudden induction of TNF- α release that coincides with the increase in viral load as HIV infection progresses. This may in turn exacerbate TNF- α neurotoxic events by secondary induction of gene expression via NK-KB activation (Zhu *et al.*, 2000; Lee *et al.*, 2005). Furthermore gp120 induces PKC-dependent phosphorylation of the CD4 receptor which results in priming of CD4+ T cells that makes them more susceptible to TNF-mediated apoptosis (Westendorp *et al.*, 1995). CD4+ T cell death is one of the key parameters measured for the assessment of progression to AIDS. Along with its involvement in driving apoptosis, these *in vitro* findings translate to the *in vivo* setting where TNF- α has also been associated with the development of neuropathic pain in various animal models (Wagner *et al.*, 1996; Zelenka *et al.*, 2005).

Despite these obvious deleterious effects, TNF- α is also an essential component in host defense and the immune system (Kollias *et al.*, 1999). TNF blockers used in the treatment of rheumatoid arthritis and other chronic inflammatory diseases have been shown to increase risk of opportunistic infections such as tuberculosis and fungal infection (Crawford *et al.*, 2008). This poses a complex challenge in our understanding; TNF- α release in response to gp120 is an obvious attempt to protect against viral infection through recruitment and activation of macrophages, natural killer (NK) cells and T cells, however chronic/ persistent release, in concert with other proinflammatory mediators, via p38-MAPK enhances tetrodotoxin-resistant (TTX-R) Na⁺ channels (Jin *et al.*, 2006), sensitises neurons and modulates NMDA-R activity among other actions.

COX2 (fold change: 134.8)

The cyclooxygenase (COX) family comprises three isoforms consisting of the constitutive COX-1, inducible COX-2 and COX-3; all rate-limiting enzymes in conversion of arachidonic acid (AA) into unstable prostaglandin endoperoxides, PGG₂ and PGH₂. These endoperoxides can then be enzymatically converted into stable prostaglandins, PGE₂, PGF₂ and PGD₂. COX-2 is not normally expressed under normal conditions in most cells but is highly inducible in conditions of inflammation.

COX-2 is another important macrophage-derived enzyme, like iNOS, which functions with dual roles; those that promote neuronal survival and those that enhance apoptotic cell death. In physiologically normal conditions, neuroinflammation is physiologically important because the production of PGs, and NO by iNOS, functions to limit overactivation of cellular immunity (Betz *et al.*, 1991) and inhibit viral replication (Hayes *et al.*, 2002) in order to prevent further cellular injury and promote neuronal regeneration. Basal release of both PG and NO has been demonstrated to protect against vascular diseases through vasodilation (Lundequist *et al.*, 2010; Weerateerangkul *et al.*, 2011) and antiplatelet activity (Zhou *et al.*, 2010; Bauer *et al.*, 2010), and enhance neuronal plasticity aiding learning and memory (Susswein *et al.*, 2004; Chen *et al.*, 2005; Yirmiya *et al.*, 2011). However, in the setting of chronic inflammation, COX-2 and PGs act with deleterious consequences. Elevated COX-2 levels commonly correlates with high levels of prostaglandin E2 (PGE2) (Almer 2002) and both are frequently evident within pathological tissue in many acute and chronic neurodegenerative diseases, including Alzheimer's, Parkinson's disease and HIV infection (Griffin *et al.*, 1994; Teismann *et al.*, 2003; Hoozemans *et al.*, 2008; Depboylu *et al.*, 2011). Studies using a mouse model of amyotrophic lateral sclerosis demonstrated a correlation between increased COX-2 and PGE2, with the degree of motor neuron loss, while COX-2 inhibition offered protection from this (Drachman *et al.*, 2002; Pompl *et al.*, 2003). A neuroprotective role of COX-2 inhibition is further supported by a study reported by Huang *et al.* who demonstrated that administration of the COX-2 inhibitor, celecoxib, was neuroprotective against LPS-stimulated macrophage toxicity (Huang *et al.*, 2005). Taken together there provide a strong indication of a role for COX-2 in neuronal degeneration.

PGs are classed in the family of fatty acid derivatives or eicosanoids together with thromboxanes and prostacyclins. PGs are released by neuronal and glial cells following an inflammatory challenge or injury and can perform a multitude of actions including vasodilation, sensitisation of nociceptors (Ferreira, 1972), calcium modulation and the enhancement of inflammation. Their potent vasodilatory effect potentiates oedema associated with inflammation due to the dilation of blood vessels allowing greater blood flow, which in turn, provides greater access and exposure to vascular permeability-enhancing mediators released in parallel. Importantly PG can increase cAMP levels and lower the threshold for activation of TTX-R Na⁺ channels that underlie the generation of action potential small diameter neurons, thereby increasing the excitability of sensory nociceptive neurons. Therefore in the context of HIV-associated neurodegeneration that may be driven by inflammatory responses, PG release may be involved in the pain commonly associated with it, acting locally at sites of neurodegeneration.

Interestingly a direct modulatory link and synergy between COX-2 and iNOS has been well reported (Depboylu *et al.*, 2011). It was first demonstrated by Salvemini *et al.* (Salvemini *et al.*, 1993) that the release of prostaglandins was driven almost exclusively by NO, through the switching on and off of the COX pathway by NO and NO donors (Mollace *et al.*, 2005). Furthermore the enzymatic activity of COX-2 can be enhanced through direct iNOS binding (Kim *et al.*, 2005). Considering that both enzymes are released jointly and a cross talk in their modulation exists, it is conceivable that synergy between the actions of PG and NO exacerbates neuronal damage.

CCL2 (fold change: 134.8)

Like the majority of chemokines CCL2 functions to recruit immune cells to sights of injury or inflammation. In addition, CCL2 has been highlighted to be among the many released factors (e.g. TNF- α , IL-1 β and histamine) that instigate and maintain neuronal abnormalities following injury (Thacker *et al.*, 2007). CCR5-dependent CCL2 secretion from human macrophages has been demonstrated in response to HIV-1 gp120 and requires PLC activation, NF- κ B signalling and that endogenous CCL2 modulates HIV-1 replication and affects cytoskeleton organisation in BMDMs (Fantuzzi *et al.*, 2008). This would correlate with the induced CCL2 gene expression I observed in the BMDM cultures following stimulation with CCR5-dependent gp120_{Bal}. Interestingly CCL2/ CCR2 signaling has been strongly implicated in the establishment of neuropathic pain-like behaviours *in vivo* (Abbadie, 2005). Supporting a role for CCL2 in pain pathways intraneural administration of CCL2 into rat sciatic nerve resulted in transient mechanical and thermal hypersensitivity that correlated with macrophage recruitment to the injection area (Thacker *et al.*, 2009). These studies demonstrated a pivotal role for CCL2 in neuro-glial interactions and in the maintenance of neuropathic pain driven largely by its ability to recruit monocytes and enhance further release of inflammatory mediators. In fact, CCL2 is being considered as a biomarker for progressive renal injury in diabetic nephropathy (Tesch, 2008), with good basis as a positive correlation between CCL2 expression and the number of infiltrated macrophages was identified in the brain following lesioning (Hausmann *et al.*, 1998).

Therefore, in this setting may be highlighted as one of the key mediators involved in maintaining the inflammatory response to HIV infection and exacerbating minor nerve injury.

Kit Ligand (fold change: 0.2)

The only gene significantly downregulated was Kit ligand (c-kit), also known as stem cell factor. C-kit is a cytokine that binds to the c-kit receptor. The resultant signalling from this interaction promotes recruitment of endothelial progenitor cells and contributes to ischemia-induced new vessel formation. Loss of function mutations and lack of expression have been associated with defective pigmentation, piebaldism (Lev *et al.*, 1994) and some types of cancer including thyroid cancer (Natali *et al.*, 1995); melanoma (Kurosawa, 1996) and breast cancer (Natali *et al.*, 1992). However, conversely over activation and expression has been associated with other types of cancer such as mast cell leukemia (Nagata H *et al.*, 1995), acute myeloid leukemia (Ning *et al.*, 2001) and gastrointestinal stromal tumours (Hirota S *et al.*, 1998). The predominant phenotype of heterozygous loss of function mutations is defective pigmentation, reduced fertility and severe anaemia (Blouin *et al.*, 1993; Lev *et al.*, 1994). These different disease states and effects of c-kit expression appear to have little connection to gp120-mediated neurotoxicity and relate closer to HIV-associated anemia and melanoma both common complications of HIV (Kreuzer *et al.*, 1997; Adias *et al.*, 2006; Pantanowitz *et al.*, 2006). Therefore the significant downregulation of c-kit following gp120 exposure may have little direct relevance to the aim of my investigations and will not be discussed further.

In summary, given the known actions of the cytokine targets investigated, the majority of genes that were upregulated were indicative of a pro-inflammatory Th1/M1 response. Few if any of the classic M2 anti-inflammatory cytokines were significantly upregulated, including IL-10 and MIF. Nor was the cytokine IL-23 that is known to induce a Th17 phenotypic response in CD4+ T cells, upregulated. Therefore there is strong evidence to indicate that gp120 induces a pro-inflammatory M1 phenotype in BMDMs that likely establishes a Th1 immune response in recruited and stimulated T cells.

Depending on the microenvironment and the presence of other cytokines or receptors, a cytokines action can range from activating gene expression and inducing cell differentiation and migration, to inhibiting viral replication or dampening the host cell immune response. The temporal course of this interplay remains complex, and I have captured just one moment in a continually changing system. However at the 4h time point, I identified the greatest gene inductions in Th1-related genes and only low fold increases for anti-inflammatory genes. Taken together, overall the cytokines reviewed here all correlate with the hypothesis that these data represent responses of a macrophage population and that 4h exposure to gp120_{Bal} induces a pro-inflammatory M1 phenotype. These data does, however, highlight a critical cytokine balance that once pushed the other way, as others have

reported with the Th1-to-Th2 switch hypothesis (for review see Clerici *et al.*, 1993), may significantly underpin the inhibition of the immune response and progression to AIDS. Certainly some of the cytokines reviewed function solely via other immune cells for example by altering their position or polarization state, however others have been clearly indicated in neurotoxicity and pain pathways via a more direct mechanism on neuronal cells.

3.5.5. Differential responses of M1- and M2-type macrophages to gp120 exposure.

A thorough characterisation of macrophage phenotype and function, on activation by HIV infection *per se* or viral-associated glycoproteins, remains absent and the many studies that have attempted to answer aspects of this have resulted in controversy, making this a subject of great debate. The induction of arginase1 and suppression of iNOS, along with increased MHCII antigen presentation and low IL-12/ high IL-10 release, is now regarded as common hallmarks of the M2 macrophage phenotype, known as the 'alternatively-activated' macrophages (Munder *et al.*, 1998; Mantovani *et al.*, 2004; Martinez *et al.*, 2009). Therefore I attempted an assessment of the macrophage phenotype in these studies and investigated how modulation of their prior activation state may affect gp120-mediated responses. Data from the cytokine array provided strong evidence for a switch to an M1 phenotype in BMDMs in response to gp120 exposure. Although not confirmed, but indicated by low basal levels of pro-inflammatory cytokine mRNA, I assumed that prior to stimulation, the induced-quiescent state reflected macrophages in a narrow spectrum of functional states that deviated slightly from the 'typical' unactivated state.

I demonstrate that 24h IL-4-primed vehicle-treated BMDMs show significantly higher basal expression levels of arginase 1 compared to unprimed macrophages supporting the proposal that, as expected, the IL-4 priming conditions sufficiently induced an M2 phenotype in the BMDM cultures. Of most interest was the finding that pre-conditioning BMDM cells in this manner with IL-4 nearly completely abolished the gp120-induced increase in TNF- α expression. In resting macrophages, there exists only weak transcription of TNF- α gene and strong repression of TNF- α mRNA translation, which results in low TNF- α synthesis. Macrophage activation, by M1-related/ inflammatory molecules such as LPS, induces TNF- α gene transcription via an NF- κ B-dependent pathway and additionally leads to derepression of TNF- α mRNA translation and secretion of functional TNF- α protein (Collart *et al.*, 1990; Read *et al.*, 1994). Comparison of TNF- α expression levels showed no difference between vehicle-treated BMDM in IL-4 primed conditions relative to unprimed BMDMs, indicating that IL-4 priming *per se* did not alter basal levels of TNF- α expression and was unlikely to

explain this lost response. To further support this, one study demonstrates that IL-4 priming alters TNF- α production only at the translational level and not at the transcriptional level (Major 2002; Suk 1996; Mijatovic 1997). Furthermore, not only was the induced TNF- α expression lost but gp120_{Bal} was also unable to override the pre-existing M2 phenotype to induce a M1 response as occurs in unprimed conditions. A phenotypic switch involves alteration of receptor expression and affects functionality of the macrophages therefore it was considered that IL-4 priming and induction of an M2 phenotype resulted in downregulation of the CCR5 receptor, the receptor required for gp120-induced activation, a theory supported by Wang *et al.* (Wang *et al.*, 1998). Downregulation of receptors in response to key signals is suggested by Mantovani as an uncoupling mechanism that acts as a stop signal to the inflammatory response (Mantovani, 1999; Mantovani *et al.*, 2001).

This may at a glance appear like a window for therapeutic intervention to prevent the gp120-induced neuroinflammatory environment however over-activation of M2 phenotypic macrophages has been highlighted to underlie chronic autoimmune diseases such as psoriasis (Djemadji-Oudjijel *et al.*, 1996), asthma (Melgert *et al.*, 2011) and rheumatoid arthritis (Szekanecz *et al.*, 1994). In addition a gradual loss of Th1/M1 responses and an increase in Th2/M2 cytokines in HIV+ patients is now becoming a common indicator of progression towards AIDS (Maggi *et al.*, 1994). Therefore although blocking cytokine induction often seems appealing it may be that this study is replicating the clinical setting following phenotypic switch and immunosuppression that leads to the associated deleterious effects of HIV. Conversely, it has been suggested that HIV-1 gp120 may drive this switch (Mellado *et al.*, 1998) however I show here, at least at this time point, gp120 induces a clear M1 profile. Therefore with these results and in view of the hypothesised M1 to M2 switch in HIV pathogenesis it makes interpretation of the cytokine data difficult, which indicated gp120-induced an M1 response and not an M2 response. Many of the gene targets induced by gp120 have been implicated in neurotoxicity, neuronal sensitisation and excitotoxicity. As an initial response this aims to offer more protection than harm, acting to provide the best method to contain the negative effects of the HIV virus. However due to the chronic state of HIV infection and continuous gp120-stimulation, a equilibrium may occur between the multiple but opposing responses until eventually one overweighs the other to lead a downward state of immunosuppression, lost protection, cell death and nerve degeneration. Therefore it would be interesting to investigate what drives the eventual switch from a Th1/M1 to Th2/M2 profile in the clinical setting, which may provide greater therapeutic opportunities in the prevention or control of HIV-associated cell death and neuropathogenesis. It would be interesting to explore the same profiles across a greater time course and in response to chronic gp120 exposure. Some targets I identified certainly have the ability to switch from pro-inflammatory functions to anti-inflammatory, so assessment following chronic

exposure may highlight the temporal profile for these cytokine targets and elucidate at what point a switch occurs and which, if any, may be mediating this switch. This work does also highlight how the immune profile of the host can greatly affect their vulnerability to HIV and indicates that a greater understanding of this field may elucidate why some people are more susceptible to neuropathies and pain resulting from HIV, and/ or show faster progression to AIDS. Immune profiling also becomes particularly relevant when considering therapy or vaccine regimes because it was shown that *Schistosoma* parasite-infected mice, with a pre-existent Th2 immune profile, failed to induce a protective Th1 immune response to HIV vaccines (Ayash-Rashkovsky *et al.*, 2002). Effective Th1 immune responses are an essential requirement for successful protection from any HIV vaccine (Burton *et al.*, 1998; Heeney *et al.*, 1999; Levy, 2001).

In conclusion, therapeutic intervention to prevent the chronic and over-activation pro-inflammatory response to initial HIV infection would provide many beneficial effects to the patient and potentially protect from HIV-mediated nerve damage. However an appropriate degree of immune manipulation would be required to achieve neuroprotective effects without creating too powerful immunosuppression. It would be imperative to ensure that the host's immune system does not switch to a dominant, and more importantly, chronic M2 immunosuppressive state.

3.5.6. HIV-1 gp120 induces expression of cytokines in Schwann cells and the subsequent release of neurotoxic compounds.

In addition to macrophages, I also investigated the involvement of Schwann cells in gp120-associated neurotoxicity. A role for Schwann cells in the neuropathogenesis of HIV has been previously investigated, as too has their involvement in neuropathic pain. It has been reported that 24h exposure of embryonic DRGs, co-cultured on a Schwann cell monolayer, to dual-tropic gp120_{MN} (1 pg/ml–1 µg/ml) results in significant reduction of neurite outgrowth in a concentration dependent manner (Keswani *et al.*, 2003). Exposure to gp120_{MN} also caused a significantly increased level of RANTES (CCL5) in the culture media, therefore the authors hypothesised that the neurotoxicity was mediated via gp120 binding to Schwann cells that resulted in the release of neurotoxic agents, including RANTES, resulting in neuronal toxicity. As an important role for Schwann cells had already been highlighted the effects of gp120 on Schwann cells did not become the focus of my project. However, as previously noted, many of the previous studies were performed on embryonic or neonatal-derived Schwann cell cultures and using a different gp120 strain therefore these studies

were performed to gain further clarification and to be able to correlate the responses to those of BMDMs and neurons.

In my own studies I found, in the setting of a mixed adult neuronal-glial culture, application of gp120_{MN} also resulted in a reduction of mean neurite outgrowth. Only a minority of neuronal cells internalised gp120 and yet a global reduction in outgrowth was observed. Internalisation was observed within 1hr of application and yet first evidence of neurotoxicity occurred only after 24h of exposure.

Together these data indicate that a direct mechanism of action of gp120 on neuronal cell seems unlikely and that involvement of non-neuronal cells is required. As stipulated previously there are profound differences between embryonic, neonatal and adult primary DRG cultures. In light of the fact that the majority of patients suffering from HIV-SN are adults, it is surprising that, to the best of my knowledge, the effect of gp120-activated Schwann cells on adult primary sensory neurons has not been previously assessed with these effects having only been assessed in embryonic and neonatal culture systems. Therefore as the most abundant contaminating non-neuronal cell in my cultures were Schwann cells, I aimed to ascertain whether the gp120-stimulated Schwann cells released compounds that were neurotoxic to adult DRG cultures.

I demonstrate that compounds released, into the culture media, by Schwann cells treated with gp120, are neurotoxic to adult cultured DRG neurons in that they induce a reduction in mean neurite outgrowth, to a similar degree as direct application of gp120 to mixed neuronal cultures. Neuronal neurite outgrowth was unaffected by application of culture media from Schwann cells treated with denatured gp120. This indicates that gp120 specifically stimulates Schwann cells through a receptor-mediated pathway.

As with the BMDM supernatant study, I attempted to limit the possibility of gp120, from the Schwann cell media, being carried over to the neuronal culture by passing the Schwann cell culture supernatant through a 50 kDa Vivaspin column. On the chance that gp120 was carried over, I knew that gp120 is an unstable molecule and its protein structure is very vulnerable to thermal changes. Therefore I was intrigued to determine the activity of gp120, following 24h at 37°C in cell-free culture media and in turn deduce whether gp120 carried over from Schwann cell cultures remains biologically active. I found that 24h pre-incubation of gp120 in cell-free culture media at 37°C completely abrogated its ability to induce neurotoxic effects on DRG neurite outgrowth. This adds support to thermal instability of the gp120 glycoprotein, but it also indicates that, although cultures were treated with gp120 for 24h, the gp120 is likely to have lost activity by the end of this

incubation. Therefore the neurotoxic pathways are likely to have been activated within the first few hours. This highlights an important result because the effects to neurite outgrowth were only visible after 24h, and not earlier despite early gp120 interactions. Therefore gp120-mediated axonal degeneration occurs significantly later after the first activation of potentially neurotoxic pathways. This indicates that more than one cell type (i.e. neuronal and non-neuronal cells) and a multitude of complex pathways are likely to be involved.

While I demonstrate that Schwann cell-secreted compounds instigate a reduction in neurite outgrowth, the degree was more subtle (mean 20.9 ± 1.6 % reduction from vehicle) than previous effects seen in chapter 2 (mean 75.3 ± 5.6 % reduction from vehicle) and compared to the effects reported by Keswani *et al.* (Keswani *et al.*, 2003). I offer numerous explanations for this, relating first to the time points chosen. I incubated the Schwann cells for 24h as I felt this may maximise the amount of Schwann-cell secreted compounds accumulated in the media before applying to neuronal cultures. However, in light of the parallel assessment of gp120 activity after 24h incubation at 37°C it may be these Schwann cells were stimulated for only a proportion of their incubation time before gp120 denatured and thereby halting its stimulatory effect. Time following the gp120 denaturation may be seen as recovery time in which mRNA expression is normalised and secreted compounds may be scavenged or degraded under their tight regulation. Unfortunately, I did not confirm or identify any of the Schwann cells-secreted compounds released, however other studies have reported TNF- α and CCL5 production by gp120-activated Schwann cells *in vitro* (Keswani *et al.*, 2003). Chemokines and cytokines have variable half lives and may be released at different rates; therefore I have no indication of the components within the culture media, at the time point at which it was collected. Another potential explanation for this subtle effect may be the quantities I applied. As I was unable to determine the composition of compounds equally I therefore did not know the concentration in the media. Again a dilution of 1:1 (Schwann cell-conditioned media:neuronal culture media) was used however if the initial concentrations were low this further dilution may have resulted in concentrations that had little biological activity.

Finally, if this study is to be taken as a true representation of the responses of adult neurons to Schwann-cell secreted compounds then it would indicate strong differences in the susceptibility of embryonic neurons versus adult neurons, to gp120-mediated neurotoxicity. This may certainly be true, especially considering the different requirements and physiology of these populations. However slight caution should also be taken when considering the effects reported by Keswani *et al.* I spent considerable time and effort deciding on the most appropriate technique for measuring neurite outgrowth to maintain accuracy and to yield biological relevance, as discussed in Section

2.5.2. To re-examine; in Keswani's study, no explanation was provided on how images were acquired, how the images were selected or how the immunocytochemistry was quantified or normalised. They recorded neurite outgrowth from approximately 10 neurons per coverslip, which, considering the variability that can be experienced in neurite outgrowth from one cell to the next and considering they plated 13,000 neurons per coverslip, seems a very under-representative selection.

This experiment, together with other reports, certainly indicates a role for Schwann cell-secreted compounds in axonal degeneration however I remain unable to comment on the identity of the compounds involved or which cell-types they were acting on. As stipulated previously, the neurite outgrowth studies are performed in the presence of non-neuronal cells, and once again this study still allows for Schwann cell-released mediators to act directly on neurons or indirectly via the non-neuronal cells present.

I was also keen to establish and compare the relative activation ability and function of two different gp120 tropic strains in Schwann cells *in vitro*. Assessing protein levels is hampered by the sensitivity of the techniques available and the vulnerability of proteins to degradation before analysis is performed. Therefore, using qPCR and a comparative C_T analysis method, I investigated the gene expression changes in pure primary adult Schwann cell culture monolayers, for 4 targets commonly cited for their involvement, at the protein level in inflammation and neurotoxicity and for their identification in the macrophage cytokine array, IL-1 β (Brabers *et al.*, 2006), TNF- α , IL-6 and iNOS (Rothwell, 1997; Lipton, 1998; Brabers *et al.*, 2006). Therefore I felt this offered a useful tool to assay 1) whether I used a valid culture system and 2) that the Schwann population was responsive to gp120 allowing us to compare the relative responses to different gp120 strains.

I was able to confirm which gp120 tropic strains were biologically active and that the Schwann cells in culture were responsive to LPS and gp120 exposure in a way that correlated with previous reports. I demonstrate that primary cultured Schwann cells from adult rats rapidly and significantly upregulate mRNA expression of IL-1 β , TNF- α and iNOS on exposure to the M-tropic (R5-utilising) gp120_{Bal}, an effect that was not seen on exposure to the denatured form. This indicates that this response occurs through the specific activation of a receptor expressed on the Schwann cell, most likely CCR5, and not a result of exposure to a foreign highly glycosylated protein. On that note, intriguingly the dual tropic gp120_{MN} strain, which can target and bind to both CCR5 and CXCR4, showed no significant induction in the genes assessed. The responses induced by gp120_{Bal} support previous studies that observed increased TNF- α , IL-6 and iNOS expression in Schwann cells after exposure to recombinant protein (Bolin *et al.*, 1995; Keswani *et al.*, 2003) and infection of HIV virus

(Jones *et al.*, 2005). This confirms that these studies can be correlated to other studies and may highlight additional important targets in gp120-mediated neurotoxicity.

There are two possible explanations for the differential activation of gp120_{MN} and gp120_{Bal}. Firstly, as previously stipulated in section 3.5.1, gp120 is one of the most heavily glycosylated proteins in nature and the combination and complexity of glycans bound plays a significant role in the physiological activity of gp120 (for review see Marth *et al.*, 2008). Therefore the variability in glycosylation state may provide gp120_{Bal} with an enhanced binding ability that is not afforded to gp120_{MN}.

The second point relates to the different tropisms of the two glycoproteins used in this study. Gp120_{Bal} targets predominantly the CCR5 receptor, while gp120_{MN} can target either CXCR4 or CCR5 depending on the cell type, often acting through just one even when both are present, although what determines this coreceptor selectivity is still unknown. It has been postulated, in fact, that gp120_{MN} targets and acts via CXCR4 on Schwann cells (Keswani *et al.*, 2003). In light of the fact that I also saw no mRNA induction following SDF-1 exposure, the endogenous ligand for CXCR4, this may support the idea that gp120_{MN} acts through CXCR4 also in adult cultures. Therefore this lack of gene expression may be due to lower availability of CXCR4 than CCR5, however this seems unlikely because my immunocytochemistry data indicated the opposite and this is supported by others who claim basal CXCR4 levels are greater than CCR5 (Oh *et al.*, 2001). Alternatively it may simply be due to the different functional roles of these chemokine receptors and the recruitment of different secondary messengers that induce different pathways, including the targets of gene expression modulation. This may therefore indicate CCR5 as the sole driver of neurotoxic effects mediated through Schwann cells.

These experiments were performed a while after the first experiments assessing direct application of gp120_{MN} to neuronal cultures, therefore there is the possibility that the activity of gp120_{MN} diminished over time during storage, as has been stipulated to occur.

3.5.7. Gp120 induces cytokine expression changes *in vivo* following intraplantar injection.

The upregulated cytokine expression identified in plantar tissue 5h following intradermal injection of gp120_{Bal} gave biological validation to my *in vitro* data. The portion of tissue extracted would have contained numerous cell types however this study did not address or determine in which cell type(s) these gene expression changes occurred. The epidermal and dermal layers consist predominantly of

keratinocytes, merkel cells, Langerhan cells and inflammatory cells, and macrophages, fibroblasts and adipocytes, respectively (McGrath JA *et al.*, 2004; Dupasquier *et al.*, 2004). However considering what is known about the cell types that gp120 can target (they must express CCR5 or CXCR4) and the cells that express the genes targeted, it is possible that macrophages, Langerhan cells or keratinocytes were responsible for the expression changes observed. However, the induction of enhanced iNOS expression is suggestive of macrophage infiltration due to the predominantly macrophage-specific expression of iNOS.

Furthermore the observation that gp120_{Bal} induces a rightward shift, or increase in the melting temperature (T_m) of the iNOS PCR products in *in vivo* samples adds further biological validity to my *in vitro* studies. This phenomenon suggests that a iNOS splice variant exists in normal naive conditions and the rightward shift would indicate newly expressed iNOS mRNA before splicing, which gives it the larger size and hence more energy required to denature it. Interestingly however unlike the *in vitro* studies, which demonstrated this rightward shift only in samples treated with gp120_{Bal}. Denatured-gp120_{Bal} samples also showed evidence for increased iNOS T_m . This may suggest that intradermal injection *per se* activates some inflammatory processes however gp120_{Bal} then actively exacerbated these effects.

Intradermal injection of 250 ng gp120_{IIIb} has been shown previously to induce mechanical hypersensitivity as indicated by a significant reduction in the withdrawal threshold to mechanical vF stimulation, with rapid onset (Oh *et al.*, 2001). It was also reported that immediate and pronounced vocalisation followed the intradermal injection. These effects were observed with 10mins of injection and were apparent after 3h, the last time point recorded in this study. Upregulation of algogenic cytokine gene expression is unlikely to be responsible for the hypersensitivity observed immediately following intradermal injection, which is driven either by directly activation of nociceptive fibres by gp120 at the peripheral terminals or gp120-activated macrophages locally release algogenic compounds. However it is interesting to consider whether the gene expression changes I observed in my studies relate to the maintenance of the hypersensitivity behaviour observed in more long-term *in vivo* experiments following a single acute application of gp120 (Wallace *et al.*, 2007b). Furthermore while neurotoxicity and hypersensitivity following gp120 exposure is becoming accepted, it still remained unknown which cytokines and chemokines produced or were involved in these effects *in vivo*. Therefore my data for the first time clarifies the mechanisms and mediators activated by gp120 in the peripheral nervous system following acute exposure and may offer investigators the opportunity to further isolate and clarify the individual roles of those highlighted.

3.5. Summary

The data presented here highlights the importance of gp120-activated non-neuronal cells in the initiation of axonal degeneration but also the complexity of pathways involved. This correlates with the insight that the severity of axonal degeneration has been shown to be linked with the degree of immune cell infiltration at the site of nerve damage. Compounds released from gp120-stimulated macrophages and Schwann cells induced neurite degeneration however to a mild degree suggesting that no single compound is the key mediator and that a multitude of complex signalling is required. Furthermore, I identified that gp120 loses its biological activity after 24h in media maintained at 37°C, indicating that gp120 is thermally unstable and that gp120-mediated events occur within the first 24h. This data also strongly indicates that gp120 induces an M1 pro-inflammatory phenotype in bone marrow-derived macrophages on first exposure. Moreover, macrophages in an M2 anti-inflammatory state lose the ability to induce TNF- α gene upregulation on exposure to gp120. I did not observe acute gp120-induced calcium modulation in macrophages however the effects to calcium levels after chronic exposure should be explored.

Chapter 4: General Discussion & Conclusions

4.1. Discussion

4.1.1. Possible mechanisms underlying gp120-mediated neurotoxicity

While many broad pathways are beginning to be elucidated and implicated among the mechanisms underlying HIV-mediated neurodegeneration, large gaps still remain. The studies presented in this thesis have broadly investigated the relative involvement of different cell types and given an insight into the temporal course of gp120-mediated neurite degeneration using an *in vitro* model. My studies have indicated that gp120-mediated neuronal toxicity is driven predominantly by the non-neuronal cells neighbouring neurons of the peripheral nervous system (resident macrophages and Schwann cells) that, when stimulated by gp120, release a diverse range of pro-inflammatory and excitotoxic compounds that induce neurotoxicity to nerves in the surrounding area. I identified direct binding of gp120 to neuronal cells however the studies performed do not explicitly demonstrate a causal relationship between these neuronal-gp120 interactions and neurite degeneration. To assess this, studies using CCR5 and or CXCR4 antagonists to block the gp120 binding, would be required to correlate the interactions with the neurite degeneration. The same applies for the implicated nuclei-specific localisation of biotinylated-gp120 in which incubation with wheat germ agglutinin, which has been shown to inhibit nuclear import (Finlay *et al.*, 1987; Yoneda *et al.*, 1987), would be required to 1) verify whether the punctate Bgp120-labelling was indicative of nuclear localisation, and 2) whether it had an involvement in neurodegeneration.

There are now many people who consider the mechanisms behind axonal degeneration to be either the same as or similar to those underlying Wallerian degeneration (Wang *et al.*, 2000);(Coleman, 2005). Therefore many insights can be gained by looking at what is currently known about the pathways activated in the initiation of Wallerian degeneration and other neurodegenerative disease states. Calcium dysregulation is considered to play an essential role in axonal degeneration. The removal of Ca^{2+} has been shown to delay axonal degeneration suggesting that upstream Ca^{2+} -dependent signalling is involved (Glass *et al.*, 1994; George *et al.*, 1995). Further evidence supporting a role of calcium signalling in axonal degeneration is given by evidence of consistent calpain activation, a Ca^{2+} -dependent protease, in several models of neuronal degeneration (Camins *et al.*, 2006; reviewed by Liu *et al.*, 2008b). Increased calpain activity has been identified in hippocampal neurons treated with HIV-infected monocyte-derived macrophages (Ehlers, 2004). Ehlers *et al.* presented data showing that NMDA receptor-stimulation in a model of HIV led to activation of the

enzyme CDK5 (cyclin-dependent kinase 5) via calpain as depicted by an increase in calpain cleavage products, increased p25 formation and CDK5 activity. CDK5 is involved in neuronal migration, control of neurite outgrowth, modulation of glutaminergic transmission and the regulation of pain signalling (Wang *et al.*, 2007) and an essential regulator of neuronal survival, neuroprotection and neuronal death pathways (Vartiainen *et al.*, 2002; Wang *et al.*, 2006). Calcium influx and NMDA-R activation have been shown previously to activate the cyclin-dependent kinase 5, CDK5, via calpain activation in response to neurotoxicity (Kusakawa *et al.*, 2000; Lee *et al.*, 2000). Treatment with NMDA-R antagonists and calpain inhibitors were shown to prevent these processes and resulted in delayed axon degeneration. CDK5 is regulated by neuronal-specific p35 (1/2 life of 20–30 min), with which it needs to bind to become activated and this association is linked with neuroprotection. However in pathological conditions p35 is cleaved by calpain to the more stable isoform p25 and p25:CDK5 complexes have been associated with neurotoxic function (Patrick *et al.*, 1999). Increased p25:CDK5 activity has been observed in neurodegenerative disorders including Alzheimer's (Pei *et al.*, 1998; Patrick GN *et al.*, 1999; Tseng *et al.*, 2002; Lopez-Tobon *et al.*, 2011), amyotrophic lateral sclerosis (Nguyen *et al.*, 2001; Patzke *et al.*, 2002), Parkinson's (Neystat *et al.*, 2001; Chergui *et al.*, 2004; Alvira *et al.*, 2008) and Huntingdon's disease (Luo *et al.*, 2005; Paoletti *et al.*, 2008). Further evidence demonstrating the similarities between these neurodegenerative diseases and HIV-neuropathy came from the identification of increased [p25] in midfrontal cortex autopsy tissue from HIV patients with HIV-associated dementia (HAD) (Wang *et al.*, 2007). Wang *et al.* also verified that NMDA-R stimulation and calpain activation led to cleavage of p35 and enhanced p25:CDK5 activity, in an *in vitro* model of HIV that used primary rat cortical cultures exposed to supernatants derived from HIV-infected macrophages (Wang *et al.*, 2007).

Gp120, acting through CCR5 and CXCR4, has been demonstrated to release Ca^{2+} from intracellular stores (Boutet *et al.*, 2001), activate MAPK p38 signalling pathways and induce NMDA-mediated excitotoxicity (Meucci *et al.*, 1996; Meucci *et al.*, 1998). Increased p25 concentrations have also been identified in autopsy tissue from HIV-infected individuals (Wang *et al.*, 2007). Therefore it is conceivable that a common link exists between signalling pathways activated by gp120 exposure and those recognised in several neurodegenerative disease states, which may involve calcium dysregulation leading to calpain activation. The eventual results of these effects could lead to mitochondrial toxicity, a dysfunctional ubiquitin-proteasome system and energetic problems, as highlighted in my review of the current literature Figure 1.5.1. Whether these effects then in turn lead to neurite degeneration and associated pain symptoms, as suggested by their presence in multiple neurodegenerative disease states, would be of great interest and requires further investigation.

In my own studies I did not find evidence for acute calcium signalling mediated by gp120 exposure in my culture set-up; however the temporal effects of chronic and long-term exposure to gp120 remains to be investigated. Calcium dysregulation and NMDA-mediated excitotoxicity have been heavily suggested to underlie the neurotoxicity of many pathologies including HIV-SN however these occurrences are in the context of chronic infection or long-term exposure. In addition, importantly, what links the calcium dysregulation and excitotoxicity to the activation of axonal degeneration is still to be addressed, although many investigations are currently underway to answer this question. Longitudinal studies assessing both calcium influx and neuronal outgrowth over a more extensive time course may be helpful in determining the involvement and temporal profile of calcium signalling in neurite outgrowth in the context of HIV infection.

As stipulated previously, strong correlations have been identified between the degree of macrophage infiltration and extent of axonal degeneration (McArthur *et al.*, 1989; Griffin JWCT *et al.*, 1998). In turn, the level of reduction in intraepidermal nerve fibre (IENF) density in HIV+ individuals has been associated with greater intensity of accompanied pain (Polydefkis *et al.*, 2002). Therefore, the potent cytokine gene expression changes, the observation of neurite degeneration *in vitro* in my studies and signs of hypersensitivity induced in gp120 models *in vivo* (Wallace *et al.*, 2007a) suggests a link between these acute gene changes, macrophage activation and the resultant neuronal damage and associated pain. I consider it unlikely that a single secreted cytokine underlies the toxic actions of gp120 as many of the most highly upregulated genes identified by the cytokine array in gp120-treated BMDMs, such as IL-1, TNF- α , COX-2 and CCL2, have similar functions and effects (Mantovani, 1999). As reviewed by Mantovani *et al.*, a great redundancy exists in cytokine signalling. All four of these cytokines have been shown to induce hypersensitivity in rodent models (Wagner *et al.*, 1996; Sachs *et al.*, 2002; Lee *et al.*, 2004b; Wei *et al.*, 2007; Thacker *et al.*, 2009; Vardeh *et al.*, 2009). All have been identified at high levels in various inflammatory or neuropathic disease states (Mastroianni *et al.*, 1992; Rothwell, 1998; Griffin *et al.*, 2002; Thacker *et al.*, 2009; Conductier *et al.*, 2010). Furthermore they can all amplify the immune response, through immune cell recruitment and activation of additional signalling pathways (Bankers-Fulbright *et al.*, 1996; O'Banion, 1999; Wajant *et al.*, 2003; Eugenin *et al.*, 2006; Minghetti *et al.*, 2007). Furthermore many of the mediators released from M1 phenotypic macrophages, such as QUIN and RANTES, have not only algogenic properties but also the ability to induce calcium mobilisation either via NMDA-R activation or through the release of calcium from intracellular stores, respectively. Antagonists against L-type voltage gated Ca²⁺ channels (Dreyer *et al.*, 1990) or NMDA receptors (Lipton *et al.*, 1991; Alirezai *et al.*, 2007) are protective against gp120-mediated neuronal damage. Therefore while acute calcium responses were not observed directly in the cell types investigated shortly after

exposure, it is plausible that calcium signalling could be activated at a later time point following chronic stimulation and activation of multiple pathways. Therefore, combined with potential direct neuronal activation of gp120 and insult from anti-retroviral drug therapies, the chronic stimulation by non-neuronally secreted cytokines and excitotoxic compounds may lead to a gradual increase in calcium signalling. Once these have reached a critical threshold they may act to mediate an active process of axonal degeneration.

4.1.2. The importance of macrophage signalling in gp120-mediated neurotoxicity

There is a multitude of data that now highlights the critical involvement of macrophages in gp120-mediated neurotoxicity. However despite all the supportive evidence progress in terms of identifying why some people develop HIV-peripheral neuropathy as a result of nerve toxicity and why others do not remains unexplored. There are numerous factors that can influence a person's chances of developing HIV-associated neuropathy including, 1) the HIV strain, 2) the length of time of infection, and 3) the type and time of HIV drug therapy, as external factors. However regarding internal factors, as well as height and age as risk factors, genetics can also greatly affect a person's vulnerability to neuropathy. Furthermore, although humans are, broadly speaking, physiologically similar, a person's psychology and, relevant to this discussion, their immune system, are two fields that vary dramatically. This is highlighted particularly prominently, as noted in the General Introduction, by the fact that the natural hosts, sooty mangabeys (infected with SIV_{sm}) and African green monkeys (infected with SIV_{agm}), do not progress to AIDS because of the lack of a potent immune response to SIV infection. Therefore with the evidence from my own work and that of others that indicates a prominent role for macrophages I was keen to elucidate the activation state of gp120-stimulated macrophages and to determine which phenotypic state is required in order for them to respond as they do to HIV-1 gp120. It has been postulated that in terms of protection against HIV infection, a dominant Th1 cytokine profile is more beneficial than a Th2 response (Clerici *et al.*, 1994). This is most likely due to their predominant cytolytic capabilities and the down-regulation of CD4 receptors, antagonistic effect of released CCR5-binding ligands (CCL3, CCL4 and CCL5) and the potent inhibition on HIV viral replication that occurs as a result of the Th1-M1 cytokine profile (Cassol *et al.*, 2009). It is also likely that these effects driven by the Th1-M1 phenotype are responsible for the latency between infection and progression to AIDS and therefore also offers protection against host death. However what is not often discussed in the studies that address this subject are the effects of chronic Th1-M1 microenvironments surrounding sensory

neurons in the peripheral nervous system. It has been demonstrated that an M1 macrophage phenotype predominates following traumatic spinal cord injury, frequently accompanied by poor nerve regeneration and neuropathic pain. Recently Kigerl *et al.* investigated the relative effects of M1 and M2 cytokine profiles on neurite outgrowth finding that only M1 macrophages are neurotoxic to cortical neuronal cultures, while M2 macrophages promote a regenerative growth response in adult sensory axons (Kigerl *et al.*, 2009). In these studies I have demonstrated that gp120 potently stimulates gene expression changes that are representative of an M1 immune response in both BMDMs and Schwann cells *in vitro* and produces similar effects *in vivo*. Many of the cytokines included in this profile have established algogenic and neurotoxic abilities and I showed that application of conditioned media from gp120-treated BMDMs and Schwann cells induced neurite degeneration. Therefore it is tempting to speculate that although protective against HIV infection a chronic M1-Th1 phenotypic immune activation underlies the sensitisation and sub-clinical neurotoxicity in the context of HIV infection mediated through non-neuronal cell activation by virion-bound or monomeric gp120.

To test these findings further in an *in vivo* setting it would be interesting to use an animal model of gp120-mediated neurotoxicity, such as that established by Wallace *et al.* (Wallace *et al.*, 2007b) for an acute perspective or Toggas *et al.* (Toggas *et al.*, 1994) for chronic exposure and treat animals pre- or post-surgery with clodronate-loaded liposome capsules. Clodronate depletes blood monocytes to prevent the production of macrophages through monocyte maturation. Clodronate treatment may be helpful in verifying the importance of macrophages in gp120-mediated neurotoxicity and the neuropathic pain associated with it.

Furthermore I would suggest a more representative animal model of gp120-mediated neuropathy would be a transgenic murine model, similar to that described by Toggas *et al.* However I would insert gp120 expression behind a promoter for a macrophage-specific gene such as ED1, rather than under the control of a modified murine glial fibrillary acidic protein (GFAP) gene as performed in that study. This would allow gp120 to be expressed and released by BMDMs, systemically including the PNS, rather than predominantly expressed by astrocytes in the CNS, thereby providing a closer mimic of the clinical setting.

In future studies, it would be interesting to establish cytokine array profiles of both the neuronal and Schwann cell responses to gp120 *in vitro* and intraplantar tissue, the sciatic nerve and DRGs following gp120 exposure *in vivo*, either distally or proximally to the point of application and compare these against the *in vitro* BMDM cytokine profile. A temporal profile of the cytokine changes would be

particularly useful, especially if correlated with pathological and behavioural changes in an animal model of gp120-mediated neurodegeneration.

4.1.3. Future directions in therapeutics for HIV-DSP

In this project I have made it highlighted how aberrant cytokine signalling may be a dominant cause of neuronal damage and associated pain. Therefore therapeutic ways to control the vast array of signals and mediators involved is complex. Inhibition of a single mediator is unlikely to solve the issue therefore the therapy involved must target and control the functionality of the cells involved themselves. In relation to macrophages this will consist of preventing or modifying the chronic activation and release of pro-inflammatory cytokines without causing immune suppression or removing the inhibition on viral replication that these states provide. Recent work using the long-chain n-3 fatty acids as dietary supplements following nerve injury to manipulate the immune response (Calder, 2003) may be of particular interest and relevance to this topic. Providing additional nutritional support to surgical or critically ill patients with fish oils, rich in the n-3 (omega-3) fatty acids eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA), has been found to reduce the inflammatory and immune changes induced by surgery or nerve injury (Fürst *et al.*, 2000). Lowered production of pro-inflammatory cytokines and eicosanoids (Endres *et al.*, 1989; Endres *et al.*, 1991; Senkal *et al.*, 1995; Wu *et al.*, 2001), increased lymphocyte proliferation (Cerra *et al.*, 1990), reduced wound healing complications (Gottschlich *et al.*, 1990), improved tissue repair responses (Cardoso *et al.*, 2011) and increased phagocytosis by monocytes (Shilling *et al.*, 1996; Braga *et al.*, 1996) are all findings resulting from the delivery of n-3 polyunsaturated fatty acids (PUFAs) to patients. Furthermore, *in vivo* studies have demonstrated significant neuroprotection, reduced neuronal and non-neuronal cell death, reduced monocyte recruitment and improved locomotive recovery after spinal cord compression injury in rats receiving dietary DHA (Huang *et al.*, 2007). It appears that the beneficial effects of n-3 fatty acids are gained via an inhibitory action on the cyclo-oxygenase pathway (Fürst *et al.*, 2000) that normally leads to the production of prostaglandins (PGE₂ and PGF₂α) and thromboxane A₂. The scientific basis of immunonutrition with PUFAs has been reviewed by Suchner who outlined the basic mechanisms by which PUFAs modulate pro-inflammatory biology (Suchner *et al.*, 2000). Firstly, through altering membrane fluidity, which can in turn affect G-protein activity (Fürst *et al.*, 2000) and the binding of cytokines and cytokine-inducing agonists (Stubbs *et al.*, 1984). Secondly, by replacing arachidonic acid (AA) as a substrate for the 5-lipoxygenase enzyme in the cyclo-oxygenase pathway and altering the metabolites that result,

which have been shown to have reduced activity than AA-derived metabolites (Kinsella *et al.*, 1990; Grimble, 1992; Ross *et al.*, 1999; Moreno, 2009). Therefore it is interesting to speculate that delivery of unsaturated fatty acids (EPA and DHA) to HIV patients, before the onset of a neuropathy, may be protective against nerve damage and prevent or reduce the number of patients developing HIV-DSP. Interestingly the immune modulation with EPA and DHA is well tolerated by both healthy patients and the critically ill (for review: Fürst *et al.*, 2000; Calder, 2003) and at carefully calculated dose regimes, in most patients, are immunosuppressive but not to a negative degree (Virella *et al.*, 1991; Endres *et al.*, 1993; Schauder *et al.*, 2002). However care will need to be taken to determine the correct dosing of n-3 fatty acids prescribed, the delivery method and the course of treatment to ensure negative effects of immunosuppression do not result. Therefore nutritional supplementation with unsaturated fish oil for HIV patients, early in the course of infection, may provide a natural and beneficial therapy. The important note is that this strategy modifies the M1 response but does not ablate it and therefore provides neuroprotective effects, reducing pain associated with HIV by reducing the release of algogenic compounds but without compromising the host immune defence. The few contraindications of EPA and DHA would also allow them to be used alongside other HIV- or non-HIV-related medicines the patient may be taking. In fact one study has demonstrated a role of n-3 fatty acids in the prevention of disease complications in HIV positive patients (Woods, 2005). However further studies are required to determine the effects of long-term EPA or DHA intake in the context of immunosuppressive diseases and whether the immunosuppressive effects are in terms of a dampened Th1 response or whether a switch to a dominant Th2 phenotype can occur, which would be detrimental in the context of HIV.

4.2. Conclusions

Direct neuronally-mediated mechanisms may be present in the context of HIV infection however I believe they are less prominent early in infection and become more potent following prior activation or insult. My studies demonstrated that gp120 interacts with DRG neurons in an in vitro model of an adult PNS. However together with the low expression levels of CXCR4 or CCR5, the small proportion of neurons that bound gp120 and given the apparent low binding affinity of gp120 for these receptors in the absence of CD4, mechanisms resulting from these interaction are unlikely to sufficiently explain the global reduction in neurite outgrowth observed in my studies.

I propose that the major contributors to axonal degeneration in HIV-infected individuals are gp120-stimulated macrophages and the pro-inflammatory compounds that they secrete as a result. I

suggest that the compounds secreted by macrophages, on acute exposure to monomeric or virion-bound HIV-1 gp120, fit a chronic pro-inflammatory M1 phenotype that underlies the neuronal sensitisation and sub-clinical neurotoxicity evident in HIV infection.

Gaining a complete cytokine profile of all stages of HIV infection in correlation with the pathological changes may allow researchers to understand the key mechanisms involved in neurotoxicity of HIV infection and clarify why some people are more negatively affected by HIV infection than others. These insights may provide useful knowledge to aid in the understanding of other disease states that share some common pathways.

In consideration of possible therapeutic strategies I suggest that targeting a single cytokine would not be sufficient to prevent the gp120-induced cytotoxicity. Instead the exaggerated immune response needs to be reduced or prevented from the source. It will be interesting to review how the prevalence of HIV-DSP changes over the coming years following the recent introduction of the CCR5 antagonist, Maraviroc in HIV therapy. My studies demonstrated that Maraviroc, at a dose representative of that used clinically, prevented the gp120-induced changes in gene expression. From a new therapeutic perspective I postulate that an alternative reduction of the immune response to HIV may be obtained through dietary supplementation with EPA and DHA, which can modify M1 pro-inflammatory responses without overt negative immune suppression, thereby providing a balance in the M1/M2 axis. Such a reduction could prevent the detrimental neurotoxic affects and associated pain elicited by secreted pro-inflammatory and algogenic compounds, and in turn reduce the prevalence of HIV-SN and associated chronic pain.

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