Cell signalling and feline immunodeficiency virus growth and latency

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Abstract

The replication of $CD4^+$ T cell-tropic retroviruses such as the human immunodeficiency virus-1 (HIV-1) and the feline immunodeficiency virus (FIV) is intricately linked to host cell signalling and activation status. This intimate relationship between the viruses and their respective hosts plays crucial roles in the pathogenesis of each virus. Focusing on FIV, this thesis examined several questions relating to virus replication and cell signalling. Firstly, the possible cell stimulatory effect when FIV Env binds to its primary receptor CD134 was investigated and results suggested that FIV does not trigger CD134 signalling. Next the activation status of susceptible $CD4^+$ T cells was manipulated to study FIV latency and it was shown that by removing exogenous supplement of the cytokine interleukin-2 (IL-2) from MYA-1 feline $CD4^+$ T cells 24 hours before infection, productive FIV replication is down-regulated such that only a very low level of ongoing virus replication can be detected among the IL-2-depleted cells with a sensitive qPCR assay. The phorbol esters PMA and Prostratin can stimulate high level of productive infection from these IL-2-depleted MYA-1 cells and this is mediated by a protein kinase C (PKC) dependent mechanism. Furthermore, productive replication of FIV in the presence of IL-2 is blocked by stimulation of PKC with phorbol esters, which is analogous to findings of similar experiments with HIV-1. However, inhibition of viral replication is not at the level of viral entry, contrary to the findings of HIV-1 studies. The mechanism behind the Prostratin-mediated inhibition of FIV remains elusive. It was also discovered that 'early' and 'late' strains of FIV responded differently to cell signalling manipulation and an attempt was made to map the viral genome region(s) responsible. Preliminary data showed that both *env* and the 5'UTR may mediate the inter-strain differences in replication dynamics.

Overall this thesis shows the complete dependency of FIV on host cell signalling, in particular optimum PKC activation to achieve productive viral replication. This may reflect the tropism of the virus. The similarities between HIV-1 and FIV replication and latency support the notion of using FIV and the cat as a model for HIV-1 latency in the development of novel therapeutic measures to eradicate hidden HIV-1 from the host. However, more research is required to fully characterise the differences between HIV-1 and FIV biology.

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Preface

The mammalian cell contains a vast network of signalling pathways which monitor and respond to changes in the external and internal environment. Essential processes such as cell division, metabolism and apoptosis are controlled by a delicate balance of a vast number of cellular and exogenous signals. Viruses are obligate intracellular parasites which have evolved to manipulate the host cell to their own advantage. However, the virus is also vulnerable to global changes of cellular activation. Unravelling this intimate relationship between the virus and its host is essential for us to understand the viral life cycle and may reveal weaknesses which can be targeted therapeutically.

The lentiviruses HIV-1 and FIV are global public health and veterinary concerns respectively. They are similar viruses in terms of tropism, molecular biology and pathogenesis. In this thesis I focused on FIV and investigated the potential effects of FIV on cell signalling and the potential effects of cell signalling changes on FIV replication and latency.

Acknowledgement

This thesis is only possible because of Professor Brian Willett and Professor Margaret Hosie, who generously welcomed me into their "scientific family" and subsequently provided much needed support, guidance and assessment of my work. I am forever grateful to have spent the last three and a half years with the magnificent people in the Retroviral Research Lab, namely Linda McMonagle, Nicola Logan, Hazel Stewart, Isabelle Dietrich, Pawel Beczkowski, Will McEwan, Ayman Samman, Emily Goldstein, and last but not least, Martin 'Luciferasse' Kraase. Thank you for the scientific discussion, technical support and most of all for sharing a laugh with me every day. I would like to thank Andy Stevenson and other members of the Institute of Comparative Medicine for their help and most of all patience for the last four years. Also I want to mention Dr Annette MacLeod, who initially invited me to Glasgow all those years ago to be a parasitologist; Professor Andy Tait for sharing his wisdom during all the trips up and down the hill on the bus that never arrives on time; and the ever indefatigable Alex MacKay, whose help during my 'Dunkirk' moment I will never forget. I thank the University Of Glasgow School Of Veterinary medicine for financial support.

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Author's declaration

I hereby declare that I am the sole author of this thesis and performed all the work presented here.

Chi Ngai Chan

30th July 2012

List of Abbreviations

Abbreviation	Meaning
AIDS	Acquired immunodeficiency syndrome
Akt	Protein kinase B
AP-1	Activator protein 1
AP-4	Activator protein 4
APC	Antigen-presenting cell
APOBEC	Apolipoprotein B mRNA-editing catalytic polypeptide
ATF	Activating transcription factor
BCL10	B-cell leukaemia/lymphoma 10
bp	Base pairs
CA	Capsid
CAPE	caffeic acid phenethyl ester
CARMA1	caspase-associated recruitment domain (CARD)-containing membrane-associated guanylate kinase 1
CFSE	carboxyfluorescein diacetate, succinimidyl ester
CPM	Counts per minute
CRD	Cysteine-rich domain
CRFK	Crandell Rees feline kidney (cells)
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
fe	feline (Domestic cat *Felis catus domesticus*)
FITC	fluorescein isothiocyanate
FIV	Feline immunodeficiency virus
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HIV	Human immunodeficiency virus
HMW	High molecular weight
HRP	Horseradish peroxidase
hu	human
IC_{50}	Half maximal inhibitory concentration
IKK	Inhibitor-kappa-B kinase
IκB	Inhibitory-kappa-B
IL	Interleukin
IN	Integrase
JAK/STAT5	Janus kinase/signal transducer and activator of transcription 5
LB	Lysogeny broth
LMW	Low molecular weight
LPS	Lipopolysaccharides
LTR	Long terminal repeat
MA	Matrix
MALT1	Mucosa-associated lymphoid tissue 1

MAPK	Mitogen-activated protein (MAP) kinases
min	Minutes
NFAT	Nuclear factor of activated T-cells
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	Primer binding site or Phosphate buffered saline
PBMC	peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PDK-1	Pyruvate dehydrogenase [lipoamide] kinase isozyme 1
PI3K	Phosphatidylinositol 3-kinases
PIC	Preintegration complex
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PR	Protease
Pro	Prostratin
RGV	Raltegravir
RIP	Regulation of phenobarbitol-inducible P450
RNA	Ribonucleic acid
RPE	R-phycoerythrin
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RT	Reverse transcriptase
SEB	Staphylococcal enterotoxin B
SU	Subunit of Env, Gp120
TM	Transmembrane domain of Env, Gp41
TNC	Chicken tenascin-C
TNF	Tumour necrosis factor
TRAF	TNF receptor-associated factors
TRIM	Tripartite motif protein
UTR	Untranslated region
VSV	Vesicular stomatitis virus
w/v	Weight/volume

1 Background

1.1 Discoveries and origins of the human and feline immunodeficiency viruses

In 1983, the human immunodeficiency virus – 1 (HIV-1) was discovered to be the causative agent of Acquired Immunodeficiency Syndrome (AIDS) identified among homosexual men since the late 1970s (Gottlieb et al., 1981, Barre Sinoussi et al., 1983, Gallo et al., 1984). HIV is transmitted by sexual contact, infected blood or blood contact, and from mother to infant during pregnancy, birth or breast-feeding (Fauci et al., 1984, De Cock et al., 2000). The virus preferentially infects activated $CD4^+$ T cells, causing a decline in their numbers which is associated with progression towards immunodeficiency (Lane and Fauci, 1985). More than two decades after the initial isolation of the virus, 25 million people have died from the disease and over 33 million individuals are living with the virus across the globe (Wainberg and Jeang, 2008). Despite intensive public health campaigns and advances in antiretroviral treatments the virus continues to spread around the world, with 2.7 million individuals having been infected and 2 million AIDS-related deaths having occurred in 2007 (Wainberg and Jeang, 2008). In the absence of an effective vaccine to prevent the spread of infection the global HIV-AIDS epidemic is set to continue to be a major global public health concern long into the future.

HIV-1 evolved from simian immunodeficiency virus (SIV), a primate retrovirus isolated from chimpanzees (Gao et al., 1999). It was assumed that SIV does not increase mortality or morbidity in chimpanzees due to host or virus adaptations that lower viral pathogenicity (Sharp et al., 2005, Silvestri, 2009). However, this is refuted in a recent study of chimpanzees in the wild, which reports ill-health and evidence of immunodeficiency in SIV infected individuals (Keele et al., 2009). Cross-species transmission is believed to have occurred during the hunting and butchering of chimpanzees as bushmeat by hunters in West Africa (Gao et al., 1999), and not as has been asserted by the contamination of oral polio vaccines in the 1950s (Worobey et al., 2004). The current pandemic HIV-1 group M viruses originated from Southern Cameroon (Keele et al., 2006). Based on phylogenetic analysis of viral sequences from historical samples and field isolates, it has been estimated that HIV-1 has been circulating in human

populations since the 1930s (Korber et al., 2000) but was confined within the local area due to the lack of trade and contact with the outside world. With the development of nearby Léopoldville (now Kinshasa, the Democratic Republic of Congo) as a major urban centre by the 1950s the movement of people in the region increased dramatically, allowing the virus to break out of the jungle of Cameroon and undergo its first expansion and diversification in the city, which ultimately resulted in its arrival in America by the 1970s (Worobey et al., 2008, Sharp and Hahn, 2008).

Feline Immunodeficiency Virus (FIV) was first isolated in 1986 from a domestic cat in California (Pedersen et al., 1987). FIV also targets activated $CD4^+$ T cells in cats and causes clinical signs similar to AIDS in humans (Ackley et al., 1990, Novotney et al., 1990, Barlough et al., 1991). Unlike HIV-1, FIV is mainly transmitted by biting (Yamamoto et al., 1989). FIV in domestic cats evolved and expanded globally (Hartmann, 1998) and is divided into genetically distinct subtypes or clades (from A to E) (Sodora et al., 1994, Kakinuma et al., 1995, Pecoraro et al., 1996, Bachmann et al., 1997).

Like HIV-1, FIV is also believed to have arisen in Africa and is believed to have infected the felidae family since the late Pliocene between 3.1 and 0.7 million years ago (Pecon-Slattery et al., 2006, Johnson et al., 2006). Different feline species are infected by their own species-specific FIV strains and based on phylogenetic data FIV is likely to have been introduced once into each species, which then evolved and expand within that species (Troyer et al., 2008). Studies of lion and puma-specific FIV genomes have shown that they have the highest sequence divergence between strains, which suggest a long period of co-evolution between host and virus (Troyer et al., 2008). Also, it has been assumed that FIV infection does not reduce lifespan or increase morbidity in lions or pumas, which is a sign of host-pathogen co-adaptation and signifies the ancestral status of FIV in lions and pumas (Troyer et al., 2008). However, analogous to SIV infection of chimpanzees, it is now known that lions infected with FIV do exhibit AIDS-related pathologies such as lymphadenopathy, gingivitis, tongue papillomas, dehydration, and poor coat condition (Roelke et al., 2009). These new findings may be significant in enhancing our understanding of lentiviral evolution and pathogenesis.

At present it is not possible to pinpoint a definite time for the introduction of FIV into the domestic cat population. The domestic cat (*Felis catus*) was domesticated by humans around 10,000 years ago in the Fertile Crescent region of the Mediterranean from the African wildcat *Felis silvestris lybica* (Driscoll et al., 2007). The absence of FIV from nearly all of the close relatives of the domestic cat (Troyer et al., 2005) suggests a recent introduction of the virus into the species, followed by rapid dissemination (Pecon-Slattery et al., 2006). The successful emergence of a virus in a new population relies on, among other factors, a population density high enough for efficient transmission (Troyer et al., 2008). The domestication process would have increased the population density of the domestic cats, which would aid the establishment and spread of FIV among cat populations.

1.2 The molecular biology of HIV-1 and FIV

The retroviruses are a large and diverse family of enveloped RNA viruses united by a common genomic structure and lifecycle (Coffin, 1992). HIV-1 and FIV are members of the lentivirus sub-family of retroviruses (Table 1-1). The virion consists of two copies of full length viral genomic RNA packaged in the viral capsid, which is surrounded by matrix (MA) proteins and the viral envelope (Coffin et al., 1997) (Fig. 1-1). The dimeric nature of the retroviral genome was first revealed by electron microscopy (Bender and Davidson, 1976, Bender et al., 1978). Further research revealed that the viral RNA genome not only encodes viral genes, but also sites that mediate the dimerization and packaging of the genome into nascent virions (Paillart et al., 2004, Lu et al., 2011). The dimerization initiation site (DIS) and packaging signal of both HIV-1 and FIV are found at a region between the 5'UTR and *gag* of the viral RNA (Paillart et al., 2004, Kenyon et al., 2008). The 5'UTRs of both viruses are believed to fold into secondary structures which can have 2 or more conformations that either allow access to the dimerization and packaging signals or promote translation of viral genes (Lu et al., 2011, Kenyon et al., 2011).

1.2.1 Lentiviral genome structure

All retroviruses contain three basic genes, *gag*, *pol* and *env* (Coffin et al., 1997) (Fig. 1-2). The *gag* gene encodes the major structural proteins MA, capsid (CA) and nucleocapsid (NC) (Elder et al., 1993, Elder and Phillips, 1995). The retroviral NC protein is a multifunctional protein vital for processes such as reverse transcription and viral assembly (Darlix et al., 2011). The viral enzymes such as reverse transcriptase (RT), viral protease (PR) and integrase (IN) are encoded in the *pol* gene (Elder et al., 1993, Coffin et al., 1997). The *env* gene encodes the envelope (Env) protein which mediates viral entry (Elder et al., 1993, Coffin et al., 1997). In addition the lentiviruses may also contain additional regulatory (*Tat and rev*) and accessory (*vif, vpr, vpu, nef, Orf-A*) genes which enhance viral replication or counteract host immune defences (Fig.1-2). Retroviruses that contain only the three basic genes are called simple retroviruses, whereas retroviruses that contain accessory genes are known as complex retroviruses (Table 1-1) (Coffin et al., 1997).

Sub-family	**Examples**	**Simple/ complex**
Alpharetrovirus (avian)	Avian leukemia viruses (ALV) Rous sarcoma virus (RSV)	Simple
Betaretrovrius (mice, primates, sheep)	Mouse mammary tumour virus (MMTV) Mason-Pfizer monkey virus (MPMV)	Simple
Gammaretrovirus (mammals, reptiles, birds)	Murine leukemia virus (MLV)	Simple
Deltaretrovirus (mammals)	Human T-Lymphotropic virus (HTLVs) Bovine leukemia virus (BLV)	Complex
Epsilonretrovirus (fish, reptiles)	Walleye dermal sarcoma virus	Complex
Lentivirus (mammals)	Human immunodeficiency virus (HIV) Feline immunodeficiency virus (FIV)	Complex
Spumavirus (foamy) (mammals)	Human foamy virus (HFV)	Complex

Table 1-1 – A table showing the different sub-family groups of retroviruses which divide into simple and complex retroviruses (Coffin, 1992).

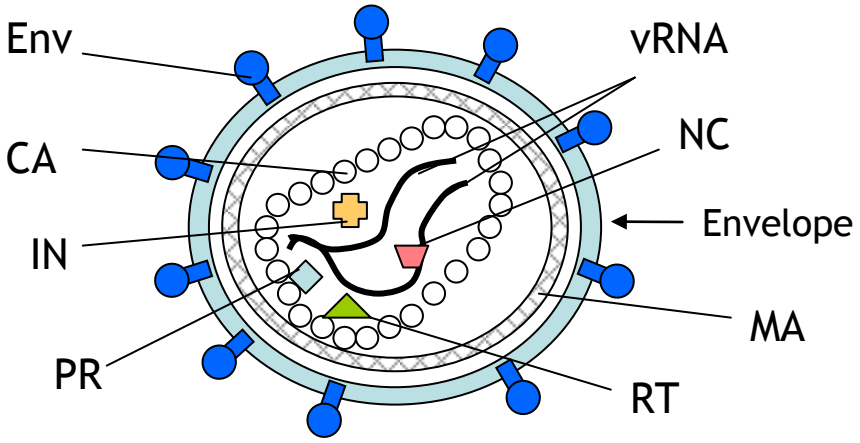


Figure 1-1 – Schematic of a retrovirus particle. vRNA = viral genomic RNA. Env = envelope protein. MA = matrix protein. CA = capsid protein. NC = nucleocapsid protein. IN = integrase. PR = viral protease. RT = reverse transcriptase.

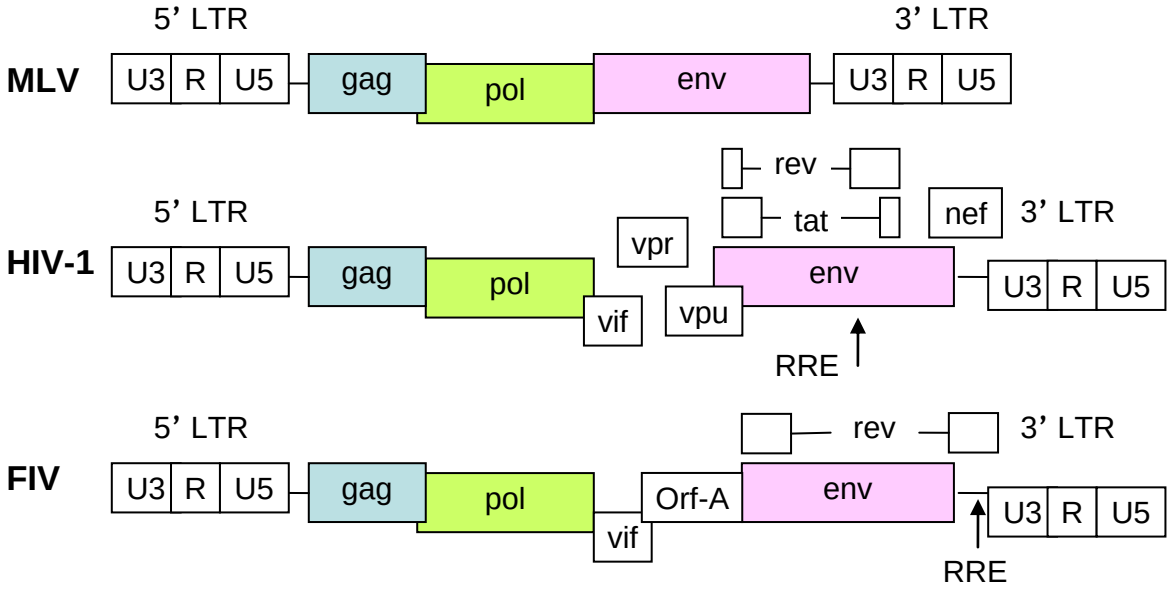


Figure 1-2 - A comparison between the genome structure of a simple retrovirus (MLV) and the complex retroviruses HIV-1 and FIV. RRE is the Rev response element.

HIV-1 and FIV share the accessory gene *vif* (which mediates the degradation of the host intrinsic restriction factor APOBEC3 proteins (Sheehy et al., 2002, Zielonka et al., 2010)) and the regulatory gene *rev* (which mediates the exit of viral RNA from the nucleus via interaction with the Rev response element (RRE) (Pollard and Malim, 1998)). However, the accessory genes *vpr*, *nef*, *vpu* and the

regulatory gene *tat* are only found in HIV-1 and not FIV; the *vpr* gene product is associated with the nuclear import of the PIC as well as the induction of cell cycle arrest and apoptosis, the modulation of viral LTR activity, the suppression of host immune response and regulation of reverse transcription (Romani and Engelbrecht, 2009); *nef* encodes a protein with multiple functions in the host $CD4^+$ T cells such as the down-regulation of cell surface CD4 and major histocompatibility class I (MHCI) molecules and the modulation of cell signalling (Foster and Garcia, 2008); Vpu protein also down-regulates CD4 expression (Willey et al., 1992) and more recently it is found to counteract the activity of tetherin, another host antiviral restriction factor (Neil et al., 2008, Van Damme et al., 2008); The *tat* gene encodes a small protein which acts as a major transactivator of HIV-1 gene expression via its interaction with the Tat-activation response element (TAR) in the 5' LTR (Taube et al., 1999). While FIV lacks these accessory genes and the Tat-TAR transactivation function, it encodes the gene for Orf-A (Orf-2), which may be a multi-functional protein affecting viral replication and host cell cycle (Gemeniano et al., 2003, Gemeniano et al., 2004). Recently it has been demonstrated that Orf-A can also down-regulate the expression of the FIV receptor CD134, mirroring a similar down-regulation of CD4 by HIV-1 Nef and Vpu (Hong et al., 2010)

Both viruses target activated $CD4^+$ T cells (Zagury et al., 1986, Yamamoto et al., 1988) but whereas the primary receptor of HIV-1 is CD4 (Dalgleish et al., 1984), the FIV primary receptor is CD134 (OX40) (Shimojima et al., 2004). HIV-1 utilizes CCR5 and CXCR4 as its secondary receptors (Choe et al., 1996, Feng et al., 1996, Deng et al., 1996) while FIV uses CXCR4 as its secondary receptor (Willett et al., 1997) (details of HIV-1 and FIV entry into host cells are discussed in section 1.4).

1.2.2 Reverse transcription

Reverse transcription (the conversion of RNA to DNA) is one of the central biological processes which define the retroviruses and is mediated by the enzyme reverse transcriptase. The viral enzyme was discovered independently by Temin and Baltimore (Temin and Mizutani, 1970, Baltimore, 1970). Before their discovery it had long been suspected that certain RNA viruses (which would go on to form the *Retroviridae* family) do not directly replicate their genomic RNA but rather go through a DNA intermediate stage. This was deduced from

studies on Rous Sarcoma Virus (RSV), which had shown its dependence on DNA-to-RNA and DNA-to-DNA synthesis (Bader, 1964, Temin, 1964).

The mechanism of reverse transcription has been studied and reviewed extensively (Katz and Skalka, 1994, Coffin et al., 1997, Sarafianos et al., 2009) (Fig. 1-3). Reverse transcriptase encompasses RNA-dependent DNA polymerase, DNA dependent DNA polymerase and ribonuclease-H activities within a single enzyme complex. A host tRNA primer is required by the RT to initiate its DNA polymerase activity (in the case of HIV-1 and FIV the primer is $tRNA_{lys3}$ (Arts et al., 1996)). This tRNA primer anneals to the 5' end of the plus-strand RNA genome at the Primer Binding Site (PBS) (Fig.1-3, step A). The RT recognizes and binds to the viral genome to initiate minus-strand synthesis towards the 5' end (Fig.1-3, step B). At the same time the RNase H activity degrades the 5' end of the plus-strand RNA from the PBS to the 5'direct repeat (R) region (Fig.1-3, step B), which exposes the single-stranded DNA product and allows the first jump (or first strand switch) to take place (Fig.1-3, step C). The minus-strand DNA hybridizes to the 3' R, which allows its synthesis from 3' to 5' to continue. Meanwhile the RNase H activity of the RT carries on degrading the positive-sense RNA genome except in a purine-rich sequence called the polypurine tract (PPT) (Fig.1-3, step D). The PPT is relatively resistant to RNase H degradation and would then become the primer for the synthesis of the plus-strand DNA, usually mediated by another RT complex (Fig.1-3, step E).

Plus strand DNA synthesis continues towards the 3' end of the minus-strand DNA until it starts to copy the tRNA primer. The RT then degrades the tRNA primer (Fig.1-3, step F), which is now on the 3' end of the viral minus-strand DNA and exposes the single stranded PBS sequence of the plus-strand DNA. This allows the second jump (strand switch) as the 5' end of the minus-strand can now anneal to the plus-strand at the PBS to form a circular intermediate (Fig.1-3, step G). DNA synthesis then continues and a process called strand displacement leads to the production a linear double-stranded DNA viral genome with the same sequences at both ends (U3, R and U5) (Fig.1-3, step H). Failure in this process may lead to the formation of 1-LTR circles (Cara and Klotman, 2006).

Despite being RNA viruses, viral DNA is detected in HIV-1 virions (Lori et al., 1992) and it has been shown recently that reverse transcription begins at the

last step of replication in the virus-producing cells (Yu et al., 2009, Mougel et al., 2009). Nevertheless the completion of reverse transcription only occurs after viral entry and is believed to initiate the process of uncoating (Arhel et al., 2007).

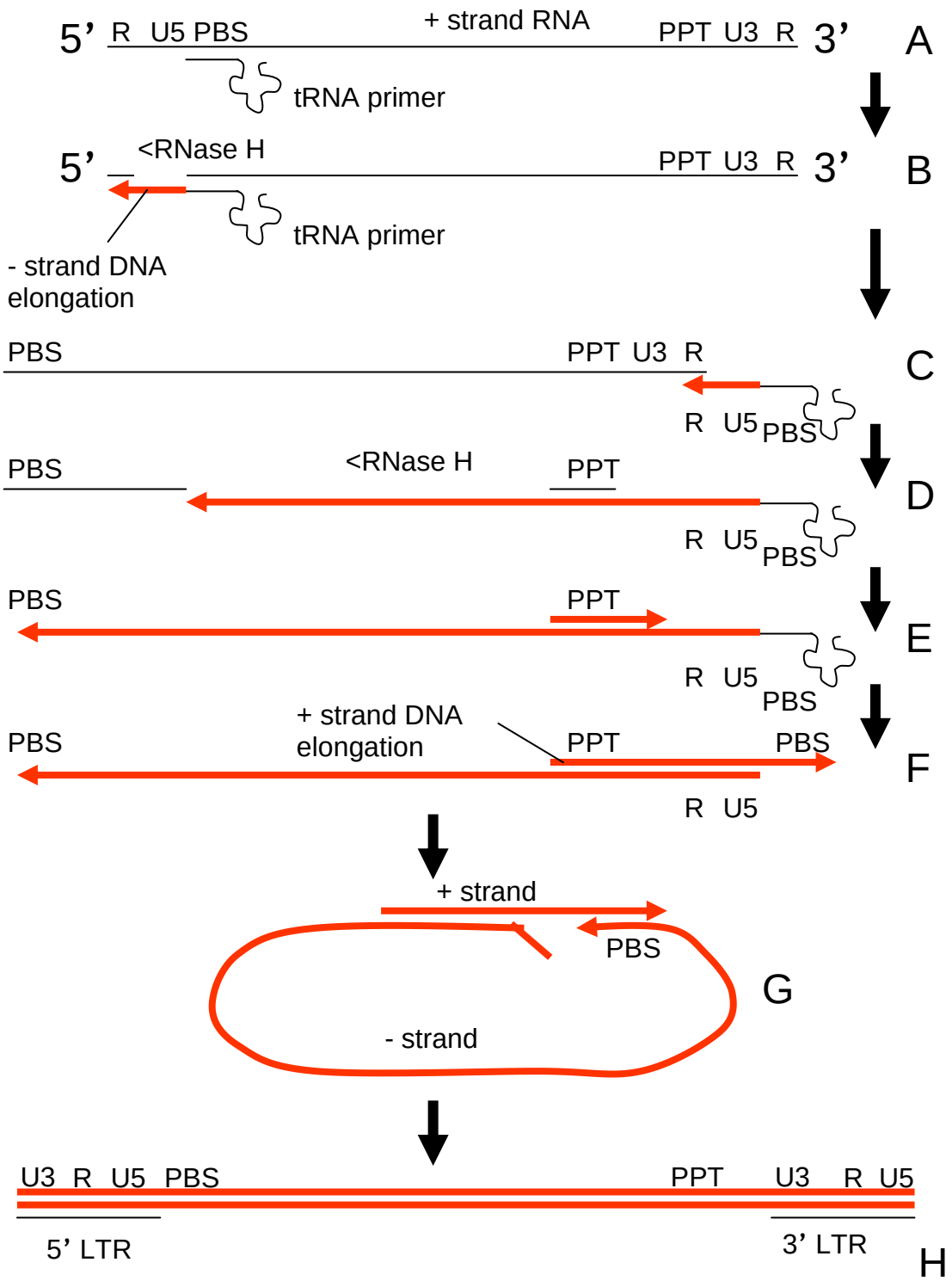


Figure 1-3 – Diagram of the reverse transcription process

1.2.3 Nuclear entry and integration

Recent experimental evidence suggest that rather than dissociating soon after viral entry, the HIV-1 capsid remains intact as it transfer the viral genome, which is undergoing reverse transcription, to the nuclear pore (Arhel, 2010). When reverse transcription is completed, uncoating then takes place (Arhel et al., 2007), allowing the direct transfer of a conglomerate of viral DNA plus viral and host proteins known as the viral pre-integration complex (PIC) into the nucleus through host nuclear pore complexes (NPCs) (Suzuki and Craigie, 2007). This allows lentiviruses like HIV-1 to cross the nuclear membrane of non-dividing cells, facilitating infection; by contrast, simple retroviruses such as murine leukaemia virus (MLV) have to wait until mitosis and the break-down of the nuclear envelope to continue their lifecycles (Harel et al., 1981, Hatziioannou and Goff, 2001).

The integration of the viral DNA into the host genome is another hallmark of the retroviral lifecycle and is mediated by the viral integrase, which is a 288 amino acid protein that comprises of 3 distinct structural domains; the amino (N) – terminal domain, the catalytic core domain and the carboxy (C) – terminal domain (Coffin et al., 1997). The active site of the HIV-1 integrase is found in the catalytic domain and consists of a three-amino-acids (Aspartic acid64, Aspartic acid116 and Glutamic acid152) motif (Coffin et al., 1997). Indeed, this Asp, Asp and Glu motif of the integrase active site is conserved across all retroviruses (Coffin et al., 1997). Following reverse transcription the integrase molecules bind to specific sequences within the long terminal repeats (LTR) at each end of the nascent viral DNA as part of the PIC (Coffin et al., 1997). The integrase then cleaves off 2 nucleotides at a conserved region on the 3' strand, generating a di-nucleotide 5' overhang and a reactive 3'-hydroxyl group at each end of the DNA (Coffin et al., 1997). Inside the nucleus the host protein LEDGF/p75 tethers the viral PIC and the host DNA together (Cherepanov et al., 2003), allowing the crucial strand transfer reaction to take place. This reaction utilizes the 3'-hydroxyl group to join the viral DNA strand to the host DNA but results in unpaired regions at the viral-host DNA junctions, which are repaired by the host DNA-repair mechanisms (Coffin et al., 1997). The integrated viral DNA is called proviral DNA.

1.2.4 Extrachromosomal retroviral DNA

For reasons still unknown, most of the reverse-transcribed retroviral DNA within a viral population is not integrated into the host genome and accumulates within the host cell (Shaw et al., 1984, Chun et al., 1997). These unintegrated or extrachromosomal viral DNAs can be found in linear, 1-LTR circular or 2-LTR circular forms. The 1-LTR circles can be the result of strand displacement failure or homologous recombination of the LTRs (Cara and Klotman, 2006, Telesnitsky and Goff, 1997), whereas 2-LTR circles are formed by the intramolecular blunt-end ligation of the linear retroviral DNA (Cara and Klotman, 2006). These extrachromosomal viral DNAs were initially thought to play little role in viral replication due to their labile nature (Pierson et al., 2002b, Zhou et al., 2005). Subsequently it was discovered that circular DNA can survive for days in resting T cells (Pierson et al., 2002a) and longer in macrophages (Kelly et al., 2008). Also these extrachromosomal DNAs are transcriptionally active (Cara and Klotman, 2006, Kelly et al., 2008) and can still replicate during co-infections with integrated viruses (Gelderblom et al., 2008). Thus these unintegrated viral species may contribute to viral pathogenesis and viral diversity within the host.

1.2.5 Virion production and egress

Viral genes are now expressed from the provirus, driven by the binding of host transcription factors such as NFκB, NFAT, AP-1 and AP-4 to the 5'LTR (Sparger et al., 1992, Thompson et al., 1994, Tang et al., 1999, Kilareski et al., 2009). For HIV-1 the Tat-TAR transactivation mechanism also regulates transcription of viral genes; in contrast FIV does not have Tat-TAR, which may explain why the basal activity of the FIV 5'LTR is higher than HIV-1 5'LTR (Sparger et al., 1992). The viral RNA produced can either be spliced and become templates for viral protein translation, or packaged into the capsids along unprocessed Gag-Pol precursors at the cell membrane. Retroviral assembly is driven by Gag (Gheysen et al., 1989) and exploits the host endosomal sorting complex required for transport (ESCRT) pathway for virus assembly and budding (Jouvenet et al., 2010). After the emergence of the immature virion from the producer cell, the Gag-Pol polyprotein precursors are cleaved by the viral protease (Vogt, 1996) into their respective single protein species. This maturation step of the nascent virion is vital for the formation of the viral capsid (Ganser-Pornillos et al., 2008) and the production of infectious virus particles (Kohl et al., 1988) (Fig.1-4).

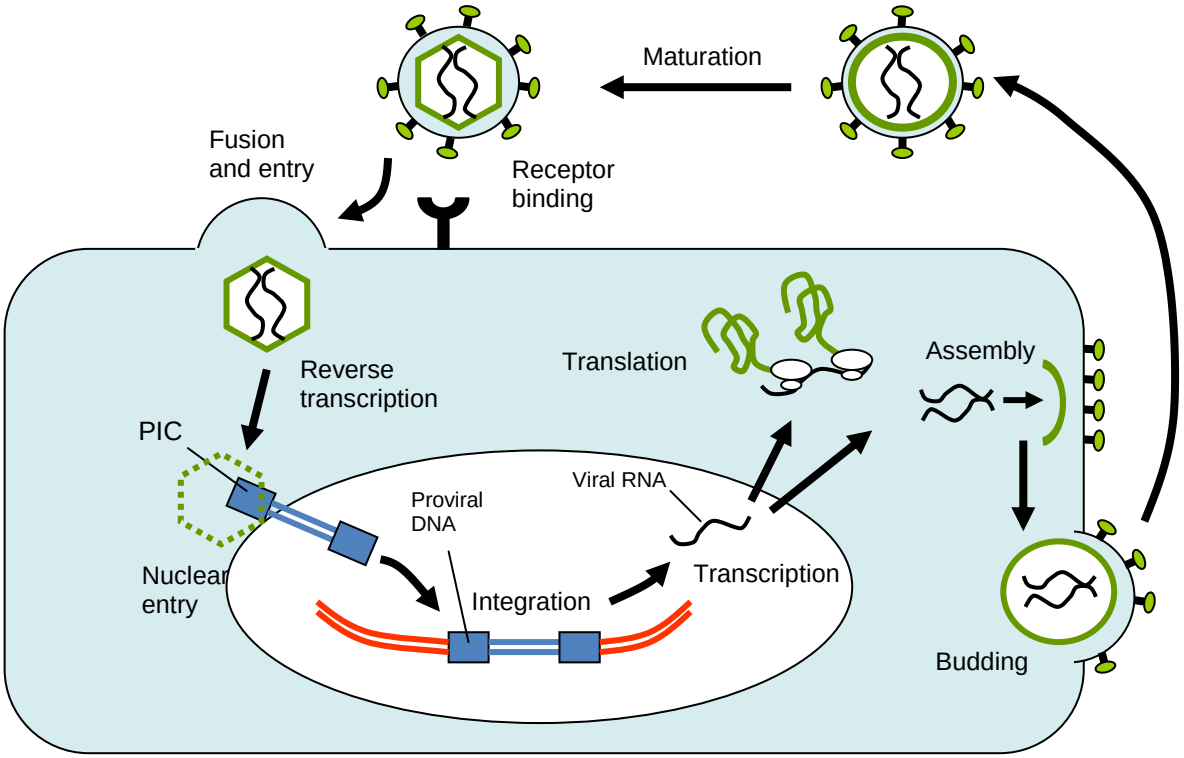


Figure 1-4 – The lifecycle of retroviruses. After entering the cell, the viral RNA genome (black) is reverse-transcribed into DNA (red) by viral reverse transcriptase and enters the nucleus. The proviral DNA is then integrated into the host genome by viral integrase. Viral gene expression then occurs, producing both genomic viral RNA and viral mRNA for the synthesis of viral proteins (Green). The nascent viral genomes are packaged into virions at the cell membrane and bud off. As part of the maturation process viral protease then cleaves the Gag-Pol precursor protein to produce Gag and Pol, allowing the formation of the viral capsid in the nascent virions.

1.3 T cell activation

The preferential target cells for both HIV-1 and FIV are activated $CD4^+$ T cells. To understand how $CD4^+$ T cells are activated and their connection with viral pathogenesis, we must first explore one of the fundamental questions of immunology: How is immune activation regulated? This apparently simple question has been occupying immunologists for the last half century (Matzinger, 2002). Theoretically, mammals can mount an immune response against all antigens, including their own tissues, due to the diversity of T cell receptors (TCRs) generated by the random assortment of the TCR loci within the repertoire of naïve resting T cells of each individual (Hannet et al., 1992, Bonilla and

Oettgen, 2010). However, autoimmunity does not occur in mammals most of the time and the theoretical models explaining how the host immune response is regulated have been evolving with experimental evidence. Initially, it was thought that the immune system recognizes self vs. non-self entities (Burnet, 1959) and evidence such as the clonal deletion of self-reactive thymocytes during T cell maturation (Kappler et al., 1987) supports this theory. However, the self vs. non-self theory is not compatible with the immuno-tolerance of fetuses, which are antigenically distinct from their mothers, and the incorporation of the concept of T cell co-stimulation (Lafferty and Cunningham, 1975), which introduced the possibility that self antigens are presented by antigen-presenting cells (APCs) and stimulate autoimmunity. To address the inadequacies of the old self vs. non-self model, the 'Danger' hypothesis of immune activation was proposed (Matzinger, 1994). The new theory suggests that APCs have to be activated by 'danger signals' before co-stimulation of T cells can take place. These 'danger signals' were later revealed to be both foreign molecules such as bacterial Lipopolysaccharide (LPS) and cellular molecules such as heat shock proteins (Hsps), which can be recognized by pattern recognition receptors (PRRs) expressed by APCs (Matzinger, 2002).

It is now established that activation of naïve T cells requires both the TCR recognizing the antigenic peptide presented within the major histocompatibility complex (MHC) molecule of APCs as well as appropriate co-stimulatory signals from APCs which have been primed by the right 'danger signals'. The co-stimulatory molecules CD27 and CD28 are constitutively expressed on T cells, while the expression of their ligands CD70, CD80 and CD86 on APCs are induced upon stimulation (Goronzy and Weyand, 2008). They provide the first wave of T cell activation signals which lead to the rapid expression of a set of activation-induced co-stimulatory receptors including ICOS, CD30, CD134 and CD137 (Watts, 2005, Goronzy and Weyand, 2008). This second wave of T cell activation signals serves to amplify T cell activation as well as to modulate T cell differentiation and memory T cell development (Coyle et al., 2000, Croft, 2003).

According to the linear differentiation model of memory T cell development, after T cell activation the antigen-specific naïve T cells undergo clonal expansion and develop into cytokine-producing effector cells. This is followed by activation induced apoptosis for the majority of the effector cells while a

small number of effector T cells remain behind as memory T cells which can be rapidly activated when the same antigen is encountered again (Ahmed et al., 2009, van Leeuwen et al., 2009).

Interleukin-2 (IL-2) is an important cytokine that regulates this process. First discovered in 1976 (Morgan et al., 1976), IL-2 is a 15kDa protein synthesised by activated T cells after the triggering of the TCR and CD28 signalling pathways; the IL-2 gene promoter contains sites for the transcription factors NFAT, AP-1 and NFκB, which are activated by the TCR signalling pathway via Protein kinase C (Isakov and Altman, 2002, Altman and Villalba, 2003). The interaction between IL-2 and its receptor has long been associated with driving the clonal expansion of effector T cells, the differentiation of $CD4^+$ T cells subsets T_H1 and T_H2 and the development of memory T cells (Malek, 2008, Hoyer et al., 2008). The IL-2 receptor consists of three subunits; IL-2Rα (CD25) contributes to the binding affinity of the ligand but not to signal transduction, whereas IL-2Rß and IL-2Rγ subunits mediate signal transduction and ligand binding (Gaffen and Liu, 2004). In addition, the IL-2Rß subunit is shared with the IL-15 receptor, while the IL-2Rγ subunit may also form part of a receptor for IL-4, IL-7, IL-9, IL-15 and IL-21, thus contributing to redundancy in cytokine signalling (Lan et al., 2008). IL-2 receptor signalling is mediated by the Janus kinase/signal transducer and activator of transcription 5 (JAK/STAT5), the mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K) pathways (Cheng et al., 2011). IL-2 signalling can be self-reinforcing since IL-2 receptor-mediated signalling up-regulates the expression of the IL-2 receptor, providing a positive feedback loop which drives T cell proliferation (Kim et al., 2001). Paradoxically, IL-2 is also vital to the growth, differentiation and survival of regulatory T cells (Treg), as demonstrated by the development of autoimmune disease in experimental animals when the IL-2 or IL-2 receptor gene is knocked-out (Sadlack et al., 1993, Willerford et al., 1995).

The cell signalling network involved in T cell activation consists of a vast number of factors and pathways. Those which are most relevant to this thesis are described below and in subsequent chapters.

1.3.1 CD134 – FIV primary receptor

The primary receptor for FIV is CD134 (also known as OX40, TNFRSF4 or ACT35) (Shimojima et al., 2004). It is an inducible co-stimulatory molecule and a member of the nerve growth factor receptor/tumour necrosis factor receptor (NGFR/TNFR) superfamily (MacEwan, 2002). In the domestic cat, CD134 is mainly associated with activated $CD4^+$ T cells (Shimojima et al., 2004, Joshi et al., 2005b, Willett et al., 2007). The normal physiological role of CD134 is to regulate the clonal expansion of both $CD4^+$ and $CD8^+$ effector T cells. CD134 knock-out animals produce fewer effector $CD4^+$ T cells following antigenic stimulation (Gramaglia et al., 2000, Maxwell et al., 2000). It exerts influence during late proliferation and enhances T cell signalling and survival after encountering antigen (Croft et al., 2009). Expression of CD134 is not constitutive but transient following T cell activation (Croft, 2010). CD134 is also central to the development of T cell memory (Gramaglia et al., 2000, Maxwell et al., 2000) and mediates the interaction between T cells and other cell types (Croft, 2010). Activation of the CD134 pathway also blocks the suppressive activity of Treg cells. CD134 signalling inhibits the induction of regulatory molecules such as CTLA4, FOXP3 and IL-10 in T cells (Prell et al., 2003, So and Croft, 2007, Ito et al., 2006, Vu et al., 2007). In accordance with CD134's role in regulating T cell expansion, the ligand of CD134 (known as CD134 ligand or CD252) is expressed transiently on professional APCs such as B cells (Stuber et al., 1995), dendritic cells (Ohshima et al., 1997) or macrophages (Weinberg et al., 1999) upon stimulation by danger signals such as bacterial LPS as well as by the CD40 ligand, a molecule associated with activated $CD4^+$ T cells and is responsible for regulating the cross-talk between $CD4^+$ T cells and APCs (Gommerman and Summers deLuca, 2011). Certain cell types associated with the lymphatic systems such as endothelial cells and mast cell also express CD134L as a mean to further augment T cell activation (Croft et al., 2009).

CD134, like other members of the TNFR superfamily, consists of extracellular cysteine rich domains (CRDs) that are linked to a cytoplasmic tail (Croft et al., 2009). Both feline and human CD134 are predicted to have three full CRDs per receptor molecule (Willett et al., 2006b). The crystal structure of human and murine CD134 receptor – ligand complex has been solved (Compaan and Hymowitz, 2006) and it showed that 3 receptor molecules combine to interact with the trimeric ligand. A model of the signalling events triggered by the

stimulation of CD134 has been constructed recently: the engagement of CD134 by its ligand leads to the recruitment of the adapter proteins TNFR-associated factors (TRAF) 2, 3 and 5, mediated by a short conserved region at the CD134 cytoplasmic tail with a QEE motif (Fig. 1-5) (Arch and Thompson, 1998, Kawamata et al., 1998, Ye et al., 1999, Croft et al., 2009). Among the recruited TRAF proteins, TRAF2 appears to play the most important role in OX40 signalling as it has been shown that TRAF2 is essential to the formation of a super-signalosome consisting of IκB kinases (IKKs), Protein Kinase C (PKC) θ (See section 1.7.5), CARD-MAGUK1 (Gaide et al., 2001) (CARMA1), regulation of phenobarbitol-inducible P450 (RIP), Mucosa-associated lymphoid tissue 1 (MALT1), B-cell leukaemia/lymphoma 10 (BCL10), PI3K, phosphoinositide-dependent kinase 1(PDK1) and Protein kinase B (Akt) (So et al., 2011b, So et al., 2011a) (Fig. 1-5). Disruption of lipid rafts (or detergent-insoluble microdomains – DIMs) inhibits the formation of the full signalosome and its signalling activities but not the association of TRAF2 and IKKs with CD134, whereas the association of CARMA1 with CD134 is independent of TRAF2 but is inhibited by lipid raft-disrupting drugs, indicating that TRAF2 recruits IKKs to CD134 outside lipid rafts, then the complex translocates to lipid rafts to join with CARMA1 and the rest of the factors to form the complete signalosome (So et al., 2011b)(Fig. 1-5).

The assembly of this signalling complex and the subsequent activation of NFκB and increased T cell survival are antigen independent events but the functional activation of the PI3K and Akt pathways is dependent on antigen specific T cell receptor activation. Thus it is suggested that in the presence of antigen, CD134 acts as a co-stimulatory receptor and supplements T cell activation via enhanced PI3K and Akt activity. While in the absence of antigen only NFκB activity is stimulated, allowing survival of activated T cells after the withdrawal of antigenic stimulation and the formation of T cell memory (Croft, 2010, So et al., 2011a, So et al., 2011b).

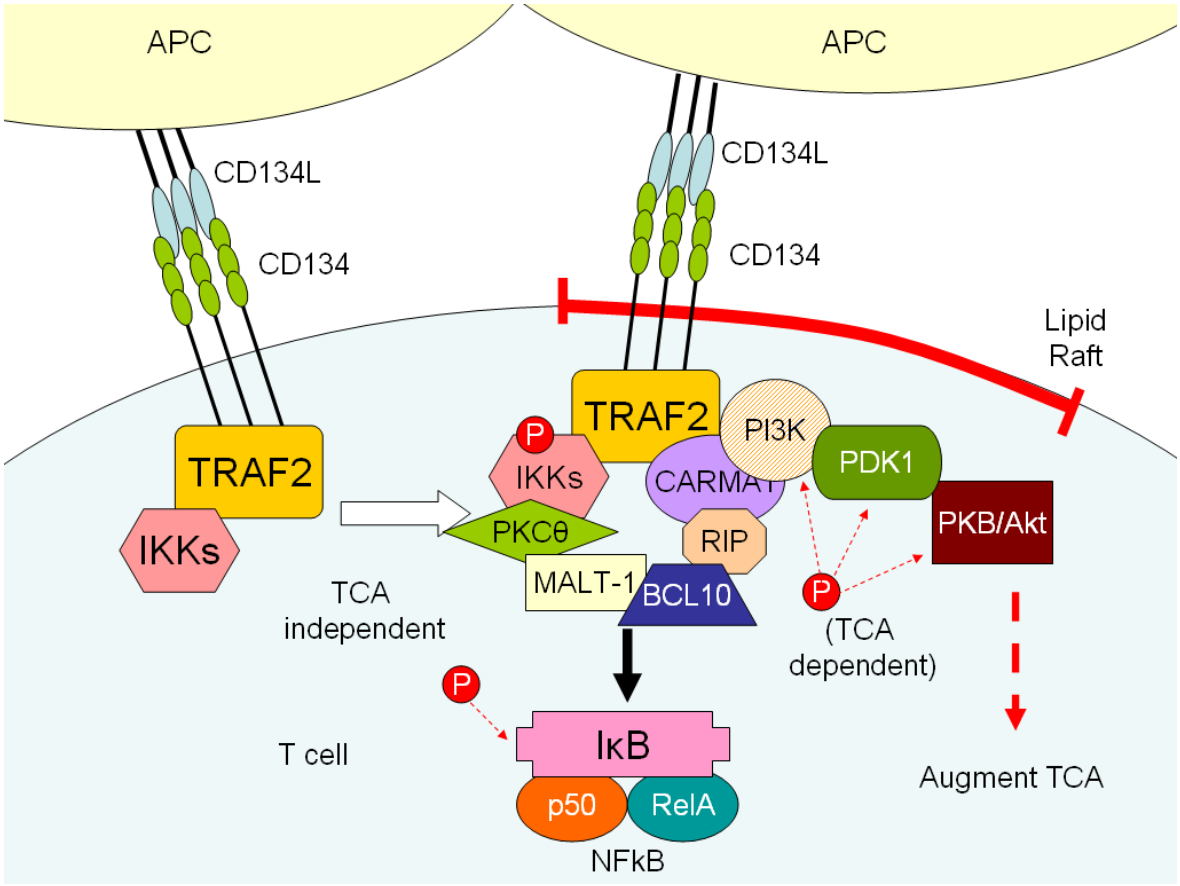


Figure 1-5 – The CD134 signal transduction pathway. T cell activation (TCA) independent events: TRAF2 binds to CD134, recruits IKKs and translocates to lipid rafts. The rest of the signalosome then assembles, which leads to IκB being phosphorylated, releasing NFκB. TCA dependent events: PI3K is phosphorylated, which lead to the activation of PDK1 and the phosphorylation of downstream kinases such as Akt. This augments T cell receptor-mediated activation. Based on data from (So et al., 2011a, So et al., 2011b).

1.3.2 NFκB

The transcription factor NFκB was discovered in 1986 and was initially identified as a B-cell specific transcription factor, binding to a specific site in the kappa immunoglobulin enhancer (Sen and Baltimore, 1986b, Sen and Baltimore, 1986a). Since then NFκB has been found in many different cell types, mediating critical biological processes such as the inflammatory response, apoptosis, innate immunity and development.

The molecular biology of the NFκB signalling pathway has been extensively reviewed (Hacker and Karin, 2006, Ghosh, 2007, Hayden and Ghosh, 2008). In mammals NFκB is an evolutionary conversed family of transcription factors which

consists of five members, p50, p52, p65 (RelA), c-Rel and RelB. The 5 members of the NFκB family form different homo/heterodimers to perform different gene regulatory functions. The subunits p65, RelB and c-Rel contain a transcription activation domain (TAD) required for up-regulation of gene expression. Meanwhile the subunits p50 and p52 do not have TADs and are associated with down-regulation of gene expression unless dimerized with a TAD-containing subunit. NFκB is normally sequestered in the cytoplasm in an inactive state by an Inhibitory-kappa-B (IκB) family protein, which blocks nuclear translocation. IκB proteins are phosphorylated by inhibitor-kappa-B kinases (IKKs) during activation, leading to the ubiquitination and degradation of the IκB protein and the nuclear translocation of the NFκB dimers. NFκB mediates the transcription of genes associated with the inflammatory responses such as cytokines, chemokines and inhibitors of apoptosis (Bonizzi and Karin, 2004).

The activation of NFκB is controlled by two pathways. The classical (canonical) pathway presides over the degradation of most IκB family members (IκBα, IκBß, IκBε) and the processing of p105, the precursor of p50. This pathway mainly relies on IKK-ß and IKK-γ regulatory subunit to phosphorylate IκB, leading to its degradation. While the alternative (non-canonical) pathway is independent of IKK-ß and IKK-γ, it relies on the IKK-α to initiate the processing of p100 by phosphorylation (Bonizzi and Karin, 2004, Dejardin, 2006). A large number of other cellular signalling pathways have been discovered to trigger NFκB activation. In T cell signalling these include PKC, P-I3K and MAPK (See later sections and chapters)

1.4 Lentiviral evolution within the host and disease progression

Before the identification of the HIV-1 secondary receptors, different primary HIV-1 strains were divided between M-tropic and T-tropic viruses. M-tropic viruses are usually found during acute/early infection, do not induce syncytium formation and can replicate in macrophages but not in T cell lines (Schuitemaker et al., 1992, Zhu et al., 1993); whereas T-tropic viruses tend to appear during the late stages of disease, do induce syncytia formation and can replicate in T cell lines but not in macrophages (Tersmette et al., 1988, Tersmette et al.,

1989). Subsequently it was shown that M-tropic viruses use CCR5 whereas T-tropic viruses use CXCR4 as their respective secondary receptors (Choe et al., 1996, Feng et al., 1996). The M-tropic, or R5 viruses as they are also called, are preferentially transmitted despite the availability of CXCR4-expressing lymphocytes in the body. Thus it is proposed that there may be multiple barriers against the transmission of T-tropic (or X4) viruses such as increased susceptibility of X4 viruses to innate antiviral proteins, lower expression of CXCR4 at different sites of viral replication or increased susceptibility of X4 virus to neutralizing antibody (Margolis and Shattock, 2006). Indeed, individuals with a rare mutation at the gene encoding CCR5 (CCR5-Δ32) are largely resistant to infection with HIV-1 (Dean et al., 1996b). Nevertheless, as the infection progresses the more cytopathic X4 tropic viruses emerge and are associated with an increased loss of $CD4^+$ T cells (Fouchier et al., 1996, Connor et al., 1997).

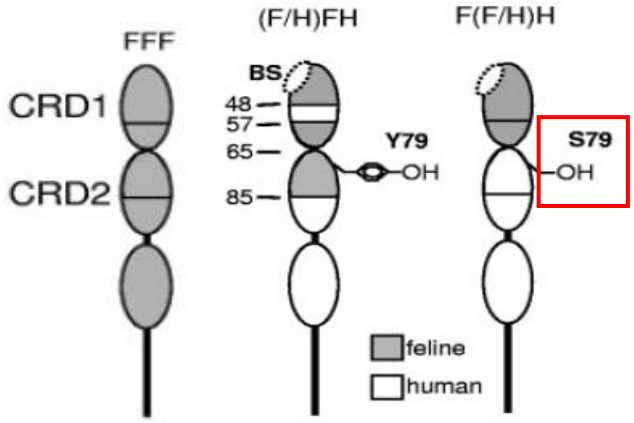
The different tropisms of the R5 and X4 strains are mediated by the viral *env* gene, which encodes the viral entry glycoproteins Gp120 and Gp41 (also known as SU and TM domain of Env glycoprotein respectively) (Cheng-Mayer et al., 1990, O'Brien et al., 1990). On the virus envelope, the glycoproteins arrange themselves into multimeric spikes: the external Gp120 forms into trimers which are held in place by non-covalent interactions with Gp41 trimers, which in turn are inserted into the virus envelope (Wyatt and Sodroski, 1998). The HIV-1 Gp120 consists of four conserved domains (C1 - C4) and five variable domains (V1 – V5), forming loop structures (Starcich et al., 1986, Modrow et al., 1987, Leonard et al., 1990, Coffin et al., 1997). The binding of HIV-1 Gp120 to CD4 induces a sequential conformational change of Gp120 (Wyatt and Sodroski, 1998, Salzwedel et al., 2000), exposing or creating a binding site at V3 (Sattentau and Moore, 1991) for the secondary receptor which leads to the binding of Gp120 to the secondary receptor (Berger et al., 1999). This allows the deployment of the Gp41 fusion protein and ultimately leads to the fusion of the viral and cellular membranes and the entry of the viral capsid into the cytoplasm (Sodroski, 1999). HIV-1 Gp120 is heavily glycosylated, masking potential antibody-binding regions on the glycoprotein (Wyatt and Sodroski, 1998). The conserved CD4 and chemokine receptor interaction domains are hidden by the variable loops, and are only exposed during binding (Wyatt et al., 1995, Kwong et al., 1998). These adaptations effectively render neutralizing antibody useless in suppressing viral replication (Wyatt and Sodroski, 1998). However, numerous broadly neutralizing

anti-HIV-1 antibodies have been discovered (Wu et al., 2010, Walker et al., 2009), which may illuminate previously unknown conserved epitopes on Gp120 and lead to the development of an effective vaccine against the virus.

The entry of FIV into feline cells is believed to be similar to HIV-1. In cats the FIV primary receptor CD134 is mainly associated with activated $CD4^+$ T cells (Shimojima et al., 2004, Joshi et al., 2005b) whereas the FIV secondary receptor CXCR4 is expressed on activated $CD4^+$ and $CD8^+$ T cells, B cells and monocytic cells (Richardson et al., 1999, Willett et al., 2003, Joshi et al., 2005b). Analogous to HIV-1 there are considerable differences in viral replication characteristics between strains isolated from the 'early' or the acute phase of infection and strains isolated from the 'late' or chronic stages of infection, demonstrated by the expansion of tropism of the 'late' stage viruses to include B cells, macrophages and CD8+ T cells (English et al., 1993, Dean et al., 1996a, de Parseval et al., 2004a, Willett and Hosie, 2008). On-going evolution of the virus *in vivo* was also demonstrated in a study that showed the emergence of more pathogenic variants of a cell culture-adapted strain of FIV, PETF14, after long term infection in cats (Hosie et al., 2002).

The overall structure of the FIV Env glycoprotein is believed to be similar to HIV Env, despite major differences in protein sequence (Pancino et al., 1995). The binding of FIV Env SU domain to CD134 initiates a sequential change which reveals a hidden binding site for CXCR4 (de Parseval et al., 2006), indicating that FIV uses a similar entry mechanism to HIV-1. Similar to the human protein, feline CD134 consists of three extracellular CRDs linked to a cytoplasmic tail (Fig. 1-6) (Willett et al., 2006b). Using a chimeric CD134 which contains both human and feline CRDs, it was discovered that strains such as FIV-PPR, which is a 'late' or chronic stage isolate (Phillips et al., 1990, Willett and Hosie, 2008), only require binding to CRD1 to mediate entry (de Parseval et al., 2005); while strains such as FIV-GL8, which is an 'early' or acute stage isolate (Hosie and Jarrett, 1990, Willett and Hosie, 2008), require additional determinants to enter cells and this was mapped to a motif at Asparagine78 – Tyrosine79 – Glutamate80 (NYE) in CRD2 (Willett et al., 2006a) (Fig. 1-6). Substituting the NYE motif into the human CRD2 restored 'early' strain binding to a cat-human CD134 chimera (FFHH) (Willett et al., 2006a). Curiously, while 'late' strains have reduced requirement for CD134 determinants, they are also more sensitive to

competitive inhibition of binding with anti-CD134 antibody or trimeric soluble feline CD134 ligand (Willett et al., 2007, Willett et al., 2009), supporting the hypothesis that the emergence of the 'late' strains only takes place when the host develops immune dysregulation and deficiency (Willett and Hosie, 2008). Interestingly, the presence of anti-CD134 autoantibodies in FIV-infected cats is associated with better overall health, which is another illustration of the important role Env plays in pathogenesis (Grant et al., 2009).



B

51 CRD1 CRD2 90

YGMESRCSGD QDTKCLQCAS GFYNEAVNYE PCKPCTQCNQ **Feline**
NGMVSRCSRS QNTVCRPCGP GFYNDVVSSK PCKPCTWCNL **Human**

Figure 1-6 – Feline CD134 (A) Diagrammatic representation of feline CD134 chimeras FHFH and FFHH , where F is feline and H is human, in comparison with wild-type CD134 (entirely feline). The tyrosine79 (Y79) in the NYE motif is highlighted. (B) Comparison of amino acid sequence between feline and human CD134 in the region covering CRD1 and CRD2. The NYE motif is highlighted in yellow. (Willett et al., 2006a). (Reproduced with permission from the Journal of Virology).

Difference in viral entry characteristic is not the only mechanism that mediates variation in replicative property between strains in lentiviruses. For example, mutations in the HIV-1 accessory gene *nef* led to the establishment of a group of long-term non-progressing HIV-1 positive patients known as the Sydney Blood Bank Cohort (Dyer et al., 1997). Also, in a study on the pathogenic determinants between different clades of FIV, it was found that inter-strain variations is mediated by multiple parts of the viral genome rather than one single region or gene (de Rozieres et al., 2008) and that a mutation at residue 813 of the FIV *pol*

gene (which forms the FIV integrase) from arginine to histidine confers an enhancement to viral growth *in vivo* (Thompson et al., 2011).

1.5 Pathogenesis of HIV-1 and FIV

During the initial phase of HIV-1 or FIV infection, the host usually experiences an acute syndrome characterised by non-specific symptoms such as fever, malaise, lymphoadenopathy and diarrhoea (Schacker et al., 1996, Yamamoto et al., 1988, Delfierro et al., 1995). This is followed by an asymptomatic period when the host attempts to clear the virus by cell-mediated and humoral responses (Pantaleo and Koup, 2004). However, without antiretroviral treatments, immunodeficiency may develop (Tindall and Cooper, 1991, Ishida et al., 1992), which would rapidly lead to fatal secondary infections or cancers. Since the early days of HIV-1 research it has been noticed that depletion of $CD4^+$ T cells and T-helper function is associated with the development of immunodeficiency (Lane and Fauci, 1985). Thus it was proposed that the direct cytopathic effect of HIV-1 on $CD4^+$ T cells was the major cause of immunodeficiency. However, there was also evidence to show that peripheral blood viraemia was low and the number of infected T cells was small throughout most stages of infection (Shaw et al., 1984, Harper et al., 1986, Chun et al., 1997); in addition most activated $CD4^+$ T cells undergo apoptosis soon after clonal expansion as part of the natural immune regulatory mechanism (Dooms and Abbas, 2006, van Leeuwen et al., 2009), indicating that a more subtle mechanism is mediating immune dysfunction in the host body which cannot be explained simply by the cytopathic effects of HIV-1.

From the beginnings of HIV-1 research it was also well known that productive virus replication requires the host $CD4^+$ T cells to be activated (Zagury et al., 1986, Stevenson et al., 1990). This led some researchers to suspect that HIV-1 pathogenesis involves the chronic activation of the immune system (Hausen et al., 1986, Fuchs et al., 1987, Ascher and Sheppard, 1988). This theory was boosted by the discovery of the selective depletion of $CD4^+$ T cells within the lymphoid tissues of the gastrointestinal tract during early infection (Brenchley et al., 2004b), allowing the chronic translocation of pro-inflammatory microbial products from the intestine to the rest of the body, causing long-term immune activation (it may also explain the protective effect of the antibiotic co-trimoxazole on HIV-1 positive children reported in a trial in Zambia (Chintu et

al., 2004) – the antibiotic may have reduced microbial translocation and the subsequent immune dysregulation). Other factors also contribute to chronic immune activation, for example the destruction of other lymphoid tissues such as the thymus (McCune, 2001, Schacker et al., 2002), the depletion of immunosuppressive Treg cells (Oswald-Richter et al., 2004b), the establishment of a latent virus reservoir which periodically seeds new infection in the body (See section 1.7), and the inability of the adaptive immune response to contain productive virus replication. This is due in part to immune evasion strategies such as glycosylation of the viral surface glycoprotein Env and the low fidelity of reverse transcriptase, promoting the generation of escape mutants (Ji and Loeb, 1992, Bebenek et al., 1993). In fact it has been estimated that in an untreated patient, every nucleotide of the HIV-1 genome would have been point-mutated in all possible ways every 24 hours (Siliciano and Siliciano, 2010). All these factors trigger the chronic activation of $CD4^+$ T cells, providing more target cells in which HIV-1 can grow and in turn stimulating the immune system even more, setting up a positive feedback cycle which eventually results in the exhaustion and dysfunction of the host immune response (Douek et al., 2008). Indeed it was found that there is an increased expression of the molecule Programmed Death-1 (PD-1), which is a negative regulator of T cell function, among HIV-1 specific $CD4^+$ and $CD8^+$ T cells in patients (Day et al., 2006, Trautmann et al., 2006).

FIV has the same cell tropism as HIV-1, thus it is expected that the host response to FIV and its pathogenesis is similar to the human virus. Cats mount both humoral and cytotoxic T cell responses to FIV infection (Egberink et al., 1992, Beatty et al., 1996, Flynn et al., 2002). However, the hosts usually fail to clear the infection and eventually succumb to immunodeficiency. Several mechanisms have been proposed to explain the dysfunction of the feline immune response during FIV infection (Tompkins and Tompkins, 2008); firstly, chronic hyperactivation of the immune system is believed to cause the dysregulation of cytokine release (Levy et al., 1998, Dean et al., 1998) and induction of apoptosis among T cell populations (Tompkins et al., 2002, Vahlenkamp et al., 2004a). As the disease progresses the activation of Treg cells has also been reported (Vahlenkamp et al., 2004b). These Treg cells suppress the antiviral functions of activated $CD4^+$ T cells. In contrast, FIV has been shown to target feline Treg cells (Joshi et al., 2005b), which may contribute to host immune dysregulation.

It has also been reported that FIV infection induces significant reduction of intestinal $CD4^+$ T cells, mirroring the results from studies on HIV-1 (Howard et al., 2010). Finally, the low fidelity of the FIV reverse transcriptase results in the generation of a diverse pool of viral variants within the host (Kraase et al., 2010), encouraging immune escape and chronic infection (Hosie et al., 2011).

1.5.1 Role of gp120 on lentiviral pathogenesis

The attachment between the HIV-1 glycoproteins Gp120 and Gp41 is not covalent, thus Gp120 can be released from virus particles or infected cells and they interact with host CD4, CCR5 or CXCR4 (Gelderblom et al., 1985). These cellular molecules are important receptors for cell signalling and the possibility of Gp120 contributing to HIV-1 pathogenesis by triggering intracellular signalling pathways have been investigated extensively (Santoro et al., 2003, Jones et al., 2011). HIV-1 Gp120 protein has been shown to activate NFκB via interaction with CD4, which signals down the Ras/Raf/MEK/ERK cascade or the PI3K – Akt pathway, stimulating the host T cell (Popik and Pitha, 1996, Briand et al., 1997, Flory et al., 1998). The binding of HIV-1 Gp120 to CCR5 stimulates focal adhesion kinase (FAK) and the cellular GTPase Rac-1, which promotes the re-organisation of cytoskeleton, allowing viral entry (Cicala et al., 1999) (Harmon and Ratner, 2008). HIV-1 Gp120 and FIV Gp120 can both interact with CXCR4 and may either induce or impair cell signal transduction (Hesselgesser et al., 1997, Hesselgesser et al., 1998, Hosie et al., 1998). The HIV-1 Gp120 interaction with CXCR4 also triggers intracellular actin re-organisation in resting T cells by activating the actin-depolymerizing factor cofilin (Yoder et al., 2008). This enables the direct infection of resting T cells by HIV-1 and contributes to the establishment of the latent virus reservoir (See section 1.7). In addition it has also been reported that HIV-1 Gp120 induces apoptosis of uninfected $CD4^+$ T cells (Jekle et al., 2003) and many studies have found that both HIV-1 and FIV Gp120 have neurotoxic effects in their respective hosts (Hesselgesser et al., 1998, Kaul et al., 2001, Meeker, 2007). It has also been reported that HIV-1 Gp120 could induce virus production from infected resting T cells (Kinter et al., 2003).

While it was believed initially that the interaction between HIV-1 Gp120 and the receptors CD4, CCR5 and CXCR4 results in the stimulation of the host cell, subsequent studies on transformed cell lines have shown that inhibiting HIV-1 co-receptor signalling has minimal effects on productive infection with HIV-1

(Cocchi et al., 1996, Farzan et al., 1997). This is corroborated by studies on primary T cells and macrophages (Amara et al., 2003, Yoder et al., 2008). The use of non-physiological experimental conditions and high amounts of HIV-1 Gp120 may also exaggerate the effects of the protein (Klasse and Moore, 2004). Moreover blocking CXCR4 signalling with the application of pertussis toxin only inhibits subsequent HIV-1 replication in resting T cells, whereas the toxin does not block HIV-1 replication in activated or transformed T cells (Yoder et al., 2008), demonstrating the relative insignificance of HIV-1 Gp120 induced signalling during the productive infection of activated T cells. Therefore, based on all the available evidence, it is reasonable to conclude that the binding of HIV-1 Gp120 to its cellular receptors induces a negligible effect on productive infection. Instead this binding is likely to contribute to the neuropathology associated with lentiviral infection (cells of the nervous system maybe more sensitive to the effects of Gp120 than T cells) and may enhance the establishment and maintenance of the reservoir of latently infected resting T cells.

In terms of FIV Env, it has been associated with the neuropathology of FIV infection (Meeker, 2007) and there has been a report of FIV Env inducing apoptosis in feline peripheral blood mononuclear cells (PBMCs) (Garg et al., 2004). Since FIV Env binds to the T cell co-stimulatory factor CD134 and chemokine receptor CXCR4, it is reasonable to hypothesise that FIV binding to these receptors could trigger activation of the host T cells. This may be advantageous to virus replication as only a low percentage of T cells are activated and conducive to infection at any given time.

1.6 Antiretroviral treatments

In the beginning of the AIDS outbreak, no effective treatments were available for physicians to stop the progression of the disease in the patients (Masur et al., 1981, Gottlieb et al., 1981). Attempts to stimulate the immune system, including the use of the then newly-discovered T cell growth factor interleukin-2, failed to reverse the immunodeficiency (Lane et al., 1984, Lane, 1989). It was not until the drug zidovudine (AZT) was licensed in 1986 that there was a successful treatment against HIV-AIDS (Rachlis, 1990). However, drug-resistant strains of HIV-1 soon emerged, which is an indication of the future problems that

lay ahead in the development and use of antiretroviral drugs (Larder et al., 1989).

Currently there are five classes of antiretroviral drugs in general clinical use (Table 1-2). These drugs are classified by their mode of action and their chemical structures. The nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) are the first class of drugs used for the treatment of HIV-AIDS. These include AZT, lamivudine and tenofovir and are the structural analogues of natural deoxynucleotides used for the synthesis of viral DNA. However, the NRTIs do not have a 3'hydroxyl group on the deoxyribose moiety, thus causing the termination of DNA synthesis when incorporated into a nascent viral DNA molecule by a DNA polymerase (Vivet-Boudou et al., 2006). The NRTIs used in antiretroviral treatment are highly selective for viral reverse transcriptase rather than human DNA polymerase so in theory host DNA synthesis should not be affected (Mitsuya et al., 1990). The non-nucleoside reverse transcriptase inhibitors (NNRTIs) are non-competitive inhibitors which bind to a site away from the active site of the enzyme (de Bethune, 2010). The viral protease is targeted by inhibitors such as lopinavir, which prevents the maturation of the virion (Hurst and Faulds, 2000, Wlodawer and Erickson, 1993). More recently, drugs targeting the viral integrase such as Raltegravir (Cahn and Sued, 2007) or viral entry such as the fusion blocker Enfuvirtide (Qian et al., 2009) have been added to the growing arsenal of antiretroviral treatments. Many of these antiretroviral drugs have also been shown to be effective against FIV (North et al., 1989, Elder et al., 2006, Savarino et al., 2007).

Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs)	Zidovudine (AZT) Lamivudine (3TC) Tenofovir (TDF)
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Efavirenz (EFV) Nevirapine Rilpivirine
Protease inhibitors	Lopinavir Saquinavir Tipranavir
Integrase inhibitors	Raltegravir (RGV)
Entry blockers	Enfuvirtide (INN) Maraviroc

Table 1-2 – Selected examples of the five classes of antiretroviral drugs currently in clinical uses.

A major problem with antiretroviral treatment is that HIV-1 can rapidly develop resistance against all the current classes of drugs (Johnson et al., 2011) and will do so against any future treatments due to its high mutation rate (Mansky and Temin, 1995). The use of a combination of three or more antiretroviral drugs, otherwise known as highly active antiretroviral therapy (HAART) makes it much more difficult for the virus to evolve resistance and has improved the prognosis of patients who can afford the treatment (Shafer and Vuitton, 1999). Antiretroviral drugs are expensive and the majority of the sufferers, who reside in the developing world still do not have access to them (Volberding and Deeks, 2010), although in a recent study it has been shown that starting HAART early rather than waiting for symptoms of AIDS to appear is a cost effective way to reduce mortality and morbidity, even in resource-poor settings (Severe et al., 2010, Koenig et al., 2011). Early treatment also prevents further transmission (Cohen et al., 2011), indirectly reducing medical cost in the future. HAART does not fully restore the health of an infected patient. For reasons which may be a consequence of drug toxicity or the chronic inflammation caused by persistent HIV-1 infection, long-term treated HIV-1 patients have reduced lifespan and increased susceptibility to non-AIDS related conditions such as cardiovascular disease, cancer, liver and kidney dysfunctions as well as neurological decline (Monforte et al., 2004, Deeks, 2011). However, the most significant drawback of HAART is that it cannot completely eradicate the virus from the body.

1.7 Lentiviral latency – obstacle to eradication

1.7.1 Establishment of the latent reservoir

Although HAART is very effective at blocking HIV-1 spread within the body, it is not a cure, as viral loads readily rebound when treatment is interrupted (Chun et al., 1999, Davey et al., 1999). Furthermore, ultrasensitive detection assays have shown that in most HAART-treated patients, a low-level viraemia of less than 5 copies per ml persists even after years of therapy (Chun et al., 2005, Tobin et al., 2005, Palmer et al., 2008). Since the half-life of the HIV-1 virion in the plasma is very short (Ho et al., 1995) (Ramratnam et al., 2000), it is believed that the persistent viraemia is either the result of the reactivation of latently infected resting T cells or on-going virus replication in 'sanctuary' sites within the body such as the central nervous system (Gonzalez-Scarano and Martin-Garcia, 2005, Churchill et al., 2006, Canestri et al., 2010), the gastrointestinal

tract (Chun et al., 2008) and the male and female genital tract (Halfon et al., 2010, Launay et al., 2011). Although improvements in drug penetration or HAART intensification may be able to eliminate persistent virus replication within these sanctuary sites in the future, it will not affect the latent viruses hiding within resting $CD4^+$ T cell populations in the body.

The first indirect evidence for HIV-1 infection of resting T cells was the discovery that the number of cells expressing HIV-1 mRNA *in vivo*, as detected by *in situ* hybridisation, is lower than the number of cells carrying viral DNA (Schnittman et al., 1989). Then it was shown that a small number of the resting T cells in HIV-1 infected individuals, even those who are receiving anti-retroviral treatments, harbour latent virus (Chun et al., 1997). Latent infection of HIV is defined as the lack of full virus gene expression as a consequence of the quiescent status of the host $CD4^+$ T cell, leading to the absence of virus production. HIV-latency is thought to be an unintended consequence of the tropism of the virus, unlike viruses such as the herpesviruses HIV-1 does not encode latency induction genes (Han et al., 2007). Most of the circulating $CD4^+$ T cells in the body at any given time are in a resting state (Berard and Tough, 2002). These can be broadly divided into cells that have not undergone antigen-stimulated expansion (naïve T cells) and the cells that have remained behind after the end of an immune response (memory T cells) (Berard and Tough, 2002). Since most of these latently infected resting $CD4^+$ T cells are $CD45RO^+$ memory cells (Chun et al., 1997, Pierson et al., 2000, Brenchley et al., 2004a, Chomont et al., 2009), it is hypothesised that the majority of the latently infected T cells come from activated $CD4^+$ T cells that were infected and then reverted back to a resting memory state before the start of virus replication (Han et al., 2007). It is also possible that resting T cells are directly infected by the virus. Direct infection of resting T cells is very inefficient (Stevenson et al., 1990, Pierson et al., 2000) due to defects in reverse transcription and delays in integration in comparison with infection of activated $CD4^+$ T cells (Vatakis et al., 2007, Vatakis et al., 2009). No method to date has been able to distinguish between latently infected memory T cells which were infected during activation or quiescence (Vatakis et al., 2010). However, latent infection of naïve T cells has been observed in patients, albeit at a lower frequency than memory T cells (Pierson et al., 2000, Chomont et al., 2009). In a recent clinical study, it has been shown that among patients receiving antiretroviral treatment, the total

amount of HIV DNA in memory T cells declined over time while the amount of HIV DNA in naïve cells remained constant, suggesting that direct infection of resting T cells may be replenishing the latent viral reservoir as the disease progresses (Wightman et al., 2010). This ties in with the observation that R5 tropic viruses (which are associated with acute infection (Zhu et al., 1993)) preferentially infect CCR5 expressing memory T cells whereas X4 tropic viruses (which are associated with late disease progression (Connor et al., 1997)) exhibit a preference for $CCR5^-$ $CXCR4^+$ naïve T cells (Bleul et al., 1997, Ostrowski et al., 1999, Wu et al., 1997). The stimulatory effects of HIV-1 Gp120 may enable the direct infection of resting T cells in the following ways: Incubation of resting T cells with HIV-1 Gp120 stimulates calcium flux and NFAT signalling down the CCR5 signalling pathway, while at the same time increases inositol triphosphate (IP_3) signalling and IL-2 receptor expression (Kornfeld et al., 1988, Weissman et al., 1997, Cicala et al., 2006). Stimulation of CXCR4 signalling by HIV-1 Gp120 induces cytoskeleton-remodelling activity in resting T cells, increasing the efficiency of subsequent infection with HIV-1 but at a far lower rate than in an activated cell (Yoder et al., 2008).

$CD4^+$ T cells are not the only cell type that can become reservoirs of latent infection. Dendritic cells can trap HIV-1 virions on the cell surface for long periods and unintegrated viral DNA can persist and maintain its biological activity in macrophages (Coiras et al., 2009, Kelly et al., 2008). Virus replication in privileged sites such as the CNS where drug penetration is poor may also prevent full suppression by HAART (Varatharajan and Thomas, 2009). In addition it has been proposed that infection of immature $CD4^+/CD8^+$ "double positive" thymocytes during thymopoiesis may generate a population of latently infected naïve T cells (Brooks et al., 2001). In contrast, in a recent case of an HIV positive leukaemia patient receiving a bone marrow transplant from a CCR5-negative donor, CCR5 positive macrophages remained after the transplant, but re-infection by HIV-1 was not detected (Hutter et al., 2009). This suggests that latent infection in cell types other than $CD4^+$ T cells may not be important in maintaining a persistent infection, at least in this one case, and that it is enough to achieve a cure by targeting the $CD4^+$ T cells alone (Allers et al., 2011, Pace et al., 2011). However, the HIV-1-resistant nature of the donor CCR5-negative $CD4^+$ T cells may mask any low-level on-going infection which would seed a new wave of infection if autologous stem cells were transplanted instead.

1.7.2 Latent reservoir leads to viral persistence

The long-term survival of memory $CD4^+$ T cell populations and their rapid expansion following re-exposure to a previously encountered antigen are cornerstones of adaptive immunity (Dooms and Abbas, 2006, van Leeuwen et al., 2009). However, in an HIV-1 infection these vital components of the immune system become its biggest liability. Latent proviruses are not affected by antiretroviral drugs and are hidden from the body's immune response until they are reactivated by stimuli such as exposure to antigens or cytokines (Coiras et al., 2009). The reactivation of the latently infected T cells is unpredictable (Siliciano and Siliciano, 2010) and in theory reactivation of even one cell carrying a replication-competent virus may lead to a new wave of infection. Even in patients who are responsive to HAART there may be intermittent episodes of detectable viraemia (Ramratnam et al., 2000) which may be caused by reactivation of latently infected cells. The size of the latent HIV-1 reservoir is very small, estimated to consist of approximately 10^6 cells (Chun et al., 1997), but due to the lifespan of these cells and the fact that latent provirus can be maintained within the memory T cell population by the homeostatic proliferation of the infected host cells, driven by IL-7 (Chomont et al., 2009, Bosque et al., 2011), it has been calculated that it would take 73.4 years of current anti-retroviral therapy to completely eliminate all the latently infected cells (Siliciano et al., 2003). For these reasons HIV-1 remains incurable in the absence of new therapeutic interventions that can eliminate the latent reservoir.

1.7.3 FIV latency

The role of viral latency in the pathogenesis of FIV-related AIDS in cats is less well understood. Experiments using feline PBMCs identified two $CD4^+$ T cells subsets which can be infected latently in the absence of exogenous IL-2: $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells (Joshi et al., 2004, Joshi et al., 2005a). In these studies stimulation by the mitogen ConA and the addition of exogenous IL-2 was required to induce p24 production, and to increase CD25 expression (a sign of cellular activation) from $CD4^+$ $CD25^-$ cells. In contrast, latently infected $CD4^+$ $CD25^+$ T cells only required the addition of exogenous IL-2 to initiate productive infections, mirroring the crucial role of IL-2 in the productive infection with HIV-1 (Oswald-Richter et al., 2004a). An *in vivo* study showed that at a low

infectious dose, cats appeared to be infected asymptomatically at 12 weeks post infection, with a low level of FIV Gp120 expressed in multiple tissues, the production of which could be stimulated by PMA (Assogba et al., 2007). More recently, it was shown that FIV can establish a latent infection within $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells in chronically infected cats, analogous to the latent infection of the resting T cell population by HIV-1 in humans (Murphy et al., 2012).

1.7.4 Molecular mechanism of latency

The mechanism of latency in HIV-1 infection has been extensively reviewed (Coiras et al., 2009, Marsden and Zack, 2009, Richman et al., 2009, Marcello, 2006, Colin and Van Lint, 2009). In general, HIV-1 latency can be divided into pre-integration or post-integration latency. Pre-integration latency refers to the partial or complete inhibition of the viral life cycle before the integration of the virus into the host genome due to due to defects in reverse transcription and delays in integration (Vatakis et al., 2007, Vatakis et al., 2009). Similar observations have been made in feline $CD4^+$ T cells where FIV entered resting $CD25^-$ cells but was unable to complete replication (Joshi et al., 2004). Although linear unintegrated DNA (See section 1.2.4) is able to complete integration after the activation of the host T cell (Bukrinsky et al., 1991), and while HIV-1 DNA has been detected in resting T cells for 2 weeks post infection (Stevenson et al., 1990), pre-integration latency is not thought to be relevant to the establishment of the latently infected T cell reservoir. This is because of the labile nature of viral DNA in the cytoplasm of the cell (Pierson et al., 2002b), which means that the unintegrated viral DNA may not be replication competent after a protracted period inside the host cell (Zhou et al., 2005, Han et al., 2007). Post-integration latency is the failure of full expression of viral genes after the integration of the provirus into the host genome. Most of the HIV-1 DNA found in resting T cells is in the linear, unintegrated form, while less than 0.05%, or approximately 10^6 to 10^7 cells, carry integrated provirus and this is the form of latency which constitutes the latent viral reservoir (Chun et al., 1997).

A variety of mechanisms are thought to contribute to the establishment and maintenance of the latent provirus. These include the low activity of the various cellular signalling pathways, thus reducing the availability of transcription factors such as NFkB, AP-1 and NFAT (Williams and Greene, 2007);

the restrictive host chromatin organisation caused by epigenetic silencing (See section 1.7.7); the insufficient expression of the viral transcription promoter Tat, causing inefficient elongation of the viral transcript (Weinberger et al., 2005); the presence of cellular transcriptional repressors, for example YY1 and LSF, which recruit suppressive histone deacetylases to the HIV-1 LTR (Coull et al., 2000); In addition, it has been demonstrated that HIV Tat and Rev transcripts are retained in the nuclei of resting $CD4^+$ T cells (Lassen et al., 2006) and that numerous host microRNAs can directly or indirectly down-regulate HIV-1 gene expression, contributing to the maintenance of proviral latency (Chiang and Rice, 2012) It is important to look at these mechanisms not as isolated entities but as part of the broad signalling and regulatory network with cross-talk, redundancy and feedback loops between different factors.

Lentiviral replication is highly integrated with the cellular signalling network that controls T cell activation. The 5'LTR of lentiviruses contains DNA-binding sites for a number of cellular transcription factors that are triggered during T cell activation, such as NFκB, NFAT, AP-1 and ATF (Tang et al., 1999, Colin and Van Lint, 2009, Sparger et al., 1992, Thompson et al., 1994). The activities of these factors are controlled by a network of signalling pathways and the triggering of one or more of these pathways would be sufficient to trigger productive viral replication (Williams and Greene, 2007). Among the plethora of pathways available, Protein Kinase C signalling has attracted much attention because of its central role in T-cell activation, culminating in the modulation of transcription factors such as NFκB, NFAT and AP-1.

1.7.5 Protein kinase C

The family of cyclic nucleotide independent serine/threonine kinases known as Protein kinase C (PKC) was discovered by Nishizuka and co-workers in 1977 (Inoue et al., 1977, Nakamura and Yamamura, 2010) and consists of three major groups or isoforms: conventional PKC (cPKC), novel PKC (nPKC) and atypical PKC (aPKC) (Fig. 1-7). The cPKC isoforms (α, ß and γ) require calcium and diacylglycerol (DAG) for activation; nPKC isoforms (δ, ε, η, θ and μ) require only DAG; aPKC isoforms (ζ and iota) require neither calcium nor DAG for activation.

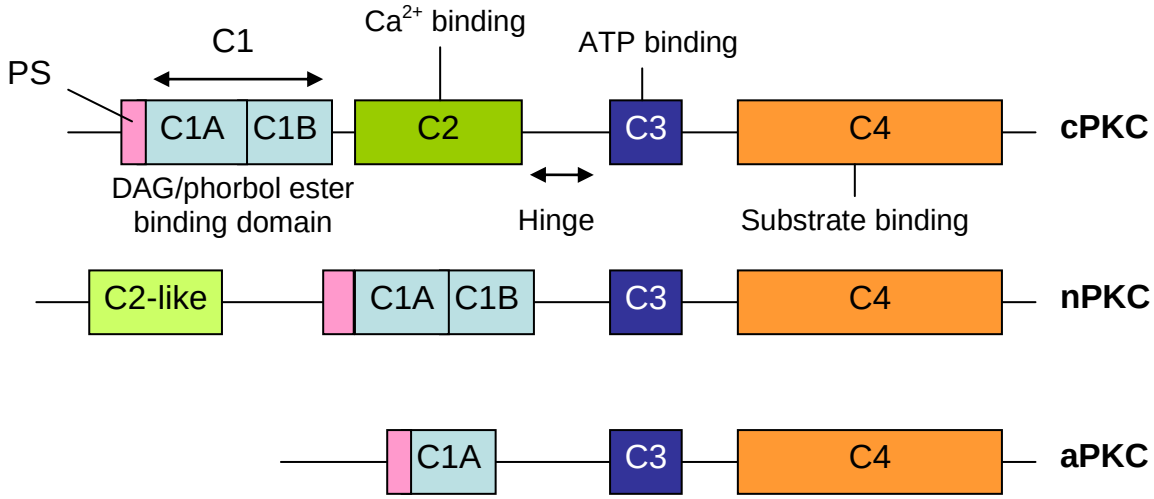


Figure 1-7 - Structure of the three main PKC isoforms. A representation of the three major PKC isoforms, showing the arrangement of the different catalytic and regulatory domains within each isoform. PS = pseudo-substrate.

All PKC isoforms consist of a catalytic and a regulatory domain, which are sub-divided into four conserved regions C1 to C4 (Fig. 1-7). C1 is the regulatory domain, with C2, C3 and C4 folded together to form the substrate binding domain (Tan and Parker, 2003). The pseudo-substrate from the regulatory domain blocks substrate access to the catalytic domain when the enzyme is unstimulated. Before activation PKC resides in the cytoplasm. In response to activation the enzyme moves to the cell membrane, its catalytic domain changes conformation, displacing the pseudo-substrate, enabling protein phosphorylation to take place.

1.7.6 Phorbol esters and PKC

The C1 DAG binding domain of the novel and conventional PKC isoform is also the binding site for a class of compounds known as phorbol esters (Ono et al., 1989, Burns and Bell, 1991). The C1 domain contains a hydrophobic surface which mediates the insertion of the enzyme into lipid membranes. In the centre of this hydrophobic surface there is a polar groove that disrupts the interaction between the protein and the membranes (Zhang et al., 1995). DAG and phorbol esters cap this groove and complete the hydrophobic surface, allowing PKC translocation and catalytic activity to take place (Zhang et al., 1995).

The first phorbol ester was isolated from the seeds of *Croton tiglium,* a member of the Euphorbiaceae (Spurge) family of plants which was used in traditional Chinese medicine (Hecker et al., 1964, Li, 1578); croton oil was well known for its ability to enhance carcinogenesis and the phorbol ester extracted from croton oil, phorbol myristate acetate (PMA or TPA) was shown to be a potent co-carcinogen (Paul and Hecker, 1969). Later Nishizuka and others demonstrated that PMA activates PKC by binding directly to its C1 domain (Castagna et al., 1982, Kikkawa et al., 1983, Niedel et al., 1983). PMA was shown to have anti-HIV-1 effects (Rullas et al., 2004, Warrilow et al., 2006) but its tumour promoting properties make it unsuitable for clinical use. However, PMA remains a major experimental tool for cell signalling and T cell activation studies.

Prostratin (12-deoxyphorbol 13-acetate) is a phorbol ester that stimulates PKC (Gustafson et al., 1992). It was first isolated as the toxic component of the shrub *Pimelea prostrata,* which was blamed for the poisoning of livestock in New Zealand (Cashmore et al., 1976). The anti-HIV-1 effect of Prostratin was discovered after a screening programme by the U.S. National Cancer Institute (NCI) looking for therapeutic compounds in a number of Samoan plant extracts. Substantial cytoprotective and HIV-1 inhibitory effects were detected in the extract of the Samoan medical plant *Homalanthus nutans*, another member of the Spurge family of plants (Gustafson et al., 1992). Prostratin has also been isolated from the traditional Chinese medicine plant "lang-du" (*Euphorbia fischeriana*) (Wang et al., 2006). From its discovery it was shown that Prostratin is different to other phorbol esters such as PMA due to its lack of tumour promoting ability (Szallasi and Blumberg, 1991, Gustafson et al., 1992). However, it was the latency reversing effect of Prostratin that attracted the attention of HIV researchers. Prostratin has been shown by various laboratories to inhibit HIV replication and to reactivate latent HIV (Kulkosky et al., 2001, Korin et al., 2002, Brooks et al., 2003b, Biancotto et al., 2004, Rullas et al., 2004, Warrilow et al., 2006). HIV-1 reactivation by Prostratin is dependent on PKC and NFκB activation, although the role of NFAT and AP-1 may be redundant, depending on the cell type used (Brooks et al., 2003a, Williams et al., 2004). Prostratin down-regulates expression of the HIV-1 receptors CD4, CCR5 and CXCR4 (Kulkosky et al., 2001, Biancotto et al., 2004, Rullas et al., 2004, Warrilow et al., 2006) and this has been proposed as the primary mechanism for the inhibitory effects of Prostratin on HIV-1 replication. Although phorbol esters

are primarily known for their PKC-stimulating activities, non-PKC receptors for phorbol esters have been discovered. These include PKD, chimaerins and RasGRPs and they were shown to mediate key proliferation and transformation processes of the cell (Yang and Kazanietz, 2003).

1.7.7 Chromatin organisation and latency

As the lentiviral provirus is integrated into the host genome, cellular histone proteins interact with the proviral DNA, forming nucleosomes. The position of the nucleosomes is vital to the modulation of latency. Studies on the HIV-1 LTR have revealed that two nucleosomes, nuc-0 and nuc-1, are positioned over key transcriptional regulatory sites of the provirus and that nuc-1 is remodelled in response to PMA, thus linking chromatin re-modelling to T cell activation (Verdin et al., 1993, Vanlint et al., 1994). Acetylation of the various histone and non-histone chromatin associated proteins is modulated by histone deacetylases (HDACs) and histone acetyltransferases (HATs) (Yang and Seto, 2007). HDAC has been shown to directly affect HIV LTR expression levels (He and Margolis, 2002). In addition, HDAC inhibitors such as valproic acid and SAHA has been shown to reactivate latent HIV-1 infection in *in vitro* models, further illustrating the role of nucleosomes and chromatin re-organisation in the maintenance of latency (Jordan et al., 2003, Reuse et al., 2009, Bosque and Planelles, 2009, Burnett et al., 2010). Methylation of DNA has also been shown to affect both HIV-1 and FIV LTR activity (Schulzeforster et al., 1990, Ikeda et al., 1996) and may play a role in the establishment of latency.

Based on the current understanding of lentiviral latency, it has been proposed that to deplete the HIV-1 latent reservoir in the body, latently infected resting T cells would be stimulated to reactivate the dormant provirus by phorbol esters or HDAC inhibitors. It is assumed that the cytopathic effects of productive infection would lead to the death of the host cell. Alternatively the synthesis of viral proteins by the reactivated infected cells would reveal it to the host immune system for cell-mediated killing. Meanwhile HAART would protect uninfected T cells in the body from any nascent virus (Fig. 1-8) (Richman et al., 2009).

1.7.8 Current models for HIV-1 latency – clues to the elusive cure

The use of stem cell transplantation to clear HIV-1 from the body has been successfully demonstrated in one patient (Hutter et al., 2009, Allers et al., 2011), but it is unlikely to be a practical or affordable cure for most patients in the near future, particularly in the developing world. A more probable strategy for the elimination of latent viral reservoirs would be to treat the patient with drugs that reactivate virus production under intensive HAART without causing global activation of uninfected T cells (Fig. 1-9) (Marsden and Zack, 2009, Richman et al., 2009). However, as viral replication is intricately tied to the cellular activation state (Stevenson et al., 1990), inducing reactivation of latent virus while avoiding T cell activation has proved challenging (Prins et al., 1999). Reversing HIV-1 latency would require a better understanding of the mechanism of lentiviral latency at both the cellular and the whole animal level. Due to the rarity of latently infected $CD4^+$ T cells in the host and the lack of surface markers to distinguish latently infected cells from the rest of the T cell population (Chun et al., 1995, Han et al., 2007), it has been difficult to use primary cells from patients in molecular biology research and almost impossible to use them in the high-throughput screening of drugs. Thus *in vitro* and *in vivo* model systems are vital to illuminate the molecular mechanisms of latency and to discover new means to reverse it. Early studies of lentiviral latency using cell lines such as ACH-2, U1 and J-Lat showed the involvement of host cytokine signalling pathways and chromatin re-organisation in modulating latency (Folks et al., 1987, Folks et al., 1989, Jordan et al., 2003), but their transformed nature means that their responses to treatments may not mimic the physiological response of primary cells. For example in the latently infected J-LAT cell line, HIV-1 preferentially integrates near the heterochromatin where transcriptional activity is low (Jordan et al., 2003). However, this preference is not observed within the latently infected resting $CD4^+$ T cells from HAART-treated HIV-1 patients. Instead, the provirus overwhelmingly favours integration into active transcriptional regions (Han et al., 2004).

Thus primary T cells form the basis of most of the *in vitro* models described. In one model naïve T cells are activated using dendritic cells grown from PBMCs and with staphylococcal enterotoxin B (SEB), and then infected with HIV-1 at a low multiplicity of infection (M.O.I) (Marini et al., 2008). After an initial cell expansion period (which also saw peak HIV-1 p24 production) the cells were

rested and were cultured with a low dose (1 ng/ml) of IL-7. Il-7 is an essential cytokine for the maintenance of memory T cells (Marrack and Kappler, 2004) and protects T cells against cell death that is induced by the withdrawal of growth factors like IL-2. These resting cells were able to be reactivated by secondary stimulation from SEB loaded dendritic cells or anti CD3/CD28 antibodies. The whole process from isolation of T cells to secondary stimulation took 68 days and involved multiple complicated steps, in which the authors tried to simulate faithfully a real immune response. However this technique leads to the loss of cell viability and an increase in the scope for error. Furthermore the use of IL-7 to maintain resting cells, albeit at a very low dose, carries the risk of reactivating the virus prematurely.

A separate model aimed to prolong T cell survival by transducing primary $CD4^+$ T cells with Bcl-2 (Yang et al., 2009). Bcl-2 is an anti-apoptotic or "survival" factor in T cells (Marrack and Kappler, 2004) which functions downstream of the cytokine IL-7. Its over-expression has been shown to restore the pro-survival effects of IL-7 in IL-7 receptor deficient mice (Akashi et al., 1997). This abolishes the need for exogenous IL-7 and may avoid accidental reactivation. Transduced T cells were cultured without T cell receptor or cytokine stimulation for 4 weeks before expansion of the remaining viable cells (which have been successfully transduced) and infection with a GFP reporter virus. The cells were then kept without stimulation for 4 weeks and GFP negative cells (which contain uninfected and latently infected cells) were sorted by FACS. These were then stimulated with various agents known to reactivate HIV-1 such as anti-CD3/CD28 antibodies, phorbol esters such as PMA and cytokines such as IL-2 and IL-7. The whole procedure took more than 8 weeks and gave a modest increase in GFP count when stimulated (from 0.15% to 2.89% using anti CD3 and CD28 antibodies). This reflects the rarity of latently infected cells *in vivo* and the authors claimed to be able to generate enough of these transduced cells for high-throughput studies, which they have demonstrated by using the model system to identify a new compound, 5-hydroxynaphthalene-1, 4-dione (5HN), that can reactivate latent HIV-1. There are two potential problems with this model: Firstly, the transduction of a survival factor into the T cells may alter the basic physiology of the cells. Secondly, the use of a highly mutated reporter virus may not reflect infections in real life since it does not demonstrate whether the system can support infection with a replication competent virus.

Direct infection of resting $CD4^+$ T cells with virus has also been investigated as a potential model for HIV-1 latency. HIV-1 engagement of CXCR4 activates cofilin, a cellular actin-depolymerizing factor, which aids the natural infection of resting T cells by the virus (Yoder et al., 2008). Furthermore, latent infection of resting T cells is more efficient when the dynamics of cytoskeleton re-organisation is stimulated artificially (Yoder et al., 2008). Other methods to increase the efficiency of direct infection of resting T cells exist; a technique known as spinoculation has been shown to assist viral entry without activation of the cells and could be useful in deducing the mechanism of latency maintenance (O'Doherty et al., 2000) (Swiggard et al., 2005, Agosto et al., 2007). Stimulation of CCR7 with the ligands CCL19 and CCL21 also increased infection efficiency and the infected resting T cells responds to post-infection stimulation with phorbol esters in a similar fashion to other T cell preparations (Saleh et al., 2011, Saleh et al., 2007).

In addition to activated and resting $CD4^+$ T cells, infected immature $CD4^+$ $CD8^+$ thymocytes may also become a latent viral reservoir after their maturation into naïve T cells. This hypothesis was developed using a SCID mouse model (Brooks et al., 2001) and from this basis an *in vitro* model of latently infected thymocytes has also be generated (Burke et al., 2007).

Animal models are essential if we are to achieve a better understanding of HIV latency and test potential therapeutic regimes that may overcome latent infection. Non-human primates, in particular rhesus macaques infected with the simian immunodeficiency virus (SIV) or a chimeric SIV containing HIV-1 reverse transcriptase have been used to model HIV-1 latency in HAART-treated patients (Shen et al., 2003, Dinoso et al., 2009, North et al., 2010). The major advantages of using non-human primates are that the persistent viral reservoirs are found in the same locations in both humans and primates (North et al., 2010), which allows comparative *in vivo* studies. Also the progression of SIV in macaques resembles HIV-1 infection in humans, with distinctive acute and chronic phases of infection that may lead to immunodeficiency (Hirsch et al., 1996). However, there are significant differences between SIV infection of non-human primates and HIV-1 infection in humans. For example, the residual viraemia for SIV in rhesus macaques during chronic infection is higher than the levels seen in humans (Brenchley and Paiardini, 2011). The progression of to

AIDS appears to be more rapid in rhesus macaques than in humans (North et al., 2010). In African green monkeys or sooty mangabeys, although high levels of virus replication are observed during the chronic phase of infection, this is not accompanied by the destructive chronic immune activation seen in rhesus macaques or humans (Brenchley and Paiardini, 2011, Chahroudi et al., 2012). Also different strains of SIV can produce different pathologies in the same host (Hirsch et al., 2000). The complexity of finding the right host and SIV strains that best mimic HIV-1 latent infection, together with issues such as ethical concerns and high cost have led to the development of other, non-primate animal models for HIV-1 infection such as the humanized severe combined immunodeficiency (SCID) mouse models (Boberg et al., 2008, Van Duyne et al., 2009, Brooks et al., 2001). These are created by transplanting human foetal thymus and liver tissues or peripheral blood lymphocytes into SCID mice to form SCID-hu Thy/Liv and SCID-hu PBL mice systems respectively (Van Duyne et al., 2009). A major drawback of using SCID-mice-based models is the failure to fully reconstitute the human immune system within transplanted animals (Rossi et al., 2001, Van Duyne et al., 2009). Further improvement to the efficiency of engraftment was achieved with the generation of the non-obese diabetic (NOD)/SCID mouse model (Hesselton et al., 1995) and later with the double knock-out of the common cytokine receptor γC and the recombinase activating gene 2 (Rag2) (Goldman et al., 1998). The transplantation of human $CD34^+$ stem cells into $Rag2^{-/-}\gamma C^{-/-}$ mice leads to the development of a functional human immune system in the body of the mouse (Traggiai et al., 2004) and forms the basis of a recent murine model of HIV-1 latency that contains infected resting T cells in the peripheral blood and lymphoid tissues (Choudhary et al., 2009, Choudhary et al., 2012). A small animal model of HIV-1 where a natural host species interacts with an immunodeficiency-causing lentivirus would offer a valuable insight into potential strategies to overcome lentiviral latency.

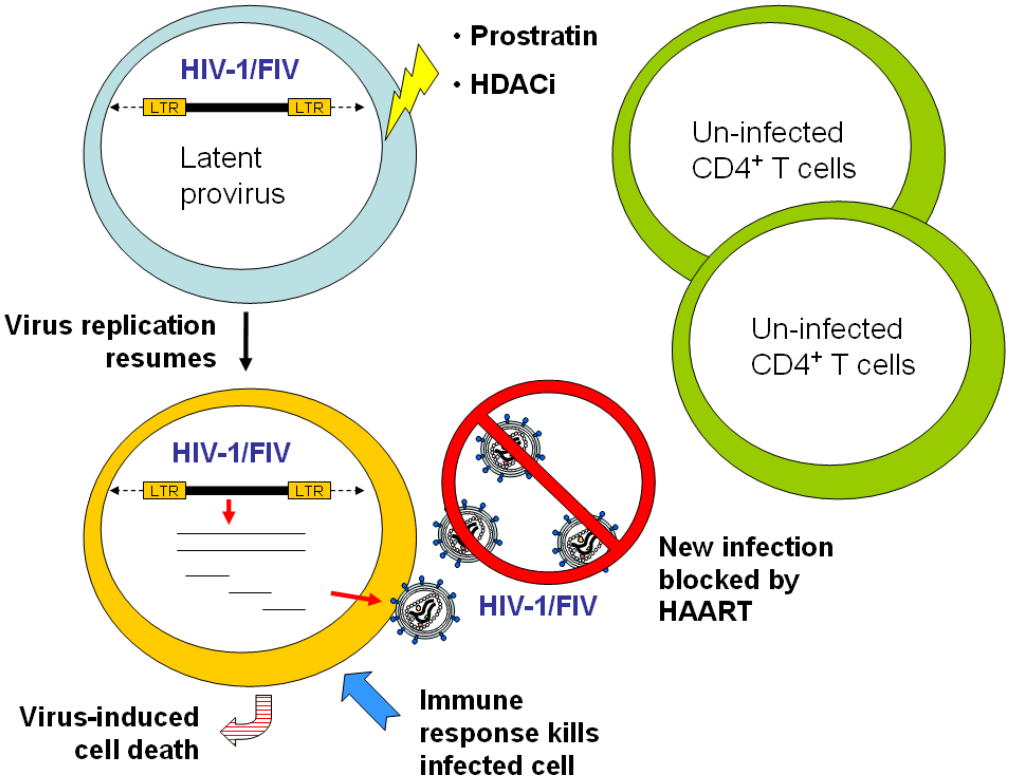


Figure 1-8 – Elimination of the latent lentiviral reservoir. Virus replication in latently infected resting T cells can be reactivated by phorbol esters (e.g. Prostratin), HDAC inhibitors or other therapeutic means. The infected cell, previously invisible to the immune system, would now be detectable and targeted by the cytotoxic T cell response. The process of virus replication may also induce cell death. Intensive HAART prevents *de novo* infection of uninfected T cells.

1.8 Scope of this thesis

The pathology of lentivirus-induced AIDS has a complex aetiology and to fully comprehend the disease process many aspects of the biology of the host have to be incorporated. The many intricate host-pathogen interactions mentioned above contribute to a state of chronic immune activation that is controlled by T cell signalling pathways. Even lentiviral latency, which is characterised by a state of low T cell signalling activity also contributes indirectly to chronic immune activation by allowing viral persistence. In this thesis I investigate different aspects of T cell signalling and their relationships with viral replication and pathology:

- In Chapter 3, the hypothesis that FIV Env binding to CD134 may trigger cell signalling is tested on *in vitro* model systems.

- In Chapter 4, the concept that FIV-infected cats can be a model for HIV-latency, as well as the hypothesis that Prostratin has both stimulatory and inhibitory effect on FIV replication similar to its effect on HIV-1 replication were tested *in vitro*.

- In Chapter 5, the hypothesis that an 'early' strain of FIV, GL8, has different replicative properties to that of a 'late' strain, PPR, in experiments that manipulate cellular signalling pathways as documented in Chapter 4 was tested *in vitro*.

2 Materials and Methods

2.1 Cells, reagents, buffers and viruses

2.1.1 Adherent cell lines

HEK-293T, NFkB/293/GFP (System Biosciences, Mountain View, CA, USA), HT1080 (Rasheed et al., 1974), NP-2 (Yamazaki, 1982), HeLa, Chinese Hamster Ovary (CHO) cells and Crandall Rees Feline Kidney (CrFK) cells (clone ID10) were cultured in Dulbecco's modification of Eagle's Medium (DMEM) with high glucose (4.5g/ml) and 110 mg/L sodium pyruvate and supplemented with 10% foetal bovine serum (FBS), 100 IU/ml penicillin and 100µg/ml streptomycin. This supplemented media will be referred to as 'complete DMEM'. All media and supplements were obtained from Invitrogen Life Technologies Ltd. (Paisley, United Kingdom) except for the foetal bovine serum, which was supplied by Thermo Fisher Scientific (Loughborough, UK). Where appropriate, culture medium was supplemented with the antibiotics G418 (400 µg/ml; Invitrogen) or puromycin (1.25 µg/ml; Sigma Aldrich, Gillingham, UK) for transgene selection. Cells were grown at 37°C until confluent and then passaged by washing with phosphate buffered saline (PBS), digested with 0.05% Trypsin for less than 5 minutes at 37°C, mixed with complete DMEM to halt trypsin activity, pelleted by centrifugation at 1000 rpm in a bench-top centrifuge, resuspended in fresh complete DMEM and re-seeded into new flasks at the appropriate density.

2.1.2 Suspension cell line

The majority of experiments described in chapter 4 and 5 were performed using the IL-2 dependent feline $CD4^+$ T cell line MYA-1, which has been cultured from a FIV-infected cats in 1989 (Miyazawa et al., 1989) and has been cultured continuously since. These cells are not considered to be primary $CD4^+$ T cells, but retain some primary cell characteristics as they remain dependent on exogenous IL-2 for growth (Joshi et al., 2005a) and undergoes phases of blasting, rapid expansion and rest unlike the continuous rapid growth often associated with transformed cell lines. The MYA-1 and canine lymphocytic leukaemia (CLL) (Willett et al., 2006b) cell lines were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 2mM glutamine, 100 IU/ml penicillin and 100µg/ml streptomycin. This will be referred to as 'complete

RPMI'. For growing MYA-1 cells complete RPMI was further supplemented with conditioned medium from a murine cell line (L2.3) transfected with a human IL-2 expression construct (equivalent to 100 U/ml of recombinant human IL-2) and 50 μM 2-mercaptoethanol. Cells were grown at 37°C until confluent and then pelleted by centrifugation at 800 – 1000 rpm with a bench-top centrifuge, resuspended in fresh complete RPMI and re-seeded into new flasks at the appropriate density. Selection antibiotic was also used where appropriate (See Adherent cell lines).

2.1.3 Plasmid vectors

The retroviral expression vector pDONAI (Takara, Tokyo, Japan) and pRetroQ-DSRed Monomer N1 (Clontech, CA, USA) were used to transduce and transfect cells with CD134 constructs FFF (feline wild-type CD134 (Willett et al., 2006b), HHH (human wild-type CD134 (de Parseval et al., 2005), FFHH (feline-human chimeric CD134 (Willett et al., 2006b). Constructs expressing FIV GL8 or PPR Env SU Fc (de Parseval et al., 2004a) peptides were cloned into the vector pTorsten (Spiller et al., 2006). The plasmids CMVi, which is a cytomegalovirus promoter-driven MLV *gag-pol* expression vector (Bock et al., 2000), and pMDG, which contains the vesicular stomatitis virus (VSV) G-glycoprotein (Naldini et al., 1996), were used to produce pseudotypes for transduction (See below). FIV GL8 or PPR *env* gene was cloned into the vector VR1012 (Vical Inc., San Diego, USA). The plasmid pNL4-3-Luc-E^-R^- (N. Landau, Aaron Diamond AIDS Research Center, the Rockefeller University) contains a defective HIV-1 NL4 provirus as well as the firefly luciferase gene. Both of these vectors were used to produce pseudotypes for luciferase assays.

FIV strains GL8 (Hosie and Jarrett, 1990) and PPR (Phillips et al., 1990) were grown in either CLL-CD134 or MYA-1 cell-lines. Productive infection was confirmed by FIV p24 ELISA (see below) approximately one week after inoculation. Cells were centrifuged at 3000 rpm for 3 minutes to break open cells and pellet cell debris. The virus containing supernatant was then purified and concentrated by a double sucrose gradient centrifuged at 28,000 rpm for 2 hours at 4°C (Beckman L8-70M Ultracentrifuge) and the virus pellets were resuspended in PBS and stored at -80°C. Fresh virus aliquots were used in each experiment.

Chimeric viruses derived from GL8 and PPR-Mlu molecular clones (See below) were produced by transfection of the molecular clone into HEK 293T cells. The 0.45 μm filtered transfection supernatant was then incubated with MYA-1 cells for 2 hours. The MYA-1 cells were then washed and cultured in complete RPMI plus IL-2 until productive infection was confirmed by FIV ELISA and the virus containing supernatant was processed in the same manner as described above.

2.1.4 Ligands and chemicals

Soluble CD134 ligand containing the trimerisation domain tenascin (TNC) and the human IgG Fc domain (Willett et al., 2007, Willett et al., 2009) was produced as described previously from the vectors pDONAI and pRetroQ-DSRed Monomer N1. Briefly, constructs were stably transduced into HEK 293T cells and the cells were grown in medium supplemented with low-IgG serum (Integra Biosciences, Hudson, NH, USA). Ligand was purified from the culture medium by affinity chromatography using HiTrap™ Protein-A Sepharose columns (GE Healthcare, Little Chalfont, UK). FIV Env SU Fc (de Parseval et al., 2004a) peptides from FIV GL8 or PPR were produced from the vector pTorsten. Constructs were stably expressed in CHO cells and the peptides were purified from culture supernatant with Protein-A Sepharose columns as above.

The NFκB inhibitors CAPE (caffeic acid phenethyl ester), BOT64, SC514, JSH23 and BAY 11-7082 were obtained from Merck Chemicals UK (Nottingham, UK). The phorbol ester Phorbol-12-myristate-13-acetate (PMA) was supplied by Merck Chemicals UK and Prostratin was supplied by Sigma Aldrich. PKC inhibitor Gö6976 was supplied by Merck Chemicals UK and Gö6850 was supplied by Tocris (Bristol, United Kingdom). The histone deacetylase inhibitors valproic acid and sodium butyrate were supplied by Sigma-Aldrich. The HIV-1 integrase inhibitor Raltegravir (RGV) was obtained from Selleck Chemicals (Houston, TX, USA) while the nucleoside reverse transcriptase inhibitor Zidovudine (AZT) was supplied by Sigma Aldrich. The activities of the aforementioned activators and inhibitors (including the feline CD134 ligand) are summarised below (Fig. 2-1).

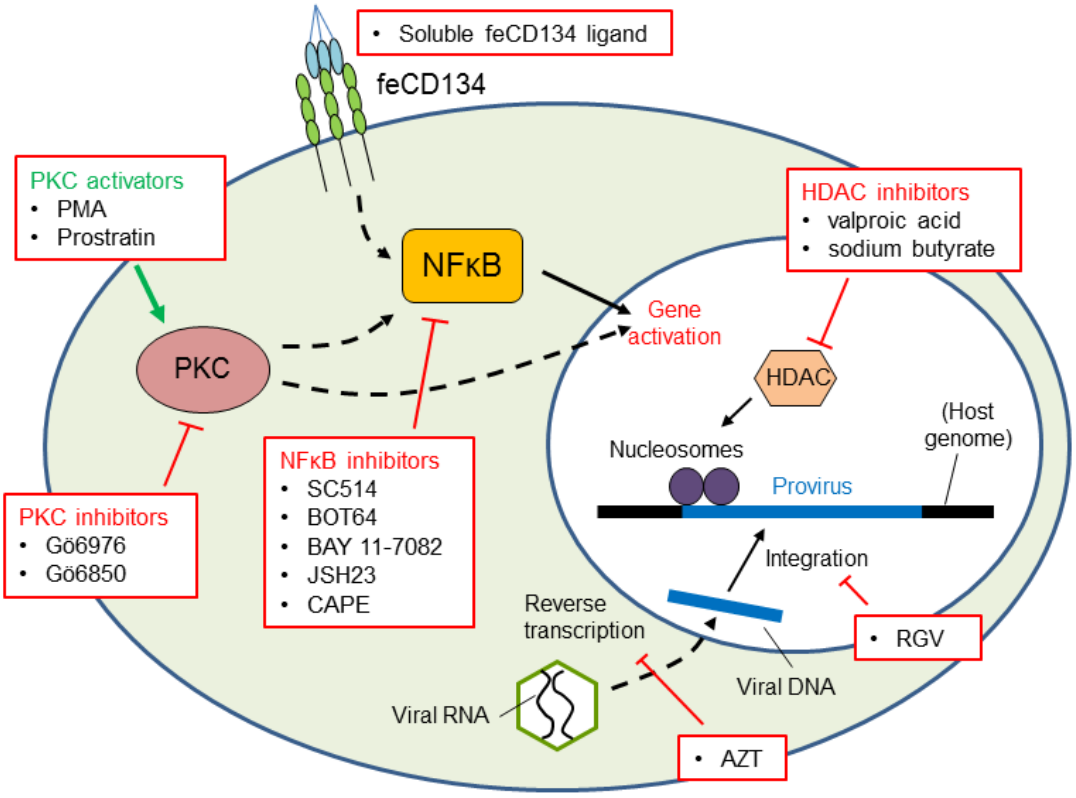


Figure 2-1 – A schematic showing the sites of action for the molecules described in this thesis which affect cell signalling or virus replication. fe stands for feline. HDAC stands for histone deacetylase. AZT stands for Zidovudine and RGV stands for Raltegravir.

The cellular stain carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Invitrogen. The non-ionic detergent Tween® (full name polyethylene glycol sorbitan monolaurate polyoxyethylenesorbitan monolaurate) was supplied by Sigma-Aldrich. The dyes methylene blue and basic fuchsin were also supplied by Sigma-Aldrich. The solvents methanol, ethanol and isopropanol were supplied by Fisher Scientific (Loughborough, UK). Phenol and chloroform were obtained from Sigma-Aldrich.

2.1.5 Transient transfection and stable transduction of cells

Transfection of adherent cells was performed using SuperFect® transfection system (Qiagen, Crawley, UK) as per manufacturer's protocol. Briefly $1.5x10^6$ cells were seeded onto $10cm^2$ cell culture dishes and cultured overnight at 37°C. Up to 12 µg of vector DNA was added to 300 µl of serum free, antibiotic free DMEM medium. 60 µl of the SuperFect reagent was added and mixed thoroughly

and incubated at room temperature for 15 minutes. Cells in culture dishes were washed with PBS. At the end of the incubation, 3 ml of Complete DMEM was added to the mixture, which was then transferred to the washed cells. The cells were incubated at 37°C for 3 hours and medium was changed. The cells were then further incubated for 72 hours before downstream processes such as antibiotic selection took place.

Stable transduction of cells involved the use of HEK 293T cells as producer of retroviral pseudotypes. HEK 293T cells were transfected with the retroviral vector pDONAI containing the gene of interest (for example, feCD134), as well as the plasmids CMVi and pMDG to produce pseudotypes. The pseudotypes-containing supernatants from the transfected HEK 293T cells were then collected and filtered with a 0.45 μm filter and added to target cells (which had been seeded $1x10^5$ cells per well in a 6-well plate the previous day and had been incubated at 37°C overnight). After 6-hour incubation the target cells were transferred to $25cm^2$ flasks to be grown to confluency and selected with suitable antibiotic.

2.1.6 Flow cytometry

Flow cytometry was used to quantify cell growth using the CFSE (Invitrogen) staining method and to measure expression of FIV receptors CD134 and CXCR4 over time (in separate experiments). CFSE enters the cytoplasm of cells and binds to intracellular molecules. During cell division the dye is transferred to daughter cells equally, which results in a reduction of fluorescence. CFSE staining was performed as per manufacturer's protocol but briefly: MYA-1 cells were resuspended in PBS with 0.1% w/v BSA that was warmed to 37°C at a concentration of $5x10^5$ cells/ml. Then 1 μl of the 5 mM stock CFSE dye was added to each ml of cells to achieve a final dye concentration of 5 μM. The cells and dye were incubated at 37°C for 10 minutes, followed by a quenching step in which five volumes of ice-cold complete RPMI was added to the cells. After a further incubation of 5 minutes on ice the cells were washed three times using fresh complete RPMI and transferred to a suitable container in preparation for treatments with IL-2 or drugs.

For the labelling of FIV receptors: 0.5 μg of primary antibodies (7D6, a mouse anti-feline CD134 monoclonal antibody (Willett et al., 2007) or the murine anti-

feline CXCR4 monoclonal antibody from R&D Systems, Minneapolis, USA) were incubated with >1x10^4 cells for 30 minutes at 4°C. Cells were washed with 1 ml of the PBAz wash buffer (1% w/v BSA (Sigma Aldrich) and 0.1% w/v of sodium azide (BDH Laboratory Supplies, Poole, UK) in PBS). Secondary staining of the receptors was performed using 0.5 µg of rabbit anti-mouse IgG R-phycoerythrin (RPE) secondary antibody (AbD Serotec, Oxford, UK) for 30 minutes at 4°C. Cells were then washed and resuspended in appropriate volumes of PBAz. Alternatively a fluorescein isothiocyanate (FITC) conjugated mouse anti-feline CD134 monoclonal antibody (AbD Serotec) was used as the primary antibody, removing the need for secondary staining. Processed cells were analysed by flow cytometry using an EPICS MCS-XL flow cytometer (Beckman Coulter, High Wycombe, UK), with 10,000 events being collected for each sample in LIST mode. Data was analysed using EXPO 32 ADC Analysis software (Beckman Coulter).

2.2 CD134 signalling assays

Before each assay, adherent cell lines HT1080, NP-2 and HeLa stably transduced with various CD134 constructs in the pDONAI retroviral vector were analysed using flow cytometry to ensure more than 90% of the cells expressed CD134. Cells were then seeded onto a 96-well plate at a density of 2x10^5 cells/ml and incubated overnight for cell adherence. To determine the length of time for stimulated cells to reach maximum IL-8 production level, feline CD134L (0.5 µg/ml) was added to the cells and supernatant was harvested after 5, 10, 20, 40, 60, 90, 120, 180, 240, 360 minutes and IL-8 concentrations of the samples were measured by IL-8 ELISA. To discover the minimum length of ligand stimulation that would induce maximum IL-8 production, cells were stimulated by CD134L for 0, 10, 20, 30, 40, 60 and 120 minutes before being washed and supernatant replaced with fresh complete DMEM. IL-8 levels in the replaced medium were measured after a further incubation of six hours. To determine the level of IL-8 production from HT1080, NP-2 and HeLa cells transduced with CD134 and stimulated with CD134 ligands, FIV virions and/or the NFκB inhibitors CAPE and SC514, supernatant was harvested 6 hours after stimulation and IL-8 concentrations measured by ELISA.

2.3 Reactivation of productive FIV infection from resting T cells

IL-2 dependent MYA-1 feline $CD4^+$ T cells were seeded at a density of $5x10^5$ cells/ml in the absence of IL-2 and 24 hours post-seeding they were infected with FIV GL8 or PPR at multiplicity of infection (M.O.I.) of 0.01. Cells were then washed twice and incubated at 37°C in fresh complete RPMI without IL-2 supplement. The IL-2-depleted cells were stimulated with DMSO (control), PMA (0.08 μM), Prostratin (1 μM), sodium butyrate (1.5 mM), valproic acid (1.25 mM), and/or the PKC inhibitors Gö6976 (2.5 μM) or Gö6850 (2.5 μM) 2 days post-infection (unless otherwise stated). Samples were collected at indicated time points and the removed volume was replaced with a matched volume of complete RPMI containing the appropriate compounds. Samples were stored at -80°C until the end of the experiment at which time virus levels in the culture supernatants were measured by either an enzyme-linked immunosorbent assay (ELISA) for FIV capsid antigen (CA, p24) (IDEXX PetCheck anti FIV antigen, IDEXX Laboratories, Maine, USA), or by non-isotopic reverse transcriptase assay (Lenti RT kit, Cavidi AB, Sweden) as per manufacturer's instructions (see below).

2.4 FIV productive infection assay

Actively growing MYA-1 $CD4^+$ T cells were seeded at a density of $5x10^5$ cells/ml in the presence of IL-2 and infected with FIV GL8 or PPR (MOI = 0.01) for 2 hours at 37°C. Cells were then washed and resuspended in complete RPMI supplemented with IL-2 and stimulated with DMSO (control), PMA (0.08 μM), Prostratin (1 μM), the NFκB inhibitors SC514 or JSH23 (Between 5 and 20 μM) and/or the PKC inhibitors Gö6976 (2.5 μM) or Gö6850 (2.5 μM) at 2 days post-infection. Samples of the culture supernatant were taken at day 8 post-infection and the virus levels in the culture supernatants were measured by FIV p24 ELISA or by assaying reverse transcriptase activity. Cell growth and viability was measured by Trypan Blue exclusion assay at day 8 post-infection.

2.5 FIV syncytium formation assay

To investigate the effect of phorbol esters on feline tetherin expression and virus spread, $3x10^5$ CRFK ID10 cells were plated onto 6-well plates and allowed to settle overnight. Cells were infected with equal doses of the cell-culture

adapted strain FIV-F14 for two hours before wash. 24 hours post infection PMA (0.08 μM), Prostratin (1 μM), feline IFN-ω (1000U/ml; Virbac Limited, Bury St Edmonds, UK) and DMSO (control) were added to the cells. Each treatment was performed in duplicate. Experiments were terminated 3 days post infection, the virus titre in the supernatant was measured by reverse transcriptase activity assay and the cells were stained with methylene blue/basic fuchsin (1% methylene blue, 0.2% basic fuchsin in 100% methanol) to visualise the formation of syncytia.

2.6 Molecular cloning techniques

2.6.1 Nucleic acid extraction from cells

To extract both the total cellular DNA and RNA from infected MYA-1 $CD4^+$ T cells for quantitative PCR, the Qiagen AllPrep DNA/RNA Mini kit was used. Cells were homogenised using the QIAShredder spin columns (Qiagen). RNA was treated with Qiagen RNase-Free DNase set on column as part of the AllPrep kit protocol to remove contaminating viral DNA. DNA was eluted in the buffer EB and RNA in RNase-free water. The Qiagen DNA Mini kit was used to extract total cellular DNA for confirmation of infection by PCR and in the viral entry quantitative PCR assay where RNA extraction was not needed. DNA was eluted in buffer AE. Supernatant FIV RNA was purified from 1 ml of culture supernatant using the Qiagen UltraSens Virus kit, as this kit is more suitable for extracting viral RNA from millilitre volumes of supernatant. An internal control in the form of total cellular RNA from uninfected MYA-1 $CD4^+$ T cells was added to all samples during the extraction process. This was to provide a way to measure the efficiency of extraction in order to adjust the estimated FIV RNA copy number per ml of supernatant. Extracted RNA was further processed by the Qiagen RNeasy Mini kit to remove contaminating FIV DNA. Contamination of the extracted RNA were not detected by qPCR in most sample at all the time points, the exceptions being samples from IL-2 supplemented, productively infected cells at day 7 and day 10 post infection. The quantities of the contaminated DNA were less in 0.01% of the quantity of FIV cDNA detected. To extract total RNA from uninfected cells, samples were homogenised using the QIAShredder spin columns (Qiagen) and RNA extracted using the RNeasy spin column kit (Qiagen). RNA was eluted in RNase-free water. Nucleic acids were quantified by a Nanodrop 2000

spectrophotometer (Thermo Fisher Scientific). Eluted DNA was stored at -20°C and eluted RNA was stored at -80°C.

Phenol chloroform extraction was also performed to extract DNA from high and low molecular weight DNA as part of the Hirt protocol. Resuspended DNA was added to equal volumes of phenol: chloroform: isopentyl alcohol mixture in a 1.5 ml Eppendorff tube. The sample was shaken vigorously for 20 seconds and centrifuged at 13,000 rpm for 5 minutes at room temperature to separate the aqueous and organic phases. The DNA-containing aqueous phase was removed by pipette and added to equal volume of chloroform: isopentyl alcohol. This was shaken vigorously for 20 seconds and centrifuged at 13,000 rpm for 5 minutes at room temperature. The DNA from the aqueous phase of the chloroform extraction was then purified using ethanol precipitation. DNA was made up to 100 µl in dH_2O and 200 µl of 100% ethanol was added, followed by 1 µl of 3M sodium acetate. The mixture was incubated at -80 °C for at least 30 minutes and then centrifuged at room temperature in a table top microcentrifuge for 15 minutes at full speed. The supernatant was removed carefully and 200 µl of 70% ethanol was added. Samples were centrifuged at top speed for a further 10 minutes. The supernatant was then removed and the DNA pellets were air-dried.

2.6.2 Polymerase chain reaction (PCR)

The presence of FIV viral DNA in infected MYA-1 $CD4^+$ T cells was confirmed by using polymerase chain reaction (PCR) and the primers (manufactured by Eurofin MWG, Germany) LTR Forward 3 (5'-GCTTAACCGCAAAACCACAT-3') and Gag Reverse 3 (5'-CAAATCTCCTGGCTTGAAGG-3') amplifying a 466bp region between the 5' LTR and *gag* of the FIV provirus. To detect FIV LTR circles, the primers Env sense 1 (5'-GGCAATGTGGCATGTCTGAAAAAGAGGAGGAATGATG-3') and Gag Reverse 3 were used. They produce two products which are approximately 1kb (1-LTR) and 1.3 kb (2-LTR) in size. The aforementioned primers are specific for FIV GL8 and PPR strains. Primers that bind to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene were used as controls for equal DNA loading (GAPDH Forward: 5'-CCTTCATTGACCTCAACTACAT-3'; GAPDH Reverse: 5'-CCAAAGTTGTCATGGATGACC-3'). All reactions used GoTaq® Flexi DNA Polymerase kit (Promega, Madison, WI, USA) as per manufacturer's protocol but with the following cycling parameters: An initial denaturation step of 3 minutes

at 95°C was followed by 35 cycles of denaturation at 95°C for 45 seconds; annealing at 57°C for 45 seconds and extension at 72°C for 1 minute. The amplification was completed with a final extension step at 72°C for 10 minutes. In certain experiments the cycle number was lowered to 27 to make the PCR more sensitive to small differences in template quantity.

Roche (Mannheim, Germany) High Fidelity PCR Master reaction mix was used to amplify specific regions of FIV genome for the construction of chimeric viruses. Between 50 - 100 ng per DNA template was used. Reactions were set up according to the manufacturer's protocol and performed as followed: An initial denaturation step of 5 minutes at 94°C was followed by 35 cycles of denaturation at 94°C for 30 seconds; annealing at 55°C for 30 seconds and extension at 72°C for between 1 to 5 minutes. The amplification was completed with a final extension step at 72°C for 10 minutes. All PCR products were visualised by 2% agarose gel electrophoresis followed by ethidium bromide staining.

2.6.3 DNA sequencing

DNA sequencing was carried out using the BigDye v1.1 system (Applied Biosystems) as per manufacturer's protocol. Between 150 – 300 ng of DNA template was used per reaction. Reactions were purified by ethanol precipitation (see the section Nucleic acid extraction from cells) and resuspended in 20 µl of HiDi Formamide (Applied Biosystems) and sequenced using an ABI 7000 genetic analyser (Applied Biosystems). Output sequence traces were analysed in DNA Dynamo (Blue Tractor Software).

2.6.4 Mutagenesis

In-vitro mutagenesis of FIV molecular clones was carried out using the QuikChange® II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Stockport, UK) according to the manufacturer's protocol. Mutagenic primers (Appendix 4) containing the desired point mutation and complementary to both strands of the vector were designed using the online QuikChange® Primer Design Program (http://www.genomics.agilent.com/homepage.aspx). Primers were synthesised by MWG Eurofins. Mutant strand synthesis reaction was then carried out using plasmid DNA template isolated from a dam$^+$ *E.coli* strain, which

produces methylated plasmid DNA (DH5α and XL-1 Blue strains are appropriate). Reactions were set up using 5 µl of 10x reaction buffer, 125 ng of each mutagenic primer, 1 µl of dNTP mix, 3 µl of QuikSolution, 50 ng of template plasmid dsDNA, 1 µl of PfuUltra HF DNA polymerase (2.5U/µl) and dH_2O up to 50 µl. Thermal cycling was performed with an initial denaturing step at 95°C for 1 minute, followed by 18 cycles of denaturation at 95°C for 50 seconds; annealing at 60°C for 50 seconds and extension at 68°C for 14 minutes. The amplification was completed with a final extension step at 68°C for 7 minutes. Reactions were cooled on ice and 1 µl of Dpn I restriction enzyme (10U/µl), which digests methylated and hemimethylated parental DNA template, was added directly to each reaction. Digestion was carried out at 37°C for 1 hour. The remaining mutated DNA was purified up by ethanol precipitation (see DNA sequencing) and resuspended in 5µl TE buffer. The entire volume of the mutagenesis was used in the transformation of XL-10 Gold® Ultracompetent cells (Agilent Technologies). After ampicillin selection, clones were grown up in lysogeny broth (LB) overnight and their plasmids were extracted using the Qiaprep Spin Miniprep kit (Qiagen). The nucleic acid sequence of each mutated construct was confirmed as above.

2.6.5 Substitution of FIV-LTR and env regions in the molecular clones

The molecular clone of FIV GL8 or PPR consists of the full proviral DNA sequence of each virus cloned into the vector backbones pBR328 and pUC119 respectively. The substitution of the 5' LTR and *env* was performed separately. Separations of restriction digest fragments were performed by electrophoresis on a 0.8-2% agarose gel, stained with ethidium bromide. DNA was visualised by UV-transillumination and bands of the expected sizes were excised using a scalpel. Gel extraction was carried out using the Qiaquick Gel Extraction kit (Qiagen). **FIV 5' LTR:** The first 509 nucleotides of the 5'LTR was excised using the restriction enzymes EcoRI (which was originally used to clone each provirus into its respective vector) and SacI (at nucleotide 509). However, the existence of downstream SacI (for GL8) and EcoRI (for PPR) sites meant that the initial digest had to be performed with another restriction enzyme that cut once downstream of the SacI site. NcoI, which has a unique site at nucleotide position 2500 was chosen. This fragment was digested again with SacI and the original 5'LTR was replaced with one from a different virus. A double ligation was then performed to reconnect the 5'LTR to the remainder of the NcoI restriction fragment and

subsequently to join the fragment with the molecular clone. **FIV *env*:** the *env* sequence from each molecular clone was exchanged by restriction digest with MluI and NdeI and subsequent re-ligation of the replacement *env* fragments; the GL8 mya molecular clone contains an engineered MluI site 256 nucleotides into the *env* gene at the L-SU junction. The PPR molecular clone does not contain a MluI cut site in the *env* gene. Mutagenesis of the vector was required to generate a MluI site at the same location as GL8 molecular clone. In total 3 mutations were performed at nucleotides 6515, 6517 and 6519 in 3 steps, resulting in an amino acid substitution A86V. The restriction enzyme NdeI cuts at a site downstream of the *env* gene in the RRE. For the GL8-LTR-env-(PPR) double chimeric molecular clone, the GL8 *env* was exchanged before the LTR. See Figure 5-7 for a schematic of all the chimeric viruses.

After an overnight ligation reaction with T4 ligase (Invitrogen), the molecular clones were transformed into competent cells and plated onto LB agar with 100 µg/ml ampicillin. Various strains of competent cells were investigated, including DH5α (Invitrogen), XL-1 Blue and XL-10 Gold (Agilent Technology); the best results were obtained when transformations were performed with XL-10 Gold. X-Gal IPTG blue/white colony screening was used when appropriate. Colonies were picked and grown in 3 – 5 ml of LB broth at 30°C in an orbital shaker set at 200 rpm overnight (a temperature lower than 37°C was used to ensure plasmid stability). Extraction of plasmid DNA was carried out using the Qiaprep Spin Miniprep kit. Large scale DNA prep was made by inoculating 300-500ml LB broth with bacterial clones and grown overnight. Plasmid DNA was extracted using PureLink HiPure Filter Plasmid Maxiprep Kit (Invitrogen). Pelleted DNA was resuspended in TE buffer, the DNA concentration was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Cultures of clones were diluted with glycerol to make 50% glycerol stock for long term storage.

2.6.6 Modified Hirt Protocol

The Hirt protocol (Hirt, 1967) was originally used to separate out low molecular weight (LMW) DNA from contaminating genomic DNA, which is much larger in size. This protocol was modified in order to isolate only the genomic DNA from infected cells and not unintegrated viral DNA. The Hirt lysis buffer for the Hirt

method consisted of 0.6% sodium dodecyl sulphate (SDS) and 10 mM EDTA pH 7.5. See chapter 4 for full description.

2.7 Quantitative methods

2.7.1 Human IL-8 ELISA

The OptEIA™ Human IL-8 kit (BD Biosciences – cat. No. 555244) was used to detect IL-8 produced by the HT1080 cells as per manufacturer's protocol. Briefly, stock capture antibody was diluted 1 in 250 with coating buffer (8.40g $NaHCO_3$, 3.56g Na_2CO_3 to 1.0 L; pH to 9.5) and 100 µl of the diluted antibody was used to coat the wells of high binding ELISA strips (Immulon® 2 HB 2x8 strip assemblies from Thermo Fisher Scientific). The strip assemblies were incubated overnight at 4°C. The strips were then washed with phosphate-buffered saline – Tween® (PBS-T) and blocked with assay diluent (PBS with 10% FBS) for 1 hour at room temperature. The strips were then washed with PBS-T and 100 µl of each sample was then pipetted into the appropriate wells and incubated at room temperature for 2 hours. Bound IL-8 was detected using the working detector prep (detection antibody and streptavidin-horseradish peroxidase (HRP) conjugate diluted 1 in 250 in assay diluent) and strips were developed using tetramethylbenzidine (TMB) substrate (Sigma-Aldrich). HRP-TMB reactions were stopped using 1M hydrochloric acid the absorbance of each well was read at 450nm using the Multiskan Ascent plate reader (Labsystems).

2.7.2 Feline IFN-γ and IL-4 ELISA

The concentrations of feline IFN-γ or IL-4 in the supernatant of MYA-1 CD4+ T cells were measured using the DuoSet® ELISA Development System (R&D Systems) as per manufacturer's protocol with the following minor modifications: Capture antibodies against IFN-γ or IL-4 were diluted in serum-free, antibiotic-free DMEM to working concentrations of 1.5 µg/ml and 4.0 µg/ml respectively and were used to coat the wells of high binding ELISA strips (Immulon® 2 HB 2x8 strip assemblies from Thermo Fisher Scientific). After washing with PBS-T, the coated wells were blocked with 200 µl of 0.2% skimmed milk powder dissolved in PBS-T overnight at 4°C. 100 µl of samples were then added to appropriate wells and incubated at room temperature for 2 hours. Bound cytokines were detected using biotinlyated detection antibody against the appropriate cytokine (anti-IFN-

γ antibody - 200 ng/ml; anti-IL-4 antibody - 300 ng/ml) and streptavidin-HRP and visualized by the addition of TMB substrate. HRP-TMB reactions were stopped using 1M hydrochloric acid and the absorbance of each well was read at 450nm using the Multiskan Ascent plate reader.

2.7.3 FIV p24 ELISA

The presence of FIV capsid antigen (CA, p24) in tissue culture medium or whole cell lysate was measured by ELISA. Anti FIV p24 specific monoclonal antibody VPG62 stock was diluted to a concentration of 2 ng/ml with serum-free, antibiotic-free DMEM. 100 µl of the diluted antibody was pipetted into each well of high binding ELISA strips (Immulon® 2 HB 2x8 strip assemblies from Thermo Fisher Scientific). The strip assemblies were incubated at room temperature for 1 hour and then washed three times with PBS-T and blocked overnight with 200 µl of 0.2% skimmed milk powder dissolved in PBS-T at 4°C. The strips were washed once with PBS-T and 10 µl of sample treatment solution (100mM Tris, 1.5M NaCl, 5% Triton, 5% deoxycholale, 1% SDS, 50mM EDTA and 0.1% azide for 10X solution) were added, followed by 100 µl of each sample. After an incubation period of 1 hour at room temperature the wells were washed five times with PBS-T and 2 drops of the anti-FIV HRP conjugate (IDEXX PetCheck anti FIV antigen, IDEXX Laboratories, Maine, USA) were added. The strips were incubated for 1 hour at room temperature and then washed five times with PBS-T. 100 µl of TMB solution were added to each well and 50 µl of 1M hydrochloric acid was used to stop the HRP-TMB reaction and the absorbance of each well was read at 450nm using a Multiskan Ascent plate reader.

2.7.4 Reverse transcriptase activity assay

Reverse transcriptase (RT) activity of the supernatant samples was measured using Lenti RT Activity kit (Cavidi AB, Sweden). This technique quantifies RT activity by its ability to reverse transcribe a polyriboadenosine template attached to the wells of a 96-well plate. Between 150 and 200 µl of reaction mixture containing a primer and the nucleotide analogue 5-bromo-deoxyuridine-5'-triphosphate (BrdUTP) was added to each well of the kit poly-A plate. Depending on the level of sensitivity required, 10 to 40 µl of sample was then added to appropriate well up to a final volume of 210 µl. The plate was sealed with adhesive cover tape and incubated overnight at 33°C. The wells were then

washed 5 times with RT wash buffer to stop reverse transcription. 100 µl of RT Product Tracer containing an alkaline phosphatase (AP) conjugated anti-BrdU antibody was added to quantify the amount of BrdU, which is directly proportional to the reverse transcriptase activity and visualized by the reaction of the AP with a colourimetric substrate. Absorbance at 405nm of each well was read by Multiskan Ascent plate reader.

2.7.5 Luciferase viral entry assay

FIV strain GL8 *env* gene was expressed from the vector VR1012 (Shimojima et al., 2004, Willett et al., 2006b). 5 µg of the FIV *env* expression constructs plus 7.5 µg of pNL4-3-Luc-E^-R^- were co-transfected into HEK 293T cells (See above). Culture supernatant from transfected cells containing the HIV (FIV) pseudotypes were collected 48 hours post transfection, filtered at 0.45 µm and stored at -80°C till required.

$5x10^4$ MYA-1 $CD4^+$ T cells were then seeded into each well of a CulturePlate™ 96 assay plate (Perkin Elmer) and cultured with 50 µl of the thawed HIV (FIV) pseudotype-containing supernatant for 72 hours. Luciferase activity was quantified by the addition of 100 µl of Steadylite HTS™ (Perkin Elmer) luciferase substrate prior to measurement by single photon counting on a MicroBetaTriLux luminometer (Perkin Elmer).

2.7.6 Quantitative PCR

Quantitative PCR was used to accurately determine the amount of FIV DNA and cDNA. Purified RNA was reverse transcribed into cDNA using the Roche Transcriptor High Fidelity cDNA synthesis kit. Manufacturer's protocol was followed and RNA was reverse transcribed using the random hexamer primer. The FIV GL8-specific primers FIV1360F (5'-GCA GAA GCA AGA TTT GCA CCA-3') and FIV1437R (5'-TAT GGC GGC CAA TTT TCC T3') plus the Taqman probe FIV1416P (5'-FAM-TGC CTC AAG ATA CCA TGC TCT ACA CTG CA-TAMRA-3') were used to amplify a 78bp section of the FIV GL8 *gag* gene. Primers and probes which amplify feline 18S rRNA (343-Fwd: 5'-CCA TTC GAA CGT CTG CCC TA-3'; 409-Rev: 5'-TCA CCC GTG GTC ACC ATG-3', and probe: 5'-FAM-CGA TGG TAG TCG CCG TGC CTA-TAMRA-3') were used as internal control. The final concentrations of primers and probes were 900 nM and 225 nM respectively. The primers,

probes and templates were combined with TaqMan Universal Master Mix (Applied Biosystems) to a final volume of 20 µl per reaction in MicroAmp Optical 96-well reaction plates (Applied Biosystems). Thermo cycling was performed with an initial denaturing step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C 15 seconds; annealing and detection at 55°C for 60 seconds and measurements were taken using an ABI 7500 thermal cycler (Applied Biosystems). Results were analysed using the Sequence Detection Software v1.4 (Applied Biosystems). Relative quantities of nucleic acid were calculated using the ΔΔCt method: ΔCt values (Ct = threshold cycles) were calculated by subtracting the FIV gag Ct value of each replicate from its 18S Ct value. Each ΔCt value was subtracted from the average ΔCt of the mock-treated cells at Day 0 post infection to obtain ΔΔ Ct. which was then converted to relative quantity using the formula: $2^{(-\Delta\Delta\ Ct)}$. The mean and standard deviation of the triplicates of each condition at each time point was plotted on graph. This assay can detect a minimum of 10 copies of spiked FIV plasmid per reaction, equivalent of a theoretical minimum of 350 copies per ml of supernatant.

2.8 Protein visualization

2.8.1 SDS-PAGE and Western blotting

To estimate the amount of FIV p24 in the virus stocks, they were processed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualised by western blotting along with samples of recombinant FIV p24 of known concentrations. Protein samples were boiled at 100°C for 5 minutes in SDS-PAGE protein loading buffer. PAGE was performed in NuPAGE® 4-12% Bis-Tris gels (Invitrogen) and the transfer of protein to nitrocellulose membrane was carried out using the iblot rapid dry blotting system (Invitrogen). The nitrocellulose membrane was blocked overnight in blocking solution consisted of 5% Marvel skimmed milk powder dissolved in PBS-T. The anti-FIV p24 primary antibody VPG50 was incubated with the membrane at a dilution of 1 in 1000 for 30 minutes, followed by 5 washes with PBS-T and incubation with the horse-anti-mouse IgG biotinylated secondary antibody (Vector Laboratories) for 30 minutes. Protein bands were visualised using the Vectorstain® ABC (avidin-biotinylated enzyme complex) system and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrates.

2.9 Statistics

Data was analysed for statistical significance using the One Way Analysis of variance (ANOVA) test with the Bonferroni and the Tukey post-hoc means comparison tests from OriginPro 8.6 (OriginLab Corp. USA) where indicated.

3 Investigation of virus binding and CD134 signalling

3.1 Summary

The primary receptor for FIV is CD134 (OX40) (Shimojima et al., 2004). CD134 is an important co-stimulatory molecule involved in T cell activation and the establishment of memory T cells (Croft, 2003, Sabbagh et al., 2007, Croft et al., 2009). The binding of CD134 to its ligand activates the NFκB and PI3K/Akt signalling pathways, which promote T cell function, expansion and survival (Song et al., 2004, Croft, 2010). FIV preferentially infects activated $CD4^+$ T cells; however, most of the T cells in the body are at a resting state (Dean et al., 1996a, Berard and Tough, 2002, Joshi et al., 2005b). Thus, hypothetically, it would be advantageous for the virus to trigger CD134 signalling by the binding of the virus or Env protein to its primary receptor, making the cell more conducive to productive infection or extending the lifespan of infected cells. Indeed it has been shown that HIV-1 Gp120 Env protein stimulates T cell signalling (Kornfeld et al., 1988, Wu and Yoder, 2009). To investigate whether FIV exploits its host cell this way, an attempt was made to detect any changes to CD134 signalling during virus binding using the following strategies:

1. To transiently or stably express feline CD134 in non-T cell lines and measure activation of CD134 by quantifying release of cytokine (IL-8) or the expression of a reporter gene under the control of NFκB.

2. To detect changes in signalling in feline $CD4^+$ T cells (MYA-1) caused by the addition of CD134L or FIV.

3.2 Results

3.2.1 No enhancement of NFκB signalling by FIV binding to feline CD134 expressed on interleukin-8 (IL-8) producing cells

IL-8 is produced by many different cell types and its expression is regulated by NFκB (Hoffmann et al., 2002). The production of IL-8 has been used as an indicator for cell signalling activity, including the activity of the human CD134 signalling pathway (Muller et al., 2008). Thus I attempted to detect changes in

cell signalling induced by FIV binding to CD134 by quantifying IL-8 release from cells. HT1080, NP-2, and HeLa cells were stably transduced with a feline CD134 construct, a human CD134 construct, a feline-human chimera construct and vector only control. After the expression of CD134 was confirmed on these cells by flow cytometry (Table 3-1), experiments were performed to measure IL-8 production from these cells in response to stimulation of CD134.

Cell line	Construct	Primary Antibody	% cells gated (PE)	Mean fluorescence
HT1080	FFF	anti-CD134 (7D6)	96.45	68.5
		no antibody	0.5	13.1
	HHH	anti-CD134 (7D6)	59.8	21.2
		no antibody	0.4	4.4
	FFHH	anti-CD134 (7D6)	79.14	43.6
		no antibody	0.04	5.6
	Vector only	anti-CD134 (7D6)	1	10.8
		no antibody	0.9	5.9
NP-2	FFF	anti-CD134 (7D6)	93.8	11.4
		no antibody	0.6	0.3
	Vector only	anti-CD134 (7D6)	0.6	0.3
		no antibody	0.7	0.3
HeLa	FFF	anti-CD134 (7D6)	98.64	78.7
		no antibody	3.94	1.9
	HHH	anti-CD134 (7D6)	90.7	40.8
		no antibody	4.5	1.8
	Vector only	anti-CD134 (7D6)	5.16	1.6
		no antibody	6.46	1.5

Table 3-1 – High levels of different CD134 are expressed by transduced HT1080, NP-2 and HeLa cell lines. Cells were transduced with expression constructs in the pDON-AI retroviral vector expressing feline (FFF), human (HHH) or feline-human chimeric (FFHH) CD134. PE = phycoerythrin.

IL-8 production by HT1080 cells has been used to measure human CD134 signalling (Muller et al., 2008), thus it was the first cell line to be tested as to whether it can be used to measure signalling from feline CD134. HT1080 cells stably transduced with feline, human, feline-human chimera CD134 or vector control were seeded onto 96-well plates and stimulated with either recombinant soluble human, feline or murine trimeric CD134 ligands (Willett et al., 2007, Willett et al., 2009). A concentration of 0.5 µg/ml for each ligand was tested initially based on the activity of the ligands in previous studies (Willett et al., 2007, Willett et al., 2009). HT1080 cells expressing feline CD134 produced IL-8 in response to the addition of feline and murine ligands (Fig. 3-1A), the latter

has been shown to bind promiscuously to both human and feline CD134 (Willett et al., 2007) and in this experiment murine ligand induced higher IL-8 production. As expected, human CD134 ligand did not trigger IL-8 production from HT1080 cells expressing feCD134, and feline CD134 ligand did not trigger IL-8 production from HT1080 cells expressing huCD34 (Fig. 3-1A and B). Murine and human ligands triggered IL-8 production in cells expressing the feline human chimera, consistent with the fact that the chimeric receptor contains human CD134 residues at amino acid positions 78 to 80, which are crucial for CD134L binding (Fig. 3-1C) (Compaan and Hymowitz, 2006, Willett et al., 2007).

Next the dynamics of CD134 signalling were investigated. To deduce the minimum period of time for stimulated cells to reach maximum production of IL-8, feline CD134L was added to the feline CD134-expressing HT1080 cells and supernatant was collected after 5, 10, 20, 40, 60, 90, 120, 180, 240, 360 minutes. Results showed that after stimulation, IL-8 production remained low during the first 100 minutes, then increased rapidly between 100 to 200 minutes post-stimulation before reaching a plateau after 240 minutes (4 hours). However it is unknown whether this plateau reflected a physiological negative feedback mechanism or rather represented the saturation of the ELISA (Fig. 3-2A). To discover the minimum length of ligand stimulation that would induce maximum IL-8 production, the cells were stimulated by CD134L for 0, 10, 20, 30, 40, 60 and 120 minutes before being washed and supernatant replaced with fresh medium. IL-8 levels in the replaced medium were measured after a further incubation of six hours. Results demonstrated that the final supernatant IL-8 concentration depended upon the length of the contact between the cells and the ligand. Efficiency of stimulation initially rose quickly, with stimulation for just 10 minutes achieving a final IL-8 concentration that was 70% of the maximum. With longer stimulation the rate of increase in final IL-8 production slowed down and eventually peaked at 60 minutes (Fig. 3-2A). Based on these results it was decided that the ligand should be applied to the cells for at least 60 minutes and supernatant from cells collected after an incubation of at least 6 hours.

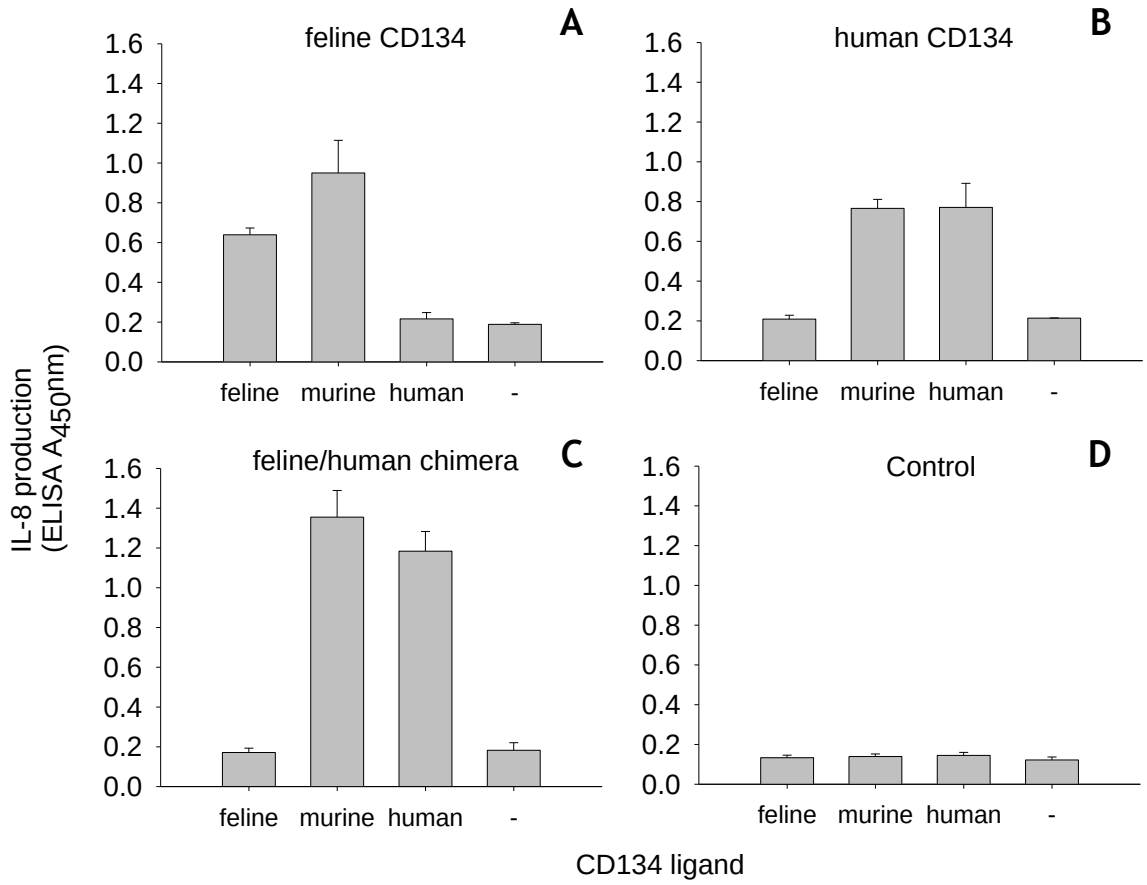


Figure 3-1 – HT1080 cells transduced with CD134 exhibit species specificity towards ligand. HT1080 cells stably expressing (A) feline, (B) human, (C) chimeric feline/human CD134 (FFHH) or (D) empty vector were stimulated with feline, murine or human CD134 ligands (0.5 µg/ml). Supernatant IL-8 concentration was measured by ELISA. Each bar represents the mean +/- standard error (n= 3).

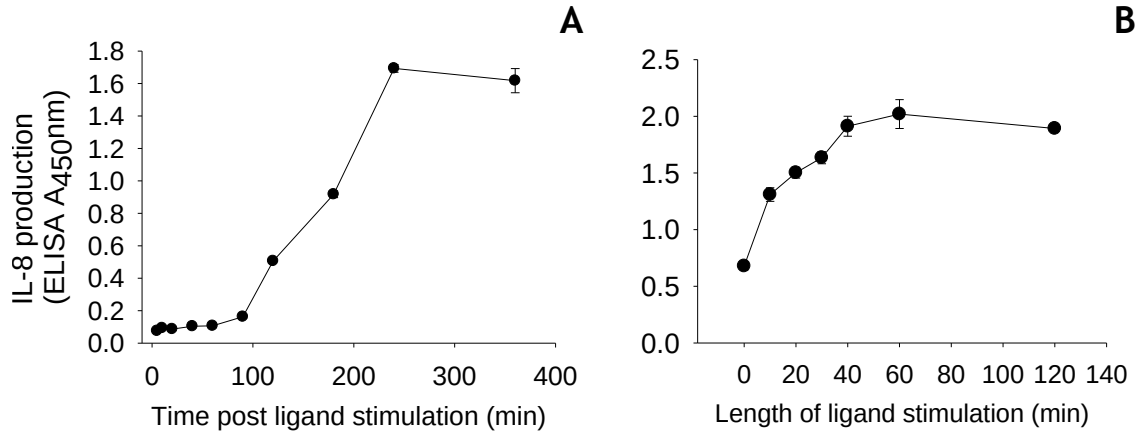


Figure 3-2 – The dynamics of CD134 signalling in HT1080 cells. (A) Cells were stimulated with feline CD134L and supernatant collected at specified times post-stimulation to be measured by IL-8 ELISA. (B) Cells were stimulated for specified times by feline CD134L and then replaced with fresh medium without

ligand. Replacement media was collected after 6 hours and processed by IL-8 ELISA. Each point represents the mean +/- standard error (n= 3).

The 'early' strain of FIV GL8 and the 'late' strain PPR were grown in MYA-1 $CD4^+$ T cells and virions were purified by double sucrose gradient to minimise contamination with cellular debris. Different concentrations of the purified virus were then added to feline CD134 HT1080 cells to investigate whether CD134 signalling is triggered by the presence of the virus. Application of FIV particles, even at high doses, did not induce an increase in IL-8 production (Fig.3-3).

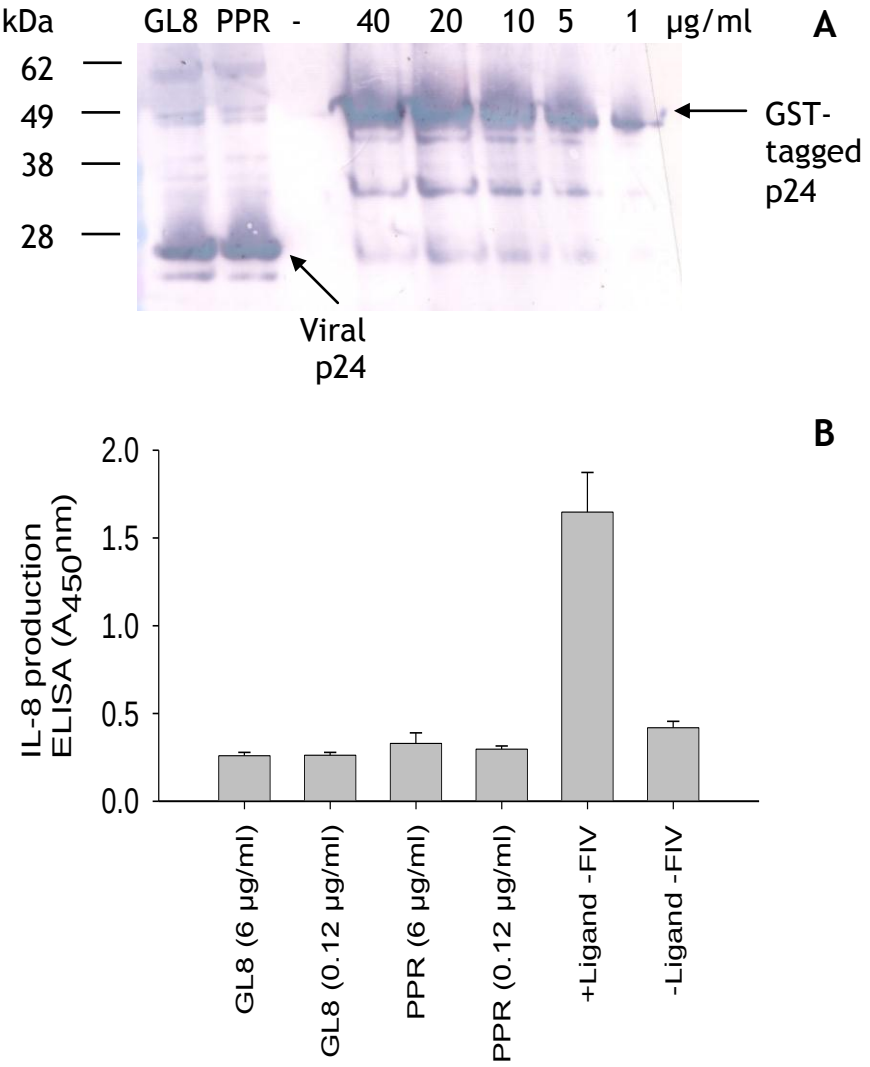


Figure 3-3 – Purified FIV does not stimulate IL-8 production in HT1080 feCD134 cells. (A) The concentrations of purified viruses were estimated by comparing with recombinant GST-tagged FIV p24 protein of known concentrations on an immunoblot. (B) FeCD134L (0.5 µg/ml) or purified FIV GL8 or PPR were incubated with the feline CD134-expressing HT1080 cells for 6 hours and IL-8

production were measured by ELISA. Each bar represents the mean +/- standard error (n= 4).

If FIV and feline CD134L bind to the same site on CD134, the virus may interfere with CD134L binding and reduce downstream signalling. Alternatively, if FIV and feline CD134L bind to distinct sites on CD134, Env-binding may augment signalling. To investigate, HT1080 cells expressing feline CD134 were pre-incubated with purified FIV prior to the addition of feline CD134L and IL-8 production measured. In 6 out of 8 repeat experiments, neither strain of virus affected ligand-induced IL-8 production (Fig. 3-4A). The same result was observed when the order of application was reversed and the cells were pre-incubated with ligand before the addition of FIV (Fig. 3-4B). However, in 2 of the repeats FIV reduced IL-8 production in a dose-dependent manner when added before ligand, with FIV PPR causing a more intense reduction of IL-8 production than FIV GL8 (Fig. 3-4C). When feline CD134L was added first, FIV GL8 increased IL-8 production by the same amount regardless of virus concentration (Fig. 3-4D). In contrast, the titre of FIV PPR was negatively correlated with enhancement of IL-8 production, with the lowest virus titre producing the largest enhancement (Fig. 3-4D).

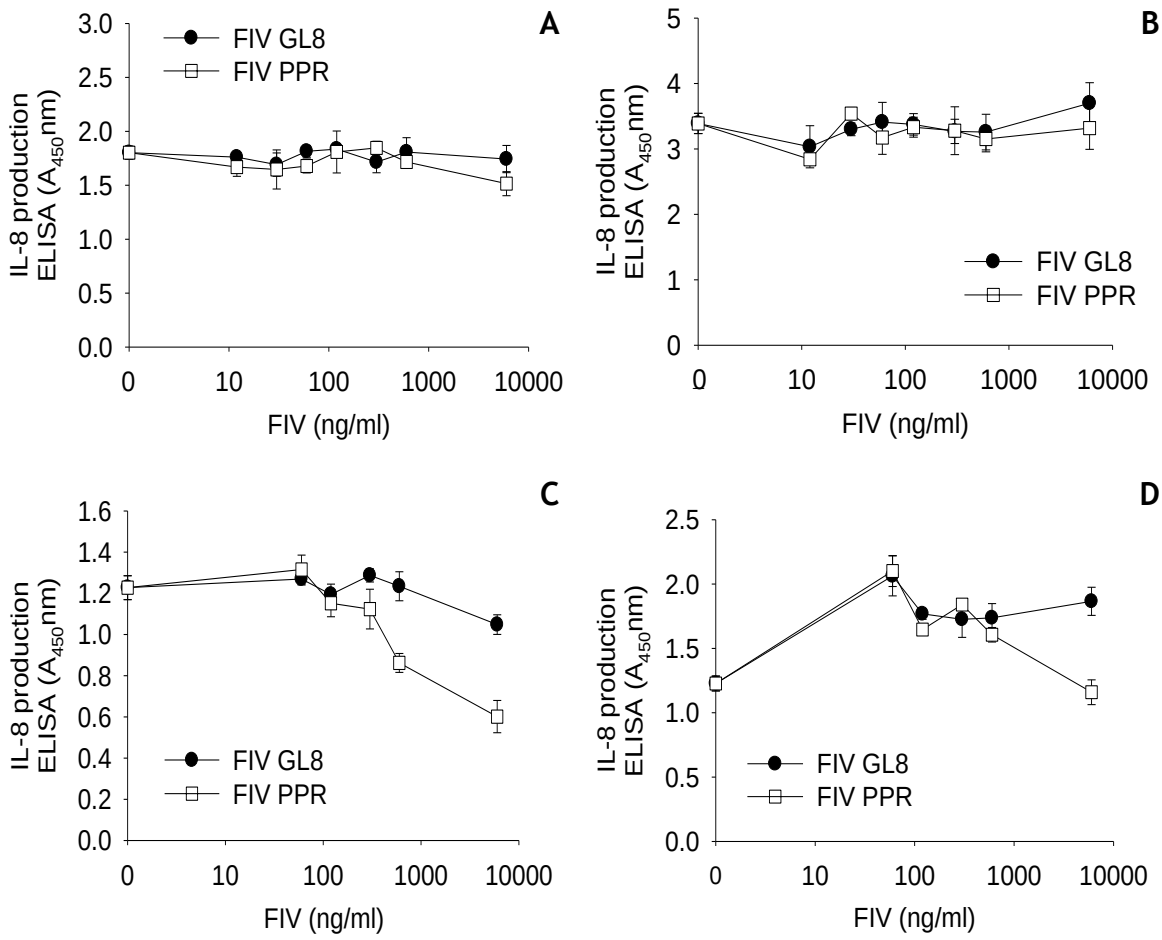


Figure 3-4 – Effect of purified FIV on the production of IL-8 by transduced HT1080 cells in response to feline CD134 ligand stimulation. Various concentrations of FIV particles were added to feline CD134-expressing HT1080 cells 1 hour before (A and C) or after (B and D) the application of feline CD134 ligand (at 0.5 µg/ml). The cells were further incubated with the virus and ligand for another 6 hours before supernatant was harvested. (A and B) are representative of 6 independent experiments while (C and D) are representative of 2 independent experiments. Each point represents the mean +/- standard error (n= 3).

Given the inconsistent observations with the feline CD134-expressing HT1080 cells, the effects of FIV on IL-8 production for two additional cell lines were investigated. The human glioma cell line NP-2 (Yamazaki, 1982) and epithelial adenocarcinoma cell line HeLa were stably transduced with CD134 as previous (Table 3-1) and their responses to CD134 ligand examined. Background IL-8 production from the transduced NP-2 cells was 7 times higher than transduced HT1080 cells (Fig 3-5A). Application of feline CD134 ligand to NP-2-feCD134 cells

induced a large increase in IL-8 production from the cells. The presence of FIV virions enhanced IL-8 production from NP-2 cells in one independent experiment (Fig. 3-5B). However, this trend was not replicated in two further independent experiments (Fig. 3-5C).

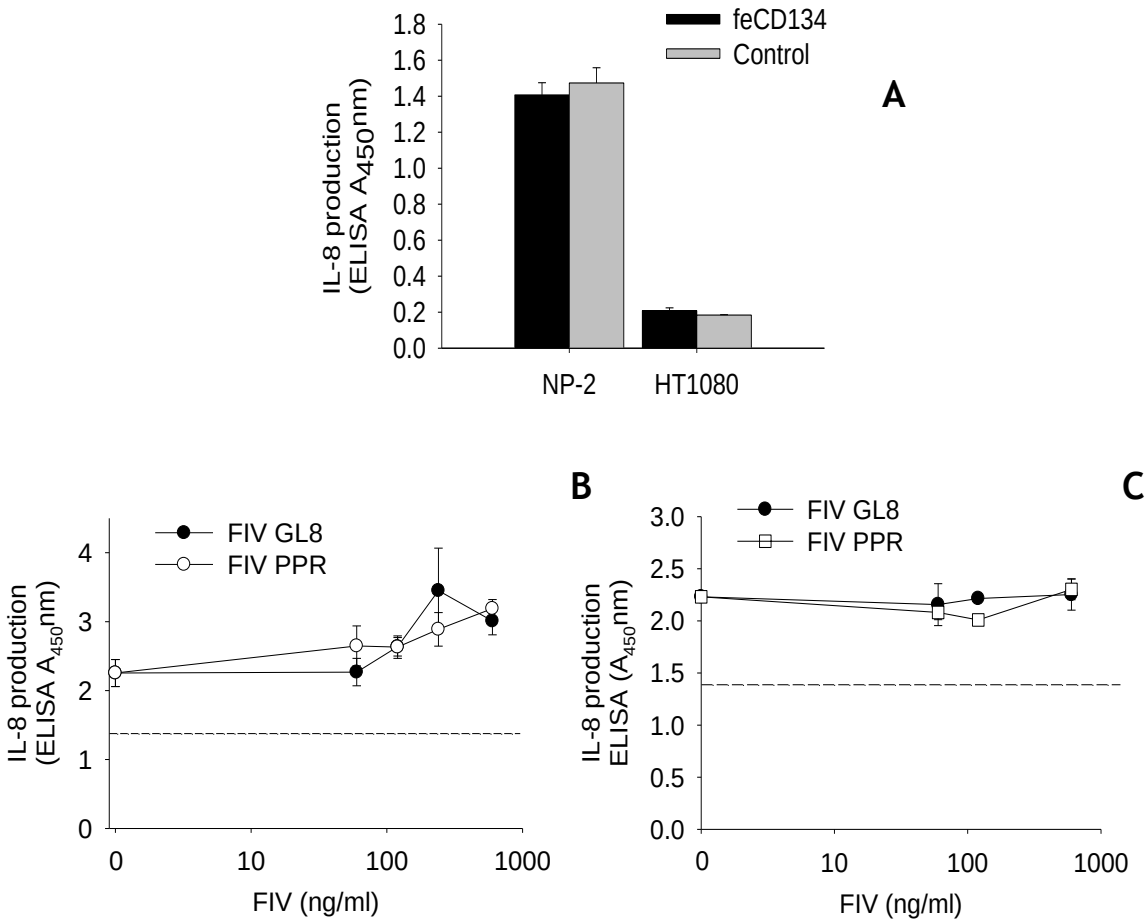


Figure 3-5 – Effect of purified FIV on IL-8 production from feline CD134-expressing NP-2 cells (A) NP-2 and HT1080 cells transduced with feline CD134 or the vector pDON-AI alone (control) were left unstimulated for 6 hours before supernatant was collected for measurement. Each bar represents the mean +/- standard error (n= 3). (B) NP-2 CD134 cells were pre-incubated with feCD134L (0.5 µg/ml) for 1 hour before the addition of purified FIV GL8 or PPR and the cells were further incubated for 6 hours post virus addition before supernatant was harvested. Dotted lines represent the background levels of IL-8 production. Each point represents the mean +/- standard error (n=3)

The response of HeLa cells to stimulation with CD134 ligand was different to the responses of HT1080 or NP-2 cells. HeLa cells transduced with feline CD134 produced very little IL-8 when stimulated with feline CD134 ligand compared with the stimulation of human CD134-transduced cells with human CD134 ligand

(Fig. 3-6). The lack of a strong response to stimulation with feCD134L by HeLa cells suggested that they were not a suitable cell line for the purpose of investigating the signalling of feline CD134.

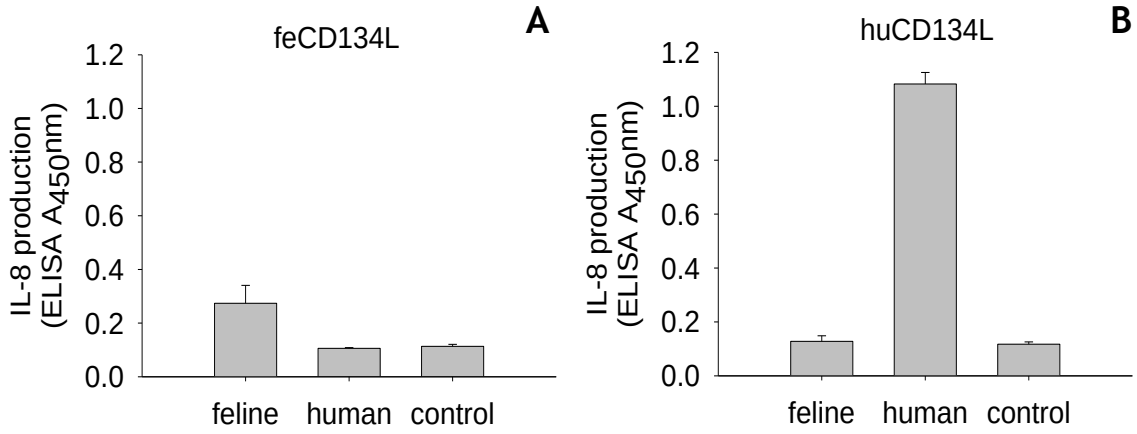


Figure 3-6 – HeLa cells transduced with feline CD134 do not respond to trigger by feline ligand. HeLa cells transduced with feline or human CD134 were stimulated with feline CD134 ligand (A) or human CD134 ligand (B). The ligands were added at a concentration of 0.5 µg/ml. Control = cells transduced with pDON-AI vector. Each bar represents the mean +/- standard error (n= 3).

The major downstream effector of CD134 signalling is NFκB (Croft, 2010). This transcription factor is also an important driver of IL-8 production (Hoffmann et al., 2002) and human CD134 activation has been shown to induce IL-8 production (Muller et al., 2008). To investigate the role of NFκB in IL-8 production by HT1080 CD134 cells, NFκB inhibitors SC514 and CAPE were added to feCD134 ligand-stimulated cells. Unexpectedly CAPE increased ligand-induced IL-8 production by 25% as well as background IL-8 production by 55% (Fig 3-7A). In contrast, addition of SC514 reduced ligand mediated IL-8 production by 50% (Fig. 3-7B), consistent with the involvement of NFκB-dependent signalling in CD134-driven IL-8 production. To demonstrate the NFκB inhibiting properties of CAPE and SC514, they were added to NFkB/293/GFP reporter cells. Results showed that the two NFκB inhibitors blocked TNF-α induced, NFκB-driven GFP production in (Fig. 3-9). The surprising effect of CAPE on HT1080 cells may be explained by the fact that in addition to affecting NFκB, CAPE also affects other factors such as the mitogen activated protein kinase p38 (Natarajan et al., 1996, Wei et al., 2008) and has both pro and anti-apoptotic effects on a range of cells

(Fitzpatrick et al., 2001, Chen et al., 2008, Wei et al., 2008). Thus it may not be appropriate to characterize it as a specific inhibitor of NFκB.

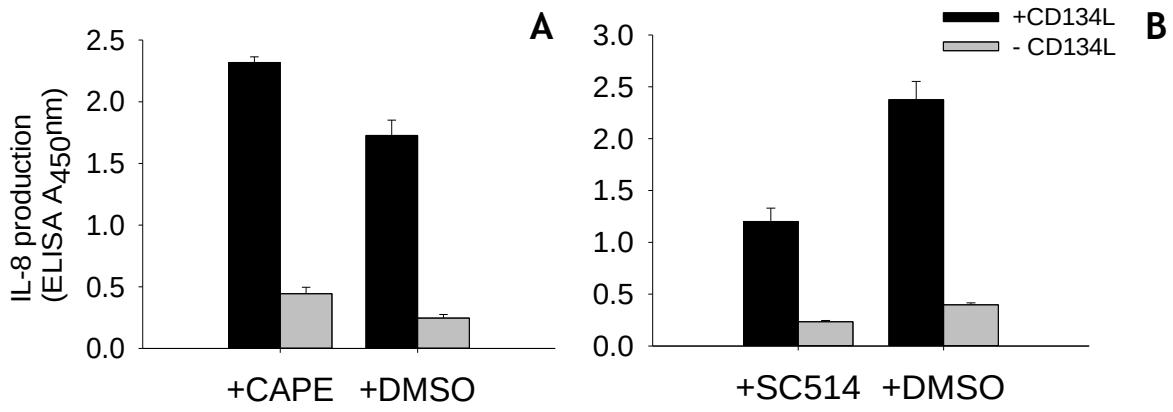


Figure 3-7 – Effect of NFκB inhibitors on IL-8 production from HT1080-feCD134 cells. Cells were pre-incubated with complete DMEM containing (A) CAPE (88 μM) or (B) SC514 (10 μM) and then stimulated by feCD134L (0.5 μg/ml). Cells were also mock-treated with DMSO for comparison. Results are representative of 2 independent experiments. Each bar represents the mean +/- standard error (n= 3).

The preceding experiments on IL-8-producing adherent cells (HT1080, NP-2, HeLa) transduced with feline CD134 did not suggest that FIV binding to CD134 triggered signalling through NFkB. However, it was possible that the effect of the virus was subtle and could be masked by the signal mediated by the CD134 ligand. Given that CAPE increased ligand-induced IL-8 production it could potentially amplify any effect mediated by virus binding. To test this hypothesis, HT1080-feCD134 cells were pre-incubated with CAPE for 1 hour before the addition of feline CD134L, followed by FIV a further hour later. Adding ligand before virus was considered more likely to demonstrate an enhancement of IL-8 production. The addition of CAPE doubled IL-8 production induced by ligand, consistent with earlier findings. However, the presence of FIV did not increase IL-8 levels further (Fig. 3-8A). In the absence of the ligand FIV did not induce extra IL-8 production from CAPE incubated cells except at the highest virus concentration (Fig 3-8B). However, this increase was very small compared with the IL-8 production induced by stimulation with feCD134L. Thus it was decided to explore other *in vitro* systems to see if they will provide a more conclusive answer.

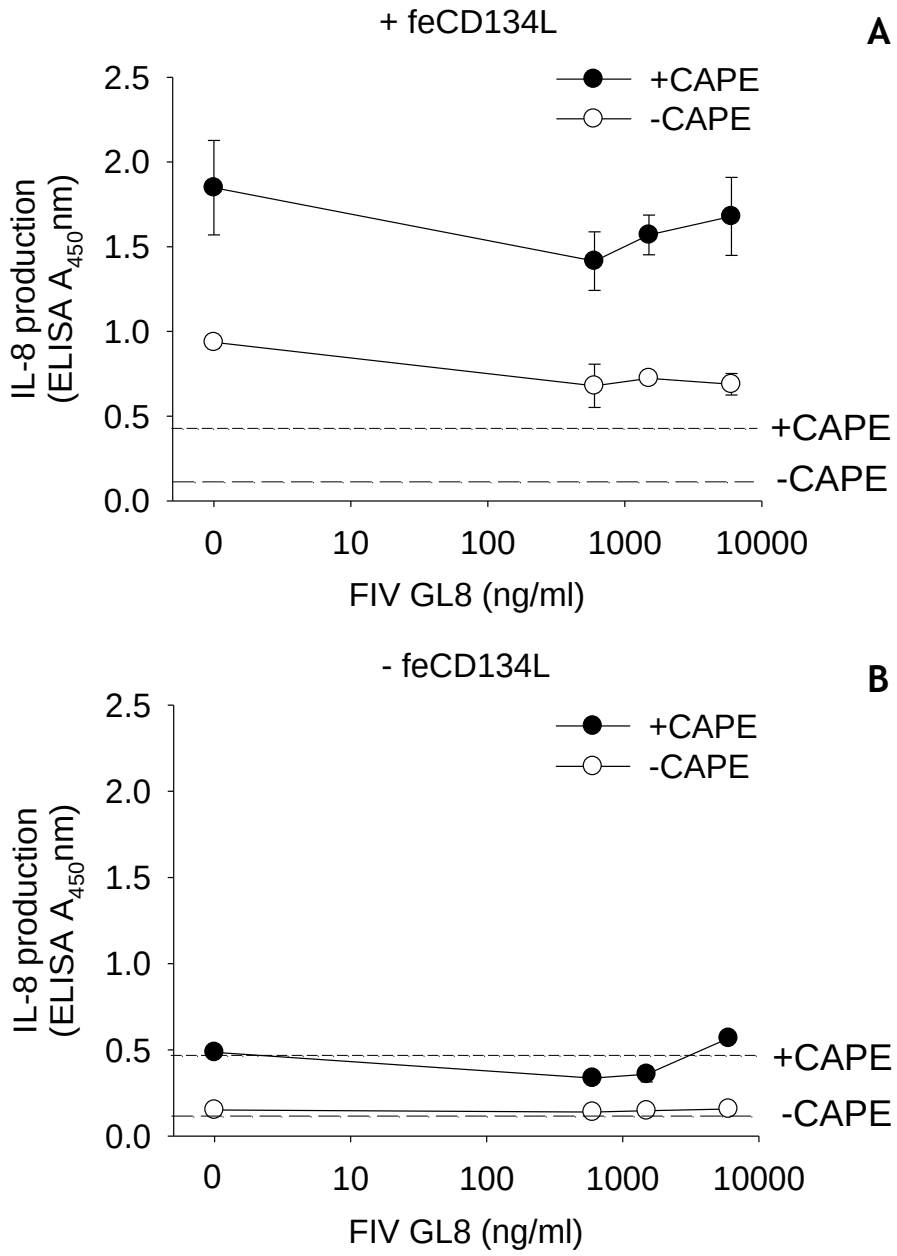


Figure 3-8 – Effect of the NFκB inhibitor CAPE on IL-8 production from HT1080-feCD134 cells treated with FIV GL8 in the presence or absence of feCD134L. (A) Cells were pre-incubated with CAPE and stimulated with feCD134L before FIV was added. Dotted lines indicate the levels of background IL-8 production in the presence or absence of CAPE. (B) Cells were pre-incubated with CAPE and then virus was added. Each point represents the mean +/- standard error (n= 3).

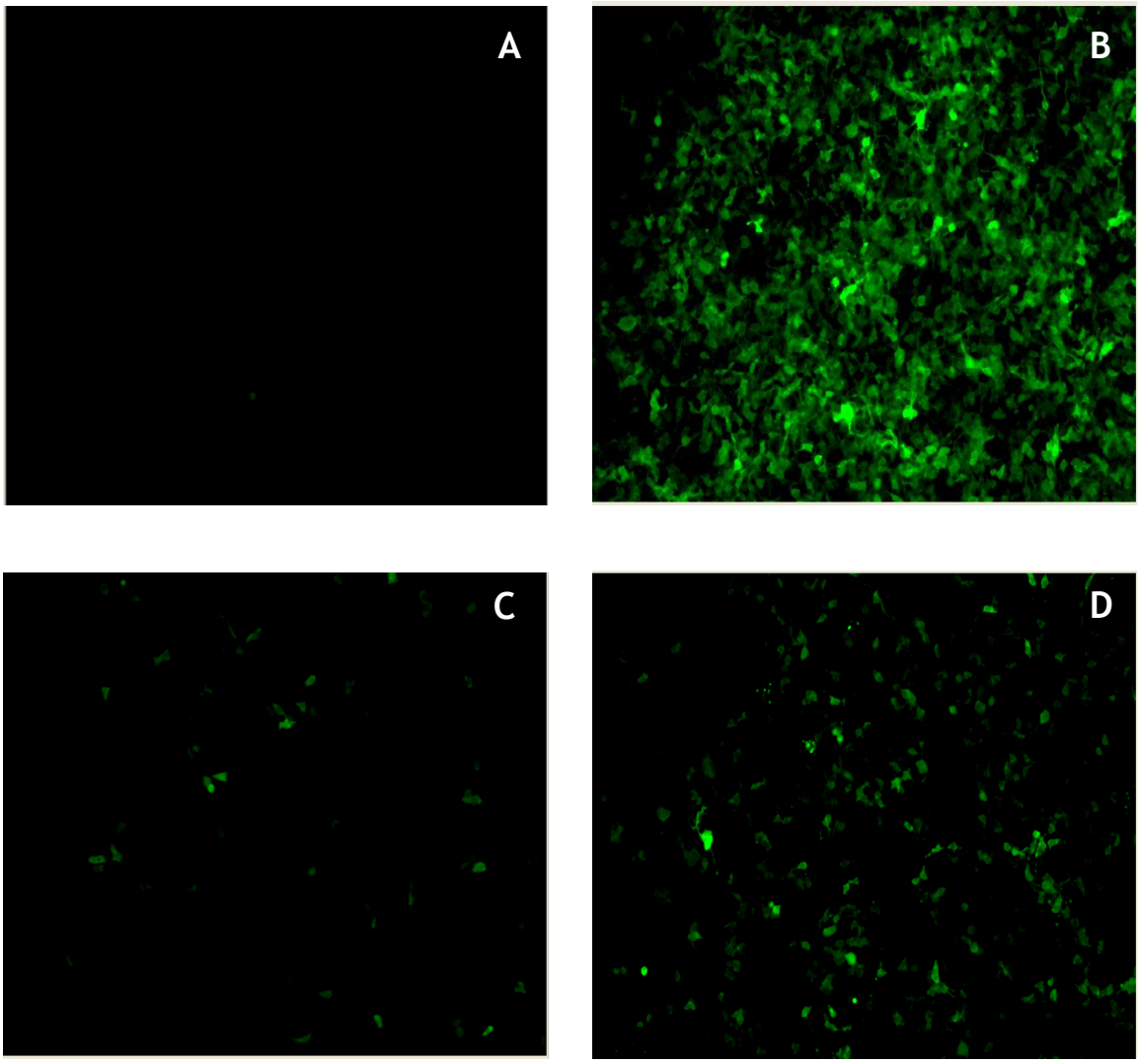


Figure 3-9 – The NFκB inhibitors CAPE and SC514 effectively inhibit TNF-α induced GFP production in NFkB/293/GFP cells. Cells were seeded in 6-well plate and were (A) mock-stimulated with DMSO, (B) stimulated with TNF-α (10 ng/ml) and DMSO, (C) stimulated with TNF-α and CAPE (88 μM) and (D) stimulated with TNF-α + SC514 (10 μM). Pictures were taken with bright-field microscopy.

3.2.2 Effect of FIV on CD134-mediated signalling in NFκB/293/GFP cells

The NFkB/293/GFP reporter cell line contains an expression vector encoding the GFP reporter gene driven by 4 copies of the NFκB consensus transcriptional response element along with a downstream minimal cytomegalovirus (mCMV) promoter (System Biosciences Cat. #TR800A-1). These reporter cells may be more sensitive to small changes in cell signalling induced by FIV binding to CD134 than measuring IL-8 production from non-human cell-lines because NFκB is further upstream of the signalling pathway and any signal generated from the

receptor has fewer obstacles to overcome to reach NFκB than to stimulate IL-8 production. First, both transient transfection and stable transduction of feline CD134 into NFkB/293/GFP cells using the pRetroQ-DSRed vector (Clontech) was successful (Table 3-2). Next, the reporter cells were stimulated overnight with feline CD134 ligand to explore whether feline CD134 engagement could trigger the GFP reporter gene. Flow cytometry revealed that transduction or transfection of feline CD134 induced a higher level of background NFκB activation compared to the transduction or transfection of the empty vector (Table 3-3). This indicated that feline CD134 may be able to interact with the intracellular signalling of NFkB/293/GFP cells. A large increase in green fluorescence was observed when TNF-α was added as a positive control (Table 3-3 and Fig. 3-10), indicating that the reporter gene was functional and responsive to stimulation of NFκB pathway by TNF-α.

Addition of CD134 ligand to the CD134-expressing NFkB/293/GFP cells did not induce NFκB activity. On the contrary, as more ligand was added to the cells, less fluorescence was generated (table 3-3). Background fluorescence was higher in feCD134 transduced cells compared to feCD134 transfected cells, perhaps a reflection of the fact that transfection is a transient method of DNA expression. More importantly, in a separate experiment the addition of FIV to the transduced cells did not induce an increase in NFκB activity (Fig. 3-10). Cells were incubated with virus alone or in conjunction with feCD134L at 0.5 and 1 μg/ml but none of the treatments increased NFκB activity above the level of the no treatment control. Since the feCD134L failed to stimulate GFP production, this model system was deemed inappropriate for this project. It was possible that NFkB/293/GFP cells display a similar species specificity in signalling to HeLa cells.

		antibody	% cell FITC gated	Mean fluorescence	% cell PE gated	Mean fluorescence
CD134	Transduced	anti-CD134 FITC	80.56	51.6	0.01	1
		no antibody	2.9	5.8	2.55	1.7
Vector only	Transduced	anti-CD134 FITC	6.5	3.7	95.87	12.7
		no antibody	5.8	4	94.81	12.2
CD134	Transfected	anti-CD134 FITC	74.9	28.5	2.18	2.7
		no antibody	3.4	5	12.05	2.1
Vector only	Transfected	anti-CD134 FITC	2.7	13.4	91.8	9.7
		no antibody	3.4	7.6	92.3	11.1

Table 3-2 – CD134 expression on transduced and transfected NFkB/293/GFP cells. PE = phycoerythrin. Expression of feline CD134 on NFkB/293/GFP cells transduced or transfected with feline CD134 in pDSRed were analysed by flow cytometry. Primary antibody was a FITC-conjugated mouse anti-feline CD134 antibody. High PE in empty vector control was attributed to DSRed monomer expression that was not disrupted due to the absence of the CD134 gene. % gated relative to the untreated, untransduced/untransfected NFkB/293/GFP cells.

	DSRed transduced			
	293T-NFκB-GFP feCD134		293T-NFκB-GFP vector only	
Treatment	% gated	mean fluorescence	% gated	mean fluorescence
NTC	13.7	4.1	0.3	11.7
TNF-α	51.8	38.2	18.5	26.6
feCD134L 1.0 μg/ml	12.5	5	0.2	15.3
feCD134L 0.5 μg/ml	16	3.6	0.2	11
feCD134L 1.0 μg/ml + TNF-α	49	35.3	17.3	24.9
feCD134L 0.5 μg/ml + TNF-α	50.2	33.2	12.4	21.9
	DSRed transfected			
	293T-NFκB-GFP feCD134		293T-NFκB-GFP vector only	
Treatment	% gated	mean fluorescence	% gated	mean fluorescence
NTC	6.6	9.4	1.16	14.3
TNF-α	18.9	16.5	24.3	21.9
feCD134L 1.0 μg/ml	4.25	8.1	0.8	7.9
feCD134L 0.5 μg/ml	5.9	6.4	0.9	7.3
feCD134L 1.0 μg/ml + TNF-α	26.9	17.8	27.29	27.6
feCD134L 0.5 μg/ml + TNF-α	22.45	14.3	28.38	26.6

Table 3-3 – Stimulation of feline CD134 transduced or transfected NFkB/293/GFP cells with ligand does not change NFκB activation status. Cells were stimulated overnight by TNF-α (10 ng/ml) alone or in conjunction with feCD134L (1.0 or 0.5 μg/ml). The green fluorescence level of the cells, which correlates to NFκB expression level, was measured by flow cytometry the next day. NTC = no treatment control. % gated relative to the untreated, untransduced/untransfected NFkB/293/GFP cells.

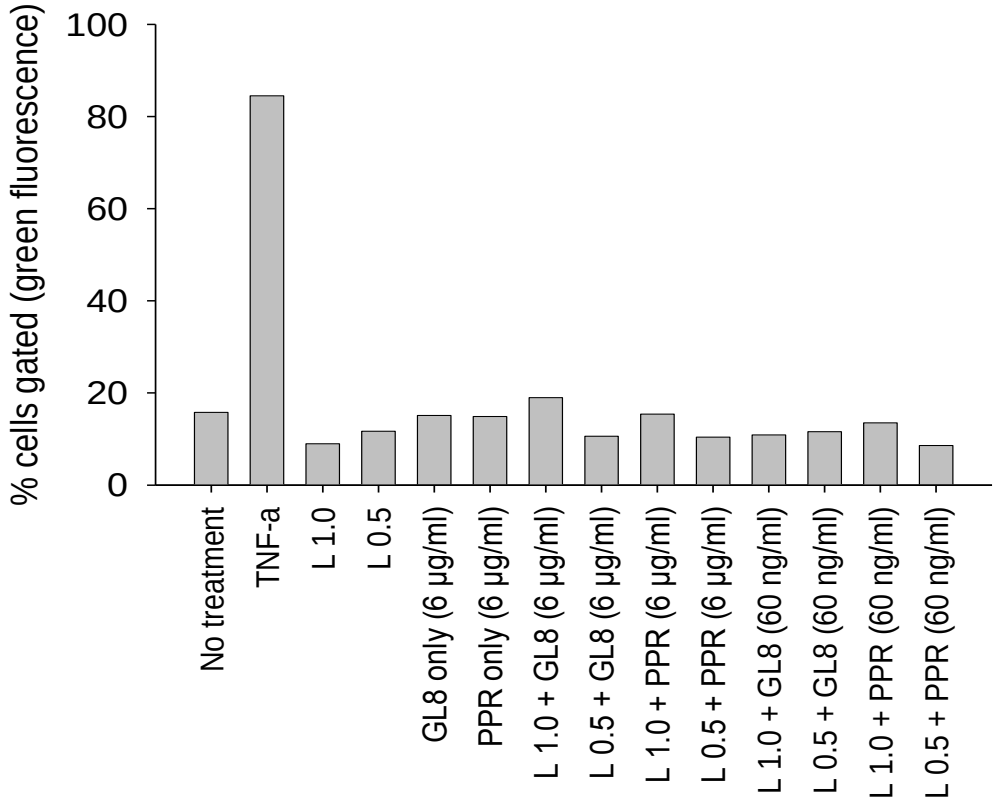


Figure 3-10 – FIV binding does not have any effect on NFκB signalling of the NFkB/293/GFP cell transduced with feCD134. Cells were stimulated overnight by TNF-α (10 ng/ml) alone or in conjunction with feCD134L (=L; at 1.0 or 0.5 µg/ml) and FIV GL8 or PPR (at 6 or 60 µg/ml). The green fluorescence level of the cells, which correlates to NFκB expression level, was measured by flow cytometry the next day.

3.2.3 An attempt to detect cell signalling changes in MYA-1 CD4$^+$ T cells by measuring cytokine released

Activated CD4$^+$ T cells can be divided into T_H1, T_H2 and T_H17 subsets, based on the expression of subset specific transcription factors and cytokines (Hirahara et al., 2011, Romagnani, 1994). T_H1 cells are known to produce IFN-γ (Abbas et al., 1996) while IL-4 is the signature cytokine for T_H2 cells (Mowen and Glimcher, 2004). Signalling via CD134 can influence the expression of both IFN-γ and IL-4 (Flynn et al., 1998, Ohshima et al., 1998, Vosskuhl et al., 2010). Thus it may be possible to detect changes in T cell CD134 signalling by measuring the amount of cytokine released after stimulation with ligand and/or virus.

The IL-2 dependent feline CD4$^+$ T cell line MYA-1 naturally expresses a high level of CD134 (fluctuating at ~50% positive over seven days) (Fig. 3-11A). To

determine the T_H subtype of MYA-1, supernatant of the T cells stimulated with the phorbol ester PMA were analysed by ELISA for feline IFN-γ and IL-4 production. Stimulation with PMA did not induce IFN-γ production from MYA-1 $CD4^+$ T cells (Figure 3-11C). In contrast a background level of IL-4 production was detected which was more than doubled after PMA stimulation (Fig.3-11B), indicating that MYA-1 $CD4^+$ T cells belong to the T_H2 subtype.

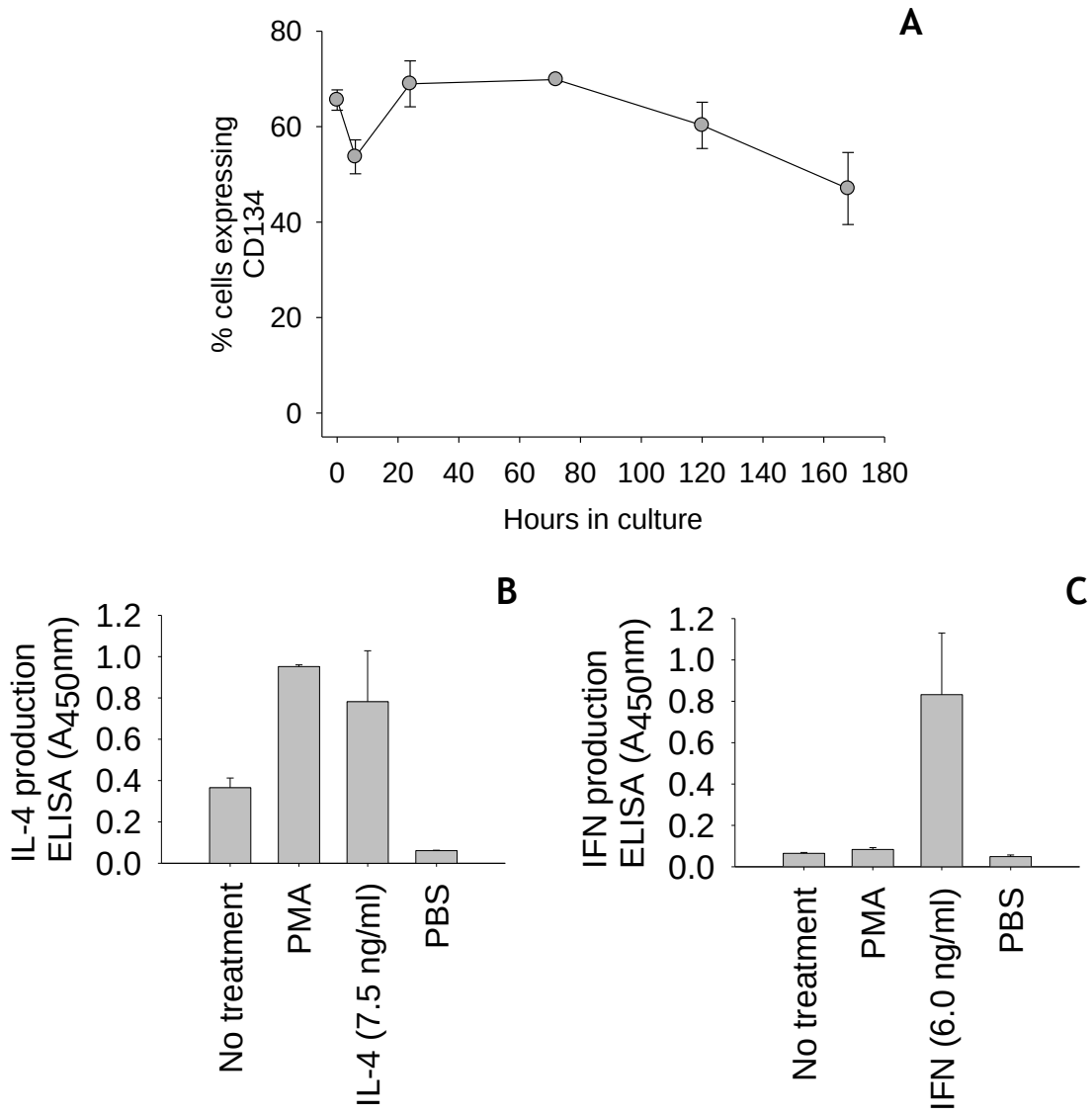


Figure 3-11 – MYA-1 $CD4^+$ T cells naturally express CD134 and produce IL-4 after stimulation. MYA-1 $CD4^+$ T cells were cultured in IL-2 supplemented medium and (A) the expression of CD134 over a 7 day period was quantified by flow cytometry of the sequential samples. Each point represents the mean +/- standard error (n= 3). Unstimulated cells (No treatment) or cells stimulated with PMA (0.08 μM) were incubated for 48 hours and the supernatant IL-4 (B) or IFN-γ (C) concentrations were measured by ELISA. Recombinant cytokines were used

as positive control for the ELISA. Data is representative of 2 independent experiments. Each bar represents the mean +/- range (n= 2).

MYA-1 $CD4^+$ T cells were then stimulated with feCD134L and supernatant IL-4 levels measured after 48 hours. The result from one experiment showed that IL-4 production by the MYA-1 $CD4^+$ T cells was reduced by ~50% by feline CD134 ligand when IL-2 was present, and more than doubled when IL-2 was absent (Fig. 3-12A and B). However, this trend could not be repeated in 3 subsequent independent experiments (Fig. 3-12C and D). Recombinant FIV Env SU-Fc proteins were added to the cells in substitute for live FIV particles and these, particularly FIV PPR, had less pronounced but similar effect on IL-4 production as the ligand in the first experiment (Fig. 3-12A and B). Again, this trend was also not found in subsequent experiments (Fig. 3-12C and D). When the MYA-1 $CD4^+$ T cells were loaded with carboxyfluorescein (CFSE) as a means of tracking cell division, none of the treatments, including the addition of PMA, produced a shift in fluorescence when IL-2 was present (Fig. 3-13A). In the absence of IL-2, PMA increased the rate of cell division while GL8 Env SU Fc incubated cells showed a drop in rate of division compared to no treatment (Fig. 3-13B). These trends suggested that in the absence of IL-2 virus or viral components may have an observable effect on cell signalling.

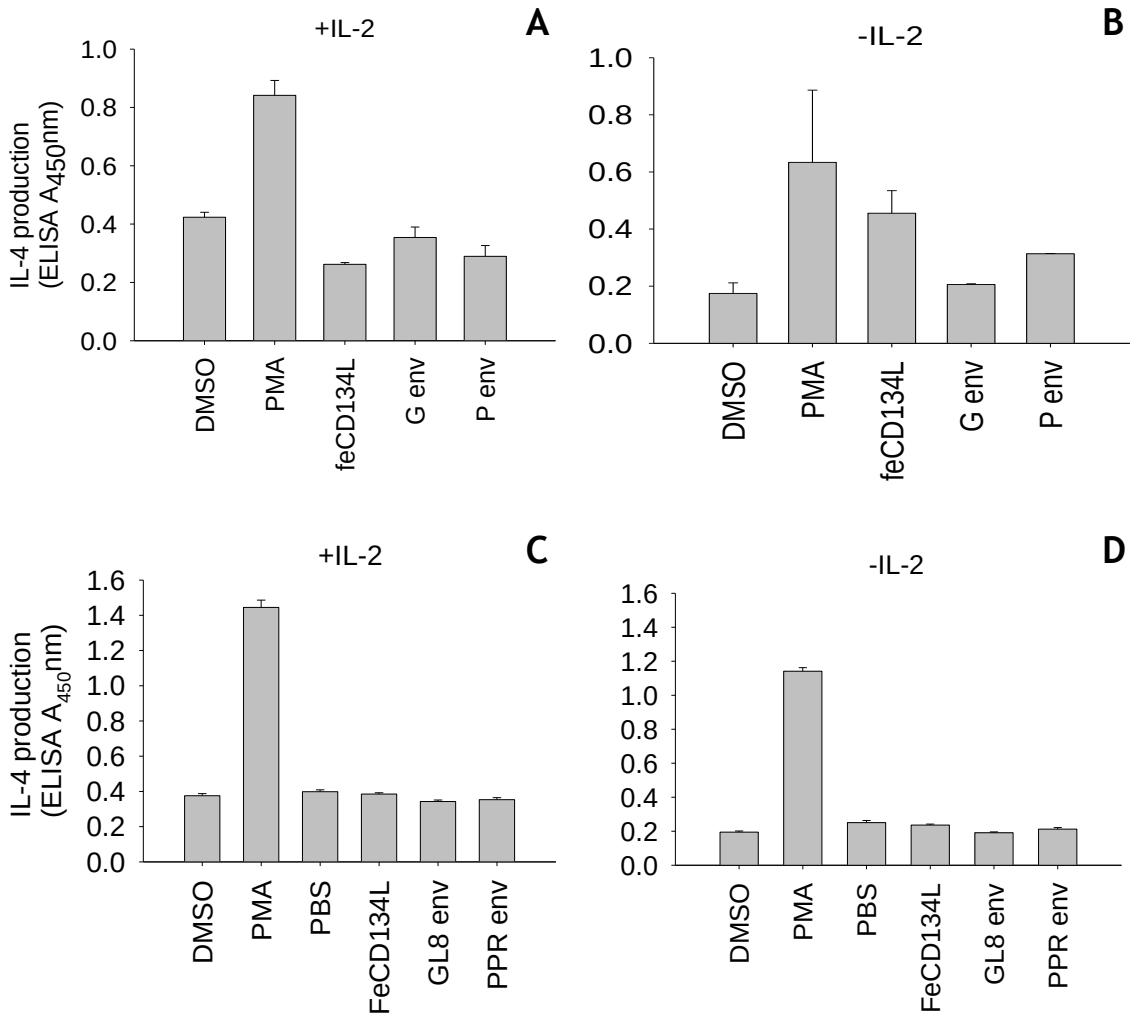


Figure 3-12 – Effect of feline CD134 ligand and FIV Env SU Fc proteins on IL-4 production by MYA-1 CD4$^+$ T cells. Cells with (A and C) or without IL-2 supplement (B and D) were subjected to incubation with PMA, feline CD134 ligand, FIV GL8 or PPR SU Fc proteins (1 µg/ml), DMSO or PBS for 48 hours before supernatant IL-4 was measured by ELISA. (A and B) are representative of 1 out of 4 experiments while (C and D) are representative of 3 out of 4 experiments. Each bar represents the mean +/- standard error (n= 3).

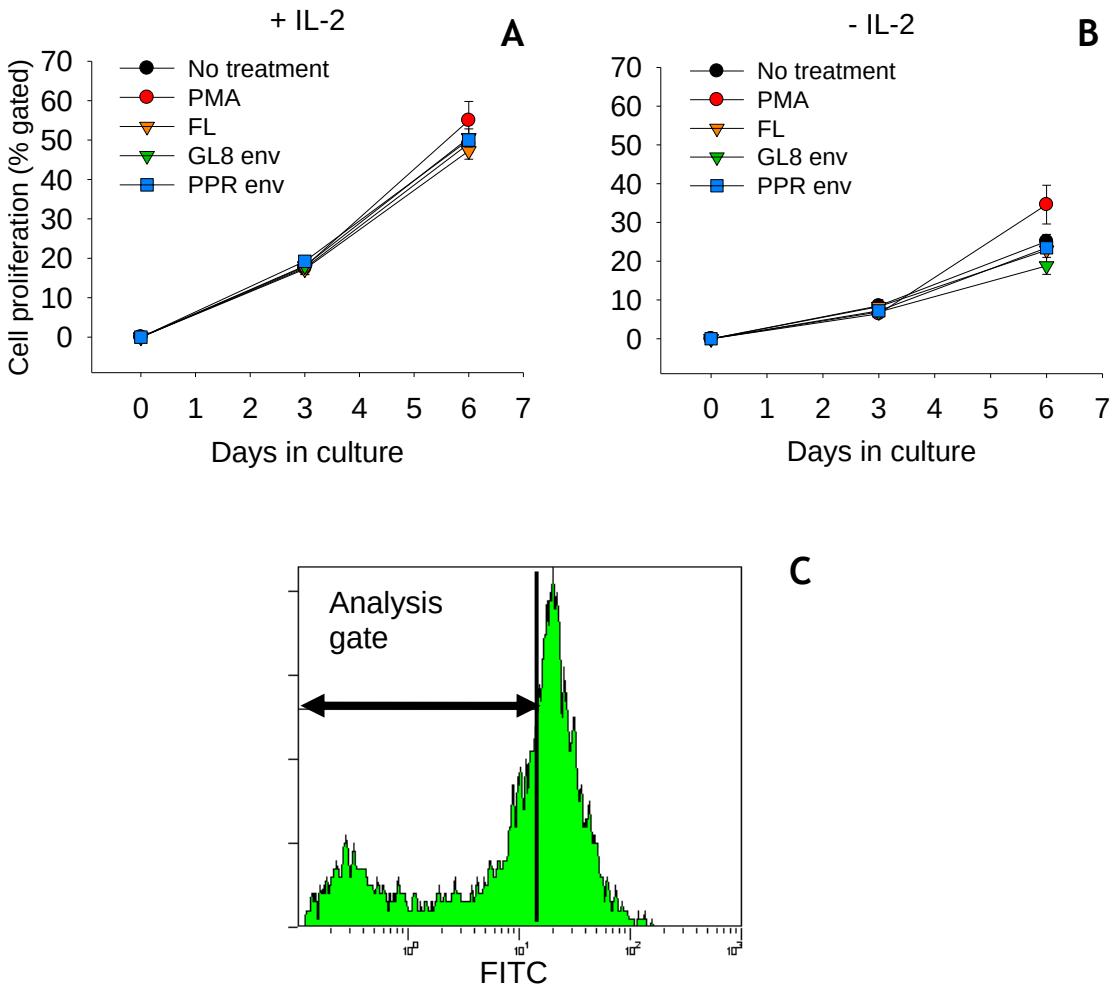


Figure 3-13 – Modest reduction in cellular proliferation is caused by GL8 Su Fc in IL-2-depleted cells. However, feline CD134 ligand and FIV SU Fc protein do not induce a shift in fluorescence in CFSE assay of MYA-1 CD4$^+$ T cells in the presence of IL-2. Feline CD134 ligand (FL), PMA, FIV Env SU Fc proteins were added to CFSE loaded cells in the presence (A) or absence (B) of IL-2 and sequential samples were taken over 6 days to monitor cell proliferation rates. Percentage of proliferating cells was estimated as the percentage of cells with fluorescence values lower in the gated region designated in (C), arbitrarily set at a value of 50%. Each point represents the mean +/- range (n= 2).

3.3 Discussion

In order to ensure successful infection, viruses have evolved to manipulate the host at every step of the viral lifecycle. The primary receptor for FIV is CD134 (Shimojima et al., 2004), a $CD4^+$ T cell co-stimulating molecule that is vital to the proliferation, survival and function of activated T cells (Croft, 2010). In theory FIV may benefit from an enhancement in CD134 signalling in activated T cells as it would increase the number of target cells susceptible to infection or promote the survival of infected cells. This chapter investigated the hypothesis that FIV binding to CD134 induced changes in signalling within the target cell.

To achieve the initial aims of the project three potential *in vitro* models of signalling were constructed and their abilities to detect signalling induced by feline CD134 ligand examined. Subsequently, they were subjected to stimulation with either FIV virions or recombinant FIV Env SU Fc proteins. Firstly, IL-8 producing adherent cells HT1080, NP-2 and HeLa cells were stably transduced with CD134 expression vectors. When HT1080-feCD134 and NP-2-feCD134 cells were stimulated by soluble feline CD134 ligand, IL-8 was produced in response. In contrast HeLa-feCD134 cells failed to respond to stimulation with feCD134L. The reason behind this incompatibility between feline CD134 and HeLa cells is unknown. More importantly, stimulation of these cells with virus particles, even at high titre, did not induce IL-8 production. When FIV and feCD134L were added to the cells simultaneously, an enhancement of IL-8 production was noted on some experiments but this observation was not reproducible. When HT1080-feCD134 cells were stimulated by the NFκB inhibitor CAPE and incubated with FIV GL8, a small increase in IL-8 production could be detected. This result was not thought to be reliable because it is still unclear as to why CAPE can induce IL-8 production, and because of the small magnitude of the increase. Other experimental approaches to examine the cell signalling events potentially induced by FIV were examined. The reporter cell line NFkB/293/GFP was transduced or transfected with feline CD134. However, while efficient surface expression of the molecule was detected, ligand binding or incubation with FIV virions did not result in the activation of NFκB. Finally, the production of cytokines from the feline $CD4^+$ T cell line MYA-1 was studied. Direct stimulation of the MYA-1 $CD4^+$ T cells with feCD134L or FIV Env SU Fc did not affect IL-4 production of the cell line.

Several studies in the past have provided evidence suggesting that HIV-1 Gp120 Env binding to CD4, CCR5 and CXCR4 triggers cell signalling and that this may promote viral replication (Kornfeld et al., 1988, Popik and Pitha, 1996, Weissman et al., 1997, Yoder et al., 2008). However, contradictory studies have concluded that HIV-1 receptor-mediated signalling has little effect on replication (Cocchi et al., 1996, Farzan et al., 1997, Amara et al., 2003, Yoder et al., 2008). Interestingly, studies which used activated or transformed cells are more likely to report that HIV-1 Gp120-induced signalling is unnecessary for virus production (Wu and Yoder, 2009). In addition, studies which claim that HIV-1 Gp120 can induce significant cell signalling activity may be undermined by the observation that cell signalling required a supra-physiological level of HIV-1 Gp120 (Klasse and Moore, 2004). The concentration of free HIV-1 Gp120 *in vivo* is difficult to measure because of interference from plasma antibody (Klasse and Moore, 2004). The concentration of Gp120 in different tissues may vary and it is difficult to quantify HIV-1 Gp120 expressed on the cell surface during cell-to-cell transmission (Klasse and Moore, 2004). A small increase in IL-8 production was recorded when CAPE-stimulated HT1080-CD134 cells were incubated with virus equivalent to 6 μg/ml of FIV p24. Is this concentration of virus physiological? It is also important to consider the binding efficiency and receptor occupancy of the viral protein in relation to the level of receptor expression (Klasse and Moore, 2004). Taken together, these data cast doubt on the hypothesis that HIV-1 Gp120 initiates cell signalling to a level that significantly enhances virus production. It is more likely that other viral proteins such as Nef and Tat may contribute to the increase in T cell activation prior to viral gene expression (Ott et al., 1997, Wang et al., 2000a, Wu and Marsh, 2001).

Based on the available data, there is no indication that FIV binding to feline CD134 triggers signalling through the NFκB cascade. If FIV Env-induced signalling is confirmed by future studies, the expected level of stimulation is expected to be small compare to the virus-promoting signals generated by activated T cells. However, it is important to acknowledge that the methods described in this chapter have potential flaws. Firstly, although it has been shown that using IL-8 production as a measure for CD134 signalling is sensitive enough to detect the effect of soluble CD134 ligand binding, is it enough to detect a potentially more subtle stimulation of CD134 by FIV virions? The stimulation threshold to generate IL-8 may be greater than the threshold to produce observable

activation of NFκB. Secondly, the physiological level of CD134 stimulation in feline cells is not known, thus if stimulation of cell signalling was detected, it would be hard to assess its biological significance. Thirdly, the behaviour of the cells has not been uniform, giving occasional positive results that were not repeatable. Lastly, there is a lack of reagents available that can be used in feline cells. It means that traditional methods to monitor NFκB stimulation such as detecting IκB phosphorylation using immunobloting are not possible. Recent discoveries have demonstrated the involvement of pathways other than NFκB in CD134 signalling such as PKC, PI3K, PDK-1 and Akt/PKB pathways (So et al., 2011a, So et al., 2011b), which may be areas for further study.

Another avenue of research that is yet to be explored is the effect of FIV Env-induced signalling on the infection of resting T cells and the implications for viral latency. HIV-1 establishes latent reservoirs within the host which persist for many years (Siliciano et al., 2003). The latently infected cells seed new infections when they periodically reactivate, preventing the full eradication of the virus despite intensive anti-retroviral treatment (Richman et al., 2009). This is an important problem by itself and will be explored in the next chapter.

It has been demonstrated that most of the latently infected cells in HIV-1 infection are memory T cells that have been infected when they were in an activated state (Chun et al., 1997, Pierson et al., 2000, Brenchley et al., 2004a, Chomont et al., 2009). In contrast, the direct infection of resting T cells (naïve T cells or quiescent memory T cells) is not thought to be important in the establishment of latent reservoirs because infection of resting T cells is very inefficient (Stevenson et al., 1990, Pierson et al., 2000, Vatakis et al., 2010). However, it has been shown recently that HIV DNA isolated from the memory T cells of patients receiving antiretroviral treatment decreases over time while HIV DNA from naïve T cells remained stable (Wightman et al., 2010). This suggested that as infected memory T cells were lost from the reservoir due to reactivation, the proportion of naïve T cells in the reservoir increased slowly due to direct infection.

One of the obstacles to the direct infection of naïve resting T cells is the static nature of their cytoskeleton. Recently, a mechanism has been put forward to explain how HIV-1 overcomes this. The binding of HIV-1 Env to CXCR4 triggers G-

protein dependent signalling which ultimately activates the cellular actin-depolymerizing molecule cofilin and facilitates the transport of the viral pre-integration complex towards the nucleus (Yoder et al., 2008). FIV can establish a latent infection in feline T cells (Joshi et al., 2004) and uses CXCR4 as its co-receptor (Willett et al., 1997). Does FIV Env trigger signalling downstream of CXCR4 in the same way as HIV-1? In one experiment the addition of FIV GL8 SU Fc proteins to feline MYA-1 $CD4^+$ T cells appeared to affect cellular proliferation as demonstrated using the CFSE assay. Further experiments should be performed to confirm these results and to deduce the signalling pathways that are involved. If it is proven that the binding of FIV to resting feline T cells can stimulate significant cell signalling, then will this signalling be required in the successful establishment of post-integration latency? If so, will this lead to a higher proportion of naïve T cells within the latent reservoirs of cats compared with humans and will this result in differential responses to latency reversing therapies between the two species? These are questions worthy of further research.

4 Dual action of phorbol esters on FIV production illustrates shared principles in the replication of both HIV-1 and FIV

4.1 Part 1 – establishment of FIV latency in MYA-1 $CD4^+$ T cells

In addition to being an interesting scientific question, the molecular mechanisms behind HIV-1 latency have huge public health implications worldwide (Richman et al., 2009). The estimated number of latently infected memory T cells within an individual is small, around 10^6 cells (Chun et al., 1997). However, this pool of latently infected cells serves as a major reservoir of virus in the host and a source of new virus following reactivation. With an extremely slow decay rate, even under intensive HAART (Finzi et al., 1999, Siliciano et al., 2003, Pace et al., 2011), this small population of latently infected cells ensures lifelong infection of the host and is a stumbling block for complete virus eradication.

The most practical strategy to attempt to eliminate the latent reservoir is to use drugs to reactivate virus production under intensive HAART without causing global activation of uninfected T cells or other undesirable side-effects (Marsden and Zack, 2009, Richman et al., 2009). However, as viral replication is intricately tied to cellular activation (Stevenson et al., 1990), reactivating the latent viruses while at the same time avoiding T cell activation has been challenging (Prins et al., 1999). In order to successfully deduce a precise method to reverse HIV-1 latency, its molecular mechanism has been studied at both the cellular and whole animal level. Yet difficulties such as a limited supply of suitable cells and the use of heavily modified virus in an unnatural host like the SCID mice remain. This part of chapter 4 presents the development of an *in vitro* model of lentiviral latency using FIV and the feline $CD4^+$ T cell line MYA-1.

4.2 Results

4.2.1 Attempts to establish latent infection in MYA-1 CD4$^+$ T cells

It has been demonstrated that factors such as low cellular activation level, the lack of cellular transcription factors and a restrictive chromatin environment contribute to the maintenance of lentiviral latency (reviewed in (Han et al., 2007, Coiras et al., 2009, Colin and Van Lint, 2009)). However, it is the level of cellular activation, more specifically the host cell NFκB activation status during infection that determines whether a productive or latent infection will result (Stevenson et al., 1990, Duverger et al., 2009). Based on this understanding it may be possible to latently infect feline MYA-1 CD4$^+$ T cells with FIV by blocking NFκB activation of the cells using NFκB inhibitors before or during viral entry. To find a working concentration for each NFκB inhibitor that would cause minimum toxicity, the MYA-1 CD4$^+$ T cells were incubated for 10 days with the NFκB inhibitors CAPE, BOT64, SC514 and BAY 11-7082 at a range of concentrations starting from their respective half maximal inhibitory concentrations (IC_{50}) (Table 4-1). Results showed that BOT64 and SC514 were relatively non-toxic, with viabilities comparable with the DMSO control even at IC_{50} concentrations (Fig. 4-1B and C). Based on these data working concentrations of 10 μM and 3 μM for SC514 and BOT64 respectively were chosen. CAPE at a concentration of 88 μM (which is the IC_{50}) showed considerable toxicity, reducing the viability of the cells to less than 50% by day 6(Fig. 4-1A). Thus a lower concentration of 44 μM was chosen as the working concentration for CAPE. BAY 11-7082 was found to be the most toxic NFκB inhibitor out of the group; when used at its IC_{50} of 15 μM, it caused a drop of 80-90% in cell viability from the start of the assay (Fig. 4-1D) and thus it was not used for further experiments.

Inhibitor			
CAPE	88 µM*	44 µM	22 µM
BOT64	3 µM*	1.5 µM	0.75 µM
SC514	10 µM*	5 µM	
BAY 11-7082	15 µM*	7.5 µM	3.75 µM

Table 4-1 - The IC_{50} (*) and other concentrations of the NFκB inhibitors tested in the viability assay of feline MYA-1 $CD4^+$ T cells. IC_{50} based on the manufacturer's protocols.

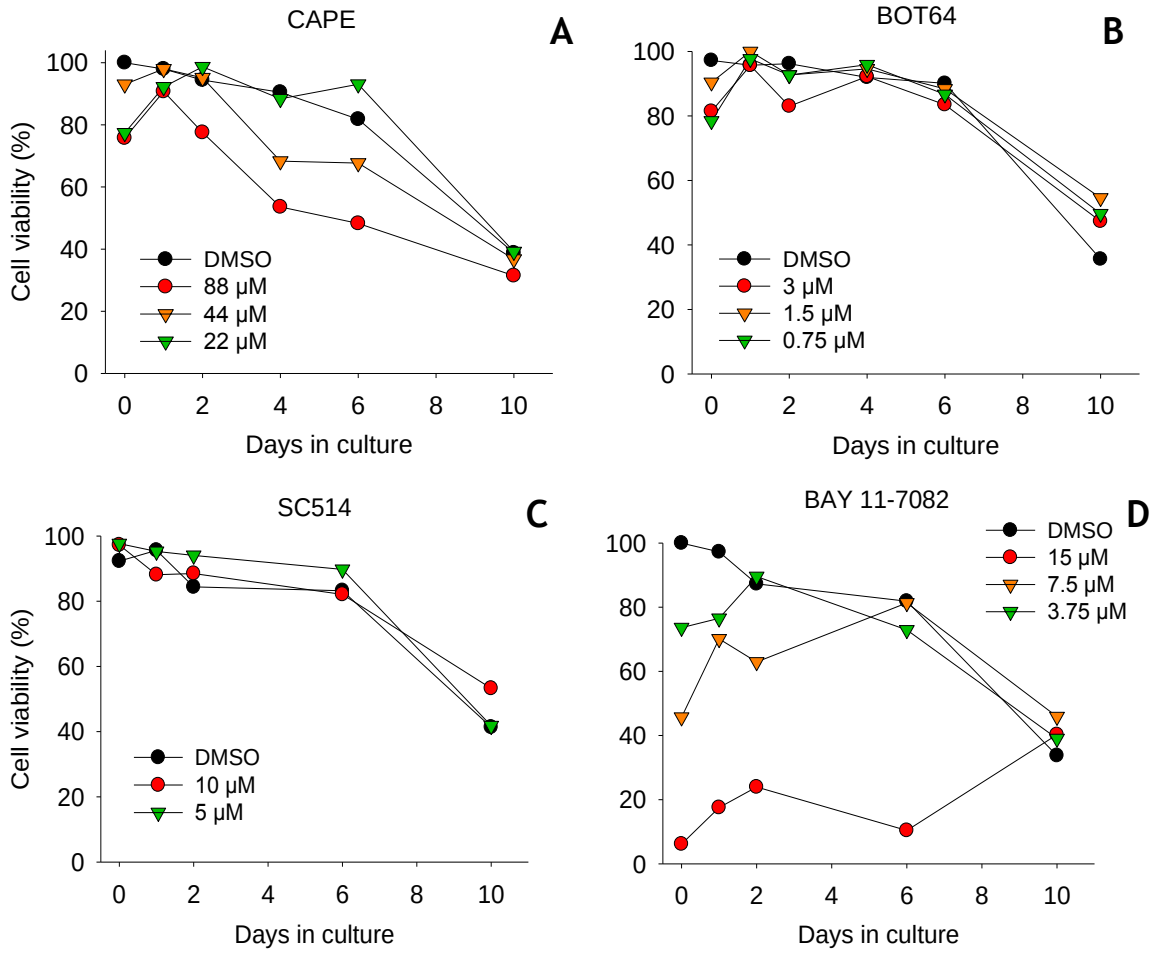


Figure 4-1 – Effect of different NFκB inhibitors on the viability of MYA-1 $CD4^+$ T cells. Feline MYA-1 $CD4^+$ T cells supplemented with IL-2 (100U/ml) were incubated with different concentrations of (A) CAPE, (B) BOT64, (C) SC514 and (D) BAY 11-7082 for 10 days. Viability was measured by trypan blue exclusion assay at day 0, 1, 2, 4, 6 and 10.

Next, the NFκB inhibitors CAPE (44 µM), BOT64 (3 µM) and SC514 (10 µM) were added to cells 24 hours before infection with FIV strains GL8 or PPR at an MOI of 0.001 to see if productive infection could be stopped. Supernatant samples

were collected at days 0, 1, 3, 5, 7 and 10 post infection and FIV production measured by an ELISA that detects the FIV p24 (Gag) protein. Productive FIV infection was almost completely abrogated in cells incubated with CAPE during the 10-day period (Fig. 4-2). The presence of BOT64 and SC514 appeared to have more than doubled the production of FIV strain GL8 by day 10 post infection (Fig. 4-2A), while the replication of FIV PPR was not affected by BOT64 and was slightly reduced by SC514 (Fig. 4-2B).

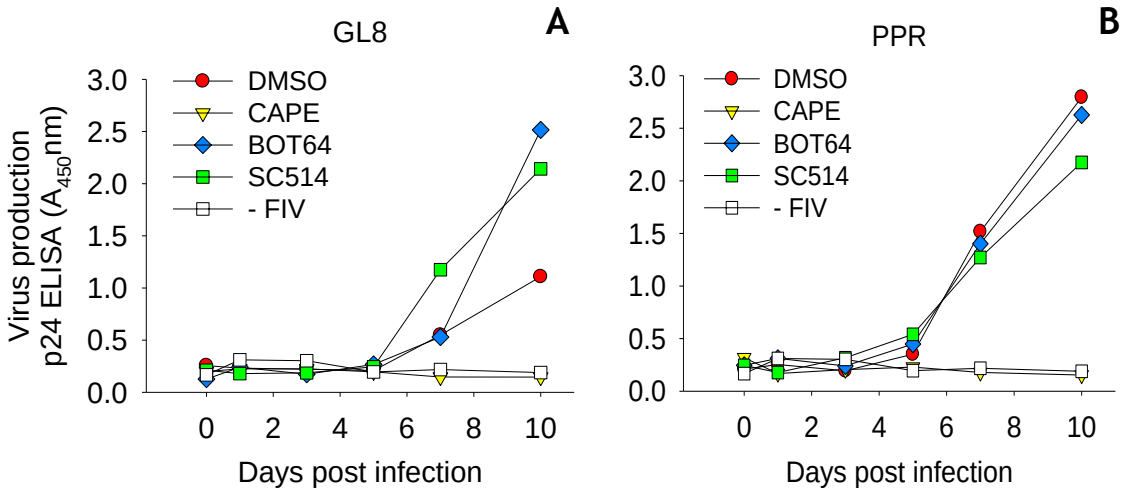


Figure 4-2 – Productive FIV infection of MYA-1 CD4$^+$ T cells is blocked by CAPE. IL-2-supplemented (100 U/ml) MYA-1 CD4$^+$ T cells were pre-incubated with NFκB inhibitors CAPE (44 μM), BOT64 (3 μM), SC514 (10 μM) and mock treated (DMSO) for 1 hour before infection with FIV (A) GL8 or (B) PPR (MOI 0.001) and virus production was monitored in the culture supernatants by FIV p24 ELISA.

To check if the FIV infection would remain latent after the withdrawal of CAPE, IL-2-supplemented infected cells treated with CAPE were washed at day 3 or day 7 post infection and resuspended in fresh, IL-2-supplemented medium without CAPE. After an extended study period of 20 days, virus production was detected in the supernatant of CAPE-treated FIV PPR-infected cells but not FIV GL8 infected cells (Fig. 4-3A) The removal of CAPE at day 3 or day 7 post infection resulted in the return of productive infection afterward (Fig. 4-3C and D), suggesting that CAPE had to be present constantly to ensure a non-productive infection. Under all the different conditions, FIV PPR was less sensitive to the effects of the NFκB inhibitors than FIV GL8 and the titre of FIV PPR always

reached a plateau before FIV GL8, illustrating the differences in viral replication dynamics between strains (See Chapter 5).

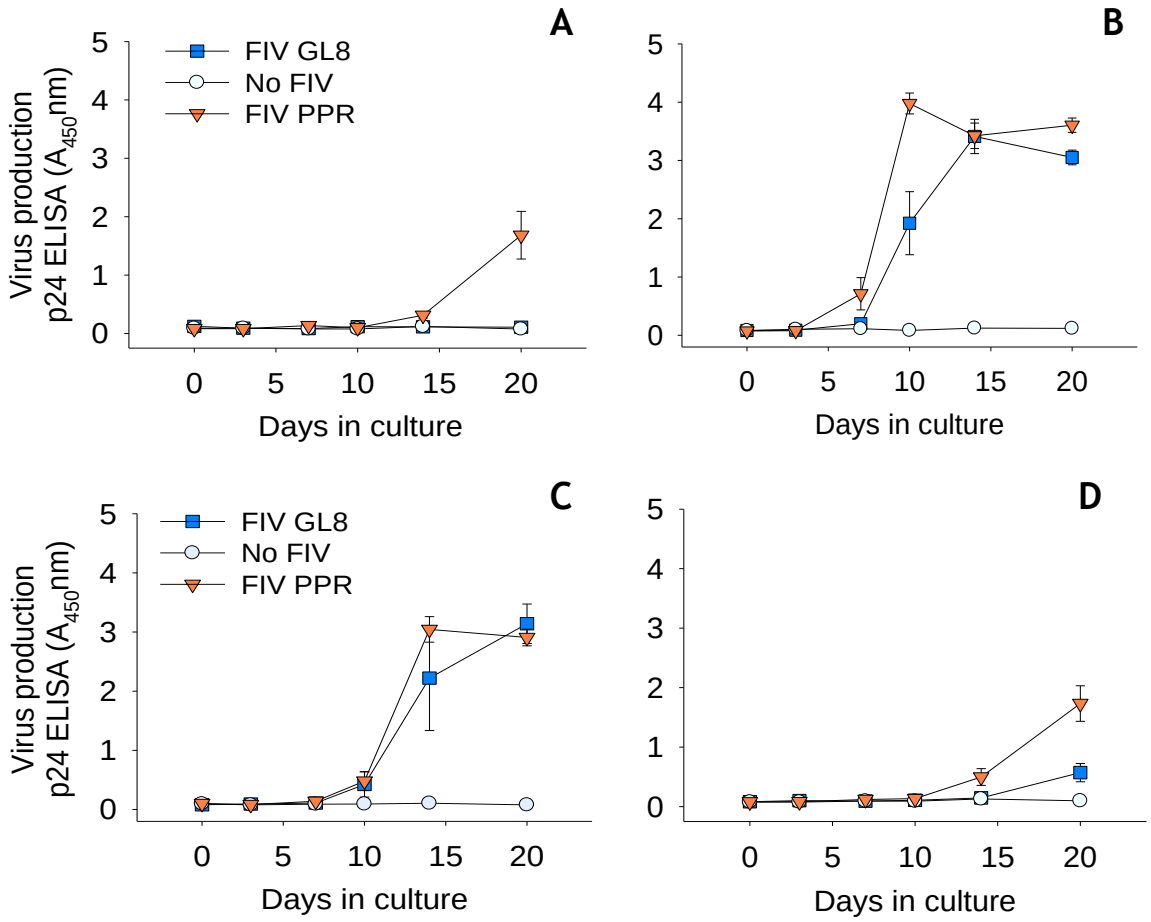


Figure 4-3 – The continuous presence of CAPE is necessary to maintain non-productive infection. Parallel experiments were performed in which IL-2 supplemented (100 U/ml) MYA-1 $CD4^+$ T cells were pre-incubated with NFκB inhibitor CAPE (44 μM) 1 hour before and during infection with FIV GL8 or PPR (MOI 0.001), after which the cells were (A) continuously maintained in medium containing CAPE, (B) kept in medium without CAPE, (C) maintained in CAPE-containing medium for 3 days or (D) 7 days. Supernatant samples were collected at intervals for up to 20 days post infection and virus production measured by FIV p24 ELISA. Each bar represents the mean +/- standard error (n=3).

It has been shown previously that PKC activating phorbol esters are potent reactivating agents of latent HIV-1 infection (Marquez et al., 2008) and among them Prostratin, with its non-tumour-promoting property, could be developed into a viable therapy to purge the reservoir of latently infected cells (Kulkosky et al., 2001, Rullas et al., 2004). To test whether the non-productive infection

maintained by CAPE was comparable to latent infection of MYA-1 T cells by HIV-1, Prostratin and PMA, a powerful, tumour promoting phorbol ester, were added to infected cells incubated with CAPE at day 2 post-infection. The phorbol esters did not rescue virus production in CAPE incubated cells during the 10 day experiment (Fig. 4-4A) and furthermore lowered the virus titre in the productively infected control cells, especially Prostratin, which reduced virus titre in both strains by more than 50%. PMA reduced the viability of the cells incubated with/without CAPE by 20% and 15% respectively (Fig. 4-4B), while Prostratin did not markedly reduce cell viability. This demonstrated that the reduction in virus titre was not due to the toxicity of the compounds added to the medium. It has been shown previously that Prostratin has the dual effect of inhibiting productive HIV-1 infection as well as reactivating latently infected viruses (Kulkosky et al., 2001, Biancotto et al., 2004, Rullas et al., 2004). The failure by Prostratin to rescue virus production from cells incubated with CAPE suggested the CAPE induced non-productive infection was not a true latent infection.

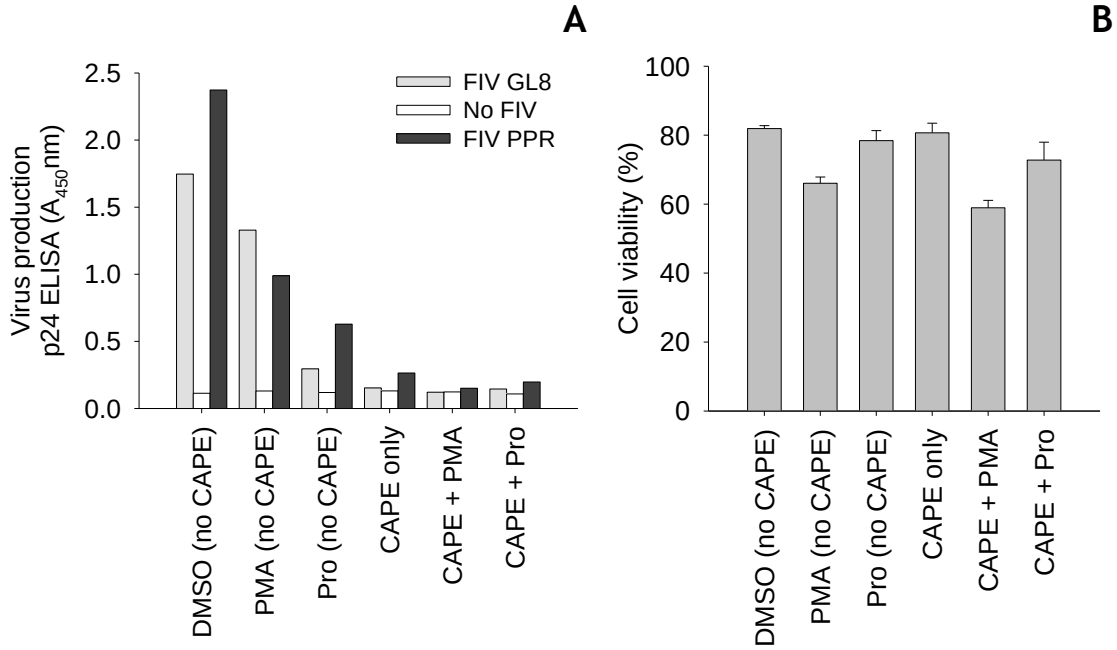


Figure 4-4 – The phorbol esters PMA and Prostratin do not rescue virus replication from FIV infected MYA-1 CD4$^+$ T cells incubated with CAPE. IL-2 - supplemented MYA-1 CD4$^+$ T cells were pre-incubated with the NFκB inhibitor CAPE (44 μM) or DMSO (no CAPE) for 1 hour before and during infection with FIV GL8 or PPR (MOI 0.001), after which the cells were resuspended either in complete medium + IL-2 with CAPE or without CAPE (no CAPE). PMA (0.08 μM) and Prostratin (Pro) at 0.5 μM were added to the cells 2 days post infection.

(A) Virus production was measured by FIV p24 ELISA at day 10 post-infection. (B) Cell viability in the treated cells in the presence of FIV infection was measured 10 days post-infection. Each bar represents the mean +/- standard error (n=3)

In the previous experiments the MYA-1 $CD4^+$ T cells were supplemented with exogenous IL-2 because it is an IL-2-dependent cell line. IL-2 is a major promoter of T cell expansion, survival and differentiation (Gaffen and Liu, 2004, Malek, 2008). IL-2 is also a known activator of latent HIV-1 and FIV infections (Prins et al., 1999, Joshi et al., 2005a). Could depleting the cells of IL-2 lead to a latent FIV infection? First, the growth characteristics of the cells in the presence or absence of IL-2 were examined. In the absence of exogenous IL-2 cell growth ceased and viability fell to approximately 20-30% within 6 days (Fig 4-5A and B). In contrast, the addition of exogenous IL-2 maintained both cell viability and cell growth, with an exponential expansion phase that lasted for approximately 6 days, after which the culture reached confluence and further expansion required sub-culture.

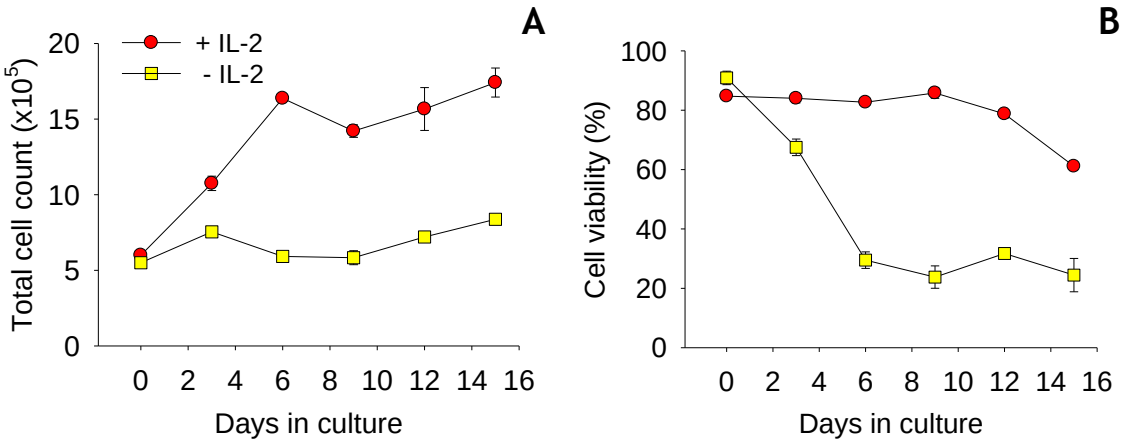


Figure 4-5 – Effect of exogenous IL-2 on the growth and viability of MYA-1 $CD4^+$ T cells. Total cell number (A) and cell viability (B) of MYA-1 $CD4^+$ T cells grown in the presence or absence of exogenous IL-2 (100 U/ml). Each point represents the mean +/- standard error (n=3).

Next the susceptibility of cells grown in the presence and absence of IL-2 to infection with FIV is compared. Since IL-2 depletion would reduce cell growth and viability, which in turn would decrease the virus production, a higher dose of FIV (M.O.I = 0.1) was used. When IL-2 was depleted 2 days post infection, productive infection of FIV PPR was detected from day 2 and FIV GL8 at day 4 in

both treatment and IL-2 positive control cells, indicating that the attempt to establish a latent infection was unsuccessful (Fig. 4-6B).

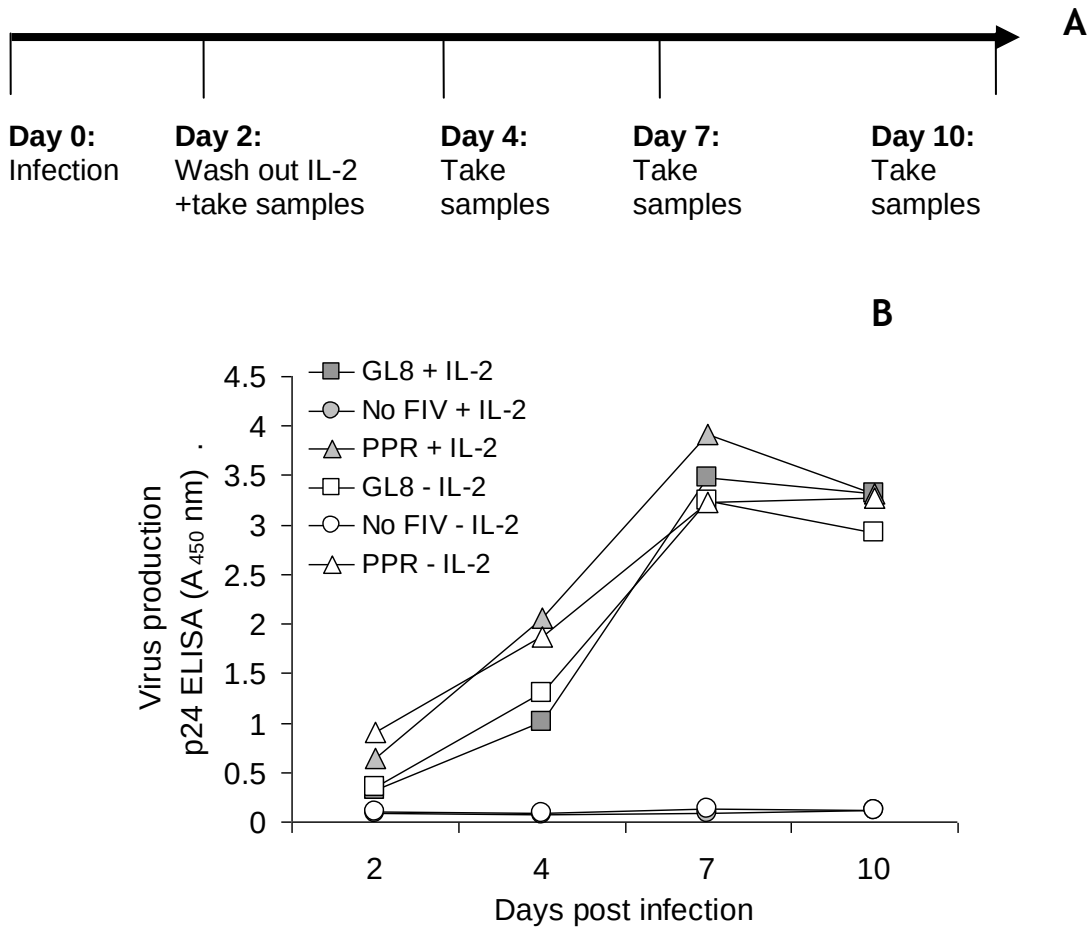


Figure 4-6 – Removing IL-2 post infection does not establish latent infection. **(A)** Experimental protocol: MYA-1 $CD4^+$ T cells were infected with FIV (M.O.I = 0.1) and IL-2 was removed 2 days post infection. **(B)** FIV p24 ELISA showing supernatant FIV levels over a period of 10 days.

When exogenous IL-2 was removed from actively growing MYA-1 cells 24 hours prior to infection, no virus production could be detected using FIV p24 ELISA (Fig. 4-7A). It has been shown that for HIV-1 infection, the majority of the latently infected CD4+ T cells are memory T cells (Chomont et al., 2009) and it is likely that most of these cells were infected when they were in an activated state and subsequently return to quiescence when they become memory cells (Siliciano and Greene, 2011). The depletion of IL-2 from activated $CD4^+$ T cells causes the cells to enter a resting state analogous to the transition of effector cells to memory cells (McKinstry et al., 2007). Does the lack of virus production, as determined by ELISA, mean that I have re-created the transition from

infected effector to latently infected memory T cell? To characterise the viral replication dynamics within these IL-2-depleted T cells, intracellular DNA and RNA were extracted over 10 days and the amount of intracellular FIV DNA and unspliced FIV RNA (Tomonaga et al., 1995) quantified using quantitative PCR (qPCR), amplifying a 78bp region at the 5' end of the FIV *gag* gene. Data was analysed using the ΔΔCt method with a primer set that amplifies the 18S rRNA sequence as the internal control. The qPCR detected less intracellular viral DNA within the IL-2-depleted resting MYA-1 T cells compared with IL-2 supplemented cells 2 hours post infection (Fig. 4-7B). Subsequently there was an increase in intracellular FIV DNA among IL-2-depleted cells, which reached a plateau four days post infection. However, this increase was noticeably smaller than the increase in intracellular FIV DNA detected from cells grown in the presence of IL-2 (Fig. 4-7B). These data indicated a defect in reverse transcription and viral spread among the infected resting MYA-1 T cells. This is consistent with previous studies that showed HIV-1 entry and reverse transcription is less efficient in resting T cells compared with stimulated T cells (Pierson et al., 2002b, Vatakis et al., 2007). The qPCR also detected the production of unspliced intracellular FIV RNA in the resting MYA-1 T cells that reached a plateau after 4 days post infection (Fig. 4-7C). The quantity of intracellular viral RNA produced within the resting cells was markedly lower compared with the quantity within the IL-2 supplemented cells. The presence of a small amount of unspliced intracellular lentiviral RNA does not demonstrate full productive infection, since a low-level of unspliced RNA can be found within HIV-1 and FIV infected resting T cells (Tomonaga et al., 1995, Lassen et al., 2004).

To assess the contribution of *de novo* infection to viral nucleic acid production and the virus titre in the culture supernatant, a parallel experiment was performed using IL-2 supplemented, FIV infected MYA-1 T cells in the presence of the retroviral integrase inhibitor Raltegravir (RGV) and the reverse transcriptase inhibitor Zidovudine (AZT) at concentrations previously shown to be effective against FIV (Savarino et al., 2007, Bisset et al., 2002). The antiretroviral drugs were added 24 hours post infection, which would allow virus production from cells infected during the initial inoculation but would block any subsequent reverse transcription and integration. Results showed that similar to the IL-2- depleted cells, the levels of viral p24 in the supernatant, as well as the production of intracellular FIV DNA and RNA were noticeably reduced in

antiretroviral treated cells compared with untreated IL-2 supplemented cells (Fig. 4-7A-C). Intracellular FIV DNA and RNA levels of the RGV+AZT treated cells reached a plateau by day 4 post infection (Fig. 4-7B and C). However, the relative quantity of intracellular FIV DNA and RNA was higher in the antiretroviral treated cells compared to the IL-2-depleted cells, which is a further indication of a latent infection within the IL-2- depleted cells.

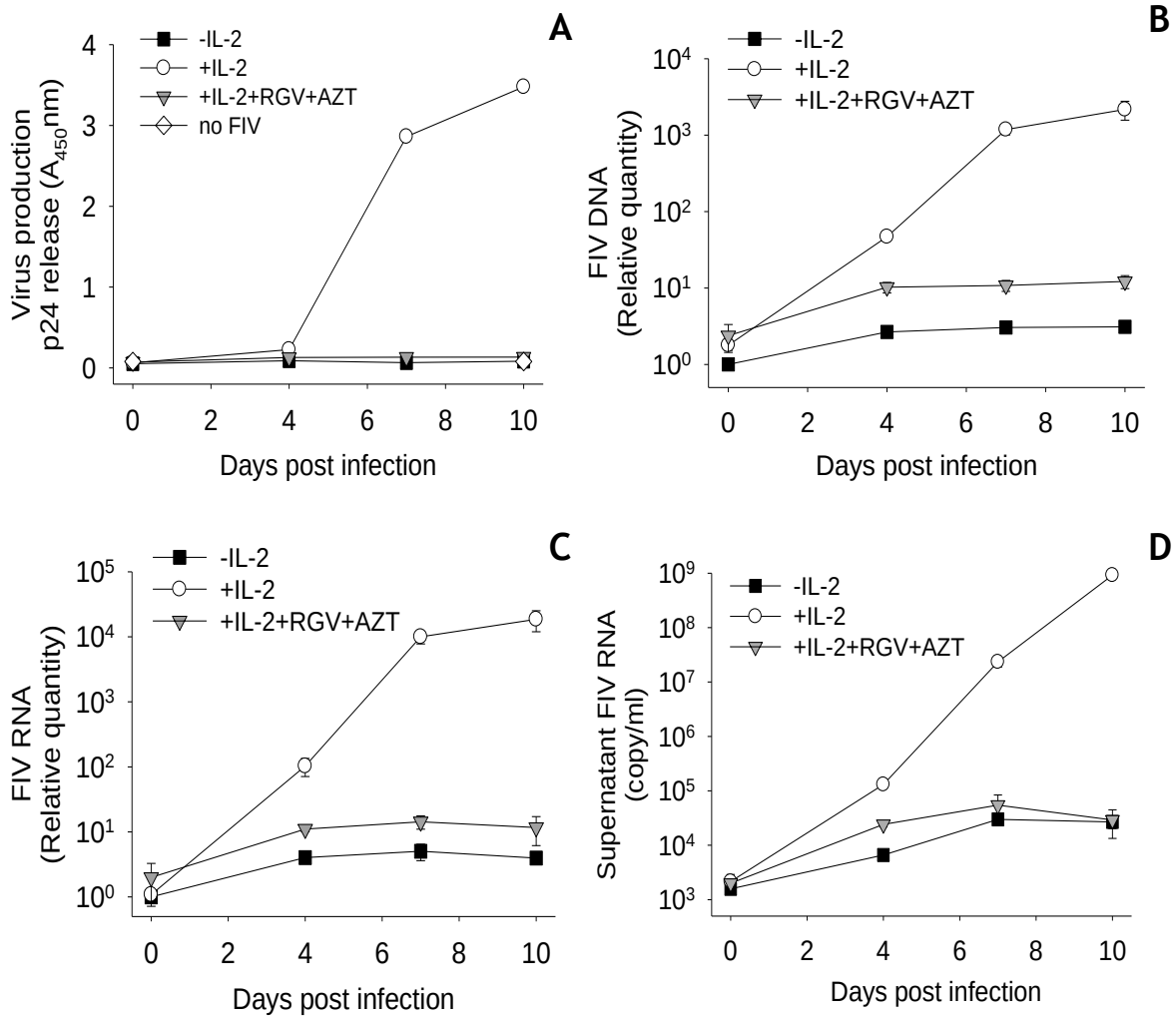


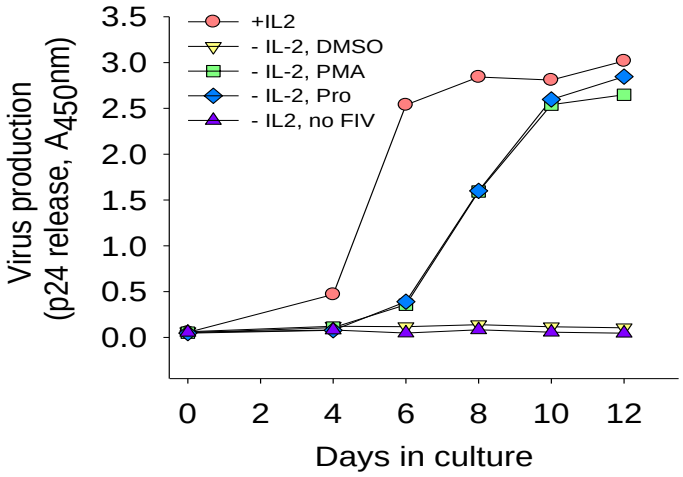
Figure 4-7 – A low level of persistent FIV replication can be detected in IL-2-depleted resting MYA-1 T cells. (A) Virus production was not detected from IL-2- depleted cells infected with FIV GL8 (M.O.I. = 0.01) or from infected, IL-2 supplemented cells treated with RGV (1 μM) and AZT (5 μM) at 24 hours post infection during the entire 10 day study period using the FIV p24 ELISA. The relative quantities of intracellular FIV DNA (B) and intracellular unspliced RNA (C) from approximately $1x10^6$ cells were quantified by qPCR. Samples for day 0 were taken at 2 hours post infection. Data was analysed using the ΔΔ Ct method, normalised to the amount of cellular 18S rRNA detected and relative to the average ΔCt values of the IL-2-depleted cells (-IL-2) at Day 0 post infection. (D)

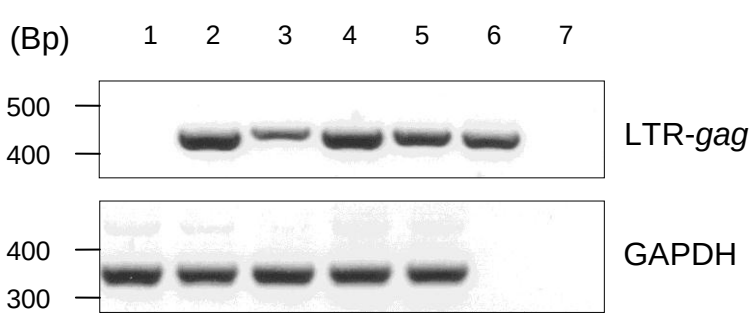
The quantities of FIV unspliced RNA in the culture supernatant over 10 days were measured by qPCR. Each point represents mean +/- standard deviation (n=3) of the calculated relative quantities/copy number for the qPCR data and mean +/- standard error (n=3) for the p24 ELISA data. Results are representative of two independent experiments.

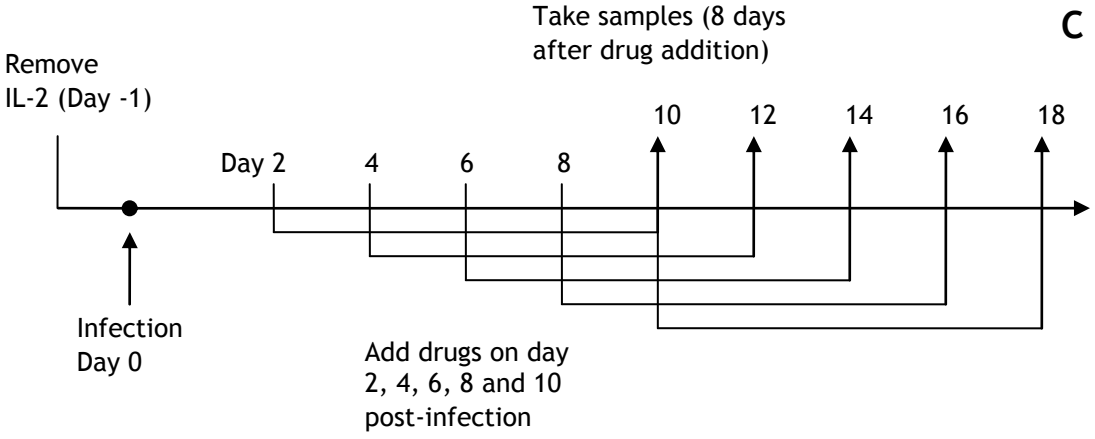
As shown previously, using the FIV p24 ELISA no virus production was detected from the infected IL-2-depleted MYA-1 T cells. However, the ELISA was not sensitive enough to detect the low level ongoing viral replication from the infected IL-2 supplemented cells that were treated with antiretrovirals (Fig. 4-7A). Thus the more sensitive qPCR was used to check whether ongoing productive infection was occurring among the IL-2-depleted cells. A background of fewer than 2000 copies of FIV RNA per ml was detected at the start of the experiment, which represented the virions from the initial inoculation that had remained behind after washing (Fig. 4-7D). The quantity of FIV RNA in the culture supernatant of the IL-2-supplemented infected cells rose dramatically during the experiment, reaching almost $1x10^9$ copies/ml by day 10 post infection (Fig. 4-7D), which is consistent with the results from FIV p24 ELISA. In contrast, a low level of virus production was occurring in the IL-2-depleted cells, peaking at approximately 30,000 copies/ml of culture supernatant by day 7 post-infection (Fig. 4-7D). During the same period the rate of virus production from IL-2-supplemented infected cells treated with RGV and AZT was also much lower compared with IL-2-supplemented cells not treated with antiretrovirals, reaching a maximum of approximately 55,000 copies/ml of culture supernatant 7 days post infection. However, this level of virus production was initially higher than the rate of virus production from IL-2-depleted cells. Virus production from the antiretroviral-treated cells began to decline 7 days post infection. Since *de novo* FIV infection was blocked by antiretrovirals, this could be an indication of the cytopathic effects of productive infection. The above data indicate that rather than establishing a latent infection whereby the infected MYA-1 T cells entered a quiescent state that led to the complete shutdown of viral production, low-level productive infection was still occurring while new infection of resting MYA-1 T cells was blocked completely.

4.2.2 Effect of phorbol esters on resting MYA-1 T cells infected with FIV

Stimulation of the FIV-infected, IL-2-depleted cells with either PMA or Prostratin post infection induced virus production from day 6 post infection onward, reaching a peak similar to the level reached by cells in the presence of IL-2 (Fig. 4-8A). Due to the loss of cell viability, the IL-2-depleted MYA-1 T cells were left unstimulated for no more than 10 days post infection during our assays. To examine the ability of Prostratin to stimulate productive infection from the resting MYA-1 T cells during this 10 day period, parallel cultures of FIV-infected, IL-2-depleted cells were stimulated with Prostratin at 2, 4, 6, 8 or 10 days post infection and cultured for an additional 8 days post-stimulation. Virus production could be induced at all time points post infection and up to 10 days post infection (Fig. 4-8C and D). Standard PCR was used to confirm the presence of FIV DNA within the total extracted cellular DNA of unstimulated, resting MYA-1 T cells, although it should be noted that this PCR did not confirm the integration of the provirus (Fig. 4-8B).


A
+IL2
- IL-2, DMSO
- IL-2, PMA
- IL-2, Pro
- IL2, no FIV
Virus production (p24 release, A450nm)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
Days in culture


B
(Bp)
1
2
3
4
5
6
7
500
400
LTR-gag
400
300
GAPDH


C
Take samples (8 days after drug addition)
Remove IL-2 (Day -1)
Day 2
4
6
8
10
12
14
16
18
Infection Day 0
Add drugs on day 2, 4, 6, 8 and 10 post-infection

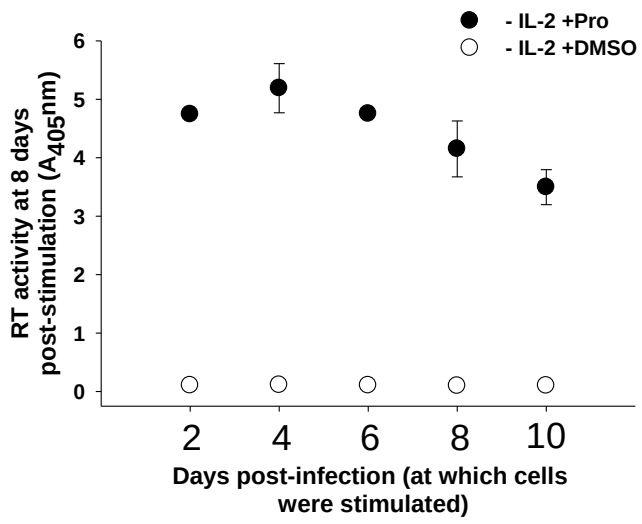

D
- IL-2 +Pro
- IL-2 +DMSO
RT activity at 8 days post-stimulation (A405nm)
6
5
4
3
2
1
0
2
4
6
8
10
Days post-infection (at which cells were stimulated)

Figure 4-8 – The phorbol esters PMA and Prostratin can stimulate productive infection from resting MYA-1 T cells. (A) MYA-1 $CD4^+$ T cells were seeded in the presence or absence of IL-2 and 24 hours post-seeding the cells were infected with FIV GL8 (M.O.I. = 0.01) and virus production was monitored by p24 ELISA. Four days post infection, the IL-2-depleted cells were stimulated with either PMA (0.08 μM), Prostratin (Pro - 1 μM) or mock stimulated with solvent (DMSO). (B) Detection of FIV DNA in latently infected cells. DNA was extracted from FIV infected cells and screened by PCR for either a 466 bp FIV LTR-*gag* product or a GAPDH control. Lanes: (1) -FIV, +IL2; (2) +FIV,+IL-2; (3) +FIV, -IL-2, +DMSO; (4) +FIV, -IL-2, +PMA; (5) +FIV -IL-2, +Prostratin, (6) FIV plasmid positive control, (7) dH_20 negative control. (C) MYA-1 $CD4^+$ T cells depleted of IL-2 supplement for 24 hour were infected with FIV GL8 (M.O.I. = 0.01) and maintained in the absence of IL-2 for 2, 4, 6, 8 or 10 days post-infection before stimulation with Prostratin. Cells were incubated for a further 8 days before culture supernatant samples were harvested and stored at -80^oC. Virus production was quantified by a non-isotopic reverse transcriptase (RT) assay (D). Each point represents the mean +/- standard error (n=3) and all results are representative of three independent experiments.

Next, the contribution of *de novo* infection to virus production following PMA and Prostratin treatment was assessed by stimulating the cells with the phorbol esters in the presence of RGV. Virus production was detected using a reverse transcriptase activity assay, which showed a dramatic reduction in virus production among RGV treated, PMA and Prostratin stimulated cells compared with cells not treated with RGV (Fig. 4-9A and B). The low level of FIV production in the presence of RGV was consistent with a similar experiment with HIV-1(Yang et al., 2009) and indicated that both phorbol esters stimulate FIV production from integrated provirus but that following stimulation, the majority of virus production then results from the *de novo* infection of adjacent cells.

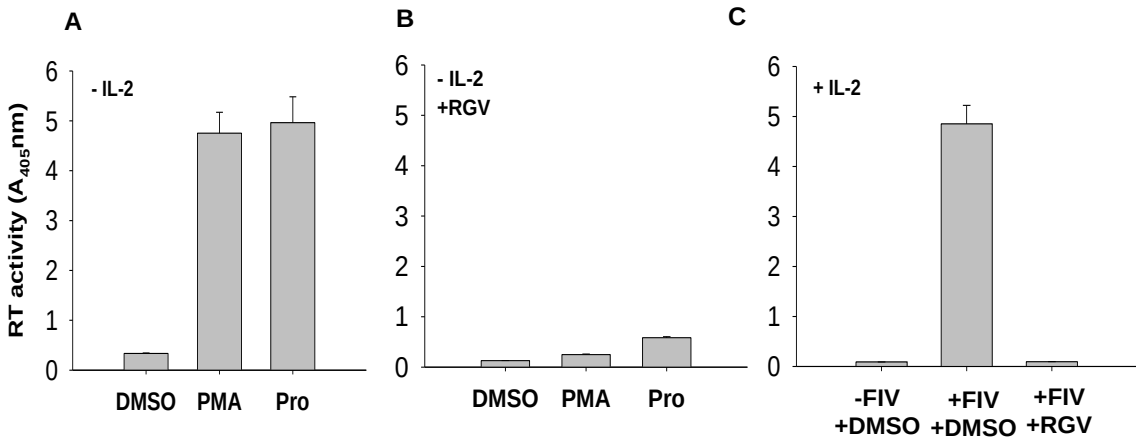


Figure 4-9 – Reactivation of integrated provirus by phorbol esters. (A) IL2-depleted MYA-1 $CD4^+$ T cells were infected with FIV GL8 (M.O.I. = 0.06). Two days post infection cells were treated with PMA (0.08 μM), Prostratin (Pro – 1 μM) or mock-treated (DMSO). Supernatant was collected at day 10 post infection and virus production was quantified by a non-isotopic reverse transcriptase (RT) assay. (B) Parallel experiment to (A) in which Raltegravir (RGV – 1 μM) was added to the FIV-infected cells 24 hours post-infection (24 hours prior to treatment with PMA or Pro). (C) Control experiment demonstrating the potency of RGV at blocking productive infection with FIV. RGV (1 μM) added to IL-2-supplemented cells in the presence 24 hours prior to infection with FIV and was maintained post-infection. Each bar represents the mean +/- standard error (n=3) and representative of three independent experiments.

PCR amplifying the LTR to *gag* region of FIV cannot distinguish between integrated or unintegrated viral DNA. *Alu*-PCR, which involves the use of a pair of primers that bind to the highly repetitive chromosomal *Alu* elements and within HIV-1 sequence, is a popular method to detect integrated HIV-1 sequences (Yamamoto et al., 2006, Han et al., 2007). Unfortunately, no suitable repeat element had been discovered in the cat genome at the time of writing to develop a similar assay.

Unintegrated viral DNA, by its nature, is low in molecular weight compared with genomic DNA and should remain as physically separate entities. An attempt was made to demonstrate the presence of integrated provirus by separating out the high molecular weight (HMW) genomic DNA from the low molecular weight (LMW) DNA containing unintegrated viral DNA. The Hirt Protocol (Hirt, 1967),

which was originally conceived to isolate Polyoma viral DNA from infected cells without contamination by genomic DNA, was modified for this purpose. $5x10^6$ MYA-1 $CD4^+$ T cells were infected with FIV GL8 (MOI 0.01) with/without pre-incubation by the integrase inhibitor RGV. The cells were then grown for five days and washed with PBS. The cell pellet was resuspended in 1 ml of Hirt Lysis buffer (0.6% sodium dodecyl sulphate - SDS, 10 mM EDTA pH 7.5) and 250 µl of 5M NaCl was then added. The mixture was kept at 4°C overnight and centrifuged at 13,000 rpm for 45 minutes at 4°C. The supernatant, which contained the LMW unintegrated viral DNA, was removed and kept as a control. The HMW DNA pellet was precipitated by SDS and was washed once by PBS before being dissolved in 500 µl of 10mM EDTA. DNA from both the LMW and HMW portions were extracted using the phenol-chloroform method. PCRs that amplify a region between the FIV 5'LTR and *gag* (which detects both integrated and unintegrated viral DNA) and 1 or 2-LTR circles (which detects unintegrated viral DNA only) were performed to confirm the presence of FIV DNA and to check for contamination of the HMW DNA by unintegrated LTR-circular DNA. A sample of HMW DNA containing integrated FIV provirus should be positive for FIV LTR-*gag* but not for FIV circles. Cells pre-incubated with RGV should be positive for FIV LTR-*gag* only in the LMW samples, because RGV should not block reverse transcription and would still produce unintegrated FIV DNA species.

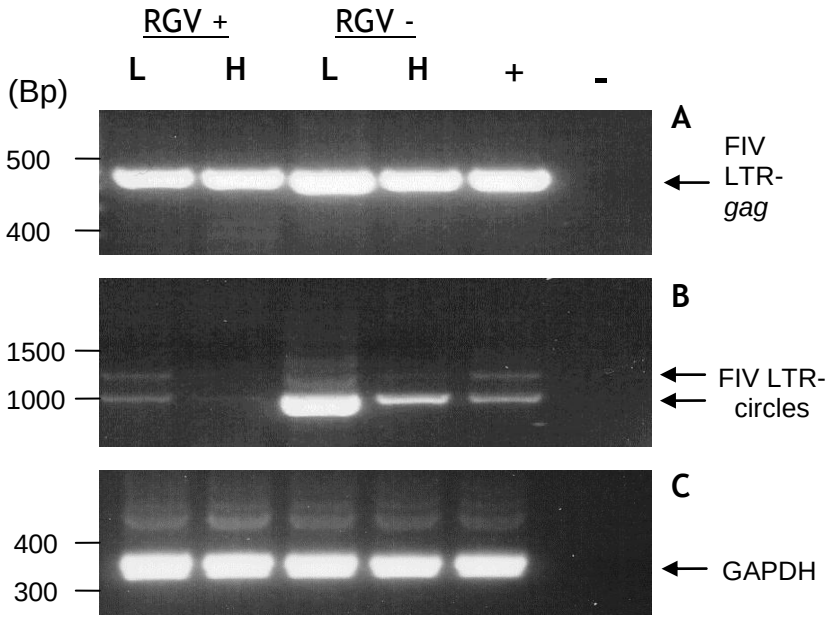


Figure 4-10 – Unintegrated FIV DNA contaminates genomic High molecular weight DNA. (A) FIV LTR-*gag*, (B) FIV LTR-circles and (C) GAPDH control PCR of DNA samples extracted from infected MYA-1 $CD4^+$ T cells using the modified Hirt method. L = LMW DNA. H = HMW DNA. (+) = Positive control is total DNA of productively infected MYA-1 $CD4^+$ T cells. (-) = dH_2O control. RGV = Raltegravir (1 μM).

Results showed that all the samples, including the RGV HMW, were positive for FIV LTR-*gag* (Fig. 4-10A). The presence of the FIV LTR-*gag* sequence from the HMW DNA of cells treated with RGV could be explained by the presence of contaminating unintegrated LTR-circles (which can be amplified by the FIV LTR-*gag* primer set), rather than the failure of the antiretroviral drug (Fig. 4-10B). Further improvements of the method were made. The HMW DNA pellet was washed 2 more times with PBS before being dissolved by EDTA. The phenol-chloroform extraction step was replaced by the use of Qiagen DNA extraction columns to simplify the protocol. To extract LMW DNA from the Hirt supernatant, 3 volumes of the Qiagen QG buffer was added to correct the pH and salt concentration for DNA extraction using the Qiagen Gel Extraction columns. To extract the HMW DNA dissolved in EDTA, the lysis buffer from the Qiagen DNA Mini kit was added to correct the pH and salt concentration and the samples were processed using the DNA Mini columns according to the manufacturer's protocol. The quality of the DNA extracted by the columns was good (determined using the Nanodrop spectrophotometer – data not shown). However, the contamination of the HMW DNA by FIV circles persisted (Fig 4-

11B), albeit at a lower level than recorded before, as shown by the weaker FIV *gag* product for the RGV$^+$ HMW sample. Due to time constraints I was unable to optimise this method of isolating HMW DNA further.

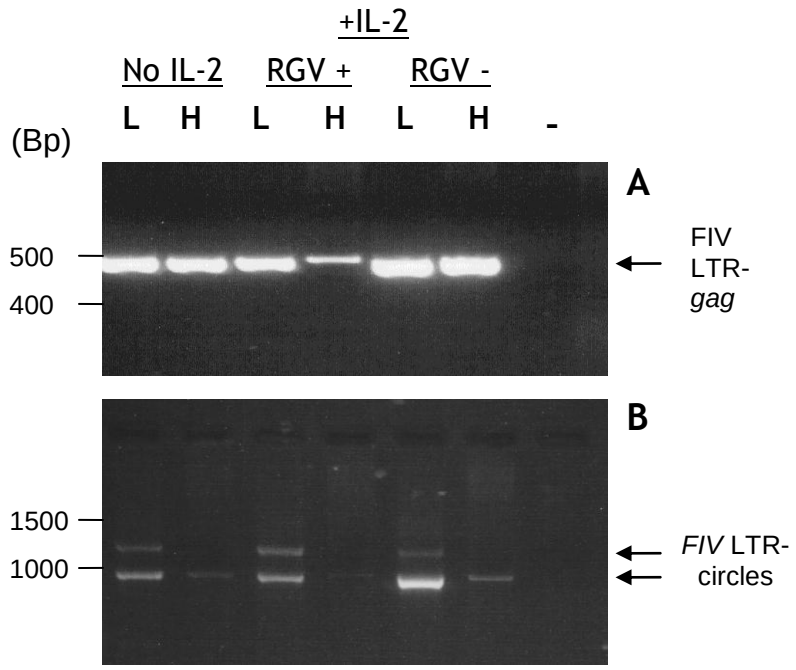
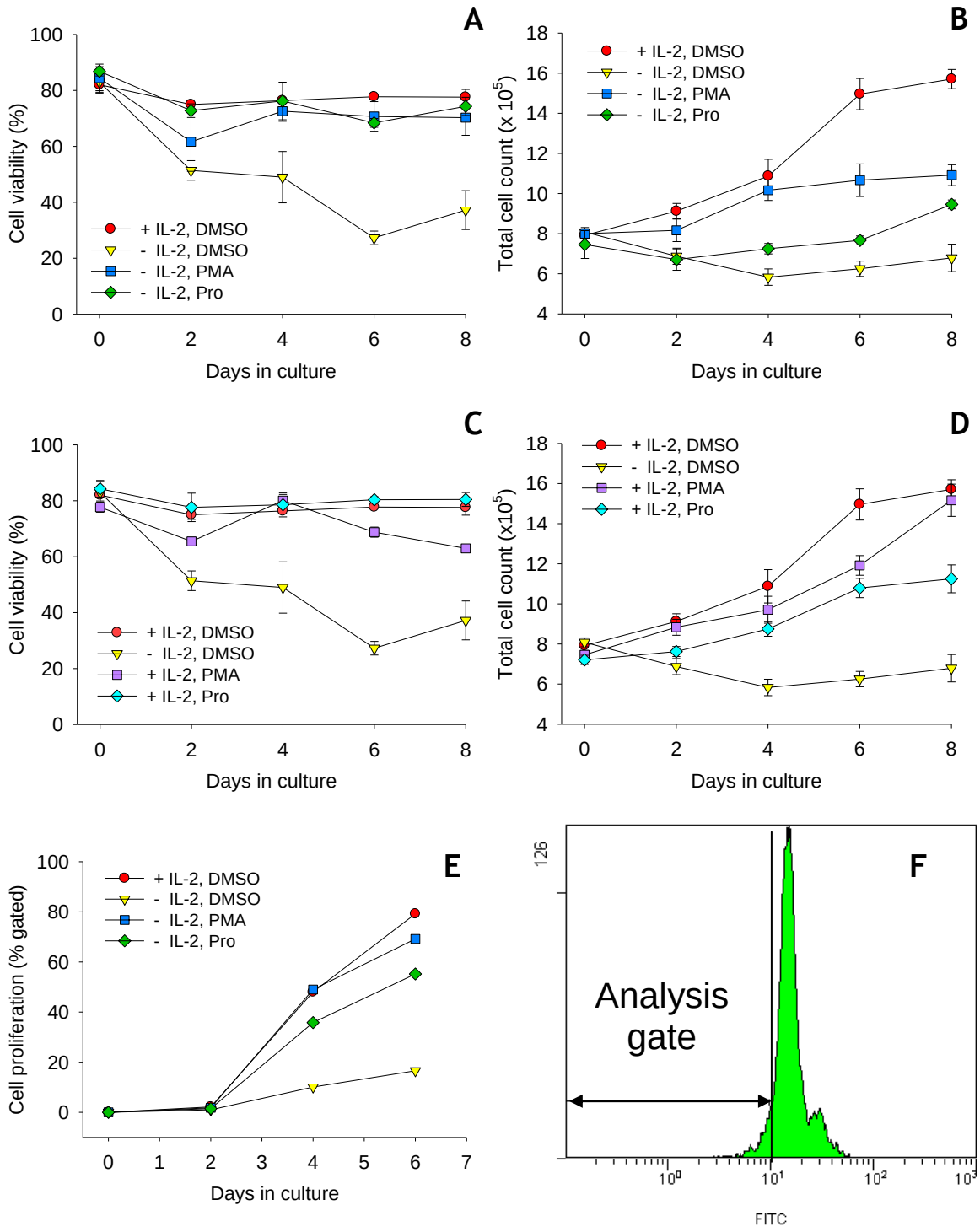


Figure 4-11 – Improvement in DNA purification reduces contamination of HMW DNA by unintegrated FIV DNA. FIV LTR-*gag* (A) and FIV circles (B) PCR of DNA samples extracted from cells using the modified Hirt method. L = LMW DNA. H = HMW DNA. No IL-2 = latently infected cells. (-) = Negative control (total DNA from uninfected MYA-1 CD4$^+$ T cells). RGV = Raltegravir (1 μM).

4.2.3 Effect of phorbol ester on cell growth and viability

PMA is a recognised tumour-promoting agent (Hecker et al., 1964) and thus would not be suitable for *in vivo* use. However, previous studies have indicated that Prostratin lacks the tumour-promoting activity associated with PMA (Szallasi and Blumberg, 1991), indicating a potential for clinical use in the reactivation of latent lentiviral reservoirs. Given that both PMA and Prostratin reactivated latent infection of MYA-1 CD4$^+$ T cells with FIV, the effects of both agents on cell growth and viability were examined. The addition of either PMA or Prostratin maintained cell viability in IL-2-depleted cells (Fig. 4-12A), viability remaining stable approximately 80% in treated cultures compared with untreated cultures where viability dropped sharply to 40% over the 8 day study period. Over this period, both PMA and Prostratin triggered a modest expansion of the cells,

reflected in an increase in cell number, most notably with PMA where cell number expanded from $8x10^5$/ml to $1x10^6$/ml. The addition of exogenous IL-2 resulted in a doubling of cell number over an eight-day period to $1.6x10^6$/ml (Fig. 4-12B). The addition of either PMA or Prostratin in the presence of IL-2 had little effect on cell viability although a modest reduction in viability was noted with PMA (Fig. 4-12C). In the presence of IL-2, both PMA and Prostratin reduced cell growth, the effect with Prostratin being more evident and the treated cultures achieving a final density of $1.1x10^6$/ml compared with $1.6x10^6$/ml in the untreated cultures (Fig. 4-12D). When IL-2-depleted cells were loaded with CFSE as a mean of tracking cell division, the proliferation enhancing and cytoprotective properties of PMA and Prostratin was confirmed (Fig. 4-12E,F), a dramatic shift in fluorescence evident in the PMA and Prostratin treated cultures and mirroring the effect of adding exogenous IL-2. These data support previous observations describing the cytoprotective properties of Prostratin (Gustafson et al., 1992, Korin et al., 2002). Curiously, stimulation of the feline MYA-1 $CD4^+$ T cells with PMA or Prostratin resulted in the acidification of the medium in the presence or absence of exogenous IL-2 (Fig. 4-12G, H). Acidification is usually associated with cell growth and metabolism, as cells without IL-2 supplement do not cause acidification (Fig. 4-12H). This result may indicate that PMA and Prostratin can stimulate cellular metabolism without causing cell growth. The addition of the phorbol esters to the culture medium in the absence of cells did not cause a change in pH over the same experimental period (Fig. 4-12I).


A
Cell viability (%)
Days in culture
+ IL-2, DMSO
- IL-2, DMSO
- IL-2, PMA
- IL-2, Pro
B
Total cell count (x 10^5)
Days in culture
+ IL-2, DMSO
- IL-2, DMSO
- IL-2, PMA
- IL-2, Pro
C
Cell viability (%)
Days in culture
+ IL-2, DMSO
- IL-2, DMSO
+ IL-2, PMA
+ IL-2, Pro
D
Total cell count (x10^5)
Days in culture
+ IL-2, DMSO
- IL-2, DMSO
+ IL-2, PMA
+ IL-2, Pro
E
Cell proliferation (% gated)
Days in culture
+ IL-2, DMSO
- IL-2, DMSO
- IL-2, PMA
- IL-2, Pro
F
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Analysis gate
FITC
+ IL-2
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G
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DMSO
PMA
Pro
Pro
PMA
DMSO

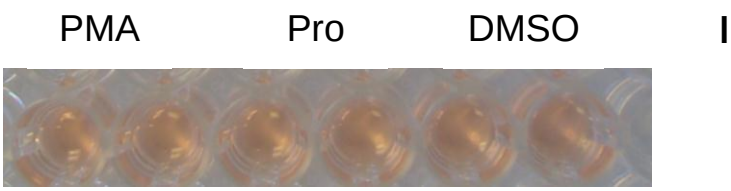


Figure 4-12 – Effect of phorbol esters on cell growth and viability. The effect of PMA and Prostratin on the growth and viability of MYA-1 $CD4^+$ T cells was examined in the presence or absence of IL-2. (A) Cell viability and (B) total cell count of IL-2-depleted cells stimulated with PMA (0.08 μM), Prostratin (Pro – 1 μM) or mock stimulated with solvent (DMSO) compared with IL-2-supplemented (100 U/ml) cells treated with DMSO. (C) Cell viability and (D) total cell count of IL-2-supplemented cells stimulated with PMA or Prostratin (Pro) or mock stimulated with solvent (DMSO) compared with IL-2-depleted cells treated with DMSO. Each point represents the mean +/- standard error (n=3) and representative of three independent experiments. (E) CFSE assay comparing proliferation of IL-2-depleted cells treated with PMA, Prostratin, DMSO, or control cells treated with IL-2. Percentage of proliferating cells was estimated as the percentage of cells with fluorescence values lower in the gated region designated in (F), arbitrarily set at a value of 10%. Results are representative of three independent experiments. The effect of stimulation by phorbol esters on the pH of the culture medium was monitored. RPMI medium contains the pH indicator Phenol red, which would turn yellow if the pH of the solution decreases and would turn bright pink if pH increases. Cells were incubated with DMSO, PMA or Prostratin in the presence (G) or the absence (H) of IL-2 for 8 days. (I) The phorbol esters were added to culture medium and incubated at the same conditions as the cells during the experiment to account for any change in pH due to possible chemical reaction of the phorbol esters with the constituents of the medium.

4.2.4 Reactivation of productive infection from resting MYA-1 $CD4^+$ T cells required PKC-mediated signalling

Phorbol esters such as Prostratin are potent activators of the PKC pathway in human cells and their HIV-1 reactivating effects have been shown to depend on this pathway (Castagna et al., 1982, Kikkawa et al., 1983, Williams et al., 2004). I therefore asked whether the specific inhibitor of PKC "Gö6850", a compound that targets both conventional and novel PKC isoforms, would inhibit the FIV stimulating effects of Prostratin *in vitro*. The application of Gö6850 (at a concentration of 2.5 μM, which should inhibit most novel and conventional isoforms of PKC (Martiny-Baron et al., 1993)) completely blocked the stimulation of productive FIV infection by Prostratin in IL-2-depleted resting MYA-1 $CD4^+$ T cells (Fig. 4-13A). The ability of exogenous IL-2 added post infection to reactivate viral replication from infected resting cells was similarly abrogated by Gö6850 (Fig. 4-13A), confirming a common mechanism of action of Prostratin and IL-2 in the stimulation of FIV replication and spread. Treatment with Gö6850 also reversed the cytoprotective effect of Prostratin in IL-2-depleted cells, suggesting a correlation between the maintenance of cellular viability and the induction of viral growth (Fig. 4-13B) and consistent with the vital modulatory role of PKC in T cell activation and IL-2-dependent signalling (Isakov and Altman, 2002) (Tan and Parker, 2003).

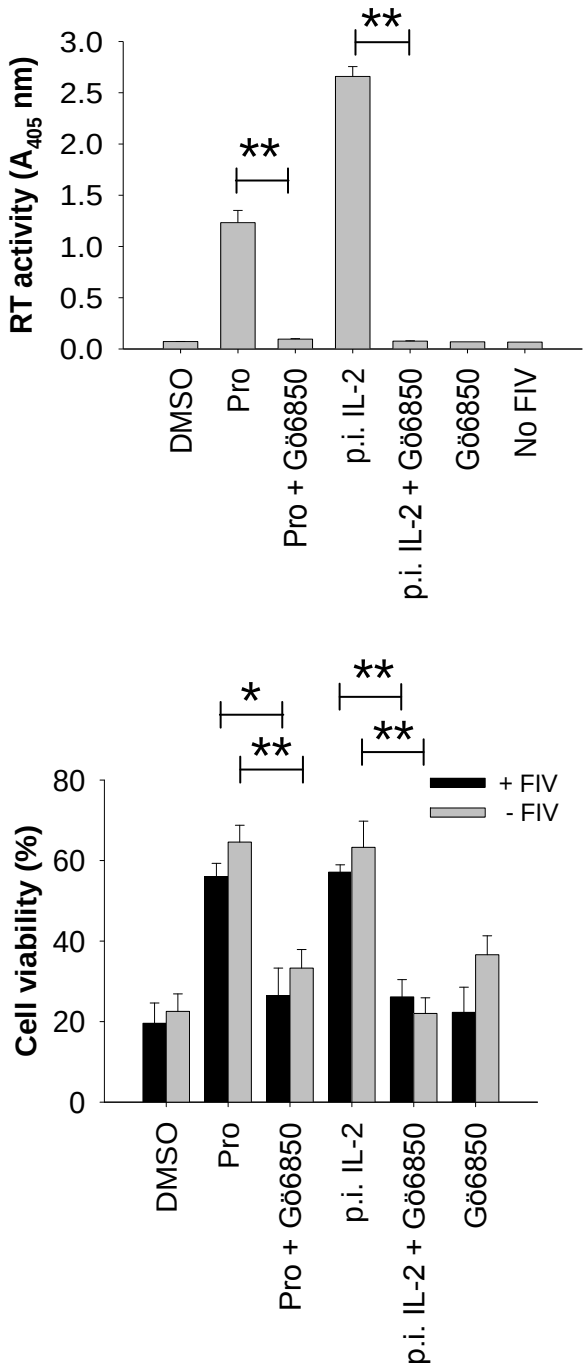


Figure 4-13 – Reactivation of productive infection from infected resting MYA-1 T cells is PKC-dependent. IL-2-depleted MYA-1 cells were infected with FIV GL8 (M.O.I. = 0.01) and virus production was monitored by a non-isotopic reverse transcriptase (RT) assay (A). Cells were treated with DMSO, Prostratin (Pro – 1 μM), Prostratin + the PKC inhibitor Gö6850 (2.5 μM), IL-2 (IL-2 100 U/ml), IL-2 + Gö6850 or Gö6850 alone 2 days post-infection with FIV. (B) Cell viability in the treated cells in the presence or absence of FIV infection as measured 8 days post-infection. (p.i.) indicates IL-2 was added post infection. Each bar represents the mean +/- standard error (n=3) and results are representative of three independent experiments (One Way ANOVA *p<0.05; **p<0.01)

4.2.5 Possible synergistic reactivation of productive FIV infection from resting MYA-1 T cells by Prostratin and the HDAC inhibitor valproic acid

As well as the regulation of PKC, the modulation of histones and nucleosomes has been shown to be important to HIV-1 latency (Reviewed in (Colin and Van Lint, 2009) and (Coiras et al., 2009)). The use of Histone deacetylase (HDAC) inhibitors to reactivate latent virus *in vitro* and in clinical trials had been performed before (Van Lint et al., 1996, Lehrman et al., 2005). Recently, a study has found that reactivation is enhanced by the use of both Prostratin and HDAC inhibitors (Reuse et al., 2009). Thus an experiment was carried out to investigate the effects of two HDAC inhibitors, valproic acid and sodium butyrate, on the infected resting MYA-1 T cells. Results showed that the application of the two HDAC inhibitors on their own did not have any stimulatory effects on virus replication. However, when valproic acid was administered together with Prostratin, an enhancement of Prostratin's activity was observed (Fig. 4-14). Both valproic acid and sodium butyrate exhibited cytotoxic effects, but viability of cells treated with valproic acid was rescued by Prostratin (Fig. 4-14B). However, due to time and resource constraints no further repeats were performed to confirm these findings.

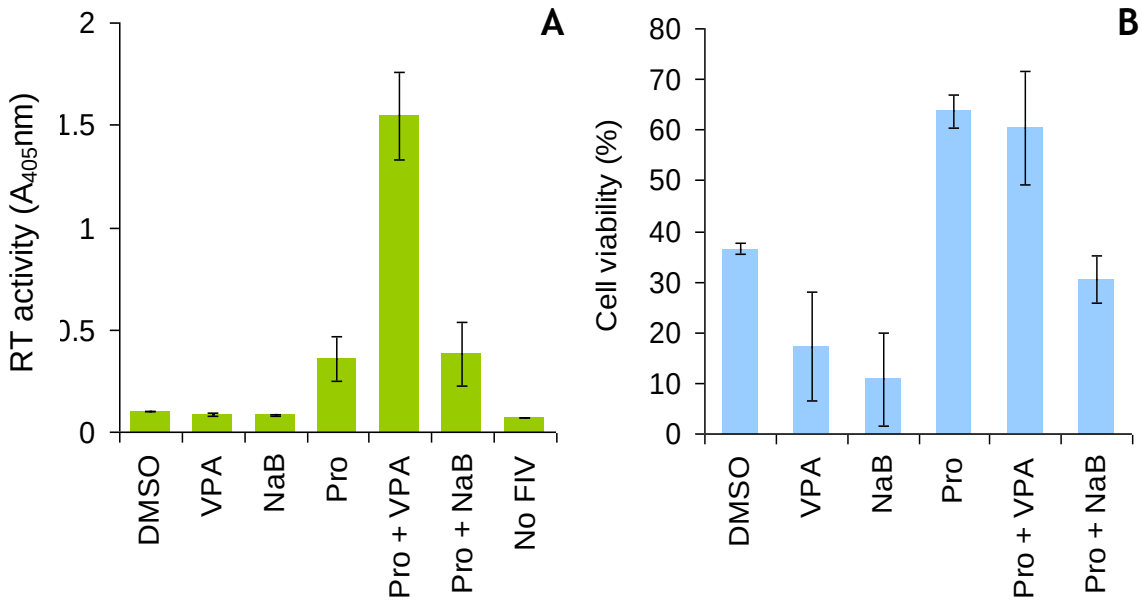


Figure 4-14 – Valproic acid enhances reactivation of productive FIV infection from infected resting MYA-1 T cells by Prostratin. IL-2-depleted MYA-1 $CD4^+$ T cells were infected with FIV GL8 and virus production was monitored in the culture supernatants by a non-isotopic reverse transcriptase (RT) assay at Day 7 post infection (A). Cells were treated with DMSO, valproic acid (VPA – 1.25 mM), sodium butyrate (NaB – 1.5 mM), Prostratin (Pro – 1 μM), Prostratin + valproic acid or sodium butyrate. (B) Cell viability measurements were carried out on day 7 post infection. Each bar represents the mean +/- range (n=2).

4.3 Part 2 – Phorbol esters inhibit productive FIV infection

Both PMA and Prostratin have been shown by multiple studies to have anti- HIV-1 replication activity (Gustafson et al., 1992, Gulakowski et al., 1997, Kulkosky et al., 2001, Biancotto et al., 2004, Rullas et al., 2004, Warrilow et al., 2006). The paradoxical dual effect of activating latent lentiviral infection and inhibiting productive lentiviral replication exhibited by the phorbol esters makes them potential candidates for therapeutic use in anti-latency treatment. In this part of the thesis I investigate whether phorbol ester Prostratin could also inhibit productive FIV infection.

4.4 Results

4.4.1 Prostratin significantly blocked productive FIV infection and this was mediated by PKC

Actively growing, IL-2 supplemented feline $CD4^+$ MYA-1 T cells were infected with FIV and 2 days post infection Prostratin was added to the cultures. Supernatant samples were taken at day 8 post infection and virus titre was measured by a reverse transcriptase activity assay. The addition of Prostratin significantly reduced FIV production by more than 50% (Fig. 4-15A, C). Furthermore, the inhibition of virus production was reversed by the simultaneous treatment of the cells with Gö6850, suggesting that the inhibitory effect required PKC-dependent signalling. An assessment of cell viability (Fig. 4-15B) indicated that Prostratin did not reduce the number of viable cells and thus the inhibitory effect on virus growth was not the result of a non-specific toxic effect. Importantly, the addition of Gö6850 on its own did not increase productive infection relative to mock stimulated cells and in two of the four independent experiments Gö6850 reduced FIV production as well as the viability of infected cells (Fig. 4-15C, D).

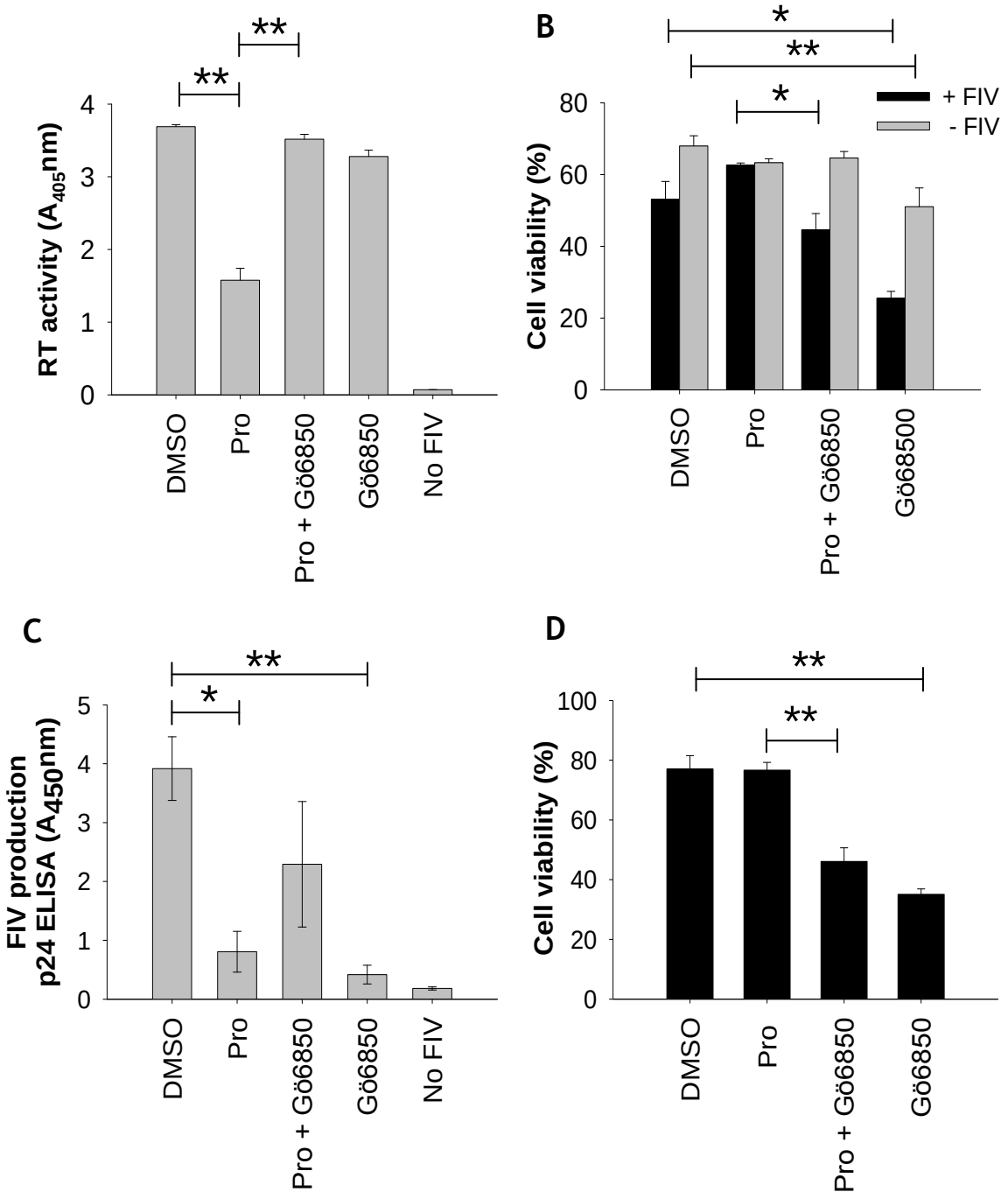


Figure 4-15 – Inhibition of productive infection with FIV by Prostratin in the presence of IL-2 is PKC dependent. MYA-1 $CD4^+$ T cells were cultured in the presence of IL-2 and infected with FIV GL8 (M.O.I. = 0.01). 2 days post-infection, Prostratin (Pro – 1 μM)), Prostratin and Gö6850 (2.5 μM), Gö6850, or DMSO were added to the cells 2 days post-infection. At day 8 post-infection, (A) Virus production was quantified in the culture supernatant by non-isotopic reverse transcriptase assay while (B) the viability of infected and uninfected cells was assessed by Trypan Blue exclusion. Each bar represents the mean +/- standard error (n=3) and is representative of 2 independent experiments. In separate experiments, virus production was quantified at day 8 post-infection by FIV p24 ELISA (C) and the viability of FIV infected cells were assessed by Trypan Blue

exclusion (D). Each bar represents the mean +/- standard error (n=4) and the data are the combination of two independent experiments (One Way ANOVA *p< 0.05; **p<0.01)

4.4.2 Manipulation of NFκB has no effect on productive infection with FIV

Earlier, in an attempt to establish latent infection various NFκB inhibitors were incubated with IL-2 supplemented feline MYA-1 $CD4^+$ T cells and surprisingly the inhibitor SC514 enhanced FIV GL8 production (Fig. 4-2A). If this could be replicated it may illustrate new principles governing host-pathogen interactions. MYA-1 $CD4^+$ T Cells were infected with FIV GL8 and incubated with different concentrations (5 μM – 20 μM) of SC514 and another NFκB inhibitor, JSH23, immediately after infection (JSH-23 was chosen because it interferes with a different step of the NFκB signalling pathway to SC514, namely the nuclear translocation process of NFκB (Shin et al., 2004) while SC514 is a selective IKK-2 inhibitor (Kishore et al., 2003)). Virus production during the subsequent 14 days was measured by FIV p24 ELISA. In the initial experiment, the titre of virus in the cell culture supernatant was doubled by the addition of 10 μM SC514 (Fig 4-2A). This was not replicated in subsequent experiments; rather the addition of SC514 did not increase the rate of virus production (Fig. 4-16A). The addition of JSH23 also did not increase the rate of virus production during the course of the experiment. Large errors were observed in the cultures incubated with NFκB inhibitors but not in DMSO solvent added culture. This persisted in every repeat experiment and the significance of this observation is unknown. If the large errors were ignored the data shows that the NFκB inhibitors actually reduced the rate of virus production during the early phase of the experiment, eventually reaching a plateau by day 10 post-infection. Based on the available data it was concluded that the application of the NFκB inhibitors SC514 and JSH23 does not have any FIV enhancement effect, contrary to my initial findings.

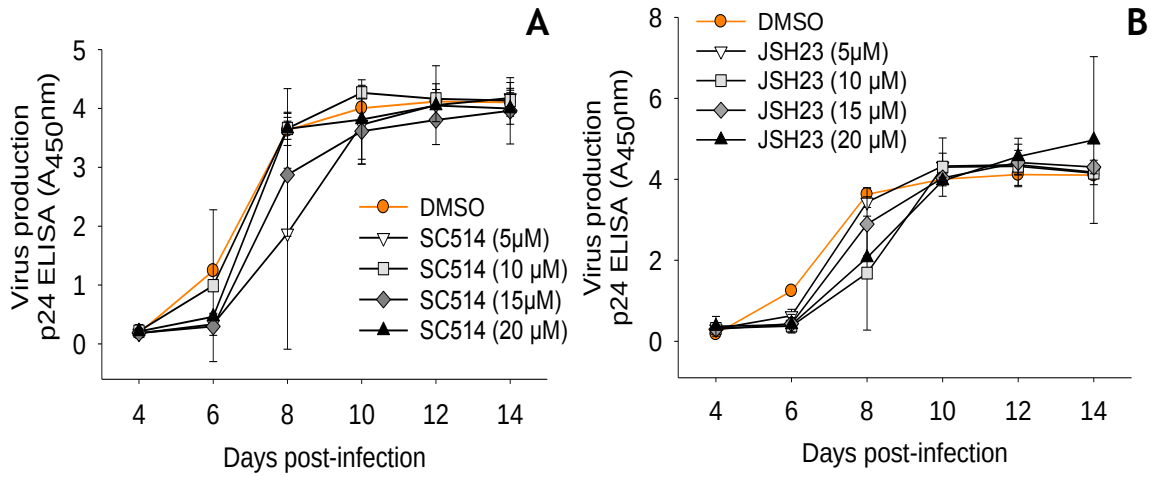


Figure 4-16 – NFκB inhibitors do not enhance productive infection contrary to initial findings. IL-2 supplemented feline MYA-1 $CD4^+$ T cells were infected with FIV GL8 (MOI = 0.001) for 2 hours, washed and incubated with the NFκB inhibitors SC514 (A) and JSH23 (B) at indicated concentrations. Samples of supernatant were taken at day 4, 6, 8, 10, 12 and 14 post-infection. Supernatant virus titre was measured by FIV p24 ELISA. Each point represents mean +/- range (n=2) and is representative of three independent experiments.

Previous studies have suggested that the inhibition of productive infection with HIV-1 by Prostratin is mediated by the down-regulation of viral primary receptor CD4 and co-receptor(s) CCR5 and CXCR4 (Kulkosky et al., 2001, Rullas et al., 2004, Warrilow et al., 2006). Thus the effect of Prostratin on the expression of both CD134 and CXCR4 (the FIV primary receptor and co-receptor respectively) on MYA-1 $CD4^+$ T cells in the presence of IL-2 was examined over a 7 day period. The expression of CD134 showed a general decline over the experimental period from ~70% to ~40% positive (Fig. 4-17A). The application of Prostratin to the cells had variable effects on the expression of CD134 between experiments, which was probably a reflection of the nature of the MYA-1 $CD4^+$ T cells as they cycle through phases of blasting, rapid expansion and then resting (Fig. 4-17A, C). However, when the cells were treated with Prostratin plus Gö6850 or Gö6850 alone the expression of CD134 was dramatically reduced to below the detection limit of flow cytometry (Fig. 4-17A, C). The expression of CXCR4 on the MYA-1 $CD4^+$ T cells was relatively low compared with CD134 (Fig. 4-17B, D). Again the effect of Prostratin on the expression of CXCR4 was variable between experiments, but crucially the expression of CXCR4 was completely down-regulated when the cells were treated with Gö6850 alone or with Prostratin plus

Gö6850, a treatment which rescued FIV replication from Prostratin-induced inhibition (Fig. 4-17B, D). Thus the ability of Prostratin to inhibit productive infection with FIV cannot be explained by the down-regulation of expression of either CD134 or CXCR4.

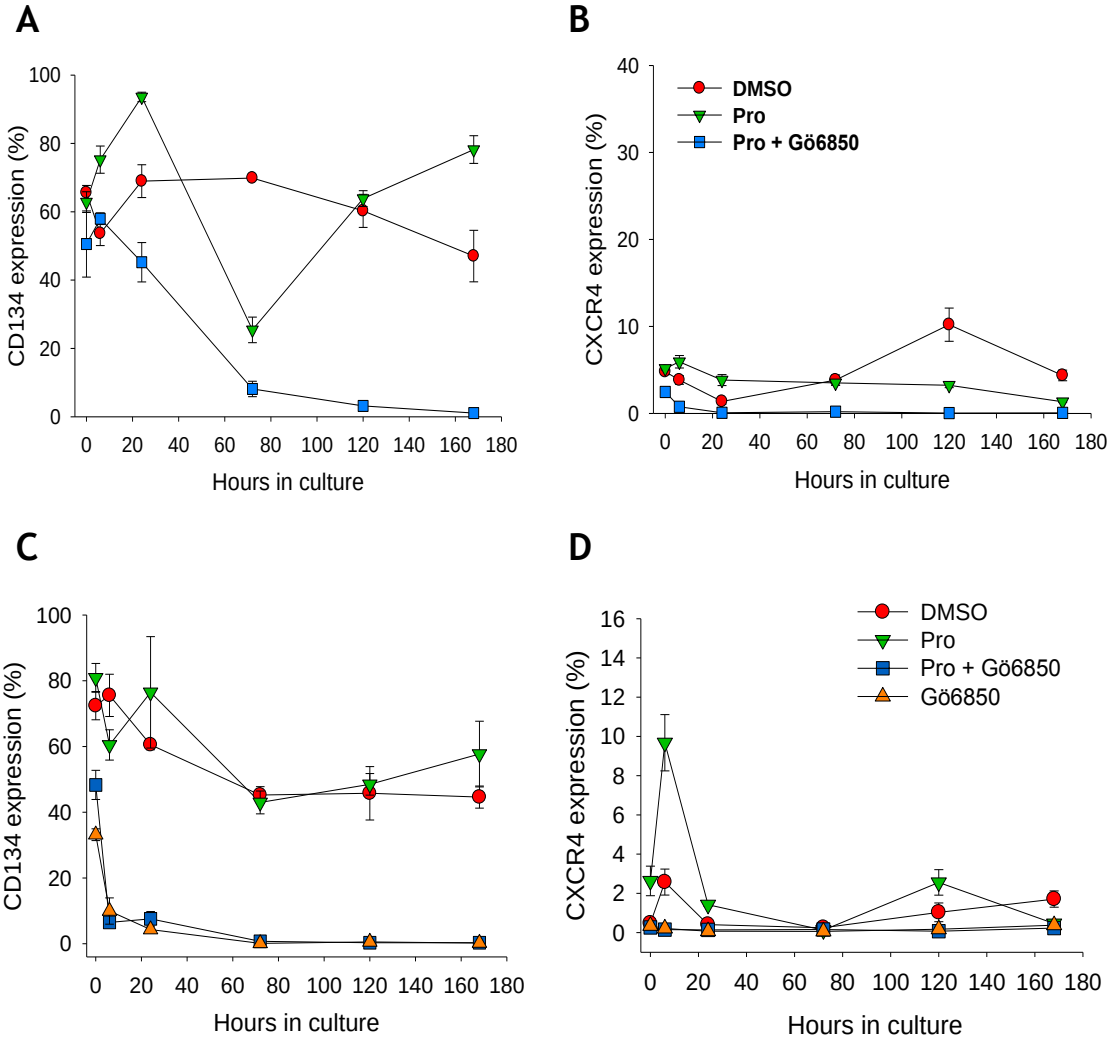


Figure 4-17 – Effect of Prostratin and Gö6850 on the expression of CD134 and CXCR4 on MYA-1 CD4$^+$ T cells. MYA-1 CD4$^+$ T cells were cultured in IL-2-supplemented medium in the presence of Prostratin (Pro – 1 μM), Prostratin plus Gö6850 (Pro + Gö6850 – 2.5 μM), or solvent (DMSO). Sequential samples were collected over a 7 day period and the expression of (A) CD134 and (B) CXCR4 was quantified by flow cytometry. Each point represents the mean +/- standard error (n=3) and is representative of three independent experiments. In an additional experiment the effect of applying Gö6850 alone on the expression of CD134 (C) and CXCR4 (D) was investigated. Each point represents the mean +/- standard error (n=3).

4.4.3 Pre-incubation with Prostratin did not reduce virus titre 8 days post infection

Next the effect of pre-incubation with Prostratin and the PKC inhibitor Gö6850 on virus production was investigated. MYA-1 $CD4^+$ T cells were pre-incubated with Prostratin, Gö6850 or Prostratin plus Gö6850 overnight before infection with FIV. Cells were then washed and resuspended in fresh medium plus exogenous IL-2 and sequential samples were supposed to be taken at day 4, 6 and 8 post infection. Due to constraints of resources only the samples from the end of the experiment at day 8 post infection were processed by reverse transcriptase activity assay. Results indicated that none of the treatments had reduced virus titre compared with solvent control at the end of the experiment (Fig. 4-18). However, since virus replication was not monitored throughout the experiment, a small defect in viral entry during the first round of infection might be masked by subsequent *de novo* infection.

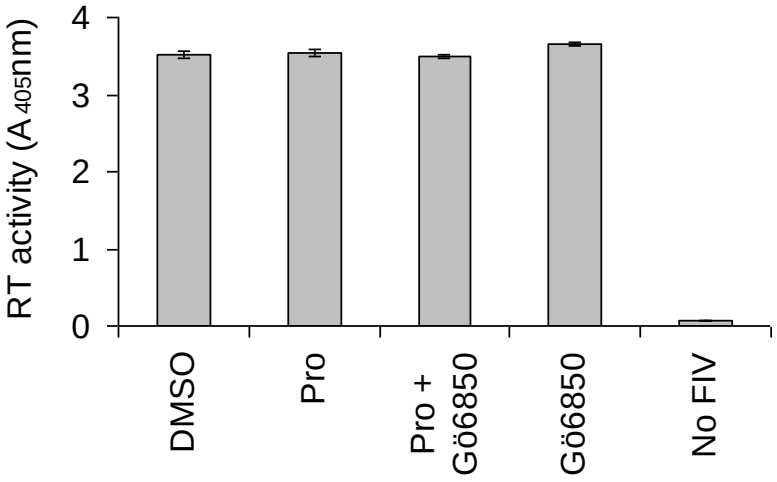


Figure 4-18 – Pre-incubation of cells with Prostratin before FIV infection does not reduce final virus titre. MYA-1 $CD4^+$ T cells were pre-treated with Prostratin (Pro – 1 μM), Prostratin + Gö6850 (2.5 μM), Gö6850 or mock-treated (DMSO) overnight before infection with FIV GL8 (MOI = 0.01). Cells were then washed twice and re-suspended in complete RPMI + IL-2 in the absence of Prostratin or Gö6850. Virus titre in the culture supernatant eight days post infection was quantified by non-isotopic reverse transcriptase assay. Each bar represents the mean +/- standard error (n=3).

4.4.4 Measuring virus entry by luciferase assay

Infection of cells with a luciferase reporter virus is an established method to study viral entry and other aspects of the viral lifecycle (Connor et al., 1995, Samman et al., 2009, Gonzalez et al., 2010). To further investigate the effect of Prostratin on viral entry, MYA-1 CD4+ T cells were infected with HIV-luciferase pseudotypes containing FIV GL8 Env and luciferase activity assays were performed. First, the cells were maintained in the presence or absence of exogenous IL-2 supplement for three days to simulate the conditions of the aforementioned resting T cell or productive infection assays. Then the cells were pre-incubated with Prostratin for 1 hour before infection. Luciferase activity of each culture was measured after a further incubation of 72 hours in the presence of Prostratin. Results showed that in the presence of IL-2, Prostratin did not noticeably alter luciferase activity (by causing less than one log difference in cpm) of the cells (Fig 4-19A). In the IL-2-depleted cells, a marked reduction in luciferase activity in the mock-treated cells was observed (Fig. 4-19B). This reduction in luciferase activity was rescued by Prostratin, which brought activity back to a level comparable to IL-2-supplemented samples (Fig. 4-19B).

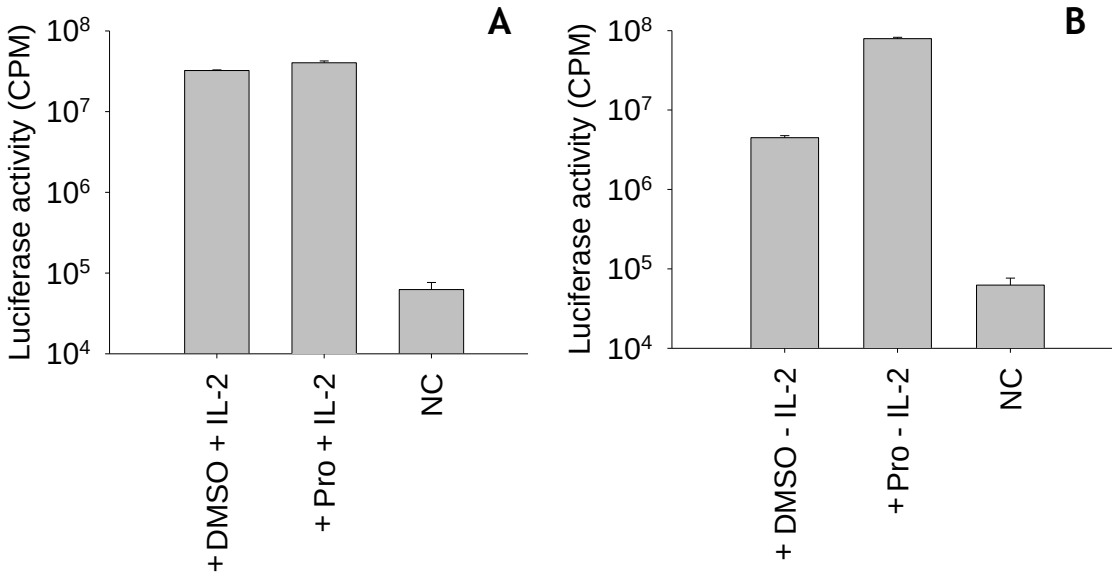


Figure 4-19 – Prostratin increases luciferase activity in IL-2-depleted MYA-1 CD4$^+$ T cells but not the luciferase activity of IL-2 supplemented cells. MYA-1 CD4$^+$ T cells were kept in the presence (A) or absence (B) of exogenous IL-2 supplement for three days, followed by a pre-incubation with DMSO or Prostratin (Pro – 1 μM) for 1 hour. Infections with HIV (FIV) pseudotypes were then

performed and the cells were maintained for another 72 hours in the presence of Prostratin or DMSO before luciferase activity was measured. NC = no pseudotype control. Each bar represents the mean +/- standard error (n=3) and is representative of two independent experiments.

The enhancement of luciferase activity in IL-2-depleted cells by Prostratin could be due to an increase in viral entry or the stimulation of cellular signalling pathways, which increases the subsequent expression of the luciferase gene. To answer this question, cells were infected with the HIV (FIV) pseudotypes and then re-suspended in fresh medium supplemented with exogenous IL-2, Prostratin or DMSO. In this arrangement viral entry was assumed to be constant, therefore any difference in luciferase activity between phorbol ester treated and untreated cells would be due to post-entry effects. Results indicated that the addition of exogenous IL-2 or Prostratin after infection markedly increased luciferase activity (Fig. 4-20). Since Prostratin had an entry-independent stimulatory effect on luciferase activity, it was concluded that the luciferase assay is an unreliable method to deduce the effect of Prostratin on viral entry.

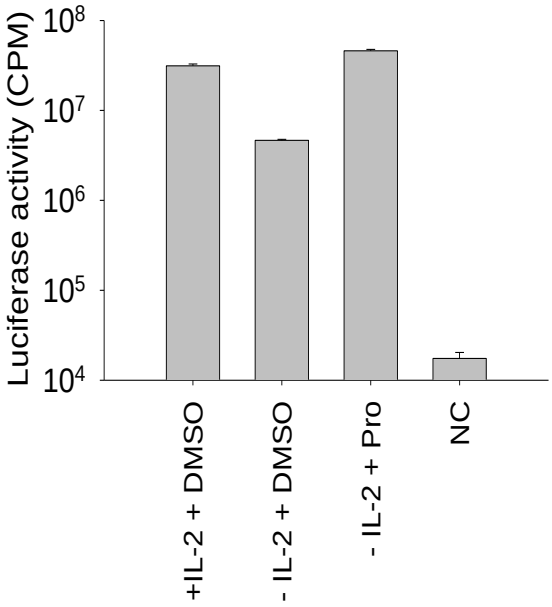


Figure 4-20 – Phorbol esters increase the activity of the luciferase assay independent of effects on viral entry. MYA-1 $CD4^+$ T Cells were pre-incubated with exogenous IL-2 supplement (100 U/ml) for three days before infection with HIV (FIV) pseudotypes. Cells were then washed and resuspended in fresh medium and were maintained in the presence of IL-2, DMSO or Prostratin (1 μM) for a further 72 hours before the luciferase activity assay was performed. NC = no pseudotype control. Each bar represents the mean +/- standard error (n=3).

4.4.5 FIV p24 ELISA to detect intracellular gag

Another method to quantify viral entry is to directly measure the amount of viral Gag protein that has entered the cell just after infection. MYA-1 $CD4^+$ T cells supplemented with exogenous IL-2 were pre-incubated with Prostratin, Prostratin plus Gö6850, Gö6850 alone or mocked-treated for 24 hours before infection with a high titre (MOI=0.1) of FIV GL8. Cells were then washed 1 hour after infection and trypsin-digested to remove surface-bound viruses. To measure internalized FIV Gag the cells were lysed and the lysates were processed by a FIV p24 ELISA. The high titre was necessary to allow detection of the viral Gag in the lysate. The results showed a small reduction of FIV p24 in the lysate of cells pre-incubated with Prostratin compared with control cells (DMSO) and cells pre-incubated with Prostratin and Gö6850 (Fig. 4-21). However, the difference was not significant (confirmed by One Way ANOVA, $p > 0.05$). Since a very high titre of FIV had to be used in order to provide enough FIV Gag to be detected by the FIV p24 ELISA, the result of this experiment may not be physiologically relevant.

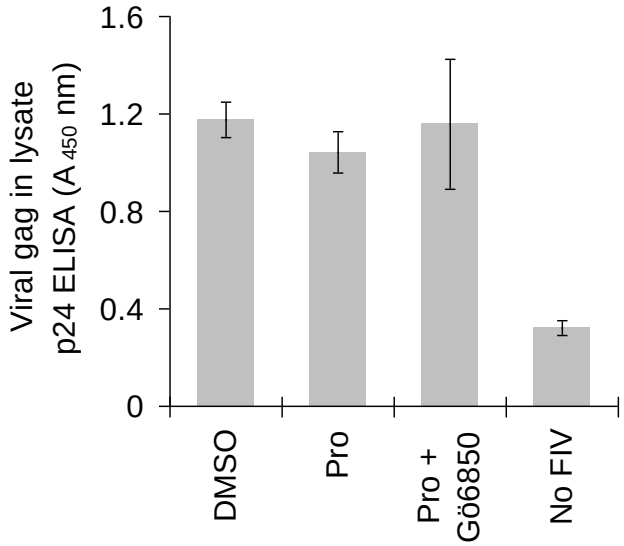


Figure 4-21 – Effect of Prostratin and Gö6850 on the amount of internalized FIV p24 post infection. IL-2 supplemented MYA-1 $CD4^+$ T Cells were pre-incubated with solvent (DMSO), Prostratin (Pro – 1 μM) or Prostratin plus Gö6850 (2.5 μM) for 24 hours before infection with FIV GL8 (MOI = 0.1). One hour post infection the cells were then washed, trypsin treated, lysed and the amount of intracellular FIV Gag was quantified by ELISA. Each bar represents the mean +/- standard error (n=3). The differences between the absorbance values of DMSO,

Prostratin and Prostratin plus Gö6850 are not statistically significant (One Way ANOVA, $p > 0.05$).

4.4.6 Quantification of intracellular FIV DNA

Quantifying the total viral DNA within cells immediately after the completion of reverse transcription is another method to measure viral entry, assuming that reverse transcription is not affected by the experimental treatment. IL-2-supplemented MYA-1 $CD4^+$ T cells were pre-incubated with Prostratin, Prostratin plus Gö6850, Gö6850 or DMSO for 24 hours before infection with FIV. Cells were harvested 2 hours post infection and total cellular DNA extracted to allow the quantification of intracellular FIV DNA by qPCR. Results from three independent experiments found no significant differences (One Way ANOVA, $p>0.05$) in the relative quantities of FIV DNA between cells subjected to the four treatments (Fig. 4-22), which further supports the hypothesis that the anti-FIV mechanism of Prostratin is post-entry.

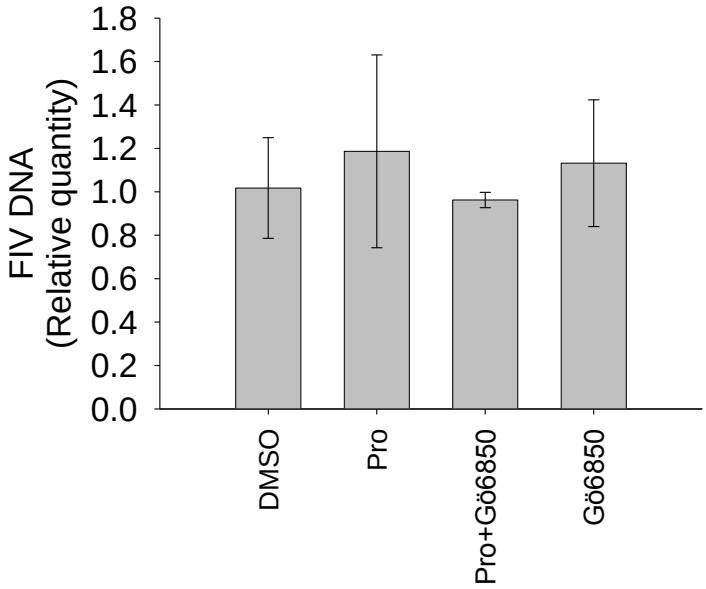


Figure 4-22 – Pre-treatment of MYA-1 $CD4^+$ T cells with Prostratin or Gö6850 does not reduce intracellular FIV DNA detected 2 hours post infection. IL-2 supplemented $CD4^+$ MYA-1 T cells were pre-incubated with Prostratin (Pro – 1 µM), Prostratin plus Gö6850 (2.5 µM), Gö6850 alone or mocked treated with solvent (DMSO) for 24 hours before infection with FIV GL8 (M.O.I. = 0.01). 2 hours post infection the cells were washed twice and total cellular DNA extracted. qPCR amplifying a region of the FIV *gag* gene was performed on the DNA samples and the results were analysed using the ΔΔCt method relative to the

average ΔCt value of the mock treated (DMSO) cells. Each bar represents the mean +/- standard deviation of the calculated relative quantities (n=3) and is representative of three independent experiments.

4.4.7 Phorbol esters and innate restriction factors

Recently, a number of intrinsic viral restriction factors have been discovered in cells; these include TRIM5α (Hatziioannou et al., 2004, Stremlau et al., 2004), tetherin (Neil et al., 2008, Van Damme et al., 2008) and APOBEC3G (Sheehy et al., 2002). These factors are expressed endogenously in host cells and are the first line of defence against viral pathogens. They can also mediate species-specific tropism of closely related viruses like HIV-1, SIV and FIV (Stremlau et al., 2005, Dietrich et al., 2011a). Furthermore, the expression and functions of these factors are connected to changes in cell signalling (Rose et al., 2004, Pertel et al., 2011). Thus, I planned to investigate the effect of Prostratin on the expression of these restriction factors.

TRIM5α belongs to the tripartite motif family of proteins and has been demonstrated to have an anti-retroviral effect before or during reverse transcription (Stremlau et al., 2004). TRIM5 gene expression is up-regulated by type I but not type II interferon signalling, indicating a connection between it and innate immune signalling (Carthagena et al., 2009). Furthermore, a recent study suggested that TRIM5α can mediate innate immune signalling by stimulating AP-1 and NFκB (Pertel et al., 2011). However, it is unlikely that feline TRIM5α plays an active anti-viral role in feline cells because of a truncation in its B30.2 SPRY domain (McEwan et al., 2009), which means that it is unable to interact with the viral capsid and thus incapable of restricting virus replication (Stremlau et al., 2005, McEwan et al., 2009).

Next, tetherin is an interferon inducible membrane protein and a restriction factor of enveloped viruses (Van Damme et al., 2008, Neil et al., 2008) that has been shown to prevent FIV particle release (Dietrich et al., 2011b). However, the expression of tetherin also increases syncytium formation in adherent CRFK (ID-10) cells infected with a cell-culture adapted FIV strain (F14), indicating an enhancement of cell-to-cell transmission. To take advantage of this phenomenon, ID-10 cells were infected with FIV F14 and stimulated with Prostratin 24 hours post infection. When feline interferon omega (IFN-ω) was

added to ID-10 cells, it up-regulated feline tetherin expression and reduced virus concentration in the supernatant while at the same time increased syncytium formation (Fig. 4-23A, B and (Dietrich et al., 2011b)). Thus if Prostratin stimulated the expression of feline tetherin in ID-10 cells, it would be expected that supernatant virus titre would be reduced while more syncytia would be observed. However, results showed that Prostratin did not reduce the release of FIV at day 3 post infection (Fig. 4-23A) and did not enhance syncytium formation (Fig. 4-23B) in comparison with solvent control cells. The data indicate that Prostratin restricted viral replication by means other than tetherin, although this only applied to ID-10 cells, which are not the natural cellular targets of FIV.

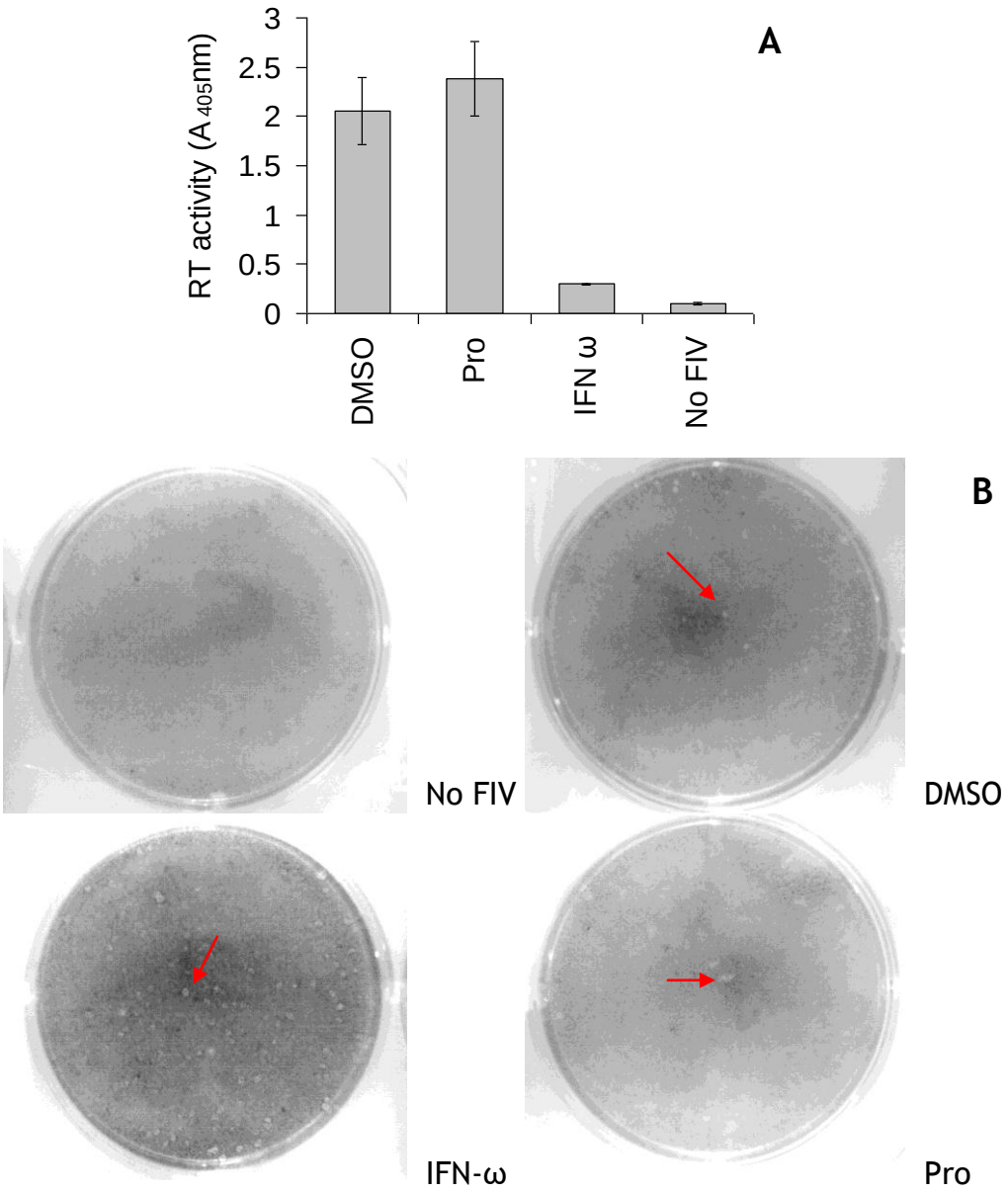


Figure 4-23 – Prostratin restricts FIV replication by means other than the up-regulation of tetherin. CRFK (ID-10) cells were plated onto six-well plates and infected with FIV F14. 24 hours post-infection the cells were stimulated with

Prostratin (Pro - 1 μM), solvent (DMSO) or feline IFN-ω for a further 48 hours before (A) virus titre in the culture supernatant was measured by a non-isotopic reverse transcriptase (RT) assay and (B) cell monolayers were stained with methylene blue/basic fuchsin to visualise the syncytia. Each bar represents mean +/- range (n=2). Representative syncytia are indicated by red arrows.

To investigate further the dynamics of viral replication when infected cells were treated with Prostratin, the viral entry experiment in which cells were pre-incubated with Prostratin before infection was continued for a further 7 days. 24 hours after infection, RGV and AZT were added to the culture supernatant. In the absence of *de novo* infection, FIV RNA in the supernatant 3 days or 7 days after infection was quantified using qPCR. Surprisingly Prostratin treated cells produced more extracellular viral RNA than either DMSO or Prostratin plus Gö6850-treated cells at day 3 and day 7 post-infection (Fig. 4-24A, B). Supernatant RT activity at day 7 was also measured and higher RT activity was detected from the supernatant of Prostratin-treated cells (Fig. 4-24C). In a parallel experiment where antiretroviral drugs were not added, Prostratin blocked virus replication but curiously Gö6850 did not rescue productive infection (Fig. 4-24D). This anomaly was attributed to the timing of the application of Gö6850 to cells. It has been demonstrated previously that Gö6850 could inhibit the effects of IL-2 (Fig. 4-13, 4-15). The addition of Gö6850 before infection may be analogous to removing IL-2 prior to infection, which leads to a non-productive infection. Based on the available results it appears that Prostratin blocked productive infection of FIV at a step other than entry or egress.

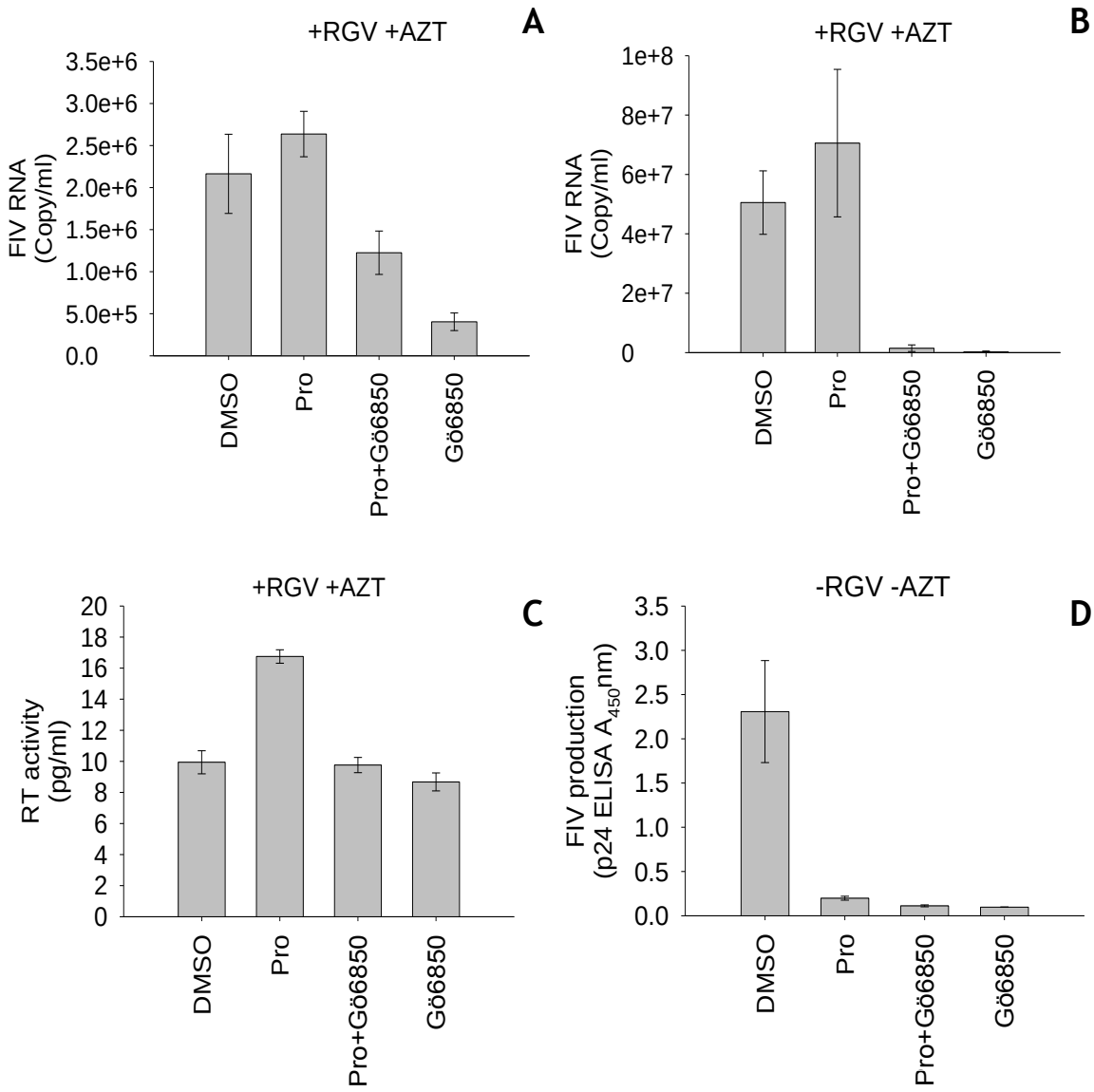


Figure 4-24 – Prostratin treatment does not reduce FIV production per infected cell. MYA-1 $CD4^+$ T cells were pre-treated with Prostratin (Pro – 1 μM), Prostratin plus Gö6850, Gö6850 alone and mock treated (DMSO) 24 hours before infection with FIV GL8 (M.O.I. = 0.01). Cells were washed and the antiretroviral drugs RGV (1 μM) and AZT (5 μM) were added 24 hours post infection. The quantity of FIV RNA in the supernatant at day 3 (A) and day 7 (B) post-infection was measured using qPCR. Each bar represents the mean +/- standard deviation (n=3). (C) Supernatant RT activity at day 7 post infection was also measured. Each bar represents the mean +/- standard error (n=3). (D) A parallel experiment without the addition of antiretroviral drugs was also performed. Virus production was measured by FIV p24 ELISA. Each bar represents the mean +/- standard error (n=3).

4.5 Discussion

The ability of HIV-1 to establish a latent reservoir within the host makes the virus invulnerable to eradication from the body by current antiretroviral treatments. In the absence of an effective vaccine against the virus, a practical method of reactivating latent viruses within the body and thus rendering them susceptible to antiretroviral drugs and the immune response may prove to be the only way to stem the global HIV-AIDS epidemic. A suitable small animal model of HIV-1 latency and reactivation is vital to the development of such therapeutic regimes and the research presented in this chapter has shown that FIV infection of the domestic cat may fulfil the necessary criteria.

Previous studies have shown that the majority of the latently infected $CD4^+$ T cells in asymptomatic patients are memory T cells (Pierson et al., 2000, Chomont et al., 2009). However, direct infection of resting T cells replenishes the latent reservoir as the infection progresses (Wightman et al., 2010). It has been well established that lentiviruses can infect resting T cells, but viral replication and further spread is very inefficient (Joshi et al., 2004, Vatakis et al., 2010). This may be due to defects at various stages of the viral lifecycle, including reverse transcription, integration and viral protein expression (Vatakis et al., 2007, Vatakis et al., 2009). This chapter described two strategies that were used to attempt to establish FIV latently infected feline $CD4^+$ T cells. The first strategy of using NFκB inhibitors to induce latency was unsuccessful, whereas the second strategy of infecting IL-2-depleted MYA-1 $CD4^+$ T cells as they slowly became quiescent was more successful. The depletion of exogenous IL-2 from MYA-1 $CD4^+$ T cells in this assay is analogous to the progression of activated T cells to quiescence during the formation of memory cells (McKinstry et al., 2007) and led to a significant defect in virus replication and spread within the cell population. However, on-going low-level virus replication among the IL-2-depleted cells was detected, indicating that rather than successfully re-creating the latently infected memory T cells, this assay may be more similar to the direct infection of resting T cells.

The low level continuous viral replication detected in my *in vitro* assay may be the result of an incomplete but uniform down regulation of viral replication by all of the infected cells or a small number of infected cells replicating virus at the normal rate as the population enters the quiescent state in a non-uniform

fashion. The assay used was unable to distinguish between these two possibilities. To eliminate this continuous viral gene expression or replication completely in an *in vitro* system of latent infection is difficult, and other models have displayed similar "leaks" of low-level virus expression (Bosque and Planelles, 2009, Yang et al., 2009, Saleh et al., 2011). The existence of low-level virus gene expression or replication may actually reflect the nature of the latent reservoir *in vivo*, as it has been proposed that there is a range of different levels of viral activation within the reservoir, analogous to the fact that the resting T cell population in the body is not homogenous in terms of cellular activation (Pace et al., 2011). Another limitation of the assay is that the qPCR primers used cannot distinguish between integrated or non-integrated viral DNA. Integrated HIV-1 provirus can be detected using techniques such as *Alu*-PCR (Sonza et al., 1996). Unfortunately no equivalent reliable method of confirming FIV integration is available at the time of writing. The attempt to separate unintegrated viral DNA from the host genome using the modified Hirt Protocol failed due to persistent contamination by the unintegrated viral DNA. Using the integrase inhibitor RGV a small increase in virus production was repeatedly demonstrated when IL-2-depleted resting MYA-1 T cells were stimulated with PMA or Prostratin compared with mock-stimulated cells. However, it was not certain whether this increase in virus production came from latently infected cells or from the cells which were persistently producing virus. These problems are not limited to this assay and represent a degree of uncertainty as to which kinds of viral DNA had been identified and where the viraemia came from in many of the published HIV-1 latency models (Han et al., 2007).

Despite these shortcomings with the *in vitro* assay, the parallels between the regulation of FIV and HIV-1 replication and spread are clear. Replication of both viruses is stimulated by IL-2 (Oswald-Richter et al., 2004a); while Prostratin can both stimulate HIV-1 and FIV replication in resting T cells and inhibit productive infection in activated T cells (Gustafson et al., 1992, Kulkosky et al., 2001, Biancotto et al., 2004, Rullas et al., 2004, Warrilow et al., 2006). This illustrates a shared reliance on PKC-dependent signalling by both HIV-1 and FIV. Lentivirus production is very dependent on the activation status of the host cell (Stevenson et al., 1990, Joshi et al., 2004). The IL-2-dependency of FIV infection of MYA-1 $CD4^+$ T cells mirrors the behaviour of $CD4^+CD25^+$ T cells isolated from feline peripheral blood mononuclear cells, which could be non-productively

infected by FIV in the absence of IL-2 (Joshi et al., 2005a). The similarities between the two systems suggest that the MYA-1 $CD4^+$ T cell culture system described herein reflects the characteristics of $CD4^+$ T cells *ex vivo*. In regard to the practicalities of investigating lentiviral latency in a laboratory setting, FIV infection of feline $CD4^+$ T cells offers a safe and accessible system in which primary, wild-type viruses may be investigated without a requirement for a high level of biological containment. These characteristics are highly desirable in a physiologically relevant small animal model of HIV-1 latency.

Although Prostratin is able to stimulate FIV production from infected resting MYA-1 T cells, its ability to block *de novo* infection could potentially make it difficult to detect reactivation. My results suggested that the ability of Prostratin to block FIV production is actually dependent on the background activation status of the host cell. Replication of HIV-1 and FIV in resting T cells is very inefficient (Joshi et al., 2004, Vatakis et al., 2010). Despite on-going low level production of virus within the model system, the spread of FIV was almost completely blocked by the depletion of IL-2. By administering the antiretroviral drugs RGV and AZT post infection, it was established that most of the virus produced in the reactivation assay came from the ability of Prostratin or IL-2 to promote *de novo* infection after an initial burst of virus production. Thus in a state of low cellular activation such as after the removal of exogenous IL-2, Prostratin becomes a substitute for IL-2 and acts to stimulate, rather than block lentiviral replication and spread. When IL-2 is present, most of the cells within the population would be stimulated in a relatively consistent manner, and it appears that further stimulation of the cells by Prostratin would have an inhibitory effect on virus replication in most of the cells. It is intriguing that phorbol esters have two apparently contradictory effects on lentiviral replication.

The effect of HDAC inhibitors valproic acid and sodium butyrate on the infected resting MYA-1 T cells was also investigated. Although reactivation of productive FIV infection was not detected when infected resting MYA-1 T cells were stimulated with either compound, valproic acid was able to enhance Prostratin-mediated reactivation. This is in agreement with a recent study which reports the synergistic activation of HIV-1 when Prostratin and HDAC inhibitors are added simultaneously (Reuse et al., 2009). The lack of reactivation by sodium

butyrate was attributed to the high toxicity of the compound. In contrast, valproic acid caused much less cell death but it only had a measurable effect on reactivation of productive infection when used in conjunction with Prostratin. The fact that stimulation of virus production from infected cells could only be reliably detected by FIV p24 ELISA or the RT activity assay after amplification by *de novo* infection may explain why adding valproic acid alone to infected resting MYA-1 T cells apparently failed to stimulate virus production. Valproic acid may stimulate viral replication but it may not be able to make the surrounding resting T cells permissive to infection in the same way Prostratin could. A previous study has shown that the administration of valproic acid does not cause the activation of human $CD4^+$ T cells or make them more conducive to HIV-1 infection (Ylisastigui et al., 2004). It is important to note that valproic acid is a weak HDAC inhibitor and a weak latency-reversing agent (Colin and Van Lint, 2009) and other more potent HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA) (Archin et al., 2009, Contreras et al., 2009) may be tested in future experiments. The use of more sensitive techniques such as qPCR should be considered when evaluating the effects of compounds which do not enhance *de novo* infection. Recently, a study has demonstrated that FIV infection *in vivo* leads to the establishment of latently infected $CD4^+$ T cells analogous to the latent infections of HIV-1 in humans (Murphy et al., 2012). Indeed, SAHA is shown to reactivate latent viruses *ex vivo* subsequently (McDonnel et al., 2012), which adds support to the idea of using FIV-infected cats as a small animal model for HIV-1 latency.

The inhibition of the dual actions of Prostratin by the PKC inhibitor Gö6850 suggested a central role for this enzyme family in the replication of FIV. The PKC family of enzymes play an important role in T cell receptor signalling and T cell activation (Isakov and Altman, 2002). Because both HIV-1 and FIV target activated $CD4^+$ T-cells, the viruses have acquired the ability to exploit the cellular pathway of T cell activation by encoding binding sites for several transcription factors of the PKC signalling cascade. For example the HIV-1 LTR contains sites for NFAT, NFκB, and AP-1, while the FIV LTR contains sites for AP-4, AP-1/ATF and possibly NFκB (Tang et al., 1999, Sparger et al., 1992, Thompson et al., 1994) (Fig. 4-25A). Using site-specific mutagenesis of the FIV LTR it has been demonstrated that the AP-1 site of the FIV LTR responds to stimulation of PKC (Sparger et al., 1992). While our results demonstrate that IL-

2 stimulation of MYA-1 $CD4^+$ T cells is sufficient to promote the replication of FIV in agreement with previous studies (Oswald-Richter et al., 2004a, Joshi et al., 2005a), it was also observed that the PKC inhibitor Gö6850 abrogated the reactivation of FIV and the cyto-protective effect of Prostratin in IL-2-depleted cells. Since Gö6850 had a similar effect on the reactivation of FIV by exogenous IL-2, these data suggest that IL-2-mediated signalling through a PKC-dependent pathway to modulate viral growth and that Prostratin may share a similar mode of action. However, the PKC signalling pathway only appeared to drive FIV replication in IL-2-depleted cells, as Gö6850 could not inhibit productive infection reliably, suggesting that in the presence of IL-2, the control of viral growth was dependent upon other cellular factors. Engagement of the IL-2 receptor by IL-2 is thought to initiate signalling through a number of pathways, including the JAK/STAT5, MAPK and PI3K (reviewed in (Cheng et al., 2011)); these pathways can also stimulate NFAT, NFκB, AP-1 and ATF, transcription factors important to HIV-1 and FIV replication (Fruman, 2004, Treinies et al., 1999, Funakoshi-Tago et al., 2003, Chang et al., 2003) (Fig. 4-25B).

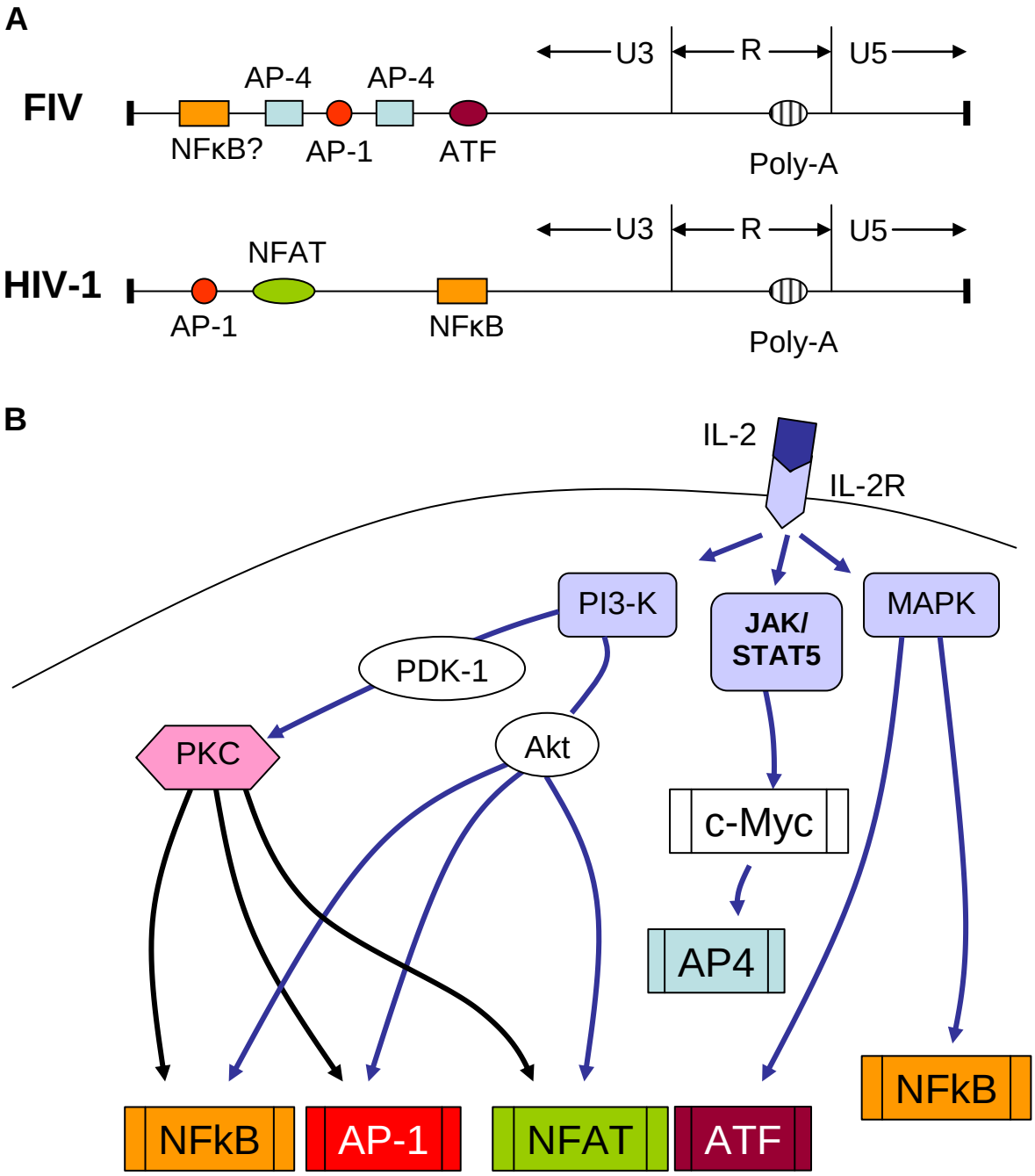


Figure 4-25 – The potential relationship between lentiviral gene expression and cellular PKC and IL-2 receptor-mediated signalling. (A) A diagram showing the 5' Long Terminal Repeats (LTR) region of FIV and HIV-1, highlighting the binding sites for transcription factors that interact with both virus and cellular PKC and IL-2 receptor-mediated signalling (Tang et al., 1999, Sparger et al., 1992, Elder and Phillips, 1995). A putative NFκB binding site was found in the FIV-Petaluma strain but it is not thought to be transcriptionally active in other FIV strains (Sparger et al., 1992, Thompson et al., 1994, Joshi et al., 2005b). (B) A diagram showing the transcription factors important in lentiviral replication interacting with PKC and IL-2 receptor (IL-2R) signalling pathways (Cheng et al., 2011). Both PKC and IL-2 receptor-mediated pathways have common

downstream targets such as NFκB, NFAT and AP-1 (Fruman, 2004, Isakov and Altman, 2002, Funakoshi-Tago et al., 2003). PKC activates AP-1 directly (Isakov and Altman, 2002, Altman and Villalba, 2003). IL-2 receptor signalling can activate PKC via the PI-3K/PDK-1 cascade (Le Good et al., 1998, Rudd and Schneider, 2003). The JAK/STAT5 pathway activates AP-4 via the cell proliferation factor c-MYC (Lord et al., 2000, Jung and Hermeking, 2009). Another IL-2 receptor-mediated pathway, ras/raf/MAPK stimulates ATF (Chang et al., 2003).

Multiple factors contribute to the maintenance of lentiviral latency in cells (Han et al., 2007, Coiras et al., 2009) but it has been shown that it is the activation state of the cell during infection that determines an outcome of latent or productive infection (Stevenson et al., 1990, Duverger et al., 2009). Based on this understanding I attempted initially to establish latent FIV infection in MYA-1 T cells using NFκB inhibitors to reduce cellular activation. The inhibitors SC514 and BOT64 were not successful in blocking FIV replication, whereas CAPE could restrict virus production only if it was applied continuously. With hindsight this was unlikely to be successful because of the following: firstly, the presence of exogenous IL-2 in the cell culture supernatant during these experiments, which would continuously stimulate the cells and would drive productive infection as soon as the inhibitor was removed; secondly, NFκB is one of many downstream effectors that are triggered by IL-2 receptor signalling or PKC activation (Fig. 4-25B), thus inhibition of NFκB could be circumvented, as demonstrated by the results; lastly, NFκB may not play an important in FIV GL8 replication as it was shown not to be transcriptionally active in this strain (Thompson et al., 1994). Nevertheless NFκB inhibitors may be useful in reducing or even stopping the residual virus replication detected after IL-2 depletion, but due to time and resource constraints I was unable to test this hypothesis experimentally. The initial results also suggested that the NFκB inhibitor SC514 increased virus production. However, this trend was not reproducible and it was concluded that SC514 does not enhance FIV production. The enhancement of virus production seen in early experiments may be caused by the lack of replicates cultures, allowing random variations in cell activation and virus production rate to generate false positive data.

Although the mechanism underlying the reliance of the MYA-1 T cells on PKC to maintain viability and drive FIV replication after IL-2 depletion remains to be established experimentally, based on the results presented in this chapter it can be envisaged that *in vivo* FIV initially targets activated $CD4^+$ T cells and replicates in dividing cells. However, as IL-2 levels diminish, coinciding with the formation of memory T cell populations, the virus will establish a latent infection. The virus would then remain latent in the memory T cell population until antigenic stimulation and IL-2-dependent expansion reactivated the latent infection.

The ability of Prostratin to inhibit FIV replication in the presence of IL-2 would seem paradoxical to its effect on the infected resting MYA-1 T cells. As the inhibition of PKC signalling by Gö6850 rescued the restriction of viral replication, the inhibitory effect would appear to be dependent upon PKC. What is the mechanism behind this PKC mediated viral restriction? Using the trypan blue exclusion assay no increase in cell death was detected after cells were treated with Prostratin in the presence of IL-2, thus it is unlikely that Prostratin inhibits virus replication by a non-specific toxicity effect. Expression of the FIV receptors CD134 and CXCR4 was monitored over seven days and it was concluded that modulation of the expression of the FIV receptors CD134 and CXCR4 cannot be invoked to explain the inhibition of FIV replication by Prostratin. This is because in the experiments the simultaneous addition of Gö6850 and Prostratin to cells led to the rapid down regulation of CD134 and CXCR4, yet the application of Gö6850 rescued productive infection from Prostratin treatment.

Other methods were also used to deduce the effect of Prostratin on viral entry. HIV-Luciferase pseudotypes with FIV Env have been previously used to investigate questions relating to virus binding and entry (Willett et al., 2006b, Willett et al., 2009). However, the application of Prostratin increased luciferase activity independent of viral entry, which compromised the reliability of the assay in this instance. Direct measurements of viral components using ELISA and PCR were also problematic. In order to obtain a positive measurement when detecting intracellular viral Gag just after infection, a high dose of virus (MOI = 0.1) had to be used, which may not be physiological and can potentially overwhelm any inhibitory effect. No significant differences between the amounts of intracellular viral DNA from the different treatments were detected

by qPCR. However, the quantities of intracellular viral DNA detected in this assay depended upon the efficiency of not only viral entry, but also virus uncoating and reverse transcription. Nevertheless, despite each of the problems mentioned, every experiment performed indicated that Prostratin had little effect on virus entry.

The down-regulation of CD4, CCR5 and CXCR4 has been observed consistently among Prostratin stimulated human $CD4^+$ T cells (Gulakowski et al., 1997, Kulkosky et al., 2001, Biancotto et al., 2004, Rullas et al., 2004). In one study the entry of pseudotypes containing HIV-1 Env was inhibited by Prostratin, but not VSV-G containing pseudotypes (Rullas et al., 2004). However, a previous study on FIV GL8 demonstrated that blockade of the FIV receptors revealed by a pseudotype assay does not translate to inhibition of productive infection (Willett et al., 2009). It is possible that Prostratin induced changes to viral receptor expression levels amongst activated MYA-1 $CD4^+$ T cells, but the magnitude of change was too small to block virus infection. Another possibility is that HIV-1 and FIV have different viral entry characteristics. In addition, the qPCR data showed that two hours after infection, Prostratin-stimulated cells contained similar amount of intracellular viral DNA compared with mock-treated cells, which suggested that viral entry and reverse transcription were not affected by Prostratin. This is contrary to a study of Prostratin's effect on HIV-1 infection of human $CD4^+$ T cells, which found that reverse transcription was partially inhibited (Biancotto et al., 2004). Furthermore this study found that HIV-1 p24 uptake was blocked and productive infection did not initiate in resting T cells pre-treated with Prostratin. Differences in the experimental protocol may account for these discrepancies (for example, it was unclear whether Prostratin was washed off after HIV-1 infection). Most studies on Prostratin's effects have focused on its ability to stimulate virus production from resting cells already infected with HIV-1 and did not address the question of whether Prostratin can promote direct infection of HIV-1 into resting T cells (Kulkosky et al., 2001, Rullas et al., 2004, Reuse et al., 2009, Yang et al., 2009, Saleh et al., 2011). However, it is possible that Prostratin causes distinct effects on human and feline cells. If FIV is to be used as a model for HIV-1 latency and growth, it is important to carry out more research to characterise the similarities and differences between HIV-1 and FIV.

HIV-1 encodes multiple accessory proteins such as Vif, Vpr, Vpu and Nef that are often dispensable during *in vitro* infection but would significantly attenuate the virus *in vivo* if absent (Anderson and Hope, 2004). This indicated that at least one of the functions of these genes is to counteract obstacles within the host cells that would block infection. Indeed the use of mutant viruses with an accessory gene knocked-out was key to the discovery of the first host restriction factor APOBEC3G (Sheehy et al., 2002). This spurred on the discovery of other restriction factors such as TRIM5α (Stremlau et al., 2004), tetherin (Neil et al., 2008, Van Damme et al., 2008) and more recently SAMHD1, a dendritic and macrophage specific restriction factor (Hrecka et al., 2011, Laguette et al., 2011). Could the stimulation of the activity of these restriction factors be the mechanism behind Prostratin's anti-FIV effect? A previous study of feline TRIM5 has shown that due to a truncation in its B30.2 SPRY domain, it is unable to bind to viral capsid and thus unlikely to play an active anti-viral role in feline cells (McEwan et al., 2009, Stremlau et al., 2005).

The effect of Prostratin on feline tetherin was then tested. Feline tetherin has been shown previously to not only inhibit the release of FIV virions, but also promote syncytium formation (Dietrich et al., 2011b), supporting the idea that lentiviruses can shift their mode of transmission from cell-free to cell-to-cell in order to overcome or exploit tetherin mediated restriction (Dietrich et al., 2011a). Using a previously established assay of tetherin activity (Dietrich et al., 2011b), I showed that Prostratin did not reduce viral release or increase syncytium formation, indicating that the phorbol ester has no effect on tetherin activity. However, this assay was performed in CrFK ID-10 cells, which are not the natural host cell of FIV.

Next, I demonstrated that when *de novo* infection was blocked by antiretroviral drugs added post-infection, the rate of virus production in IL-2 supplemented, Prostratin-stimulated cells was higher than mock-stimulated cells. A possible future experiment to confirm this result would be to treat cells that are productively infected by FIV with Prostratin or Gö6850 under conditions which prevent de novo infection (for example, by adding antiretroviral drugs post infection) and to detect any subtle changes in the initial wave of virus production in the cell supernatant using the sensitive qPCR technique outlined in this chapter. If these preliminary findings are confirmed, it means that rather

than blocking virus replication at the entry, production or exit step, Prostratin somehow affected the ability of the released virions to infect other cells. One family of restriction factors that inhibits viral replication by reducing the infectivity of nascent virions is the APOBEC proteins. In humans APOBEC3G restricts lentivirus infection by being incorporated into nascent virions and hyper-mutating the retroviral genome once the virus infects another cell (Zhang et al., 2003, Mangeat et al., 2003). The feline homologues of APOBEC3G are the APOBECs 3C, 3H and 3CH (Munk et al., 2008) and the FIV Vif protein counteracts the anti-lentiviral activities of feline APOBEC proteins by mediating their degradation (Munk et al., 2008). Activation of PKC by phorbol esters has been shown to up-regulate the expression of APOBEC3G (Rose et al., 2004, Rose et al., 2005), could it be that the combined stimulation of IL-2 and Prostratin may induce sufficient expression of APOBEC proteins to overcome Vif-dependent antagonism? Whether high levels of APOBEC expression can overcome Vif remains to be proven (Vetter et al., 2009, Mussil et al., 2011, Zielonka et al., 2010) and more research is urgently needed to address this and to replicate the effects of Prostratin on primary $CD4^+$ T cells. If this theory is proven in cat cells does it also apply to human $CD4^+$ T cells?

To my knowledge, this study is the first demonstration of the anti-viral effect of Prostratin against a non-primate lentivirus, indicating that the phenomenon of PKC-mediated viral restriction is conserved across mammalian species. Further research into this PKC-mediated retroviral restriction effect would not only enhance our understanding of the complications and subtlety of cell signalling, it may lead to advancements in the treatment of lentiviral infections if the antiviral mechanism proved to be amenable to therapeutic intervention.

5 Investigation of viral growth differences between strains of FIV

5.1 Summary

FIV strains that have been isolated at different stages of disease exhibit different biological characteristics, such as an expansion in tropism and increased pathogenicity after chronic infection *in vivo* (Hosie et al., 2002, Willett and Hosie, 2010). The variations between strains isolated in 'early' stage (or acute phase) and 'late' stage (chronic phase) infection may reflect disease progression and viral evolution under the selective pressures of the host immune system (Willett and Hosie, 2008). This chapter reports the differences in viral replication in MYA-1 $CD4^+$ T cells between the 'early' FIV strain GL8 (Hosie and Jarrett, 1990) and the 'late' FIV strain PPR (Phillips et al., 1990) (both subtype A viruses) as well as an attempt to map the viral genomic region associated with these differences.

5.2 Results

5.2.1 Experimental conditions differentially affect the replication kinetics of FIV GL8 and PPR

First the dynamics of virus replication of the early and late strains of FIV (GL8 and PPR respectively) was monitored over a 10-day period. MYA-1 feline $CD4^+$ T cells supplemented with exogenous IL-2 were infected with matched doses of FIV GL8 or PPR. Samples of cell culture supernatant were taken at day 0, 3, 7, and 10 post infection and virus titres of the supernatant were measured by an ELISA that detects the FIV p24 (Gag) protein. Results from three independent experiments showed that the rate of FIV PPR replication was higher than FIV GL8 (Fig. 5-1).

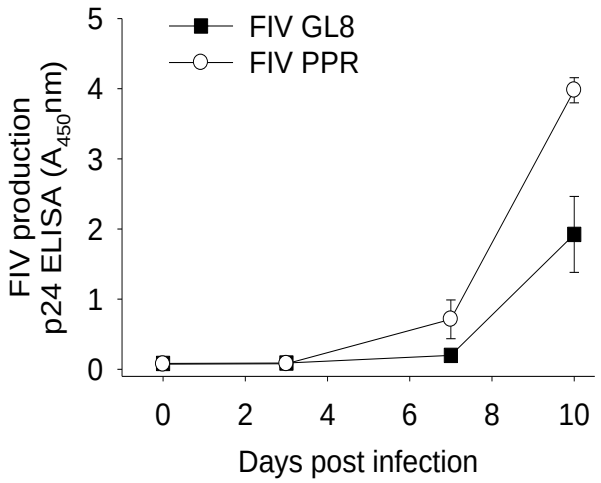


Figure 5-1 – FIV PPR replicates at a higher rate than GL8 in MYA-1 CD4$^+$ T cells. In the presence of exogenous IL-2, MYA-1 CD4$^+$ T cells were infected with FIV PPR or GL8 (M.O.I. = 0.001) and virus replication was monitored over 10 days post-infection by FIV p24 ELISA. Each point represents the mean +/- standard error (n=3) and was representative of three independent experiments.

Next, MYA-1 CD4$^+$ T cells were seeded in the absence of IL-2 and 24 hours post-seeding they were infected with the two strains of FIV. 48 hours post infection the infected resting MYA-1 T cells were stimulated by the phorbol esters PMA and Prostratin, which resulted in productive infection (Fig. 5-2). Virus production from GL8-infected cells stimulated by PMA or Prostratin proceeded at a similar rate, whereas virus production from PPR-infected cells stimulated by PMA occurred at a higher rate than virus production from PPR-infected cells stimulated by Prostratin. Virus production from PMA-stimulated, PPR-infected cells reached a peak by day 8 post infection, but by day 10 virus titres from GL8-infected cells also reached a similar level with the PMA stimulated, PPR infected cells. Without stimulation the background p24 signal from PPR-infected cells was higher than GL8-infected cells.

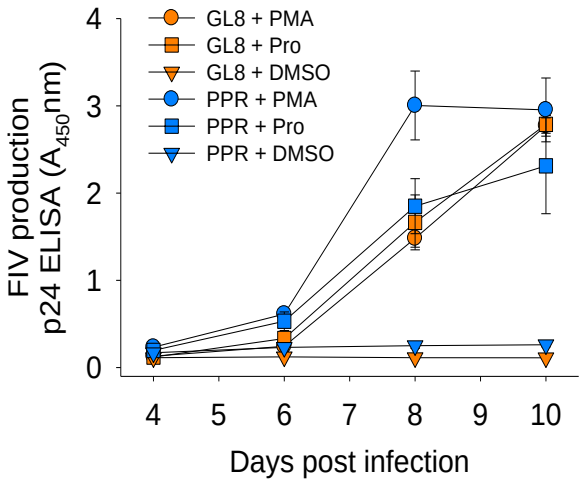


Figure 5-2 – Reactivation of productive infection by phorbol esters is more pronounced among FIV PPR-infected cells. IL-2-depleted cells were infected with FIV GL8 or PPR (M.O.I. = 0.01) and virus production was monitored by p24 ELISA. 2 days post-infection, the cells were stimulated with either PMA (0.08 µM) or Prostratin (Pro – 1 µM). Each point represents the mean +/- range (n=2) and is representative of three independent experiments.

To determine the contribution of viral spread to the variation in virus replication between the two strains, IL-2-depleted MYA-1 T cells infected with FIV GL8 or PPR were stimulated by phorbol esters in the presence of the integrase inhibitor Raltegravir (RGV). RGV blocks *de novo* infection, which allows the measurement of the rate of virus production from the initial inoculum only. The mock-stimulated infected resting MYA-1 T cells retained a very low level of basal FIV replication activity (Fig. 5-3), as demonstrated previously (Fig. 5-2 and previous chapter); this was more pronounced in PPR-infected cells compared with GL8-infected cells. PMA and Prostratin stimulated productive infection from the infected resting MYA-1 T cells, as demonstrated by the higher RT activity in the supernatant, with higher RT activities detected from the supernatant of PPR-infected cells compared to the supernatant of GL8-infected cells (Fig. 5-3). When *de novo* infection was blocked by the presence of RGV, higher virus titres were observed from the supernatant of reactivated PPR-infected cells compared with GL8-infected cells. These results suggested that FIV PPR has a higher rate of virus production per infected cell compared to FIV GL8.

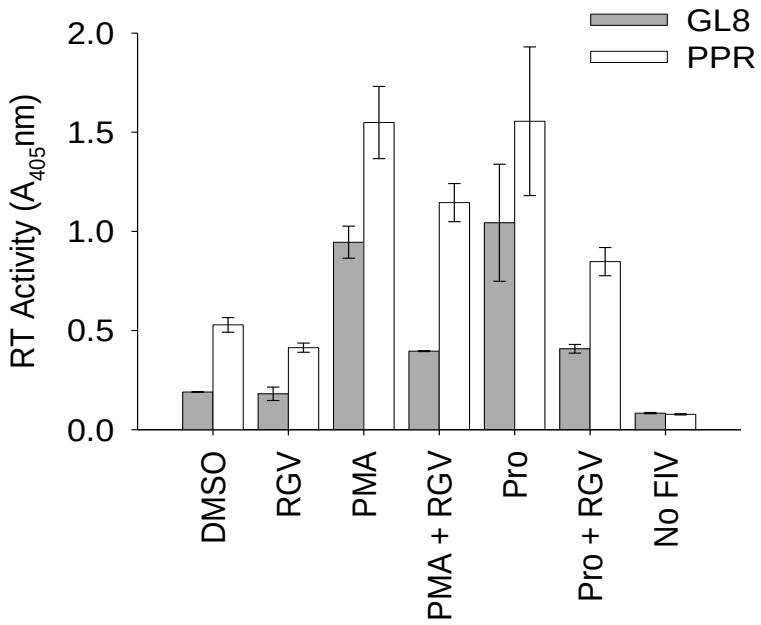


Figure 5-3 – FIV PPR demonstrates higher rates of basal replication and reactivation than FIV GL8. IL-2-depleted resting MYA-1 T cells were infected with FIV GL8 or PPR (MOI = 0.06). 24 hours post infection the integrase inhibitor RGV was added to the appropriate cells and at day 2 post infection cells were stimulated with PMA (0.08 μM), Prostratin (Pro – 1 μM) or mock-stimulated with solvent (DMSO). At day 5 post infection supernatant samples were taken to quantify virus replication using a non-isotopic reverse transcriptase (RT) assay. Each bar represents the mean +/- range (n=2). Trends are representative of 2 similar independent experiments using RGV but with different M.O.I. and end points.

PKC has been shown to play a crucial role in FIV replication (See previous chapter). To investigate if the two strains of FIV responded differently to inhibition of PKC, the PKC inhibitors Gö6976 or Gö6850 and phorbol esters were simultaneously added to IL-2-depleted MYA-1 T cells infected with FIV GL8 or PPR. Gö6850 has been shown to inhibit both conventional and novel isoforms of PKC, with IC_{50} values of 8.4, 18, 210, 132 and 5800 nM against PKC isoforms α, β1, δ, ε and ζ respectively (Martiny-Baron et al., 1993). In contrast Gö6976 is a more potent and specific inhibitor of conventional PKC isoforms, with IC_{50} values of 2.3 and 6.2 nM against PKC α and β1 respectively and no inhibitory activity against PKC δ, ε and ζ isoforms (Martiny-Baron et al., 1993). Gö6850 abrogated phorbol ester-stimulated production of FIV GL8 and PPR (Fig. 5-4). In contrast, Gö6976 caused complete restriction of FIV GL8 replication but only a partial restriction to the production of FIV PPR (Fig. 5-4). Since both Gö6850 and

Gö6976 were used at 2.5 µM, a concentration much higher than the reported IC_{50} for most of the PKC isoforms, complete, or near complete down-regulation of conventional or novel and conventional PKC isoforms was assumed. These results indicated that virus production from cells infected with FIV GL8 was dependent on both novel and conventional PKC isoforms, whereas virus production from FIV PPR-infected cells only had a requirement for novel isoforms of PKC.

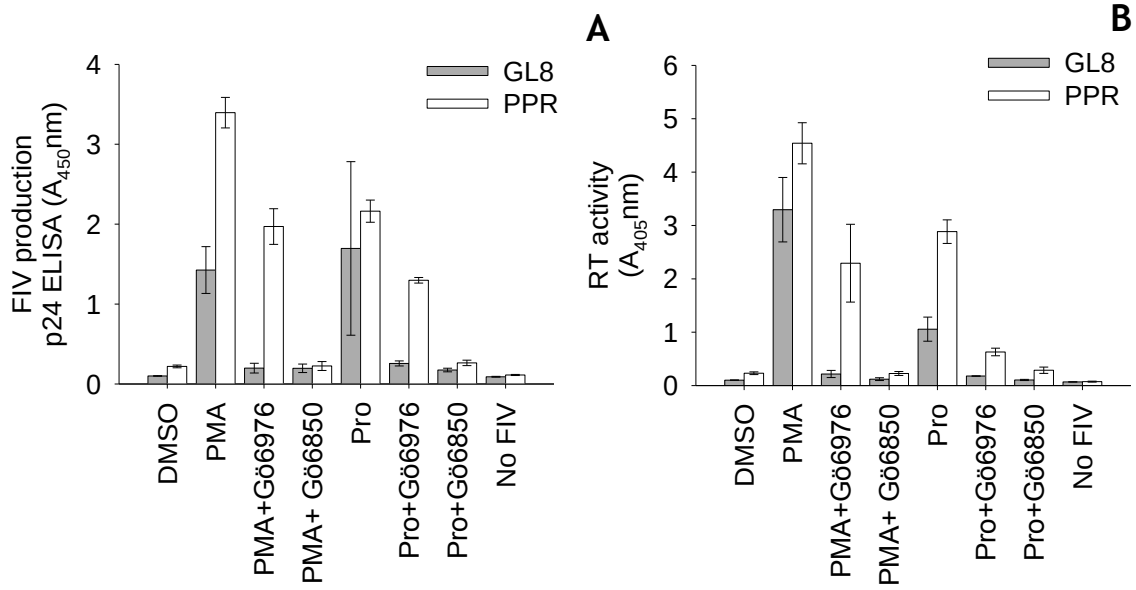


Figure 5-4 – Replication of FIV PPR from infected resting MYA-1 T cells stimulated with phorbol esters is less sensitive to the inhibitory effect of PKC inhibitor Gö6976 than FIV GL8. IL-2-depleted cells were infected with FIV GL8 or PPR (M.O.I. = 0.01). 2 days post infection, the cells were stimulated with either PMA (0.08 µM) or Prostratin (Pro - 1 µM) in combination with PKC inhibitors Gö6976 (2.5 µM) or Gö6850 (2.5 µM). Two independent experiments were performed and virus production was measured at day 8 post infection by (A) FIV p24 ELISA and (B) a non-isotopic reverse transcriptase (RT) assay. Each bar represents the mean +/- range (n=2).

As shown in the previous chapter, IL-2-dependent productive infection of feline $CD4^+$ MYA-1 T cells by FIV GL8 was inhibited by Prostratin and this viral restriction effect could be reversed by the addition of the PKC inhibitor Gö6850. A parallel experiment with FIV PPR showed that PMA and Prostratin inhibited replication of both FIV GL8 and PPR, although the restriction of PPR was not as complete as the restriction of GL8 (Fig. 5-5). Whereas Gö6850 completely reversed the inhibitory effect of PMA and Prostratin, Gö6976 could not rescue

virus replication entirely (Fig. 5-5) suggesting that PKC α and β1 played only a minor role in mediating the restriction of FIV. A further level of complexity was revealed when the PKC inhibitor Gö6976 failed to reverse PMA-induced inhibition of FIV GL8 replication completely, while the replication level of FIV PPR was fully restored by Gö6976 (Fig. 5-5). In summary, these data showed that the conventional PKC isoforms are less important in mediating phorbol ester-induced inhibition of FIV production and that replication of FIV PPR was less sensitive to manipulation of the host PKC activation level than FIV GL8.

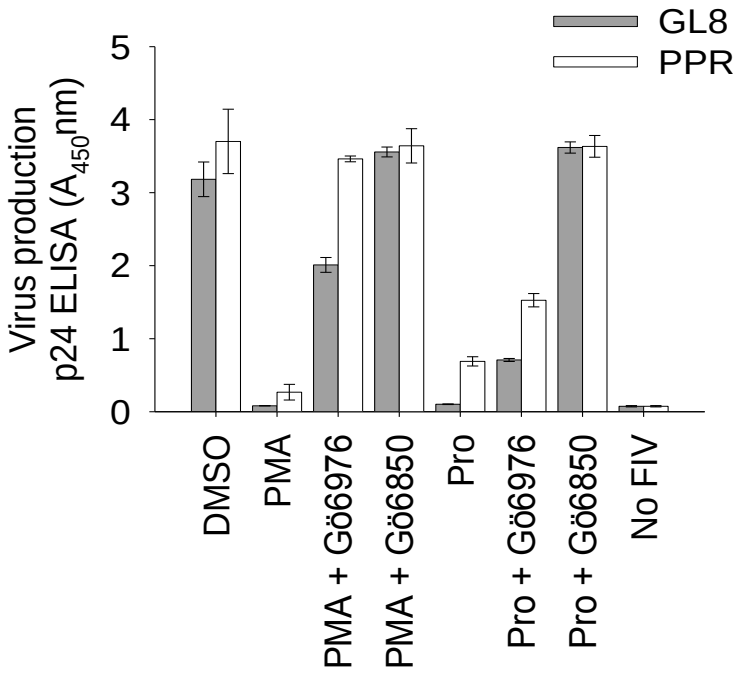


Figure 5-5 – FIV PPR is less sensitive to phorbol ester-mediated inhibition of productive infection than GL8 in MYA-1 CD4$^+$ T cells. IL-2 supplemented MYA-1 CD4$^+$ T cells were infected with FIV PPR or GL8 (M.O.I. = 0.01). 2 days post infection, the cells were stimulated with solvent (DMSO), PMA (0.08 μM), Prostratin (Pro – 1 μM), PMA or Prostratin and Gö6976 (2.5 μM), PMA or Prostratin and Gö6850 (2.5 μM). Virus production at day 8 post infection was measured in the supernatant by FIV p24 ELISA. Each bar represents the mean +/- range (n=2).

Next, attempts were made to map the region(s) of the viral genome that mediate the differences in virus replication characteristics between the two strains of FIV, based on the approaches by de Rozieres *et al* in their study of FIV inter-clade variations (de Rozieres et al., 2008). The nucleotide sequence of FIV GL8 was 92% identical to FIV PPR (Table 5-1); however, the 8% difference was

enough to cause the aforementioned measurable differences in viral replication between the two strains. FIV PPR had a higher basal replication rate than FIV GL8. This could be due to higher viral gene expression mediated by the FIV 5' LTR, which contains the binding sites for cellular transcription factors such as AP-1, AP-4 and ATF (Sparger et al., 1992, Thompson et al., 1994) (and Fig. 5-6). There are 13 single nucleotide variations between the U3 regions of FIV GL8 and PPR, only one of these is within a known transcription factor binding site (ATF - Fig. 5-6). Two single nucleotide variations were detected in a putative NFκB site, but previous binding and mutation studies have shown that this is unlikely to play an active role in FIV transcription (Sparger et al., 1992, Thompson et al., 1994, Joshi et al., 2005b).

The two strains of viruses also differ in receptor usage, mediated by sequence variations in the *env* gene. "Early" strains have stricter requirements for CD134 (Willett et al., 2006b), while "late" strains are less dependent on CD134 and are able to infect previously non-permissive cells when they over-express CXCR4 (de Parseval et al., 2004b). Alternatively, variations at other regions of the viral genome could be responsible for the differences in virus replication between the strains. To investigate whether the differences of growth characteristics between FIV GL8 and PPR were due to different viral gene expression levels or entry requirements, the GL8 5' LTR or *env* in the GL8 molecular clone was substituted with PPR 5' LTR or *env* to assess whether the substitutions would modify the GL8 growth profile to resemble that of PPR (Fig. 5-7C, D)

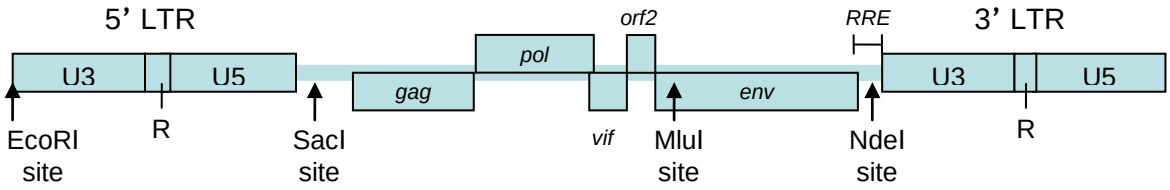
Whole genome (9469 bp)	92.00%
5'LTR → *gag* ATG (0 – 626)	95.00%
gag (626 – 1978)	96.00%
pol (2146 – 5241)	92.00%
Between *pol* → *env* (5241 – 6263)	88.00%
env (6263 – 8831)	89.00%

Table 5-1 – Sequence homology of different regions of the viral genome between FIV GL8 and PPR. Percentage homology calculated using CLUSTALW2 alignment program. Numbers in bracket represents nucleotide positions in FIV GL8 genome.

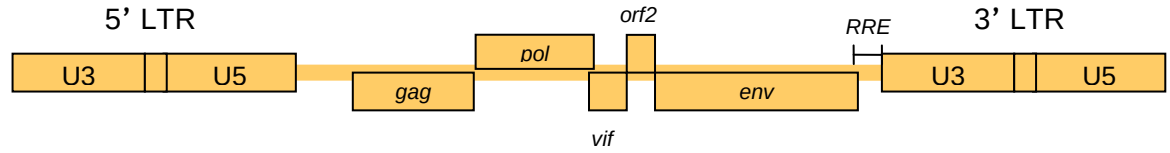
Figure 5-6 – Sequence variations between FIV GL8 and PPR in the U3 region of the 5'LTR affect only one of the known transcription factor binding sites of the FIV LTR. CLUSTALW sequence alignment of the U3 region, based on the sequence of the GL8 molecular clone and the published genome of PPR (Phillips et al., 1990). 13 nucleotide variations were found and those which fall within known binding sites of transcription factors (Sparger et al., 1992, Thompson et al., 1994) were highlighted in blue. The NFκB site is found in FIV-Petaluma but is not thought to be transcriptionally active in FIV GL8 and PPR (Sparger et al., 1992, Thompson et al., 1994).

The chimeric viruses were transduced into MYA-1 $CD4^+$ T cells and expanded in culture. The chimeric viruses produced by MYA-1 $CD4^+$ T cells were resequenced and it was found that only the R-U5 section of the 5' PPR-LTR was exchanged, despite a full LTR swap in the molecular clone (Fig.5-8). This was caused by the mechanism of reverse transcription: the reverse transcriptase binds to the 5' primer binding site of the viral RNA and reverse transcribed up to the R region, before transferring to the 3' R and continuing the reverse transcription of U3 at the 3' end (See section 1.2.2). Since the 3'LTR was not altered in the molecular clone of the chimeric viruses, the 5' LTR U3 region was unchanged in the virus produced (Fig. 5-8)

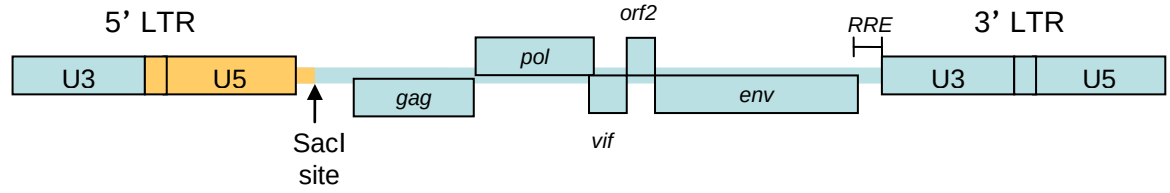
(A) FIV GL8



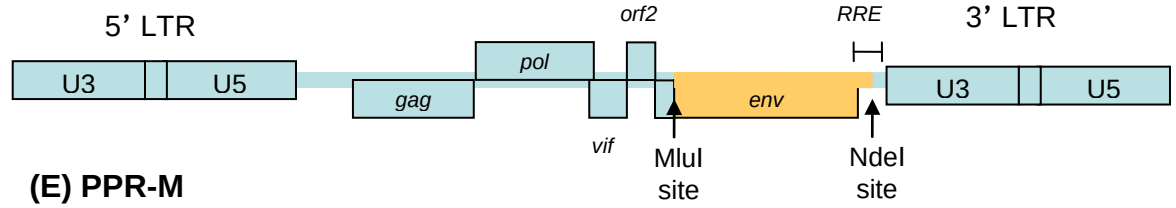
(B) FIV PPR



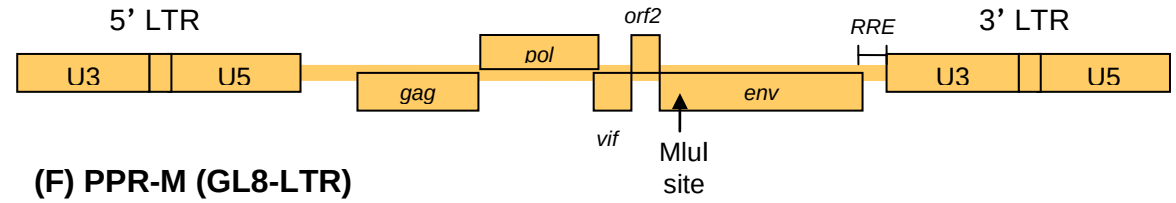
(C) GL8 (PPR-LTR)



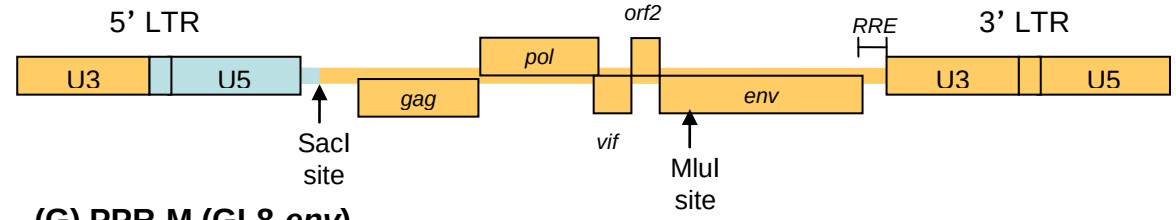
(D) GL8 (PPR-*env*)



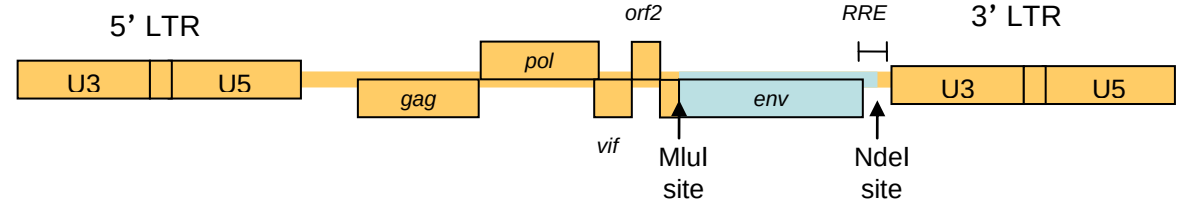
(E) PPR-M



(F) PPR-M (GL8-LTR)



(G) PPR-M (GL8-*env*)



(H) PPR-M (GL8-*env*-LTR)

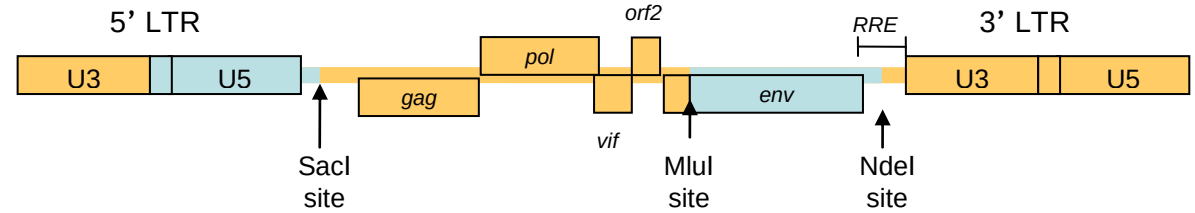


Figure 5-7 – Schematics of all the chimeric FIV described in this chapter. Diagrams (A) and (B) represent the 'wild-type' FIV strains. RRE is Rev-response element. GL8 sequences are in blue. PPR sequences are in yellow. The arrangements of the actual viruses rather than the molecular constructs are shown. LTR substitutions were performed by cutting at the EcoRI site just before U3 within the molecular clone and at the SacI site between U5 and *gag*. *Env* substitutions were performed by cutting at the MluI site within the *env* gene and at the NdeI site within the Rev response element (RRE). Wild-type PPR does not contain a MluI site and mutagenesis has to be performed to generate PPR-M. Since only the 5'LTR was substituted in the molecular clone, after reverse transcription the U3 region of the 5'LTR was not substituted.

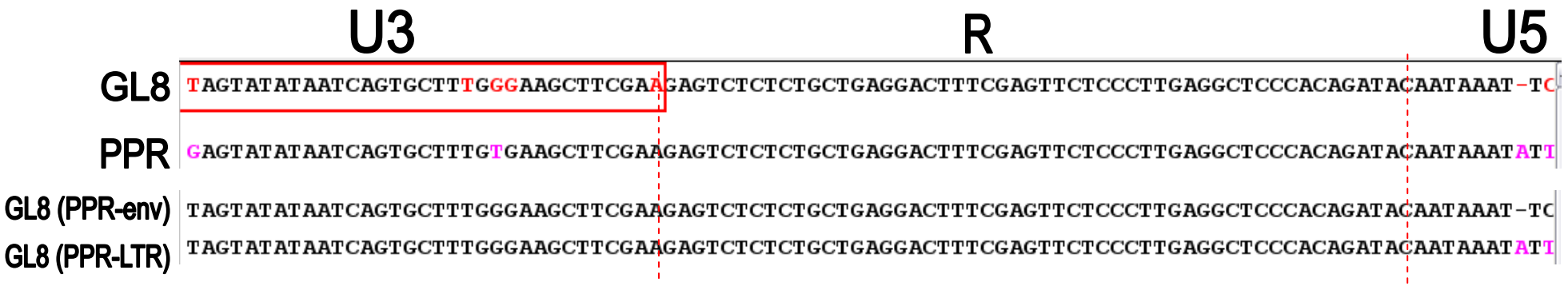


Figure 5-8 – Substitution of the 5' LTR in the molecular clone of GL8 with PPR LTR results in only the partial substitution of the 5' LTR in the provirus. DNA from infected cells was extracted and the region between the 5' LTR and *gag* was sequenced. DNA from cells infected with wild type GL8 and PPR was also sequenced for comparison.

Curiously, when this initial batch of chimeric viruses was used in infections of MYA-1 $CD4^+$ T cells under experimental conditions that had previously produced a clear distinction in virus replication rate between strains (i.e. productive infection driven by IL-2 as well as stimulation of infected resting cells with PMA and Gö6976), both of the chimeric viruses with a GL8 backbone showed higher virus titres at the end of the assay that were comparable with the titres of PPR (Fig. 5-9). In particular when the infected cells were stimulated with PMA in the absence of IL-2, substitution of GL8 LTR or *env* with their PPR counterparts reversed the sensitivity of GL8 to Gö6976 (Fig. 5-9B).

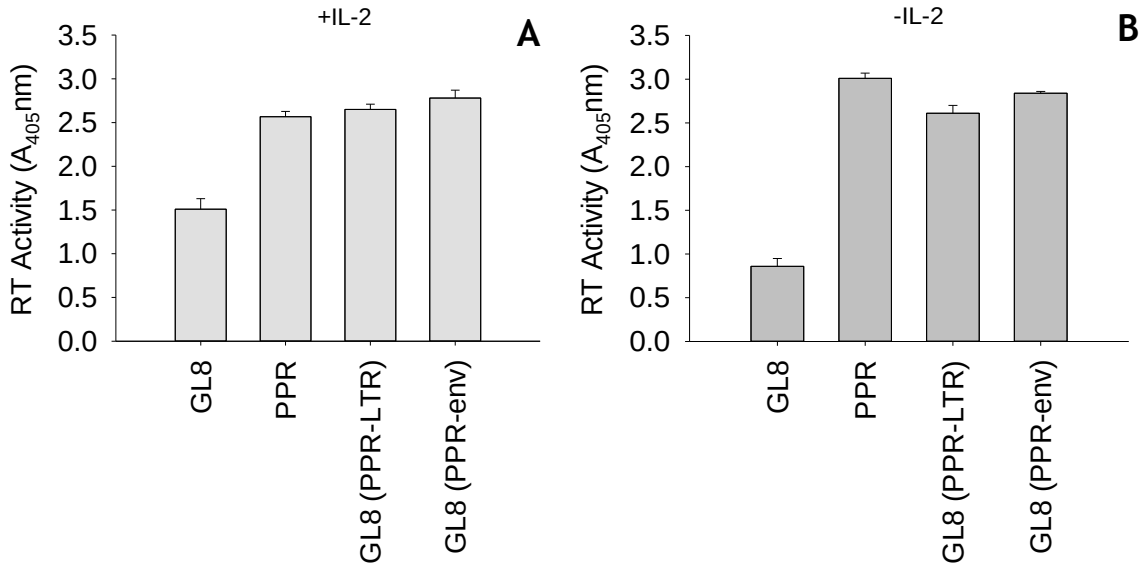


Figure 5-9 – Substitution of GL8 5'LTR or *env* with their PPR counterparts increases the replication rate of the chimeric virus. MYA-1 $CD4^+$ T cells were seeded in the presence (A) or absence (B) of IL-2. 24 hours post seeding, cells were infected with wild-type GL8, wild-type PPR, GL8 (PPR-LTR) or GL8 (PPR-*env*) at the same M.O.I (0.01). (A) Productively infected cells were maintained in the presence of exogenous IL-2 throughout the experiment while (B) infected resting MYA-1 T cells were stimulated with simultaneous treatment of PMA (0.08 μM) and Gö6976 (2.5 μM) 48 hours post infection. Virus titre in the supernatant at day 8 post-infection was measured by a non-isotopic reverse transcriptase (RT) activity assay. Each bar represents the mean +/- standard error (n=3).

Next, more chimeric viruses were constructed, this time substituting PPR LTR and *env* sequence with their equivalents from GL8 in order to further deduce the role of these regions in the dynamics of virus replication (Figure 5-7E to H). Because the substitution of either LTR or *env* appeared to be sufficient to alter

the growth dynamics of FIV GL8 to resemble those of FIV PPR, it is possible that in order to make PPR growth resemble that of GL8, both the LTR and *env* of GL8 would have to be substituted with those of the PPR molecular clone. To achieve the replacement of PPR *env* with the GL8 equivalent, the PPR *env* gene was mutated to generate a novel MluI restriction site at the start of the gene. PPR molecular constructs which had undergone this change were named "PPR-M" (Fig. 5-7E). These new viruses were transduced into MYA-1 $CD4^+$ T cells and expanded in culture. A new batch of MYA-1 $CD4^+$ T cells were then infected with all the chimeric viruses under the same conditions as described previously. Unexpectedly, results from this repeat were the opposite of the previous findings (Fig. 5-10); GL8 appeared to replicate at a higher rate when compared with PPR. The expression levels of the viral receptors CD134 and CXCR4 on this batch of $CD4^+$ T cells were assessed by flow cytometry and it was found that while the level of CD134 expression was within the expected range (59.2% positive), the level of CXCR4 expression had dropped below detection threshold compared with the normal CXCR4 expression level of between 1 and 10%. However, the CD134 and CXCR4 expression levels of the cells were not measured for the cells used in the first chimeric virus infection assay, thus it was not certain that CXCR4 expression was higher during the initial experiment.

Although the results from these two preliminary experiments were not directly comparable, comparisons within the dataset of the second chimeric virus experiment could still be made: in the presence of IL-2, PPR-M infected cells produced a lower viral titre than cells infected with the PPR wild-type virus (Fig. 5-10A). When IL-2-depleted cells were treated with PMA and Gö6976, there was no difference between the virus titres of the supernatant from PPR wild-type and PPR-M-infected cells. In the presence or absence of exogenous IL-2, substitutions of both the *env* and the LTR regions from PPR-M with GL8 sequences resulted in an increase in virus replication, but this was not as pronounced as when only the GL8 *env* sequence was introduced into PPR-M (Fig. 5-10). Furthermore the introduction of the PPR 5' LTR into GL8 dramatically reduced virus production as detected in the supernatant (Fig. 5-10), even though the chimeric virus contained GL8 *env*.

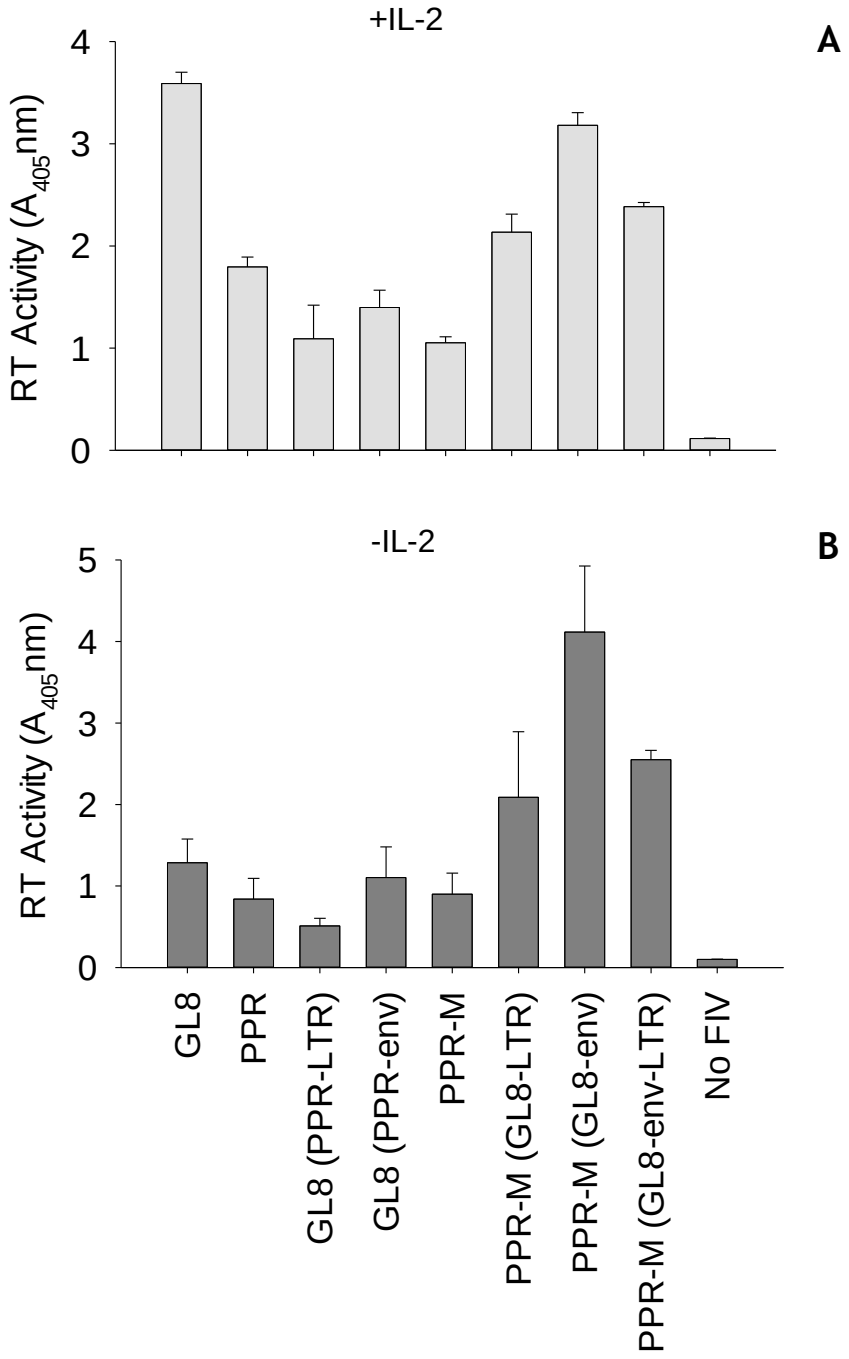


Figure 5-10 – Both the LTR and *env* influence viral replication efficiency in MYA-1 T cells. MYA-1 $CD4^+$ T cells were seeded in the presence (A) or absence (B) of exogenous IL-2 (100 U/ml). 24 hours post seeding cells were infected with wild-type GL8, wild-type PPR, PPR-LTR-GL8, PPR-*env*-GL8, PPR-M, GL8-LTR-(PPR-M), GL8-*env*-(PPR-M) or GL8-LTR-*env*-(PPR-M) at the same M.O.I (0.01). Cells were either supplemented with exogenous IL-2 (A) or treated with PMA (0.08 μM) plus Gö6976 (2.5 μM) 48 hours post infection (B). Virus titre in the supernatant at day 8 post-infection was measured by a non-isotopic reverse transcriptase (RT) activity assay. Each bar represents the mean +/- standard error (n=3).

5.3 Discussion

This chapter describes the different growth characteristics of the 'early' GL8 and 'late' PPR strains of FIV. Under different experimental conditions *in vitro* PPR consistently reaches peak virus titre sooner than GL8. When *de novo* infection was blocked by integrase inhibitor, reactivation of latent PPR produced more virus in the culture supernatant than GL8, indicating that the rate of virus replication per infected cell for PPR is higher than GL8.

The progression to AIDS in HIV-1 infected individuals has been associated with a shift in co-receptor usage of the virus from CCR5 to CXCR4 (Connor et al., 1997), which is largely determined by the sequence of the *env* gene (Cheng-Mayer et al., 1990). During FIV infection there is an analogous change in receptor preferences of different viruses strains which is mediated by the interaction between Env and CD134 (Willett and Hosie, 2008); entry of an 'early' FIV strain into host cells require CD134 and CXCR4, whereas 'late' strain viruses exhibits less requirement for CD134 (de Parseval et al., 2005, Willett et al., 2006b). Attempts were made to map the regions within the FIV genome that are responsible for the inter-strain differences by creating chimeric viruses which contained the LTR and/or *env* sequences from a different strain of FIV. Two preliminary experiments were performed; in the first experiment the PPR wild-type, as well as the GL8 chimeric viruses containing PPR-LTR or PPR *env* replicated at a higher rate than the GL8 wild-type virus, which was in concordance with established trends; in the second experiment GL8 wild-type virus replicated at a higher rate than PPR. This was contrary to previous results and corresponded with a drop in CXCR4 expression level of the cells below the detection limit of flow cytometry.

The first question that needs to be addressed is the cause of the sudden change in viral replication dynamics in the *in vitro* system using the MYA-1 $CD4^+$ T cells. In most of the experiments FIV PPR consistently replicated at a higher rate than FIV GL8. The reversal of the viral replication characteristics can be attributed to changes to the efficiency of viral entry because of the following two observations: firstly, the expression of CXCR4 on MYA-1 $CD4^+$ T cells during the second chimeric virus infection fell below the normal range of expression, although expression of the viral receptors was not checked before the first chimeric virus infection, so no direct comparison between the two experiments

was possible. Secondly, the substitution of PPR *env* with the GL8 *env* led to a large increase in virus replication. Thus it can be hypothesized that the dynamics of virus replication are influenced by the expression level of the viral receptors only when CXCR4 expression is very low. In the previous chapter it was demonstrated that FIV GL8 does not require a high level of receptor expression in order for efficient productive infection to take place. The data from the second chimeric virus experiment also agreed with the above conclusion. Furthermore, the results indicated that PPR replication was more sensitive to the lack of CXCR4 than GL8, which is contrary to a previous report of PPR infection being less sensitive to blockage of CXCR4 than GL8 (Willett et al., 2009). However, more experiments with the chimeric viruses should be carried out before a final conclusion is drawn.

The experiments involving the chimeric viruses were terminated at the preliminary stage of development. However, these early experiments have provided valuable insights into the nature of the *in vitro* system that was used. It has been shown that the expression of CD134 and CXCR4 on MYA-1 cells fluctuates over time (Fig. 4-17). As mentioned previously this may be a reflection of the change in the physiology of the MYA-1 cells as they undergo phases of blasting, rapid expansion and rest. Thus it would be important to monitor the expression levels of the viral receptors closely in future experiments with MYA-1. Alternatively viral binding to the receptors may be manipulated to deduce its contribution to the subsequent productive infection by different viruses.

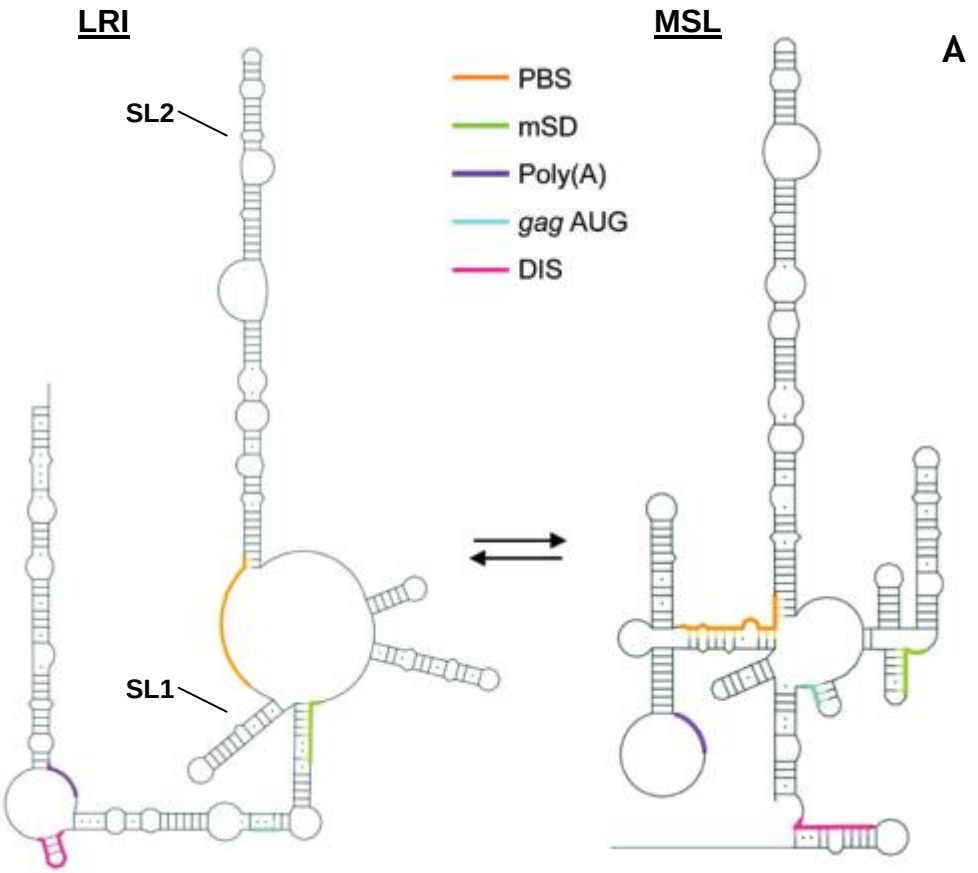
These preliminary results also illustrated the possibility that factors other than viral entry influence viral replication dynamics: firstly, both the substitution of GL8 *env* or LTR regions with the PPR counterparts led to an increase in virus replication in the first chimeric virus experiment. Secondly, the substitution of both the *env* and the LTR regions of PPR with their GL8 counterparts resulted in an increase in virus replication in the second chimeric virus experiment, but this increase was not as high as the increase caused by a substitution of the *env* alone; thirdly, in the presence of IL-2, the introduction of the PPR LTR into the GL8 genome markedly inhibited its replication despite the fact that the GL8 *env* was retained by that particular chimera. These observations indicated that the introduction of a LTR from a foreign strain can affect virus replication rate.

Sequencing of the chimeric viruses showed that replacing the entire 5'LTR in the molecular clone leads to the partial replacement of the 5' LTR in the viral genomes produced. This is due to the mechanism of reverse transcription as explained in the introduction (Section 1.2.2). In FIV the binding sites for cellular transcription factors such as AP-1 and ATF are located in the U3 region and were not substituted in the chimeric viruses. These sites are vital to the transcriptional activities of the FIV 5' LTR (Ikeda et al., 1998). However, as the U3-unchanged, R-U5 substituted viruses exhibited altered replication kinetics, it suggests that LTR transcription efficiency does not contribute to the variation in viral replication between strains. Although the aforementioned data were preliminary, the suggestion that the efficiency of viral transcription does not mediate variation between strains concurs with the conclusion of a similar study on FIV (de Rozieres et al., 2008). In this study of variations between a clade A (PPR) strain and a more pathogenic, clade C (C36) strain of FIV, it was found that the transcription of chloramphenicol acetyl-transferase driven by the LTR of the two strains are the same. Furthermore, all the chimeric viruses are less virulent than the wild-type C36 strain, while FIV PPR chimeras containing C36 elements did not enhance viral replication compared with wild-type PPR in *ex vivo* experiments. This led to the conclusion that variations at multiple regions of the viral genome, rather than variations at a single site contribute to the inter-strain differences in viral growth. Our limited data also supports such a conclusion.

A possible explanation that may account for these observations is that the sequence variations between strains have led to difference in the viral RNA secondary structures. R-U5 falls within the 5' untranslated leader region (UTR) of the viral RNA genome, which forms secondary structures such as stem loops and contains sites that are vital to virus replication such as the primer binding site (PBS), the polyadenylation (poly A) site, the major slice donor site (mSD) and the dimerization initiation site (DIS) (Fig. 5-11A). It has been well-established that the secondary structures at the 5' UTR of the retroviral RNA genome mediate dimerization and packaging of the viral RNA, processes that directly affect the efficiency of viral replication and infectivity (Das et al., 1997, Greatorex, 2004, L'Hernault et al., 2007). In FIV the initial 511 nucleotides of the viral RNA are essential for packaging, but there is also a bi-partite packaging signal which consists of the first 150 nucleotides of the 5' UTR and the first 100

nucleotides of the *gag* gene (Kemler et al., 2002, Browning et al., 2003, Kemler et al., 2004, Mustafa et al., 2005). Within the packaging signal several conserved RNA stem loops (SLs) and other secondary structures have been identified (Kenyon et al., 2008, Kenyon et al., 2011). These structures have been shown to form a long-range interaction (LRI) conformation that facilitate dimerization and packaging or the viral genome, or an alternative multiple stem loop (MSL) conformation that favours translation of the viral genes (Rizvi et al., 2010, Kenyon et al., 2011) (Fig. 5-11A).

The 5' UTRs of GL8 and PPR contain a number of nucleotide variations, which are located in the PBS, SL1, poly-A and SL2 (Fig. 5-12). Mutating the nucleotide sequence of the 5' UTR has been demonstrated to disrupt RNA secondary structure and its associated functions (Rizvi et al., 2010, Kenyon et al., 2011). Thus it can be speculated that the sequence variations between the strains led to differences in the nature and stability of the viral RNA secondary structures, and that substitution of the R-U5 region between different strains may cause instability of the RNA secondary structure as the incoming sequence may form a structure not evolved to co-ordinate with the rest of the viral packaging signal. Such an explanation has been invoked previously to explain similar results from a similar experiment (de Rozieres et al., 2008). In contrast during the first experiment, the replacement of GL8 LTR by the PPR LTR increased virus titre at day 8 post-infection. This does not fit with the aforementioned hypothesis on RNA secondary structure. However, samples of the supernatant were taken only at the end of the experiment, thus it is possible that LTR-substituted chimeric viruses did exhibit growth defects, but by day 8 post infection all the virus titres have reached plateau and the defects were hidden. This emphasises the need for more repeats and more frequent sampling of virus titre during experiments.



B

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                                                    U3 ←    R   →
FIV-GL8     TAATAGTATATAATCAGTGCTTTGGGAAGCTTCGAAGAGTCTCTCTGCTGAGGACTTTCG 240
FIV-PPR     TAAGAGTATATAATCAGTGCTTTGTGAAGCTTCGAAGAGTCTCTCTGCTGAGGACTTTCG 239
            *** ******************** **********************************

                                ←    R   →          Poly-A              (U5) →
FIV-GL8     AGTTCTCCCTTGAGGCTCCCACAGATACAATAAAT-TCTGAGATTGAACCCTGTCAAGTA 299
FIV-PPR     AGTTCTCCCTTGAGGCTCCCACAGATACAATAAATATTTGAGATTGAACCCTGTCAAGTA 299
            ************************************ * ********************

                                                  SL1                 PBS
FIV-GL8     TCTGTGTAGTCTTTTCTACCTGCGAGGTCTCGGAATCCGGGCCGGAACTTCGCAGTTGG 359
FIV-PPR     TCTGTGTAGTCTTTTCTACCTGTGAGGTCTCGGAATCCGGGCCGAGAACTTCGCAGTTGG 359
            ********************** ********************** ***************

                        PBS                 SL2
FIV-GL8     CGCCCGAACAGGGACTTGAGGAAGAGTGATTGAGGAAGTGAAGCTAGAGCAATAGAAAGC 419
FIV-PPR     CGCCCGAACAGGGACTTGAGAAAGAGTGATTGAGGAAGTGAAGCTAGAGCAGTAGAAAGC 419
            ******************** ***************************** ********

                                    SL2
FIV-GL8     TGTTAAGCAGAACTCCTGCTGACCTAAATAGGGAAGCAGTAGCAGACGCTGCTAA-CAGT 478
FIV-PPR     TGTTAAGCAGAACTCCTGTTGACCTAAATAGGGAAGCAGTAGCAGACGCTGCTAAACAGT 479
            ****************** ************************************ ****

                                SL2      SacI site
FIV-GL8     GAGTATCTCTAGTGAAGCAGACTCGAGCTCATAATCAAGTCACTGTTTAAAGGCCCAGAT 538
FIV-PPR     GAGTATCTCTAGTGAAGCAGACTCGAGCTCATAATCAAGTCACTGTTTAAAGGCCCAGAT 539
            ************************************************************
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Figure 5-11 – Multiple sequence polymorphisms between FIV GL8 and PPR are found within or near RNA secondary structures in the 5' UTR. (A) A diagram from (Kenyon et al., 2011) showing the LRI and MSL conformation of the RNA secondary structures. SL = stem loops. PBS = Primer binding site. mSD = major splice donor. Poly (A) = polyadenylation site. *gag* AUG = start codon of the *gag* gene. DIS = Dimerization initiation site. Permission obtained from Oxford University Press. (B) CLUSTALW sequence alignment of the UTR from R to the Sac-I restriction site, based on known sequence of the GL8 molecular clone and the published sequence of PPR, as well as viral DNA amplified from infected cells using PCR. Nucleotide variations are highlighted in green. The labelled and underlined sequences represent the locations of RNA secondary structures (Kenyon et al., 2008, Kenyon et al., 2011). Viral RNA species do not contain U3 and would begin at the start of the R region. FIV LTRs in the molecular clones were substituted by restriction digest with EcoRI at the 5' end (which cuts at the start of the proviral sequence) and with SacI at the 3' end (which cuts at nucleotide 491 of the FIV proviral genome).

Variations in viral entry efficiency and RNA secondary structures are not the only possible mediators of inter-strain differences in viral replication. Mutations or polymorphisms at accessory genes can produce dramatic alterations in viral growth and pathogenesis, as illustrated by the *nef*-defective HIV-1 strain responsible for the Sydney Blood Bank Cohort (Dyer et al., 1997). By monitoring the sequence of a chimeric FIV-PPR/C36 virus as it was passaged *in vivo*, residue 813 of the FIV integrase was identified as vital to *in vivo* viral growth (Thompson et al., 2011). This residue was subjected to sequential mutations that altered the characteristics of the virus, as it was passaged first *in vitro*, then *in vivo*. This observation illustrated the effect of passaging on the viral sequence, an aspect of strain difference that has not been addressed in this thesis. It is known that lab-adapted strains of HIV-1 exhibit different viral entry requirements compared with primary strains (Kabat et al., 1994). Also it has been shown that long term infection of cats with a low virulence strain (FIV-Pet $_{F14}$) leads to the emergence of more pathogenic variants of the parental strain (Hosie et al., 2002). These are all potential areas of future research.

Another observation from the chimeric virus experiment illustrated a recurring principle throughout this and the previous chapter – there is an entry-independent, PKC-mediated virus restriction mechanism within the $CD4^+$ T cells of cats. The potential identity of this mechanism has been discussed already. In this chapter it has been demonstrated that the two different strains of FIV react very differently to this PKC-driven restriction.

Using the PKC inhibitors Gö6976 and Gö6850 my experiments had demonstrated the involvement of PKC in the dual action of the phorbol esters on FIV replication. In all of the experiments, FIV PPR was less sensitive to the effects of Gö6976 than FIV GL8 while both strains were sensitive to the effects of Gö6850. Since Gö6976 is a specific inhibitor of conventional PKC isoforms such as PKC α and ß1 while Gö6850 inhibits both the conventional and novel PKC isoforms (such as PKC δ, ε and ζ), it can be speculated that the replication of the two FIV strains is controlled by different PKC isoforms. FIV PPR was also less sensitive to the inhibitory effects of PMA or Prostratin when grown in the presence of IL-2, although in the absence of IL-2, either phorbol esters could stimulate the replication of FIV PPR more robustly than they could stimulate FIV GL8. This could be the result of the higher basal replication rate of PPR, which allows it to outgrow GL8 when the situation allows. Previous studies have shown that the AP1/AP4 sites, as well as the ATF site are essential for basal FIV promoter function (Sparger et al., 1992, Thompson et al., 1994). The AP-1 site is also shown to be necessary for activation by PMA (Sparger et al., 1992). Sequencing of the LTR showed that while there is no sequence variation within the AP-1 site of both GL8 and PPR, single nucleotide variations occur around the AP-1 site and within the ATF site (Fig. 5-6). However, as mentioned earlier the U3 region of the LTR in the chimeric viruses was unchanged, thus the sequence variation within U3 cannot account for the differences in viral production between strains. The mechanism behind the different sensitivity to PKC activity demonstrated between FIV GL8 and PPR remains elusive. The implication of these findings to viral pathogenesis is also unknown at this stage. A complete profile of PKC-sensitivity for each FIV strain could be produced using knock-down experiments or employing other specific PKC inhibitors in infection assays. The data generated from these experiments would be crucial to our understanding of this phenomenon.

Lastly, the data from these experiments showed that PMA and Prostratin activate novel and conventional PKC isoforms differently in MYA-1 $CD4^+$ T cells. Phorbol esters bind to PKC at a hydrophilic cleft at the C1 domain (Ono et al., 1989, Burns and Bell, 1991, Zhang et al., 1995). This interaction results in the translocation of PKC to lipid membranes, which is essential for the activation of the enzyme (Zhang et al., 1995). The molecular structures of the phorbol esters determine their lipophilicities and biological activities (Wang et al., 2000b). The structural differences between PMA and Prostratin that mediate preferences for different PKC isoforms are outside the scope of this thesis, but it would be an important consideration in the rational design of PKC modulating compounds that target latency.

In summary, the experiments described in this chapter show that 'early' and 'late' strains of FIV have different replication dynamics under experimental manipulation of host cell signalling. Attempts were made to map the viral genome sequence(s) responsible and found that both the *env* and 5'UTR may jointly mediate the differential responses between strains. This finding, if confirmed, would illustrate a new relationship between the viral RNA secondary structure and host cell signalling which can affect virus replication and pathogenesis.

6 Concluding remarks

Viruses fall within the twilight zone between living and non-living entities (Moreira and Lopez-Garcia, 2009). All viruses, including the 'quasi-autonomous' Mimivirus, are obligate intracellular pathogens that are completely reliant on the host cell for replication (Claverie and Abergel, 2010). This makes virus replication very sensitive to changes in cell signalling. In this thesis I have explored the different ways in which cell signalling can affect FIV replication. Firstly, I have investigated the hypothesis that FIV Env triggers CD134-mediated cell signalling. My results did not support this theory and I have discussed an alternative hypothesis in which FIV binds to the co-receptor CXCR4 and triggers signalling. Next I investigated latent infection with FIV further, and showed that by depleting IL-2 from IL-2 dependent MYA-1 $CD4^+$ T cells productive infection with FIV is almost completely stopped, mainly due to a block of virus spread among the uninfected resting T cells. Moreover stimulation by the phorbol esters PMA or Prostratin induced the resting MYA-1 T cells to become conducive to productive infection. Paradoxically in the presence of IL-2, productive infection with FIV can be inhibited by the same phorbol esters. The dual-activity of PMA and Prostratin is dependent on PKC and is analogous to findings from HIV-1 studies, illustrating a reliance on a similar cell signalling pathway for the replication of both HIV-1 and FIV. However, the data indicated a post-entry mechanism mediates the anti-FIV effect of Prostratin, contrary to findings from HIV-1 studies. Lastly I demonstrated measurable biological differences between 'early' and 'late' isolates of FIV by the manipulation of host cell signalling and speculated on the significance of these differences to viral pathogenesis. I also attempted to map locations on the viral genomes that are associated with the biological differences between strains. The preliminary data showed the involvement of the *env* gene and the 5' UTR of the virus in determining strain differences, and I speculated that the sequence differences between FIV GL8 and PPR resulted in differences in the viral RNA secondary structures that translate into phenotypic variations between the two strains.

The results presented in this thesis illustrate an important aspect of FIV host-pathogen interactions; the almost complete dependency of viral replication on the activation status of the host cell. This is not the case for other viruses such as influenza, which encodes the viral protein NS-1 to activate the PI3K pathway

in its target cells in order to enhance infection (Hale et al., 2006); or the human papillomavirus (HPV) which induces cell proliferation by targeting host cell cycle regulators p53 and retinoblastoma proteins for degradation using the viral proteins E6 and E7 (reviewed in (Moody and Laimins, 2010)). The reliance of FIV replication on the activation of host cell signalling, in particular PKC, is a function of the cell tropism of the virus: in order for the host $CD4^+$ T cell to function it must become activated, which also makes it conducive to infection by FIV. In other words, there is no need for the virus to induce cellular activation. Even in the instances when virus infection does not immediately lead to efficient viral replication, the long term fitness of the virus is not affected because latent infection of $CD4^+$ T cells leads to viral persistence and increases the chance of transmission in the future. However, FIV replication is inhibited when PKC is stimulated above an optimum level (Fig. 6-1). Although the over-stimulation of PKC by phorbol esters is an artificial scenario probably not encountered in nature, does this indicate a potential weakness, a therapeutic "Achilles heel" of the virus which we can exploit?

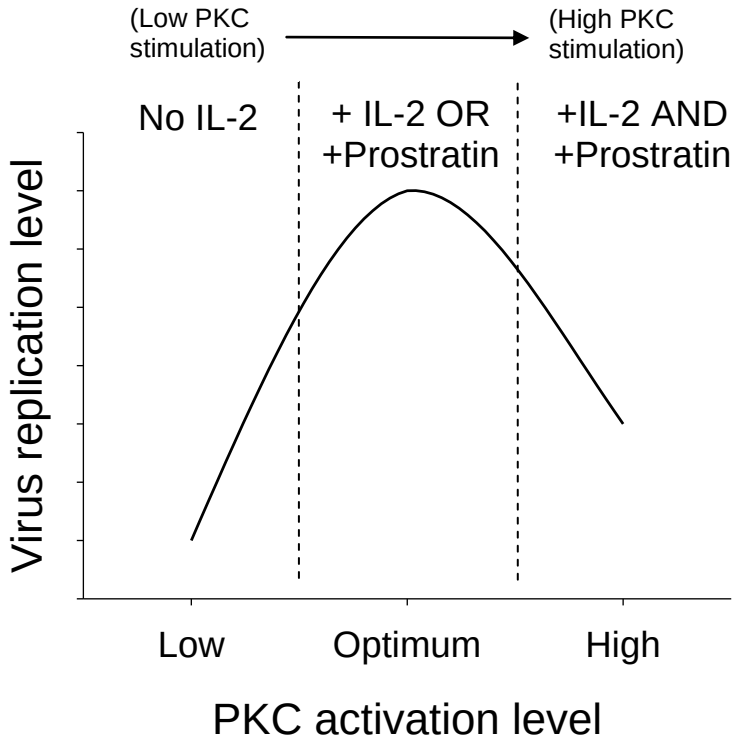

Figure 6-1 – FIV replicates best at an optimum level of PKC activation. Results from this thesis have indicated that the activity of PKC is a central determinant between productive and latent FIV infection; when PKC activation

level is low, virus replication is impaired, whereas increasing the level of PKC activation past the optimum range an anti-viral response is triggered.

The findings of this thesis also reflect the role host responses play in the pathogenesis of viral diseases: infection with HIV-1 and FIV leads to chronic activation and dysregulation of the host immune system, causing the selective destruction of the mucosal barrier and lymphoid tissues, which promote the translocation of microbes and chronic inflammation (See Chapter 1). The host immune response plays a large part in its own destruction; other notable examples of host mediated pathogenesis include the highly lethal viral haemorrhagic fever caused by viruses such as Ebola, Marburg and the Crimean-Congo hemorrhagic fever (CCHF) virus, as well as the recent outbreak of severe acute respiratory syndrome (SARS); haemorrhagic fever viruses target host endothelial cells as well as macrophages and dendritic cell, which damage host blood vessels directly and indirectly through the release of proinflammatory cytokines, coagulation factors and vasodilators. (Hoenen et al., 2006, Hensley et al., 2011, Whitehouse, 2004). The result is massive damage to the host vascular system that triggers a devastating condition known as disseminated intravascular coagulation (DIC), leading to uncontrollable bleeding, shock and death (Hoenen et al., 2006, Hensley et al., 2011, Whitehouse, 2004). SARS is caused by a novel coronavirus thought to originate from bats (Peiris et al., 2003) (Li et al., 2005). In severe cases the host interferon responses become dysregulated, impairing the switch from innate to adaptive response and triggering a destructive "cytokine storm" that causes potentially fatal lung damage (Huang et al., 2005, Cameron et al., 2007).

The investigation of the molecular mechanisms behind lentiviral latency is vital to the discovery of a cure to HIV-AIDS, which remains a major public health concern across the world despite the advent of HAART (Richman et al., 2009). At the time of writing, a method that can potentially induce sterilising immunity against HIV-1 has just been announced (Balazs et al., 2011). Instead of relying on the conventional adaptive immune response against a foreign antigen, this technique utilized an adeno-associated gene therapy viral vector to express high titres of a HIV-broadly neutralizing monoclonal antibody from muscle cells. The expression of the antibody completely blocked HIV-1 infection in a humanized mouse model system (Balazs et al., 2011). Even if an effective way to immunise

against HIV-1 is established and virus transmission is finally halted, there remain the millions of infected individuals who will have to rely on antiretroviral drugs for the rest of their lives unless a practical cure is developed. A suitable small animal model of HIV-1 latency and reactivation is vital to unravel the complex networks of cell signalling involved in lentiviral latency and may lead to the development of therapeutic regimes with minimum side effects to reverse latent infection. The research presented in this thesis has demonstrated similarities between HIV-1 and FIV, supporting the assertion that FIV infection of the domestic cat has potential as an animal model for HIV-1 infection of humans in terms of latency. However, FIV is a different virus to HIV-1, and the data presented here revealed some of these differences. Further research is required to identify which characteristics are common to both viruses to ensure that findings from any future feline model are comparable to HIV-1 infection in humans.

7 Appendices

7.1 Appendix 1: Primers

Name	Sequence (5’ to 3’)
(Detection of FIV DNA)	
LTR Forward 3	GCTTAACCGCAAAACCACAT
Gag Reverse 3	CAAATCTCCTGGCTTGAAGG
Env sense 1	GGCAATGTGGCATGTCTGAAAAAGAGGAGGAATGATG
GAPDH Forward	CCTTCATTGACCTCA ACTACAT
GAPDH Reverse	CCAAAGTTGTCATGGATGACC
(Mutagenesis)	
P-env Mlu 1	OLIGO1: GGAAATGCAGGTAAGTTTAGACGAGCAAGATTCTTGAGATATTC
	OLIGO2: GAATATCTCAAGAATCTTGCTCGTCTAAACTTACCTGCATTTCC
P-env Mlu 2	OLIGO1: GAAATGCAGGTAAGTTTAGACGCGCAAGATTCTTGAGATATTCTG
	OLIGO2: CAGAATATCTCAAGAATCTTGCGCGTCTAAACTTACCTGCATTTC
P-env Mlu 3	OLIGO1: GCAGGTAAGTTTAGACGCGTAAGATTCTTGAGATATTCTGATG
	OLIGO2: CATCAGAATATCTCAAGAATCTTACGCGTCTAAACTTACCTGC
(Quantitative PCR)	
(FIV *gag*)	
FIV1360F	GCA GAA GCA AGA TTT GCA CCA
FIV1437R	TAT GGC GGC CAA TTT TCC T
FIV1416P (Taqman probe)	5’-FAM-TGC CTC AAG ATA CCA TGC TCT ACA CTG CA-TAMRA-3’
(18S rRNA)	
343-Fwd	CCA TTC GAA CGT CTG CCC TA
409-Rev	TCA CCC GTG GTC ACC ATG
Taqman probe	5'-FAM-CGA TGG TAG TCG CCG TGC CTA-TAMRA-3'

7.2 Appendix 2: List of buffers

Hirt buffer: 0.6% SDS, 10mM EDTA, pH 7.5

LB: 10% Bacto-Tryptone w/v, 5% yeast extract w/v, 85 mM NaCl, pH 7.5

PBAz wash buffer: 10 g BSA and 1 g sodium azide dissolved in 1 litre of PBS (See below)

PBS: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4

PBS-T wash buffer: PBS plus 0.1% Tween®

Sample treatment solution (10x): 100mM Tris, 1.5M NaCl, 5% Triton, 5% deoxycholale, 1% SDS, 50mM EDTA and 0.1% azide

TBE (10x): Tris base 890 mM, Boric acid 890 mM, EDTA 2 mM

Publications and posters

Publication

Chan, C. N., MCMONAGLE, E. L., HOSIE, M. J. & WILLETT, B. J. 2012. Prostratin exhibits both replication enhancing and inhibiting effects on FIV infection of feline CD4+ T-cells. *Virus Research* (http://dx.doi.org/10.1016/j.virusres.2012.11.004).

Chan, C. N., Dietrich, I., HOSIE, M. J. & WILLETT, B. J. 2013. Recent developments in HIV-1 latency research. *Journal of General Virology* (Accepted for publication).

Posters presented

May 2010 — **Investigation of the relationship between cell signalling and the growth and latency of FIV -** 10th International Feline Retrovirus Research Symposium (Charleston, SC, USA)

April 2011 — **Feline immunodeficiency virus (FIV): a model for human immunodeficiency virus -1 (HIV-1) latency?** - Society for General Microbiology Spring Conference 2011 (Harrogate, UK)

May 2011 — **Feline immunodeficiency virus (FIV): a model for human immunodeficiency virus -1 (HIV-1) latency?-** Cold Spring Harbor Retroviruses (New York, USA)

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