Lentiviral gene therapy for HIV-1 using TRIM-Cyclophilin restriction factors

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Declaration

I, Emma Chan, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Lentiviral vector delivery of anti-HIV elements could provide the basis of alternative therapies against HIV, potentially providing long term protection after a single intervention. Some primate species have evolved restriction factors formed by the fusion of TRIM5α and Cyclophilin A (TRIM5Cyp) following retrotransposition of CypA cDNA into the TRIM5 gene, which provide potent resistance against certain lentiviruses. We have designed humanised versions of these proteins combining both TRIM5 and TRIM21 with CypA, and investigated their potential for use in gene therapy against HIV-1. Both TRIM5- and TRIM21-Cyp fusion proteins provided strong restriction of HIV-1 in all of the systems tested, including primary human T cells. However, TRIM5Cyp was shown to disrupt the antiretroviral effect of endogenous TRIM5α and rescue murine retrovirus infection, whereas TRIM21Cyp caused no interference. In contrast, neither TRIM5CypA nor TRIM21CypA expression affected the antiviral activity of endogenous TRIM21.

In addition to TRIMCyp restriction factors, a second anti-HIV strategy was investigated using zinc finger nucleases (ZFNs) to knockout the HIV-1 co-receptor, CCR5. ZFNs introduce a double stranded break into the CCR5 gene, which can be restored by homology directed repair. Provision of a green fluorescent protein (GFP) or TRIM21Cyp donor template exploits this repair mechanism to allow site specific integration at the CCR5 locus, although at low efficiency. Using integrating vectors, we have shown that TRIMCyp mediated restriction is so potent that no additional inhibition was conferred by CCR5 knockout.

In conclusion, delivery of TRIMCyp genes using lentiviral vectors could form the basis of an intracellular vaccination strategy against HIV-1, with TRIM21Cyp having benefits by maintaining endogenous TRIM function. With further optimisation to improve efficiency, this could be combined with ZFNs for site specific integration of the transgene and knockout of CCR5 to provide a dual method of HIV-1 inhibition.

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Abbreviations

aa	amino acid
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
APOBEC3	Apolipoprotein B mRNA-editing catalytic polypeptide 1-like protein 3
bp	base pair
CA	Capsid
cDNA	Complementary DNA
CRFK	Crandell Rees feline kidney
CsA	Cyclosporine A
Cyp	Cyclophilin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphide
DNA	Deoxyribonucleic Acid
DSB	Double stranded break
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme linked immunosorbant assay
env	Envelope
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FIV	Feline immunodeficiency virus
Fv1	Friend virus susceptibility factor 1
HAART	Highly active retroviral therapy
HDR	Homology directed repair
HIV-1	Human immunodeficiency virus-1
HRP	Horseradish peroxidase
HSC	Haematopoietic stem cell
IFN	Interferon

IL	Interleukin
IN	Integrase
IRES	Internal ribosomal entry site
IU	Infectious units
kb	Kilobase
kDa	Kilodaltons
LTR	Long terminal repeat
MA	Matrix
Mac	Macaque
MFI	Mean fluorescence intensity
min	Minutes
ml	Millilitre
MLV	Murine Leukaemia virus
MOI	Multiplicity of infection
NC	Nucleocapsid
NHEJ	Non-homologous end joining
NILV	Non-integrating lentivirus
p24	HIV-1 p24 gag capsid protein
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline/primer binding site
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PIC	Preintegration complex
PGK	Phosphoglycerate kinase
polyA	Polyadenylation
PR	Protease
PPT	Polypurine tract
qPCR	Quantitative real time PCR
RCL	Replication competent lentivirus
rhTRIM5α	TRIM5α of Rhesus macaque
RING	Really interesting new gene

RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute-1640
RRE	Rev responsive element
RT	Reverse transcriptase
s	Seconds
SCID	Severe combined immunodeficiency
SFFV	Spleen focus-forming virus
SIN	Self-inactivating
SIV	Simian immunodeficiency virus
TCR	T cell receptor
TCyp	TRIM-Cyclophilin/TRIMCyp
TRIM	TRIpartite motif
VSV-G	Vesicular stomatitis virus glycoprotein G
WPRE	Woodchuck hepatitis virus post transcriptional regulatory element
YFP	Yellow fluorescent protein
ZFN	Zinc finger nuclease

1 Introduction

Since the widespread introduction of highly active antiretroviral therapy (HAART) for the treatment of human immunodeficiency virus-1 (HIV-1) there has been a significant decline in morbidity and mortality (1). The categories of drugs currently available include nucleoside/nucleotide analogue reverse transcriptase inhibitors, non-nucleoside analogue reverse transcriptase inhibitors, protease inhibitors, integrase inhibitors and fusion inhibitors. However, there are numerous associated drawbacks with drug treatments, including cost, toxicity and problems with adherence to the strict drug regimen. In addition, once therapy is halted, there is often viral rebound. Also, this mode of treatment does not offer the possibility of curative therapy. All of these issues have driven research into finding a longer term solution. This includes an alternative approach to drug treatment based on gene therapy, which theoretically could provide a one-off treatment against HIV-1.

There are numerous possible anti-HIV-1 genes which could be employed in gene therapy, targeting both viral and cellular molecules, at different stages of the viral lifecycle. These strategies for restriction can be broadly categorised into two main groups: RNA-based and protein-based, examples of which include short hairpin RNA (shRNA) and neutralising antibodies respectively. There are also different methods of delivery to consider, including adenoviral and retroviral vectors, with lentiviral vectors derived from HIV-1, HIV-2, simian immunodeficiency (SIV) and feline immunodeficiency (FIV) becoming increasingly attractive.

One basic strategy for treating HIV-1 infection by gene therapy would be to modify a population of susceptible cells, for instance T cells, with an anti-HIV-1 gene to confer resistance, allowing them to function as normal in the presence of infection. If these HIV-1 resistant cells have a survival advantage *in vivo*, they would replicate and be able to repopulate the immune system, relieving the patient of the pathology associated with the dramatic reduction of the T cell population.

Some anti-HIV gene therapies have reached clinical trials testing both the safety and efficacy of various strategies.

The aim of this project is to develop the use of restriction factors formed by the fusion of TRIM proteins with the HIV-1 binding enzyme, cyclophilin A. These are based upon the naturally occurring TRIM5-Cyclophilin fusion proteins found to provide some primate species with resistance to HIV-1, suggesting that they are an ideal candidate for a therapeutic agent.

1.1 The HIV-1 genome

HIV is a member of the Retroviridae family. This family can be divided into the simpler viruses, such as murine leukaemia virus (MLV) and the more complex lentiviruses, which includes HIV-1 and -2. HIV shares many of its genes with those of other retroviruses. Common to all retroviruses are the four major structural genes: gag (group specific antigen), pro (protease), pol (polymerase) and env (envelope).

In addition to these, HIV-1 also carries six regulatory and accessory genes: Both tat (transactivator of transcription) and rev (regulator of virion expression) are critical for viral replication. The accessory genes vif (viral infectivity factor), vpr (viral protein R), vpu (viral protein unique) and nef (negative factor), although not compulsory for infectious virus, are all involved in multiple roles in the viral lifecycle and enhance infectivity (Figure 1.1).

Once integrated into the host genome, the viral genome is termed provirus and is flanked by two identical long terminals repeats (LTR). LTRs are further divided into unique U3, repeat (R) and U5 regions. Transcription is initiated from the 5'LTR to produce full length viral transcripts.

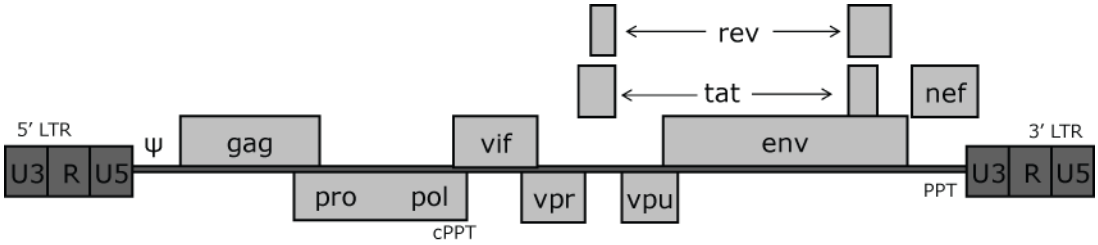


Figure 1.1 Schematic of the HIV-1 provirus
The HIV-1 genome encodes the structural genes gag, pro, pol and env, which are common to all retroviruses. In addition, there are the regulatory genes, tat and rev, and accessory genes, vif, vpr, vpu and nef. The provirus is flanked by two identical long terminal repeats (LTRs), which are subdivided into the U3, R and U5 regions. The genome also includes the packaging signal (Ψ), polypurine tract (PPT) and central PPT (cPPT).

1.2 HIV lifecycle

Virus binds and enters a target cell via interaction with cell surface receptors. Once in the cell, the viral particle is disassembled to reveal its inner viral core through a complex process termed uncoating. The viral core is where reverse transcription of the viral RNA genome takes place, producing double stranded (ds) DNA capable of integration into the host genome. The viral complex, now referred to as the pre-integration complex (PIC) enters the nucleus and integrates into the host DNA to form a provirus. Transcription and nuclear export of the RNA transcripts occur, followed by translation and assembly of viral particles at the cell membrane. Virions eventually bud from the host cell and maturation occurs within this particle, which involves proteolytic cleavage of the viral structural polypeptides and rearrangement to form a mature virus particle capable of infecting other cells. These steps are discussed in further detail below.

1.2.1 Attachment and cell entry

The first step of the HIV-1 lifecycle is attachment to target cells. The main receptor for entry is CD4 (2, 3), a member of the immunoglobulin (Ig) superfamily, expressed on thymocytes, certain mature T lymphocytes and macrophages. CD4 functions to stabilise interactions between the T cell receptor (TCR) and major histocompatibility complex (MHC) class II molecules on antigen presenting cells and is involved in signal transduction downstream of the TCR.

After the identification of CD4 as the receptor for HIV-1 cell entry, further work suggested the requirement of one or more additional co-receptors for cell entry as mouse cells expressing human CD4 alone could not be infected with HIV-1(4). In 1996, two co-receptors for HIV-1 were identified, CCR5 (5-9) and CXCR4 (10). These chemokine receptors are both G protein coupled receptors expressed on haematopoietic cells and play a role in cell migration and inflammation. CCR5 is found on macrophages and some T cell subsets, such as memory T cells. CXCR4 is

broadly expressed on virtually all cells of haematopoietic origin, particularly T cells and $CD34^+$ haematopoietic progenitor cells.

HIV-1 attachment to target cells is mediated by the viral glycoprotein Env. Env is made up of two subunits, glycoprotein 120 (gp120) and gp41. A trimer of gp120 on the viral surface is covalently associated with a trimer of transmembrane gp41.

The two subunits are transported to the cell surface membrane where they are arranged in a trimer of gp120 on the virus surface (11), non-covalently associated with a trimer of transmembrane gp41 (12). On average, 10 trimers are incorporated per virion (13).

Initially, CD4 is bound by gp120, which allows the subsequent interaction between gp120 and the co-receptor on the host cell, either CCR5 or CXCR4, depending upon the viral tropism. This binding induces a conformational change in the gp120-gp41 complex, revealing two heptad repeats and a hydrophobic region called the fusion peptide. This is inserted into the target cell membrane promoting membrane fusion. The heptad repeats are reassembled into a hairpin structure, bringing together the cellular and viral membranes to allow fusion.

1.2.2 Reverse transcription

Following fusion, the viral core is released into the host cell cytoplasm where it is partially disassembled in a process known as uncoating. Although this process is poorly understood, correct timing is thought to be critical for productive infection (14). The stability of the viral core is important, and mutations that either increase or reduce core stability can lead to non-infectious virions. Uncoating occurs sometime between cell entry and nuclear import, with conflicting evidence suggesting different time points. It is at this early stage in the virus life cycle, before reverse transcription occurs, that host cytoplasmic proteins TRIM5α and Cyclophilin A interact with the virus. These interactions and their importance in the HIV-1 lifecycle will be discussed further in sections 1.5 and 1.6.

Uncoating produces the reverse transcription complex (RTC). Reverse transcription, the defining feature of retroviruses, occurs within in the RTC to produce dsDNA

from the single stranded viral RNA genome. The enzyme responsible, reverse transcriptase (RT), is composed of two subunits, p66 and p51 (15). p66 is responsible for the enzymatic function of the protein, whereas p51 has a structural role (16). The two key enzymatic functions of RT are RNA- and DNA- dependent DNA polymerisation and RNase H activity to degrade RNA in a RNA-DNA duplex. Two polypurine tracts, the 3' PPT (17) and central PPT (18), are resistant to RNase H activity, and therefore remain after degradation to function as primers for plus strand DNA synthesis.

Reverse transcription begins with the synthesis of the minus strand of DNA using a lysine tRNA packaged into the virion from the producer cell as a primer (19). At its 3' terminal, $tRNA_{Lys3}$ has 18 nucleotides that are complementary to the primer binding site (PBS) located towards the 5' end of the viral RNA. $tRNA_{Lys3}$ binds and DNA synthesis is carried out to the 5' end producing the minus strand strong stop DNA (-sssDNA), which consists of the U5 and R regions of the LTR. Homology between the 5' and 3' R region allows minus strand transfer of the -sssDNA to the 3' R element of either the same strand of RNA or the second strand that was packaged within the virion. RNA dependent DNA synthesis (RDDP) of the minus strand is continued from here to the 5' end of the RNA, whilst the RNA strand of this RNA-DNA duplex is degraded by the RNase H activity of RT. The exception is at the two PPT which are resistant to RNase H activity and remain to act as primers for DNA polymerisation of the positive (+) strand (20). The DNA dependent DNA polymerase (DDDP) function of RT occurs from the major primer at the 3' PPT and from another primer at the cPPT. Synthesis continues up until the $tRNA_{Lys3}$, where the first 18 nucleotides at the 3' end, which are complementary to the PBS, are reverse transcribed, restoring the PBS. Synthesis stops at a modified nucleotide of the tRNA, with the resultant DNA called positive strand strong-stop DNA (+sssDNA). The tRNA and the RNA PPT are degraded by RT, leaving an overhang of the PBS on the positive strand DNA.

Positive strand transfer occurs with the +ssDNA fragment binding to the homologous -DNA strand PBS. Positive strand DNA synthesis continues to the

cPPT, terminating at the central termination sequence (CTS), where it displaces a 99 nucleotide DNA sequence of plus strand DNA to form the 'DNA flap' (21). The relevance of this flap has not been fully elucidated, and HIV-1 mutants disrupted in this area can still be infectious *in vivo* suggesting it is not vital for infection (22).
Bidirectional DNA synthesis occurs in both directions to the ends of the LTRs using the DDDP function of RT by strand displacement. Finally, this forms a complete double stranded DNA viral genome, which is capable of integration.
During RT, the RTC moves towards the nucleus in preparation for nuclear import.

1.2.3 Nuclear import

Once reverse transcription has taken place the RTC is renamed the PIC. Although there is little distinction between the two, the key difference is that reverse transcription is not complete in the RTC and therefore contains RNA or a RNA-DNA intermediate. In the PIC, reverse transcription has been completed and consequently the nucleic acid consists solely of dsDNA capable of integration.

Although retroviral PICs must wait for the breakdown of the nuclear envelope during mitosis to gain access to the genome for integration (23), lentiviruses are able to actively cross the nuclear membrane to successfully infect non-dividing cells (24). Passage across the nuclear membrane occurs even in actively dividing cells, showing the importance of this mechanism for lentiviral infection (25). The diameter of the PIC greatly exceeds that of the nuclear pore, so nuclear entry does not simply occur by diffusion through these pores. The mechanism utilised by HIV-1 to overcome this and enter the nucleus is still not understood, but many viral and host molecules have been suggested to be involved.
The viral elements MA, CA, IN, Vpr and cPPT and host proteins LEDGF/p75, importins and nucleoporins have all been implicated in driving nuclear import, either independently, or in association with each other. However, results supporting the involvement of these proteins have often been conflicting.

Active nuclear import involves recognition of proteins containing a nuclear localisation signal (NLS) by importins. This complex subsequently interacts with nuclear pore complexes (NPC) which transport proteins into the nucleus.

Initially MA was thought to be involved in nuclear import due to its nuclear localisation signals (NLS) (26), but has since been shown to be non-essential for viral replication (27).

Vpr alone predominantly localises in the nucleus, despite its lack of classical NLS (28). It has also been shown to aid docking of the PIC to NPCs (29) and can bind to importin α and nucleoporins, leading to its accumulation at the nuclear membrane (30) and transport through the NPC (31). However, like MA mutants, HIV-1 lacking Vpr is still able to infect non-dividing macrophages *in vitro* (32).

Several NLSs have been identified in IN (33) but their actual importance is debatable. In addition to its NLS, the interaction of IN with host importins may be crucial for nuclear import (34, 35).

Viral DNA structure may also play a role in nuclear entry. Some groups have shown that mutants lacking the DNA flap synthesised during reverse transcription accumulate at the nuclear membrane and are unable to enter the nucleus (36-38). Conversely, other groups suggest HIV-1 with a mutant or absent cPPT was still capable of infection equal to that of wild type virus (39, 40).

However, replacement of each of these karyophilic elements of HIV-1 with that of MLV suggests that it is the HIV-1 CA that is essential for nuclear import (41-43). This is further supported by the demonstration that certain HIV-1 CA mutants are defective for nuclear entry in cells after cell cycle arrest (44).

CA is able to directly interact with nuclear pore protein 358 (Nup358), a component of the NPC. The interactions with both host cyclophilin A and Nup358 influences the mode of nuclear import to utilise a Nup358/Nup153 dependent pathway. Some CA mutations disrupt these interactions, promoting integration through alternative pathways and consequently altering the genomic region of integration to less favourable locations (45). This implies that CA is present in the PIC and plays an important role in nuclear entry through interaction with host proteins.

Not all PICs enter the nucleus and are integrated into the host genome. Non-productive transcripts remain in the cytosol, and HIV-1 exploits the host exonuclease, TREX1, to degrade surplus DNA. In TREX knockout cells, this degradation is absent allowing the extra viral DNA transcripts to be recognised and promote innate type I IFN signaling, restricting viral replication (46).

1.2.4 Integration into the host genome

IN is the principal enzyme responsible for the integration of viral DNA into the host genome. Its two major catalytic functions are 3' processing of the viral genome and strand transfer to integrate into the host genome. IN is made up of three domains; N terminal zinc binding domain, catalytic core domain and C terminal DNA binding domain which are joined by linker regions and are all essential for catalytic activity. IN acts as a multimer and mutant IN can still support integration if combined with wild type IN (47).

IN binds to attachment sites at the end of the LTRs and cleaves 3' of a conserved CA dinucleotide at the end of the viral DNA to produce 3' hydroxyl groups (48) soon after reverse transcription and prior to nuclear localisation. This is called 3' processing and occurs in the cytoplasm. Once the PIC is imported into the nucleus, strand transfer occurs whereby each 3'-hydroxyl group of the viral LTRs carries out a nucleophilic attack on a phosphodiester bond on opposing strands of DNA of the host genome. IN catalyses the ligation of the 3' viral DNA ends to the 5' ends of the host chromosomal DNA. This results in a five base pair (bp) single stranded region of host DNA and a two bp overhang on the 5' viral DNA at either end. The viral DNA overhang is cleaved before extension from the 3' end of genomic DNA by host machinery. This results in five bp duplications flanking the integrated provirus.

The host sequence has only a mild influence on the site of integration with no strict consensus sequences identified (49, 50). In addition to sequence recognition, integration site is influenced by other factors, such as proximity to genes and transcriptional start sites, and their activity. HIV-1 preferentially integrates into

transcriptionally active genes (51), an ideal location for transcription and therefore viral production.

Host proteins are also thought to be involved in and promote integration, through interaction with IN and/or DNA. Several proteins have been identified; of particular importance is the nuclear protein lens epithelium-derived growth factor/transcription co-activator p75 (LEDGF/p75). LEDGF/p75 is able to bind both IN and chromatin, promoting integration of the viral genome (52). However, this protein is not essential for integration as shown in LEDGF knockout cells (53). Instead, the typical pattern of HIV-1 integration within actively transcribed genes is disrupted and there is increased integration within transcriptional start sites and CpG islands (53, 54).

1.2.5 Transcription of the HIV-1 genome

Once integrated into the host genome, viral transcripts can be produced from the provirus using host machinery, including RNA polymerase II (RNA pol II). Like host mRNA, viral transcripts are subject to 5' capping and 3' polyadenylation. Transcription can be initiated when chromatin is relaxed, allowing access of host enzymes to the provirus. Transcription begins at the U3-R junction of the 5' LTR, and terminates at the polyadenylation signal just after the R region of the 3'LTR.

U3 is further divided into the core promoter, enhancer and modulatory regions. U3 contains various elements that guide RNA pol II to the viral DNA as well as promoter and enhancer sequences (55). There is an ever growing list of transcription factors that have been shown to interact with the LTR and influence transcription. Of particular importance are the three binding sites for Specific protein (Sp) transcription factors (56) and a TATA box (57) found in the core promoter. Upstream of the core is the enhancer region, which contains nuclear factor κB (NFκB) binding sites (58), and the modulatory region, which recruits various factors that are able to either enhance or inhibit transcription.

Initial basal levels of viral transcription are low in the absence of Tat. Before Tat expression, Vpr enhances transcription from the 5'LTR. As Vpr is packaged into

virions through its association with the C terminal of immature Gag (59, 60) it can promote transcription early after infection before viral protein synthesis has occurred, through interaction with Sp1 (61).

This low level of Vpr mediated transcription results in synthesis of the viral Tat protein, which is essential for efficient transcription and plays a critical role in transcription elongation (62). A stem-loop structure is formed by the transactivation response element (TAR) at positions +1 to +59 after the transcriptional start site in the LTR (63). Tat binds to this secondary structure, recruiting P-TEFb. P-TEFb is a protein kinase complex made up of a regulatory subunit, Cyclin T1 (CycT1) and the CDK9 catalytic subunit. Tat makes contacts with both subunits (64). CDK9 phosphorylates the carboxyl terminal domain of RNA pol II, leading to transcriptional elongation.

1.2.6 RNA nuclear export mediated by Rev

Transcription of the provirus results in over thirty alternatively spliced viral RNAs through the use of several splice donor and splice acceptor sites (65, 66). These transcripts can be grouped into three main categories: 9kb full length RNAs which are translated into Gag and GagPol and are also packaged into virions as genomic RNA, 4kb partially spliced mRNAs that lack gag-pol and encode Env, Vif, Vpu and Vpr, and finally, 2kb mRNAs encoding Rev, Tat and Nef which have been fully spliced to remove gag-pol and most of env.

These mRNAs must be exported from the nucleus, but under normal circumstances unspliced or partially spliced RNA containing introns is sequestered in the nucleus through interaction with host pre-mRNA splicing factors (67). HIV-1 has avoided this by using the accessory protein Rev. Rev, and also Tat and Nef, are expressed from the fully spliced transcripts that are exported out of the nucleus to the cytoplasm by normal host mRNA export pathways (68). Once expressed, Tat and Rev are imported back into the nucleus via their arginine rich NLS (69, 70) where they assist in transcription and export of unspliced and partially spliced transcripts respectively.

Rev is able to transport longer transcripts by binding via its N terminal domain to a conserved cis-acting RNA sequence called the rev responsive element (RRE). This sequence of approximately 200bp forms stem loop secondary structures and is located on unspliced mRNA within the env gene of viral transcripts (71). Subsequent oligomerisation of up to eight Rev molecules at the RRE is required for export (72). The C terminal leucine rich domain of Rev functions as a nuclear export signal (NES) which, in combination with its NLS, enables it to shuttle between the nucleus and cytoplasm (70, 73-75). Through its NES, Rev is able to mediate an interaction between incompletely spliced transcripts and host proteins involved in nuclear export, such as chromosome maintenance region 1 (CRM-1) to allow nuclear export. CRM-1 mediated nuclear export is usually used for export of proteins, small nuclear RNAs and ribosomal RNAs and uses a different export mechanism to that normally employed for export of host mRNAs, or fully spliced HIV-1 transcripts (76-79).

1.2.7 Translation, viral assembly and budding

In the cytoplasm, host ribosomes synthesise viral proteins. Ribosomal frameshifting occurs at the gag-pol junction where the ribosome slips back to -1 to translate the full length Gag-Pol polyprotein which encodes the viral enzymes RT, PR and IN (80).

Gag is the main polyprotein involved in virion assembly and its expression alone is sufficient to promote viral assembly, budding and release of an immature virus particle. The MA domain of Gag is primarily responsible for localisation of Gag at the plasma membrane through myristoylation of an N terminal glycine (81) and an N terminal basic region (82) providing a signal for trafficking to the membrane. Interaction between negatively charged phosphoinositides and the basic region of MA can lead to the accumulation of Gag with phosphatidylinositol (4,5) bisphosphate (PIP_2) which is concentrated on the cytoplasmic side of the plasma membrane (83). Gag proteins accumulate and multimerise at the plasma membrane

through its interaction (I) domain forming a roughly spherical shape with the N terminal associated with the membrane and C terminal at the sphere's centre (84).

Full length RNA transcripts include a 5' packaging signal (Ψ) which spans the 5' UTR into the Gag coding sequence (85). This sequence forms a stable secondary structure of four stem loops, which interacts with Gag to mediate incorporation into the virion. Specifically, it is the NC region of Gag that binds the packaging signal, leading to its recruitment into budding virions. This signal also enables dimerisation of RNA resulting in the packaging of two strands of RNA per virion. The packaging signal is removed during splicing to ensure that only full length transcripts are packaged into virions.
Also incorporated into the budding virion are a collection of host and viral proteins. These include Vpr (60), Vif (86) and Nef (87), $tRNA_{Lys3}$ (19) and the host protein, cyclophilin A (88, 89).

The Env precursor glycoprotein, gp160, is synthesised in the rough endoplasmic reticulum (RER), where it undergoes extensive glycosylation of certain asparagine residues. Gp160 is inserted into the lumen of the RER and forms trimers which are transported to the Golgi apparatus where they are subject to mannose trimming (90) and cleavage into the subunits gp120 and gp41 by furin (91) which is essential for function. gp41 anchors gp120 to the membrane surface non-covalently in a trimeric structure. The complexes are transported to the cell surface via the secretory pathway and incorporated into virus particles with an average of 10 per virion. The method by which Env is localised to the site of viral budding has not been elucidated. HIV-1 virions are able to incorporate envelope proteins from unrelated viruses in a process called pseudotyping, suggesting that there is not simply an interaction between viral proteins and Env, but that there is also an involvement with cellular structures.

As the virion is forming and budding from the host cell, it is coated in the host cell plasma membrane, which must subsequently be excised at the neck of the bud to

allow release. To do this, HIV exploits host machinery, namely the endosomal sorting complex required for transport (ESCRT) pathway, which is normally involved in multivesicular budding and cell division. Components of this pathway are recruited to the budding virion via late or L domains in the p6 region at the C terminal of Gag and mutation of this domain results in an accumulation of virions trapped at the cell surface (92).

1.2.8 Virus maturation

During or shortly after budding, viral maturation occurs and involves a structural rearrangement from a spherical structure to a cone shaped core. Gag polypeptide is cleaved by PR to form MA, NC and CA monomers, as well as spacer peptides SP1, SP2 and p6. Gag-Pol cleavage produces the viral enzymes RT and IN. These molecules reassemble to form the mature virus particle which consists of an outer layer of lipid membrane associated MA with a CA conical core surrounding NC complexed with the RNA genome, and IN and RT.

The Fullerene cone core consists of approximately 1500 CA monomers, assembled into 250 hexamers via its N terminus. The hexamers in turn are associated with each other by their C terminal domains (93). In addition, a Fullerene cone includes 12 pentamers of CA, five at the narrow end and seven at the wide end, which are required to allow the curvature and closure of the structure at either end (94).

Maturation is essential to produce infectious viral particles.

1.3 Gene therapy

1.3.1 History of gene therapy

The basic principle of gene therapy is the introduction of a therapeutic transgene into target cells. Depending upon the disease being treated, the vector could carry a wild type gene to replace an endogenous, mutated copy in a genetic disease. Alternatively gene therapy could be used to express a novel protein that would be advantageous,

for instance, by conferring resistance to particular pathogens, or enabling cells to target and destroy cancer cells.

There are different mechanisms that can be employed to deliver transgenes into target cells, both viral and non-viral. Retroviruses have characteristics that make them highly desirable as gene therapy vectors, including their integration into the host genome to mediate long term gene expression, susceptibility to genome manipulation to enable insertion of novel genes and efficient infection of a range of different target cells. Consequently these vectors have been used extensively for research and are being developed for therapy of a vast array of diseases.

The first approved gene modification studies were initiated in 1990 with the modification of tumour infiltrating lymphocytes using a retroviral vector carrying the neoR gene (95). This trial demonstrated safety of the procedure with normal functioning of T cells, no insertional mutagenesis and no recombinant replicating virus observed. Modified cells were still detectable several months after introduction into the patient.

A second gene therapy clinical trial began to treat severe combined immunodeficiency (SCID) by delivery of a gamma retroviral vector expressing the adenosine deaminase (ADA) gene to $CD34^+$ cells. The procedure was well tolerated in terms of safety and there was detectable increase in ADA levels in modified cells. However, the effect was short lived with only transient transgene expression (96, 97). Despite a dramatic rise in the number of gene therapy clinical trials being performed throughout the 1990s, most studies failed to show sustained efficacy

In 2000, a gamma retroviral vector carrying the interleukin (IL) γ chain gene was shown to mediate *ex vivo* transduction of bone marrow HSCs of X-linked SCID (SCID-X1) patients in a multicenter study. There was an increase in T, B and NK cells and improved immunological function and sustained benefit (98, 99). However, unanticipated adverse events were uncovered when several trial participants developed leukaemia due to insertional mutagenesis (100, 101).

1.3.2 The development of HIV derived vectors

Gamma retroviral vectors, such as MLV, are being tested in clinical trials for various diseases. However, the development of leukaemia in five patients due to insertional mutagenesis in the SCID-X1 trials demonstrated the safety concerns of these vectors (100, 101). Modifications of the vector used in the SCID-X1 trial by removal of enhancer elements and use of an internal promoter rather than the viral LTRs, can improve safety (102). Lentiviral vectors, such as those derived from HIV-1, are also in development and share advantageous characteristics with gamma retroviral vectors in that they are both able to integrate within the host cell genome, theoretically providing stable, continuous transgene expression. Gamma retroviral vectors also have a preference for integration into transcriptional start sites and regulatory regions (103). In comparison, although HIV-1 has a tendency to integrate within active genes, it does not target promoter regions, reducing the likelihood of insertional mutagenesis (51). Significantly higher copies of SIN lentivirus are required than SIN gamma retrovirus to induce oncogenesis in a tumour prone mouse model used to measure insertional mutagenesis. This is most likely to be due to the differences in integration profiles of the two vectors (104).

Lentiviral vectors have several additional advantages over retroviral vectors, including their larger packaging capacity of potentially up to 10kb (although this is generally accompanied by a decrease in titre) and their ability to efficiently transduce both dividing and non-dividing cells. This significantly increases the possible target cell populations to include muscle cells, neuronal cells and various haematopoietic cells including haematopoietic stem/progenitor cells and minimally active T cells. In contrast, gamma retroviral vectors will only transduce fully activated T cells, which could have deleterious effects on their lifespan, immune function, and repertoire.

Lentiviral vectors are frequently derived from HIV-1. Using a vector based on a pathogenic virus has obvious safety concerns, and extensive modifications of the HIV-1 genome have improved the safety profile to reduce the likelihood of

recombination events producing replication competent lentivirus (RCL). Protein expression is not required for the early stages of the lentiviral lifecycle, as RT and IN are carried within virions into target cells. This means that the majority of the lentiviral genome, apart from some critical cis-acting sequences, can be removed from the transfer vector plasmid (Figure 1.2A). Viral protein expression is then limited solely to the packaging cells, rather than in patient target cells. Only the transfer vector contains the necessary cis-acting sequences required for packaging into virions in the packaging cells. Separation of the cis-acting sequences of the transfer vector from the viral genes results in virions which are only capable of a single round of infection (105).

The first generation of lentiviral vectors consisted of three plasmids; the packaging plasmid carrying all HIV-1 genes except *env*, the envelope plasmid which frequently encoded the G envelope glycoprotein of VSV (VSV-G), and the transfer vector plasmid which carried the transgene of interest and cis-acting elements of HIV-1 required for packaging, reverse transcription and integration. The presence of all HIV-1 genes, except *env*, was a cause for concern, as it would only require minimal recombination events to produce a full length genome and functional virulent particle.

Therefore the second generation packaging system, in addition to *env*, also deleted the non-essential accessory genes *vif*, *vpr*, *vpu* and *nef* from the packaging plasmid (105, 106) (Figure 1.2B). These genes are not required to produce high titre lentiviral vectors capable of both *in vitro* and *in vivo* gene delivery, but their deletion improved the safety of the vector. This second generation packaging system is already in widespread use, but additional alterations to the lentiviral vectors have been made to further reduce the risk of recombination and the formation of RCL.

The third generation system has further separated HIV-1 genes onto four separate plasmids, which are transfected into packaging cells to produce vector particles (Figure 1.2C). In this system, U3 of the 5’ LTR in the transfer vector plasmid is replaced with a constitutively active promoter, removing the requirement of Tat for sufficient transcription. Furthermore, Rev is expressed in trans from the fourth

plasmid, which maintains a high titre of vector whilst limiting Rev expression exclusively to the packaging cells (107).

Enhancer and promoter sequences have been deleted from U3 in the 3' LTR to develop self-inactivating (SIN) vectors. During reverse transcription the 3' U3 is the template for both LTRs, so the deletion is found in both provirus LTRs resulting in their inactivation and the absence of transcription of full length viral RNA. This reduces the likelihood of activation of adjacent genes or interference with the internal promoter through the promoter and enhancer activity of the LTRs (108).

As the promoter activity of the LTR is abolished in SIN vectors, an internal promoter is used to drive transgene expression. The Spleen focus-forming virus LTR (SFFV) is commonly used as a promoter as it provides high, constitutive expression. Different promoters can be used to alter the level of expression and for tissue specificity. In a clinical setting, SFFV is not favourable as it is derived from a gamma retrovirus, causing safety concerns, and it is prone to methylation induced silencing (109). The ubiquitously expressed human phosphoglycerate kinase (PGK) promoter can be used to drive high levels of expression in human cells and has been approved for use in clinical vectors.

In many cases it is desirable to express two different genes from one vector. Often this includes the transgene of interest alongside a marker gene, such as green fluorescent protein (GFP) or an antibiotic resistance selectable marker. This can be achieved by different strategies, including the use of two promoters, or separation of the transgenes by an internal ribosomal entry site (IRES) or a 2A peptide. The most common method is the use of an IRES, which promotes ribosomal access to mRNA to allow translation of a second protein from a single transcript (110, 111). The 2A peptide is a 18-22 amino acid sequence that is readily cleaved as translation occurs, separating the two proteins (112). This leads to equal amounts of the co-expressed proteins, which is not always observed when using an IRES.

Certain HIV-1 elements must be maintained in cis on the transfer vector to allow vector production and reverse transcription and integration following transduction. These include a promoter and polyA signal in the vector genome, the packaging signal, the PBS, PPT and R region required for reverse transcription and the correct

repeat ends at the end of the LTRs to facilitate integration. Inclusion of the cPPT increases the vector transduction efficiency by promoting nuclear import of the viral transcript (113-115).

As well as the removal of HIV genes to improve safety, other elements are added to vectors for different functions. The woodchuck hepatitis virus post transcriptional regulatory element (WPRE) can be added at the 3' non-coding region of a vector to increase vector titre and gene expression (116). However, the native WPRE has enhancer and promoter activity, potentially driving expression of a peptide fragment of the woodchuck hepatitis virus protein X and this is thought to be oncogenic (117). Therefore, mutant variants have been developed that delete these regions, yet maintain their ability to enhance titre and gene expression (118).

Alternative envelope proteins expressed in trans can be used in place of HIV-1 Env, a process termed pseudotyping, to alter the tropism of the vector. VSV-G is commonly used as it very stable; allowing concentration of vector by ultracentrifugation and its broad tropism allows transduction of multiple cell types (119). Particular envelope proteins can be used to target vectors to specific cell types, and recombinant envelope proteins are being developed to further enhance cell transduction efficiency and specificity.

In 2006, details of the first clinical trial using lentiviral vectors were published (120). This phase I trial against HIV-1 used a vector to deliver an antisense transgene targeting the HIV-1 envelope to autologous T cells *ex vivo*. In contrast to most examples, this gene therapy vector was conditionally replicating, utilising the wild type HIV-1 LTR. This meant that transgene expression was upregulated by Tat upon HIV-1 infection and that transcripts could be packaged using wild type HIV-1 proteins, allowing mobilisation of the transgene to other $CD4^+$ T cells. There was detectable gene marking in patients and $CD4^+$ cell levels increased in some patients. Consequently, this trial is still ongoing and has progressed into HSCs. These results provide important information about the efficiency and safety of the procedure, assisting the development of further trials.

Since then, lentiviral vectors have been tested in clinical trials for other diseases, including X-linked adrenoleukodystrophy (ALD) (121), β-thalassaemia (122), and Wiskott-Aldrich syndrome (WAS) (123) which are normally treated with HSC transplants if human leukocyte antigen (HLA) matched donors can be found.

ALD is a severe demyelinating disease caused mutations in the ABCD1 gene leading to a lack of ALD protein. Two patients received autologous $CD34^+$ cells transduced with a lentiviral vector carrying the ABCD1 gene. Following transplant, cells of all haematopoietic lineages were found to express ALD, and there was significant reduction in demyelination similar to that seen following an allogenic HSC transplant (121). This trial is important in showing that lentivirally modified HSCs can mediate sustained benefit.

Another lentiviral vector trial that has shown clinical benefit is in a patient with β-thalassaemia, a disease caused by mutations in the globin gene leading to the requirement of regular blood transfusions. Autologous $CD34^+$ cells were transduced *ex vivo* and delivered back to the patient. However, in this patient there has been clonal dominance of cells with integrated vector copies in the HMGA2 gene. Target sites for let-7 microRNAs, which bind and promote RNA degradation, were absent from the resulting truncated mRNA, leading to its increased stability. In addition many copies of integrated vector had deletion of one of the vector cHS4 insulators (122). Although this incident of clonal expansion has not progressed to transformation, this trial has demonstrated the potential risks of adverse events also occurring in lentiviral gene therapy and further studies must proceed with caution.

In early phase trials are lentiviral vectors for Wiskott-Aldrich syndrome (WAS), an inherited immunodeficiency caused by mutations in the WAS gene. Previously, gamma retroviral vectors have been used in trials for this disease and have caused transactivation of LMO2 leading to leukaemia (124), but recently an HIV-1 derived WAS vector has been developed and has entered clinical trials (123).

In addition to HSC gene therapy, lentiviral trials have also targeted T cells. A chimeric tumour specific antigen receptor has been designed to recognise CD19, the expression of which is restricted to normal and malignant B cells. Delivery of this receptor to T cells using lentiviral vectors has been used to treat a patient with

chronic lymphoid leukaemia (125). Modified cells were detectable for at least 6 months following infusion and there was a specific loss of $CD19^+$ B cells.

These trials demonstrate that there is significant clinical progress in the use of lentiviral vectors in gene therapy for a range of diseases.

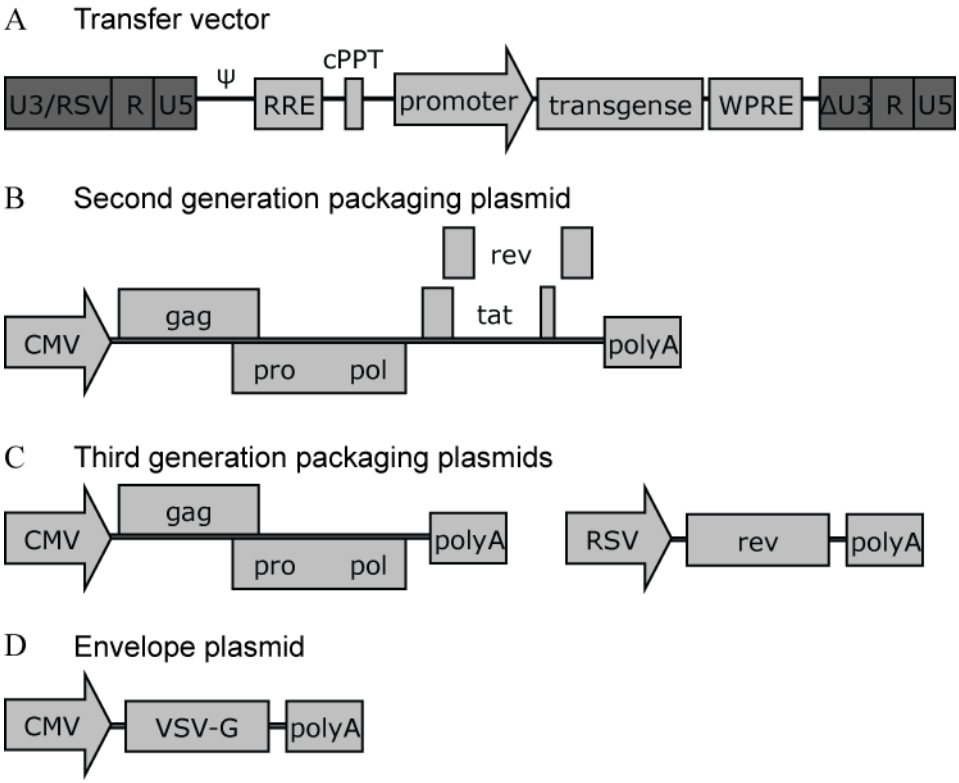


Figure 1.2 Second and third generation lentiviral vectors

A. Self-inactivating lentiviral transfer vector. The second generation system uses the native HIV-1 U3, whereas the third generation packaging system replaces U3 in the 5' LTR with the Tat independent Rous Sarcoma virus (RSV) promoter. RRE=rev responsive element, cPPT=central polypurine tract, WPRE=Woodchuck hepatitis virus post transcriptional regulatory element

B. Second generation lentiviral packaging plasmid. CMV= Cytomegalovirus promoter

C. Third generation lentiviral packaging plasmid. The rev gene is provided in packaging cells in trans using the RSV-rev plasmid.

D. Envelope plasmid with vesicular stomatitis virus glycoprotein (VSV-G)

1.3.3 Gene therapy against HIV-1

Due to advancements in gene therapy and the requirement of a different approach to treat HIV-1 infection in patients who are unable to continue, the field of anti-HIV gene therapy research is extensive. There are many different transgenes being generated and tested *in vitro,* targeting both viral and host components at different

points of the viral lifecycle. Modeling suggests that the most effective transgenes will inhibit HIV-1 early in its lifecycle, either at the point of viral entry, or prior to integration (126). This reduces the opportunity for HIV-1 to develop mutations as reverse transcription is not occurring, limits cytotoxic effects on infected cells and prevents establishment of a latent reservoir. Alongside analysis of anti-HIV transgenes, vector development and transduction protocols are being tested and improved. HIV gene therapy has progressed into the clinic, with various different preclinical, phase I and phase II clinical trials currently underway.

Early work was primarily involved with the use of gamma retroviral vectors to deliver transgenes to target cells; either HSC or T cells and consequently these vectors have been more extensively tested. Typically, these vectors carried transgenes that inhibited late stages in the HIV lifecycle, such as inhibiting Rev and Tat, and RNA interference (RNAi) of viral genes to preventing translation. Some of these transgenes have reached clinical trial using retroviral vectors to transduce both HSC and T cells. However, as a more effective therapy, transgenes targeting early stages in the viral lifecycle have been developed. This includes stages prior to integration and formation of the provirus, such as restriction of viral entry.

Although these retroviral vectors initially appeared safe, clonal expansion of modified cells in clinical trials for SCID-X1 (100, 101), WAS (124) and CGD (127) have created a move towards lentiviral vectors. These have an improved integration safety profile and also have the ability to transduce non-dividing cells, many of which are natural targets of HIV-1. These include HSCs, T cells, macrophages and dendritic cells. As gamma retroviral vectors can only transduce dividing cells, it is necessary to activate T cells and HSCs to allow transduction, which can affect their function and pluripotency upon infusion back into a patient. Extensive research is being performed testing lentiviral vectors carrying various anti-HIV transgenes *in vitro* and in mouse models, and some lentiviral vectors are now in clinical trials. A summary of several anti-HIV clinical trials that have been performed to date are presented in table 1.1.

Preliminary results from these trials have provided safety data for the procedure and important information about persistence of the transgene *in vivo*. In some cases,

there are also promising results relating to the efficacy of the treatments, including a survival advantage of modified cells, reduced viral load and an improvement in T cell numbers, which support further investigation of this new therapeutic technique.

To help prevent mutagenic escape of HIV from the therapeutic transgene, vectors are being designed that express combinations of antiviral genes. For example, a triple transgene vector encoding a chimeric TRIM5α, CCR5 shRNA and a TAR decoy has been tested *in vitro* (128). Some of these multi-transgene vectors are now in clinical trial. For instance a lentiviral vector carrying three transgenes, Tat/rev shRNA, TAR decoy and CCR5 ribozyme, has been used to modify autologous HSC (129). As well as *in vitro* viral restriction, the *in vivo* efficacy of these transgenes must now be demonstrated to determine how effective gene therapy could be for the treatment of HIV-1.

Target	Vector	Results	Reference
Env	Antisense targeting env, conditionally replicating LV vector into $CD4^+$ T cells	Some increase in $CD4^+$ counts, vector mobilisation	(120)
Tat/rev, CCR5, TAR	Tat/rev shRNA, TAR decoy, CCR5 ribozyme	Low level marking for 2 years in multiple lineages	(129)
Rev	Dominant negative Rev (huM10), RV vector in $CD34^+$ cells	Marked cells dropped below detection after a few months, increased marking in one patient when viral load increased	(130)
Tat/rev	Anti-Tat ribozyme, RV vector in $CD4^+$ T cells	Survival of marked cells for 4 years. No adverse effects	(131)
Rev/TAR	Trans-dominant Rev/antisense TAR, RV vector in $CD4^+$ T cells	Long term survival of cells, survival advantage of marked cells in patient with high viremia	(132)
Fusion	C46 fusion inhibitor, RV vector in T cells	Long term, but low gene marking, increased $CD4^+$	(133)
Tat/vpr	Anti tat/vpr ribozyme (OZ1), RV vector in $CD34^+$ cells	No significant difference in viral load, but some other markers improved	(134)
CCR5	ZFN, AdV vector in T cells	Modified cells detected in circulation and GALT	(135)
RRE decoy	RRE decoy, RV vector into $CD34^+$ cells	Low level gene marking in periphery only for a few months. No safety problems	(136, 137)

Table 1.1 Examples of anti-HIV-1 clinical trials.
Information includes the viral target, the vector and cell type used, brief summary of results of the trial and reference for the trial. AdV-adenoviral, GALT-gut associated lymphoid tissue, LV-lentiviral, RRE-Rev response element, RV-retroviral, shRNA-short hairpin RNA, TAR-trans-activation response element, TCR-T cell receptor, ZFN-zinc finger nuclease.

1.4 Restriction factors of the innate immune system

1.4.1 Antiretroviral restriction factors

Higher organisms have evolved a complex immune system where the innate immune system acts quickly after infection to provide protection against pathogens, allowing time for the adaptive immune system to mount a response. The innate system includes interferon (IFN), pattern recognition receptor (PRR) and secreted soluble molecules such as toll-like receptors or complement. Adaptive immunity is more complex and found only in vertebrates and can take days to come into effect. It involves the activation and expansion of T and B lymphocytes and production of pathogen specific antibodies. Although innate immunity begins to control infection much sooner than the adaptive response, it still requires some signaling, often IFN mediated, which can take time to upregulate.

One aspect of the innate immune system involves the expression of a variety of antiviral restriction factors. Unlike conventional immunity, this system does not involve signaling and relies on constitutive expression of a number of proteins that are able to act immediately upon viral infection, in the absence of any cell signaling or cell-cell interaction, providing the first line of defence against invading viruses (138). The expression of restriction factors is now far more widespread than initially thought and a wide range of mammals have now been shown to express these antiviral proteins.

Retroviruses have evolved systems to avoid inhibition by these restriction factors in a species specific manner. Typically, HIV-1, HIV-2 and SIV are not significantly inhibited by restriction factors of their natural host species. Host restriction factors and retroviruses have co-evolved, each trying to gain advantage over the other, resulting in high levels of selective pressure as viruses evolve mechanisms to avoid inhibition by host restriction factors. Viral tropism is therefore often determined by the panel of restriction factors expressed by host cells and the target specificity of these factors. In fact it is the tropism of different retroviruses that has been a vital tool in the identification and study of these restriction factors.

Due to the extensive research that has been performed on retroviruses, most of the knowledge obtained on restriction factors is related to inhibition of these viruses.
Four groups of proteins that fall into the category of antiviral restriction factors include APOBEC proteins, tetherin, SAMHD1 and Fv1/TRIM5α proteins.

1.4.2 The APOBEC family

The apolipoprotein B mRNA-editing catalytic polypeptide-like 3 (APOBEC3) family are capable of restricting HIV-1, the most efficient member being APOBEC3G. APOBEC3G is a cytidine deaminase and is thought to target HIV ssDNA (139, 140). Specificity for ssDNA, not double stranded or RNA-DNA hybrids, results in deamination predominantly of the minus strand DNA, which is synthesised first during reverse transcription and is the principle form of single stranded viral DNA in an infected cell. These mutations are then copied over to the plus strand DNA, ultimately resulting in a G to A mutation of the viral genome (141).
APOBEC3G also inhibits Vif deficient HIV-1 in a deaminase independent way. Mutant protein in which the cytidine deaminase domain is no longer functional is still capable of causing significant restriction (142). The mechanisms of the cytidine deaminase independent restriction have not been fully elucidated, but are thought to target reverse transcription leading to reduced levels of viral DNA (143). There has been evidence that various processes during reverse transcription are affected, including interference with primer annealing (144, 145) and strand transfer (146).
APOBEC3G is incorporated into Vif deficient virions through interactions with both Gag, specifically the NC (147), and viral RNA (148). Once the virion infects a new cell, APOBEC3G remains associated with the mature viral proteins and RNA to enable deamination during reverse transcription.
The inhibitory effects of APOBEC3G and APOBEC3F were found to be counteracted by the viral Vif protein, restoring replication (149). Vif prevents incorporation of APOBEC3G into budding virions (150) and reduces APOBEC3G protein levels by interfering with translation and reducing its half-life (151). Loss of

protein is primarily a result of ubiquitin mediated degradation. Vif acts as an adaptor molecule, binding both APOBEC3G and an E3 ubiquitin ligase complex consisting of cullin-5, elongins B and C and Rbx1. This allows polyubiquitinylation of APOBEC3G leading to proteasomal degradation (152). Vif is also able to prevent APOBEC3G incorporation into virions in a proteasome independent manner, as shown by the exclusion of a degradation resistant APOBEC3G from budding virus particles (153).

1.4.3 Tetherin

Vpu was shown to be required for HIV-1 release from cells in a cell specific manner. In some cell lines, Vpu deficient virion particles were seen to accumulate at the cell surface and endosomal compartments. Accumulation at the endosome was due to endocytosis of membrane trapped virions and could be prevented by inhibition of membrane to early endosome transport. Viral particles accumulated at the cell membrane could be released by protease treatment, indicating a protein was responsible for this accumulation, rather than prevention of membrane scission (154, 155) . In 2008, two groups identified tetherin, also called bone marrow stromal Ag 2 (BST2), as the protein responsible for this restriction (156, 157).

Tetherin is a type II membrane glycoprotein that inhibits the release of many enveloped viruses, including HIV-1. It functions as a general antiviral restriction factor without obvious species specificity, as it interacts with the host membrane rather than a viral factor. Originally identified on B cells, tetherin has now been shown to have a broad expression pattern. However, HIV-1 target cells, including $CD4^+$ T cells, dendritic cells and macrophages, were not found to express tetherin (158). This casts doubt on the importance of the interaction between tetherin and HIV-1 *in vivo*, although as this group only examined healthy donors and cancer patients it is possible that there is upregulation after HIV-1 infection and type I IFN signaling.

The protein has an unusual structure as it is anchored at both ends to the plasma membrane via an N terminal transmembrane domain and a C terminal glycosyl-phosphatidylinositiol anchor (159). Through insertion into the plasma membrane, tetherin is able to crosslink neighbouring virus particles and host cell membranes causing an accumulation of budding virions (160).

HIV-1 has evolved a mechanism to avoid this restriction through its accessory protein Vpu. Vpu is an 81 amino acid phosphoprotein that is expressed from a bicistronic mRNA with the Env gene. Its transmembrane domain is known to multimerise, most likely in a pentamer, to form cation channels (161).

Vpu is thought to abrogate the antiviral activity of tetherin through endosomal trafficking and subsequent lysosomal degradation. This process requires the host protein βTrCP (162, 163). In addition to this, Vpu can abolish tetherin mediated restriction without decreasing cellular levels of the protein, and this involves sequestering of tetherin in a perinuclear location that contains trans-Golgi network markers (164, 165).

1.4.4 SAMHD1

The most recently identified restriction factor is the sterile alpha motif and histidine/aspartic acid domain containing protein-1 (SAMHD1) (166, 167). SAMHD1 was originally identified as its mutation causes Aicardi-Goutières syndrome, an encephalopathy that manifests early in childhood and often leads to a variety of physical and neurological problems (168).

SAMHD1 was subsequently determined to be an HIV-1 restriction factor and is responsible for the inability of HIV-1 to infect dendritic cells and its reduced infectivity in macrophages. It functions as a dNTP triphosphohydrolase (169) and has been shown to deplete dNTP levels sufficiently to prevent reverse transcription (170). However, HIV-2, SIVmac and SIVsm (sooty mangabey) counteract this restriction through expression of the accessory protein Vpx, which promotes proteasomal degradation of SAMHD1 (166, 167).

1.5 TRIM proteins

1.5.1 Identification of TRIM5α as an antiretroviral restriction factor

The final restriction factor to discuss was actually the first to be identified. Studies were carried out investigating the susceptibility of different strains of mice to the Friend strain of MLV. The responsible genes were named the Friend virus susceptibility (Fv) genes, and Fv1 in particular was studied further. Two main Fv1 alleles were identified; $Fv1^n$ and $Fv1^b$, which were found to restrict B-tropic MLV (B-MLV) and N-tropic MLV (N-MLV) respectively. A third viral tropism, NB-MLV was able to infect both strains of mice.

The mode of restriction mediated by Fv1 was novel and interesting characteristics were observed. Fv1 restriction of MLV could be saturated by the addition of sufficient viral particles simultaneously or prior to infection. The virus particles could be non-infectious and genome deficient, as long as they were of the correct tropism (171). Fv1 targets MLV CA and the two strains of virus have an amino acid difference at position 110 in the CA, which determines their susceptibility to Fv1 (172, 173). In addition to amino acid 110, surrounding residues in the CA also play a role in determining susceptibility to Fv1 (174). Protection from different MLV viruses of different tropisms was inherited dominantly. These observations suggested that Fv1 encoded a saturable factor that was able to restrict incoming virus recognised by its CA sequence. Once the Fv1 gene had been identified and cloned, it was found to be closely related to the gag gene of an endogenous retrovirus suggesting a retroviral origin (175).

Fv1 mediates restriction early in infection, after reverse transcription and prior to integration, as identified by the presence of reverse transcripts, but lack of integration into the host genome (176, 177). Regions at the C and N terminus of the protein, and specific regions within the major homology region are required for restriction, although a large portion of the internal sequence is non-essential. Antiviral specificity is determined by sequences within the C terminal (178). The

protein functions as a dimer and requires dimerisation at the N terminal to restrict MLV (179).

Pseudotyping with VSV-G provides MLV with a broad tropism and allowed the identification of another restriction factor in human cells, which was named resistance factor 1 (Ref1). Similar to Fv1, Ref1 can be saturated by high titres of restricted virus, but in contrast to Fv1, Ref1 restricts incoming virus before reverse transcription and reverse transcripts cannot be detected in non-permissive cells (180, 181). These two restriction factors use different restriction mechanisms and when expressed in the same cell, compete for restriction of incoming MLV (182). The human Ref1 restriction factor was shown to restrict both N-MLV and equine infectious anaemia virus (EIAV) (183, 184).

Studying restriction in primate cells found sensitivity to a set of retroviruses distinct to both human and murine cells. The simian restriction factor was named lentivirus susceptibility factor 1 (Lv1) and provided African green monkeys and rhesus macaques with a resistance to HIV-1. Lv1 had many functional similarities to Ref1; the reduction of viral reverse transcripts, the block to infection being most effective at low multiplicities of infection and saturation with large quantities of virus (185-187).

In 2004, the cytoplasmic body component TRIM5α was identified as the restriction factor responsible for the resistance of Old World monkeys to HIV-1 (188). Shortly after this discovery, TRIM5α was confirmed to be responsible for the restrictive activities previously accounted for by the restriction factors Ref1 and Lv1 (189, 190).

This TRIM protein family member caused a species specific block to retroviral infection as previously described. TRIM5α is typically ineffective against retroviruses exogenous to the host species, but often restricts those from other species, illustrating its important role in prevention of zoonotic transmission of retroviruses. For instance, TRIM5α from Rhesus macaque (rhTRIM5α) is a strong inhibitor of HIV-1, but not SIVmac, whereas in humans N-MLV and EIAV are

strongly restricted. Human TRIM5α (huTRIM5α) mediates mild restriction of HIV-2 (191), but does not cause a significant inhibition to HIV-1. These species specific differences in restriction are attributed to CA sequence variation between viruses and subsequently, the ability of TRIM5α to recognise and bind the virus. Alteration of CA amino acid 110 will alter specificity of huTRIM5α between B- and N-MLV (192). There are even different restriction specificities within a species, for example in Rhesus macaques there are different TRIM5 alleles which have activity against different retroviruses. Throughout evolution selective pressure from viral infection has driven diversity in this gene (142, 193). Within humans, different TRIM5α polymorphisms or expression levels have little or no effect on HIV-1 infection or disease progression (194-196).

Delivery of rhTRIM5α into human cells using lentiviral vectors as an *ex vivo* gene therapy has been proposed (197) but this method is likely to lead to problems with immune rejection as it is a foreign protein. However, huTRIM5α can be modified to provide specificity to HIV-1. Either transfer of a patch of rhTRIM5α B30.2 (198) or a single amino acid change at 332 in the human protein is sufficient to allow restriction of HIV-1 by huTRIM5α (199-201). Although HIV-1 is not susceptible to restriction by TRIM5α of some species, unlike for APOBEC and tetherin, the virus has not been able to evolve a mechanism to avoid the general restrictive effects of TRIM5α, making it a good therapeutic possibility.

TRIM5α is a member of the large family of TRIM proteins, which has around 70 human proteins with diverse roles. There are a similar number in mice, whereas in worms and flies there are approximately 20 and 10 members respectively, suggesting that the TRIM family has dramatically expanded throughout evolution (202).

This family is characterised by its TRIpartite Motif (TRIM), which consists of a RING, B-box and coiled coil domains (RBCC). In addition to the RBCC domains, TRIM5α also includes a C terminal B30.2 or PRYSPRY domain. It is the B30.2 domain that is responsible for binding of restricted virus CA and therefore the proteins specificity (200, 201, 203, 204). TRIM5 is alternatively spliced to produce

other isoforms, which are truncated from the C terminal. These include TRIM5β, TRIM5δ, TRIM5γ and TRIM5ε (205). The TRIM5δ and - γ isoforms, which lack the B30.2 domain, can act in a dominant negative fashion against TRIM5α by forming heteromultimers with TRIM5α, abolishing its antiretroviral activity (182, 192). These other isoforms may be involved in regulating TRIM5α activity.
Subsequently, antiretroviral TRIM5α proteins have been identified in other non-primate species, including rabbit (206), cattle (207, 208) and hare (209), that restrict various retroviruses in a species specific way.

TRIM5 is ubiquitously expressed in all tissues throughout the human body, including stimulated, but not unstimulated peripheral blood lymphocytes which are the target of HIV (205, 210). Expression levels in many of these tissues are low, but TRIM5 expression is upregulated by IFN through a putative interferon-stimulated response element (ISRE) (211). As expression is IFN inducible, it supports the role of TRIM5 as an important factor in the innate immune system as IFNs have a key role in this system and inducing gene expression in response to viral infection.

1.5.2 Protein structure of TRIM proteins

1.5.2.1 The RING domain

At the N terminus of the TRIM protein is the Really Interesting New Gene, or RING domain, which is a zinc finger binding domain present in a diverse range of different protein molecules. RING domains are typically 40-60 amino acids and are defined by the consensus sequence CX2CX(9–39)CX(1–3)HX(2–3)C/HX2CX(4–48)CX2C, with cystidine and histidine residues interacting with two zinc ions (212).
RING domains have been shown to have E3 ubiquitin ligase activity and be involved in mediating specificity of E2 dependent ubiquitinylation and proteasomal degradation of proteins (213). Specifically, the RING domain of TRIM5α has been shown to be capable of self-ubiquitinylation, and can also be ubiquitinylated by another member of the TRIM family, TRIM21 (214). In addition to TRIM5α, E3

ubiquitin ligase activity has been shown for the RING domains of several other TRIM proteins, including TRIM11, TRIM21, TRIM22 and TRIM25 (215-218).

The RING domain is required for rapid ubiquitinylation and proteasomal degradation (219). However, the role that the RING domain plays in antiviral activity of TRIM5α is unclear. Inhibition of degradation with proteasome inhibitors does not rescue viral infectivity (220-222), and truncated TRIM5α molecules with an absent RING domain experience only a partially reduced antiviral activity (223, 224).

1.5.2.2 The B Box

Similar to the RING domain, the B-box is a zinc finger binding domain and is typically involved in protein-protein interactions. There are two different types of B-box: B-box1 and B-box2. Although their sequences differ, their tertiary structures are similar to each other, and to the RING domain, suggesting a common ancestral domain. The presence of one or both B-box domains varies between different TRIM proteins. If a TRIM protein only contains one B-box, it is always B-box2, and if both are present, B-box1 precedes B-box2. Both TRIM5α and TRIM21 contain a B-box2 (205). A B-box2 is made up of an α-helix and structured loop with two antiparallel β sheets. It also co-ordinates two zinc ions.

Along with the RING domain, the B-Box is critical for rapid ubiquitinylation and proteasomal degradation (219). The B-Box 2 domain is also required for higher order multimerisation of TRIM5α (225, 226) and mutants lacking this domain do not restrict (204, 223). TRIM5α has been shown to assemble spontaneously into a hexagonal lattice from purified recombinant TRIM5-TRIM21 *in vitro (227).* However, formation was enhanced in the presence of HIV-1 CA assembled *in vitro* to mimic the viral core. Despite only a weak interaction between TRIM5α and retroviral CA monomers, the avidity between TRIM5α higher order structures and CA assembled in the core of a restricted virus is high, reflecting the importance of high order multimerisation (225).

In addition to the B-Box, the L2 linker region located between the coiled coil and B30.2 domain is also required for this high order multimerisation, without which

TRIM5α loses its antiviral activity (228). This linker region is also required for cytoplasmic body formation. Sastri et al found that mutants lacking L2, and could not form cytoplasmic bodies, were unable to restrict virus. However, as L2 is also required for higher order multimerisation, it is thought that it is disruption of these structures, rather than cytoplasmic body formation, that abrogates restriction (228).

1.5.2.3 The Coiled Coil

In TRIM proteins, the B-box domain(s) is usually followed by a coiled coil domain, which is necessary and sufficient for homomultimerisation. Deletion of this domain results in a loss of the large aggregates normally seen with intact protein, to be replaced with diffuse localisation throughout the cell (205).

In addition to the large multimers of TRIM5α that form, smaller multimers mediated by the coiled coil domain also form. It was originally thought that TRIM5α forms and functions as a trimer, but these experiments were limited by technical difficulties (229, 230). More recent biochemical analysis of purified recombinant protein consisting of TRIM5α with a TRIM21 RING domain shows TRIM5α actually forms dimers (231). Other studies have identified dimers and trimers, but more significant for restriction, higher order multimers, particularly hexamers, have been observed (232).

The coiled coil is required for the binding of CA, along with the B30.2 domain, and contains residues that have been subject to selective pressure and can influence TRIM5α specificity against MLV. The RING and B-box are dispensable for this interaction (233, 234).

1.5.2.4 The B30.2 domain

There are different splice variants of TRIM5, but the longest TRIM5α includes a C terminal B30.2 domain that is responsible for the antiviral specificity of the protein (200, 201, 203, 224). SPRY domains are evolutionarily ancient and found in a diverse range of plants, animals and fungi, but the B30.2 domain has evolved more recently and is unique to vertebrates. B30.2 is made up of the evolutionary ancient SPRY domain, but has also incorporated a PRY domain (235).

TRIM5 and retroviruses have been co-evolving together for millions of years and this restriction factor may have played an important role in the control of spread of retroviruses within and between species (236). Regions within the B30.2 domain have been subject to high levels of selective pressure supporting its key role in antiviral specificity (237). Positive selection within this domain suggests co-evolution of host restriction factors and viruses, with each providing selective pressure on the other to gain the advantage. The B30.2 is made up of two anti-parallel β sheets, also with four loops that are located on one side of the B30.2 domain and are hypothesised to interact with the viral CA (238). These loops are variable regions that exhibit substantial variation in both length and amino acid sequence, termed v1, v2, v3 and v4 (239). Variable regions v1-v3 determine the specificity of the TRIM5 antiviral activity.

Deletion of the B30.2 domain results in a truncated protein that acts in a dominant negative fashion by dimerising with full length TRIM5α and abrogating its antiviral function (204, 223).

TRIM5α is a modular protein and in some cases, it is possible for domains in the RBCC to be functionally replaced by those of other TRIM proteins. For instance, any of the RBCC domains of huTRIM5α can be functionally replaced by the corresponding domains of the paralogous proteins TRIM6 and TRIM34. Similarly, a chimeric protein generated by the substitution of the RING domain of rhTRIMα with that of huTRIM21, a more distant relative of TRIM5, retains its antiviral capabilities. However, substitution of the B-Box2 and flanking linker regions results in a protein which is unable to restrict viral infection. Therefore this domain is crucial for rhTRIM5α restriction of HIV-1 (240).

1.5.3 Mechanism of TRIM5α restriction of HIV-1

TRIM5α inhibits HIV-1 early in infection, as shown by the absence of reverse transcripts after viral entry (192). However, the exact process by which TRIM5α

restricts retroviruses has not been fully determined, although it is likely that TRIM5α mediates restrictive effects through several different mechanisms.

TRIM5α recognises retroviruses when they enter cells via its B30.2 domain. However, the interaction between TRIM5α and CA has been difficult to characterise, indicating that it is a complex interaction and that recognition between two monomers is weak (241). TRIM5α initially forms dimers. Dimers can spontaneously form hexamers, but this is greatly enhanced in the presence of incoming viral CA. This mediates more efficient capsid binding with higher avidity and is required for efficient restriction. It is suggested that the hexameric structure of TRIM5α multimers allows multiple B30.2 domains to cover and interact with the incoming viral core with high avidity (227).

Normally upon cell entry, the virus will undergo uncoating, which is a complex process that is not fully understood. Binding of TRIM5α in the cytoplasm can lead to accelerated and disrupted uncoating of the virus, preventing infection (233, 242-244).

TRIM5α can be ubiquitinylated, both by itself and other molecules (214), and degraded by the proteasome. The RING and B-box are required for proteasomal degradation of TRIM proteins. It is thought that this degradation is also carried out on the TRIM5α-virus complex, as there is proteasome-dependent degradation of TRIM5α upon infection of a restricted retrovirus (221). Inhibition of the proteasome results in formation of viral reverse transcripts and PICs in cells, but they are not detectable in the nucleus nor are there detectable 2-LTR circles. Therefore proteasome inhibition does not abrogate the antiviral activity of TRIM5α, but does rescue reverse transcription (220-222). This correlates with data that shows that proteasome inhibition or deletion of the RING domain does not abrogate antiviral activity and that TRIM5α mediates a proteasome independent antiviral activity prior to causing proteasome dependent destruction of both the virus and TRIM5.

The proteasome independent inhibition is likely to be due to sequestering of the viral genome in either pre-existing aggregations of TRIM5, or by formation of new aggregates around virus particles. TRIM5α can also be seen to leave cytoplasmic

bodies to interact with nearby virus within the cytoplasm. This supports the notion that proteasomal degradation is needed for viral clearance, but is not necessary for restriction (245). These cytoplasmic bodies form when TRIM5α is overexpressed, but are also found at low levels of expression (205). TRIM5 varies between localisation in cytoplasmic bodies and more diffuse distribution throughout the cytoplasm (246). However, disruption of cytoplasmic bodies with geldanamycin, an Hsp90 inhibitor, does not prevent viral restriction, therefore their formation is not essential (247, 248).

Recently human and Rhesus TRIM5α have been shown to shuttle between the cytoplasm and nucleus, and inhibition of CRM1 nuclear export machinery results in accumulation of TRIM5α in the nucleus. This was not seen in TRIM5α from cattle, New World monkeys or TRIMCyp. In the nucleus, TRIM5α co-localised with TRIM19 in nuclear domain 10 (ND10) structures (249). TRIM19 is thought to be involved in restriction of herpes simplex virus, so TRIM5 may be involved in this function. Accumulation of TRIM5α in the nucleus did not abrogate TRIM5 mediated retroviral restriction, although it is likely that residual cytoplasmic protein or newly synthesised TRIM is responsible for restriction. The importance of nuclear shuttling of TRIM5α is not known, and may be involved in as of yet unrecognized functions.

In addition to CA recognition and interaction with incoming virus, TRIM5α has been identified as playing a role in cell signaling in innate immunity. Independent of the B30.2 domain responsible for CA recognition, TRIM5α affects NFκB signaling through two different pathways, and the involvement of TRIM5 varies between species. Human TRIM5α and the mouse paralog TRIM30 downregulate NFκB signaling through proteasome independent degradation of TAB2, an adaptor protein upstream of NFκB. In contrast, human and Rhesus TRIM5α are able to activate NFκB expression. The relative activity of these two opposing effects upon NFκB by the human protein are thought to depend upon TRIM5α levels (250). TRIM30 also interacts with the TAK1-TAB2-TAB3 complex. It acts downstream of toll-like

receptor (TLR) activation to promote TAB2 and TAB3 degradation causing downregulation of NFκB signaling (251).

TRIM5α activates NFκB signaling in conjunction with UBC13-UEV1A, an ubiquitin-conjugating enzyme. Together they assemble unattached K63-linked ubiquitin chains that activate the TAK1 kinase complex. TAK1 subsequently stimulates AP-1 and NF_KB transcription factors involved in innate immune signaling (252). This mechanism for NFκB signaling is similar to that mediated by TRAF6, a RING containing E3 ubiquitin ligase, which also synthesises K63-linked ubiquitin chains which activate IKK and TAK1 and subsequently NFκB (253, 254).

The formation of ubiquitin chains and activation of NFκB signaling is significantly enhanced upon CA recognition. Prevention of formation of these chains through knockdown of UBC13 or UEV1A abrogates huTRIM5α restriction of susceptible retroviruses, such as EIAV. Through this function, TRIM5α and TRIM5Cyp act as a pattern recognition receptors as recognition of a restricted retroviral CA enhances the activation of NFκB signaling and innate immune response (252).

1.5.4 TRIM21

In humans there are a large number of TRIM proteins and many members of this protein family have been shown to play an important role in innate immunity. Screening the TRIM protein family has identified their role in various aspects of different retroviral lifecycles (255). Also, many of the proteins are IFN inducible, supporting the evidence that they play a role in innate immunity (256).

The defining characteristic of the family is the RBCC motif at the N terminal, with the main differences in protein structure residing in the C terminal domains. However, the conserved RBCC domains are of interest as they are often responsible for the protein's function, in particular the RING domain. The E3 ubiquitin ligase activity of the TRIM RING domain is often crucial for the general function and antiviral activity of the protein.

One member of the TRIM family which has been shown to play an important role in immunity is TRIM21 or Ro52/SS-A. Excluding the TRIM5 paralogs that are located in the same gene cluster (TRIM6, 22 and 34) (205), the most closely related protein to TRIM5 is TRIM21 (239). The TRIM21 gene is located in the same cluster as TRIM5 (11p15) (205) and the proteins have the same RING, B-box2, coiled coil and B30.2 domain structure (202).

TRIM21 was until recently most commonly known as the autoantigen in various autoimmune diseases, including Sjögrens disease and systemic lupus erythematosus (SLE) (257). Anti-TRIM21 antibodies are used as a diagnostic for such diseases, and can act as an indicator of disease progression.

TRIM21 is ubiquitously expressed in adult cells and forms cytoplasmic bodies within cells (205). These cytoplasmic bodies are highly motile, and despite the similarities, are distinct to those of TRIM5α (258). TRIM21 forms trimers (259). However the technique used to identify this could be susceptible to the same inaccurate reading as with TRIM5α, which then turned out to function and bind CA as a dimer (231). Other groups using alternative methodologies have suggested that TRIM21 forms dimers (230, 260, 261). TRIM21 has low background levels of expression, but like TRIM5 is upregulated upon IFNγ stimulation, suggesting it plays a role in immune response (262).

As well as the interaction with autoantibodies, TRIM21 also binds IgG heavy chains with high affinity in mammalian cells (263). TRIM21 binds IgG via two binding pockets within its B30.2 domain (259, 264) through a novel mechanism, which was structurally and kinetically highly conserved between species (265).

It was unexpected that an intracellular protein would bind antibodies with such a high affinity, driving speculation that TRIM21 functioned as a receptor for internalised opsonised pathogens. Recently, the mechanism that TRIM21 mediates was revealed, and its role in inhibiting infection of adenovirus was reported. This confirmed the importance of TRIM21 as an intracellular IgG receptor (266). Once antibodies have recognised and opsonised virus, the complex enters cells and the

IgG domain is bound by TRIM21. TRIM21 targets this viral complex for proteasomal degradation via its E3 ubiquitin ligase domain, most likely through autoubiquitinylation (266). TRIM21 therefore is involved in a novel antiviral mechanism that bridges both the innate and adaptive immune systems.

TRIM21 plays an additional role by its involvement in IFN signaling, which is vital to the innate immune response against both viruses and bacteria. The family of IFN regulatory factors (IRF) transcription factors play an important role in IFN expression following pathogen recognition. IRF1, 3, 7, and 9 have all been suggested to play a role in type I IFN (α/β) gene expression.

IRF3 is an important transcription factor involved in IFN production. It is constitutively expressed and involved in the initial expression of IFN following infection. *In vitro* experiments have produced conflicting results as to whether TRIM21 causes degradation of IRF3, or whether is it critical for its sustained activation (267, 268). TRIM21 interferes with the interaction between Pin1 and IRF3, preventing IRF3 ubiquitinylation and degradation. B30.2 is essential for this function, but the RING domain is dispensable, suggesting that ubiquitination is not required. Stabilisation of IRF3 promotes an antiviral response by maintaining IFN signaling (268). Alternative results suggest that TRIM21 interaction with IRF3 via its B30.2 domain, leads to its ubiquitinylation and proteasomal degradation, restricting downstream signaling via IFNβ. This can be prevented by proteasome inhibitors or depletion of TRIM21 using shRNA. This action requires both the RING and the B30.2 domains (267). It is still unclear which mechanisms TRIM21 mediates *in vivo*.

Additional evidence supporting the involvement of TRIM21 in an IFN negative feedback loop *in vitro* shows that it is able to ubiquitinylate IRF7, which has a similar structure to IRF3. This targets IRF7 for proteasomal degradation preventing prolonged immune activation via the IFNα pathway (269, 270).

E3 ligases can also play a role in processes independent of proteasomal degradation, including transcriptional regulation. For instance TRIM21 ubiquitinylates IRF8, which is a transcription factor involved in IFNγ mediated expression of pro-

inflammatory cytokines, including IL-12p40 and type I IFNs. Rather than promoting its degradation, TRIM21 mediated ubiquitinylation leads to enhanced IL-12p40 release via IRF8. Again, the B30.2 domain of TRIM21 is critical for this interaction with IRF8 (271).
TRIM21 expression is also upregulated by IFN (262) meaning that it could be involved in an IFN feedback loop when combined with its actions on different IRFs.

The role of TRIM21 was further investigated by production of TRIM21 knockout mice. Two groups independently produced mice using GFP to replace TRIM21 exons, allowing study of the TRIM21 expression pattern. TRIM21 was found to be broadly expressed, with particularly high expression in the lymphoid compartment of spleen, lymph node and thymus, with little expression found in non-immune tissue (272, 273). Despite the similarities in expression pattern, the two studies produced mice with different phenotypes, the reasons for which are not clear.
Yoshimi et al found that knockdown of TRIM21 did not affect viability, growth or fertility, and development of the immune system occurred as normal. Embryonic fibroblasts were seen to have increased pro-inflammatory cytokine production through TLR induced NFκB signaling suggesting it plays a role in modulating signaling (273). Subsequently, TRIM21 was shown to negatively regulate NFκB signaling through monoubiquitinylating IKKβ, targeting it for autophagosomal degradation (274, 275). Upregulation of related TRIM molecules was also seen in embryonic fibroblasts, suggesting compensation of TRIM21 by related proteins TRIM12, -30 and -34. There was reduced ubiquitinylation of IRF3 and IRF8 in knockout mice, but this did not lead to an alteration in expression of IFN induced genes.
This phenotype was in contrast to that seen in the other TRIM21 knockout generated by Espinosa et al (272). Although generally there appeared to be no differences between wild type and knockout mice, after ear notching knockout mice developed severe dermatitis around the wound. This led to uncontrolled inflammation and development of systemic autoimmunity mediated through the IL-23-Th17 pathway. IRF5 was also identified as being ubiquitinylated and degraded by TRIM21 (272).

The reasons for these differences are not known, but it is though that the different gene knockout strategies used could be responsible. Yoshimi replaced TRIM21 exons 3-5, which included the translational start site, with GFP resulting in eradication of TRIM21 transcripts. Espinosa et al replaced exons 5-8 with GFP, which theoretically could lead to production of a truncated protein encoded by exons 1-4. This truncated protein may interfere with normal TRIM21 function and act in a dominant negative fashion (273).

1.6 Cyclophilins

1.6.1 The cyclophilin family

Another protein that interacts with HIV-1 and plays an important role in its lifecycle is the peptidyl-prolyl isomerase (PPIase), cyclophilin A (CypA). Proline residues in proteins can exist in either a cis or trans conformation, and the intrinsic switch between the two forms is very slow, unless catalysed by PPIases. These enzymes can be divided into four structurally distinct families called cyclophilins (Cyps), FK506-binding proteins (FKBPs), the parvulins and Ser/Thr phosphatase 2A activator (PTPA) (276). PPIases are a conserved family of proteins found in all bacteria and eukaryotes. This high level of conservation suggests that they play a role in a fundamental process conserved between species.

Humans express at least 17 Cyps, including proteins that contain cyclophilin-like domains. They vary in size and some contain additional domains to the Cyp moiety. The smallest is the 18kD CypA and the largest is the nuclear pore protein Nup358, also called RanBP2, which in addition to its Cyp domain includes Leu-rich, zinc finger and Ran-binding domains.

Particular interest has been shown towards cyclophilins as they interact with cyclosporine A (CsA), a fungal metabolite that is used as an immunosuppressive drug following allogeneic transplantation (277). CypA is the predominant cyclophilin involved in CsA mediated immunosuppression, as CypA knockout mice require greatly elevated concentrations of the drug to illicit an effect (278).

CsA interacts with the binding pocket in CypA, interfering with substrate binding substrates and preventing PPIase activity. This CypA-CsA complex also inhibits calcineurin, a calcium activated serine/threonine phosphatase (279). Calcineurin is responsible for dephosphorylation of the nuclear factor of activated T cell (NFAT) family of transcription factors downstream of the TCR involved in T cell activation (280, 281). Once dephosphorylated, the NFAT transcription factors become activated and are translocated to the nucleus. Inhibition of calcineurin prevents NFAT dephosphorylation and consequently prevents signaling downstream of the TCR, such as cytokine expression. This restricts T cell activity, preventing organ rejection after transplant. This function is independent of the PPIase activity of CypA.

CypA is a widely expressed, highly conserved protein found in both the cytosol (282) and nucleus (283). It is a globular protein consisting of a β barrel made up of eight anti-parallel β sheets, capped at either end by an α helix. A hydrophobic pocket forms the binding site and catalytic site for proline peptides (284).

Different natural functions of Cyps have been proposed. They are thought to be involved in the correct folding of a range of proteins as cis/trans isomerisation is often the rate limiting step in protein folding, with the trans isomer the energetically favoured state. Cyps have been shown to mediate folding of a range of proteins, including collagen (285), carbonic anhydrase (286) and ribonuclease T1 (287). Independently of their PPIase activity, they have been shown to act as protein chaperones. They prevent aggregation of incorrectly folded proteins leading to an increased yield of correct folded protein, in addition to catalysing protein folding through PPIase activity (286, 288). However, these activities have only been observed *in vitro*.

Another proposed role of Cyps is in cell signaling, and this is supported by *in vivo* data. CypA knockout mice have normal development and lifespan, but some mice develop spontaneous allergic disease driven by Th2 cells. CypA downregulates Th2 cytokine expression by interacting with the IL-2 tyrosine kinase, Itk. CypA interacts with a proline residue in the SH2 domain of Itk, which acts a molecular switch by

promoting homodimerisation of Itk, preventing it from mediating downstream signaling (289).

1.6.2 Role of cyclophilins in the HIV-1 lifecycle

CypA is probably the most extensively studied PPIase due to its ability to interact with HIV-1, and the key role that it plays in the virus lifecycle. CypA and B were found to bind to HIV-1 Gag protein (290), although only the interaction with CypA has been recorded *in vivo*. CypB is localised to the endoplasmic reticulum and is not thought to have the opportunity to interact with either incoming or outgoing Gag (291). The active site of CypA binds to the G89-P90 peptide bond located on a nine amino acid flexible loop (P85-P93) in the N terminus of the CA, and catalyses cis/trans isomerisation of the bond (292, 293). Nuclear magnetic resonance studies show that this bond can be found in either the cis (14%) or the trans (86%) conformations (294). G89-P90 is the bond primarily responsible for CypA binding, and mutation of either G89 or P90 will abrogate CypA binding. The surrounding residues P85, V86, H87, A88, P93 are also involved in binding (295).

The interaction between CA and CypA results in its incorporation into newly synthesised HIV-1 virions at a ratio of ten CA molecules to one CypA. Incorporation can be abrogated by treatment of producer cells with CsA, mutating the G89-P90 proline peptide bond within the CA or knockdown of the CypA gene, leading to a block in HIV-1 infection early after cell entry (88, 89, 295, 296). Despite its inclusion in virions, the presence of CypA in the target cell, rather than in the virus producing cell, is required for infectivity (297, 298). The relevance of virion incorporated CypA has not been elucidated. However, treatment of producer cells with CsA decreases infectivity of virions in a CypA independent manner, even when producing G89V mutant virions that do not bind CypA (297). Binding of CypA is not a conserved ability between all lentiviruses, but HIV-1, SIVagm and FIV have been shown to be targeted by CypA.

Although it is now known that it is CypA in the target cell that influences infection, the role that the enzyme plays in host cells is not known. CypA acts upon incoming

HIV-1 particles soon after entry and before reverse transcription, at the same time as TRIM5α restriction in non-human primates occurs (299). It is thought that CypA binding to incoming HIV-1 CA protects the virus from restriction and that altering the isomerisation state of the proline bond may make the CA more recognisable to restriction factors such as TRIM5α. This is supported by the fact that CypA causes a range of effects on infectivity, both cell type and species specific, indicating interaction with other proteins and not solely on CypA assisting viral uncoating (300). Disruption of the CypA-CA interaction through CsA treatment or knock down of CypA by shRNA leads to HIV-1 restriction in human cells independently of TRIM5α (301).

Generally binding of CypA to incoming HIV-1 CA is required for proper infectivity. However, it is now known that the interaction between CA and CypA is much more complex.

Culture of HIV-1 in $CD4^+$ HeLa cells in the presence of CsA results in the evolution of viral strains bearing mutations within the CypA binding loop of Gag; A92E and G94D. Although these mutants retain the ability to bind CypA, unlike wild type HIV-1 they are CsA dependent. In this instance, CA-CypA interactions appear to be detrimental to virus infectivity. Disruption of the CA-CypA interaction with CsA, knockdown of CypA or additional mutations that prevent CypA binding, rescue viral infection. Removal of CsA from culture results in reversion back to the wild type, CsA sensitive phenotype (302, 303). These CA mutants are therefore susceptible to CypA mediated restriction. The mechanism is not known, but it occurs after nuclear entry and before integration, as shown by the increased buildup of 2-LTR circles. This restriction is enhanced by cell cycle arrest (304-306).

An important feature of the HIV-1 lifecycle is the ability to infect non-dividing cells. The factors responsible for this have still not been established, but CA has been suggested to play a role. Substitution of HIV CA with MLV CA or introduction of single amino acid mutations in the CA resulted in virus that showed reduced infectivity in arrested cells (41, 43). The enhancement of CypA restriction of CA

mutants upon cell cycle arrest provides further evidence of the involvement of CA in infection of non-dividing cells.

Interestingly, the CsA dependence of these CA mutants is only seen in infection of particular cell lines, for instance HeLa and H9 cells. Jurkat, human osteosarcoma (HOS) and TE671 cells are able to support replication of these mutants even in the absence of CsA (298).

One proposed explanation for the variability seen between cells of the effects of CypA and CsA on infection is thought to be due to different CypA expression levels in cells. HeLa cells have higher levels of CypA than Jurkat and TE671 cells. CA mutants are CsA dependent in HeLa cells, which is thought to be due to the high CypA expression levels. CypA has been proposed to be involved in the regulation of CA uncoating (307) and may target the mutated CA more efficiently leading to disruption of normal uncoating. Infection could be restored to wild type levels in HeLa cells treated with CsA or by siRNA knockdown of CypA. This indicates that the block to infection is CypA dependent. Increasing CypA levels in TE671 cells results in the same restriction pattern seen in HeLa cells (306).

However, CypA levels alone are not able to explain the biphasic response to CsA dose seen in viral infection by primary isolates. In cells with similar CypA expression levels increasing CsA dose may or may not cause an increase in infectivity. This implies the involvement of other factors in addition to CypA. Similarly, TRIM5α levels and CA polymorphisms alone cannot explain the pattern of inhibition seen by treatment of cells with high doses of CsA. This suggests that there may be two factors with opposing effects within a cell that are influenced by the CA-CypA interaction. The effect that a particular dose of CsA has on infectivity depends upon the relative levels of these factors within the particular cell type (308).

There could be a host restriction factor that is able to target the CA-CypA interaction. For instance in heterokaryons of CsA dependent and independent cells, the dependence is conferred between cells, supporting the hypothesis of a dominant cellular restriction that targets CA-CypA (309).

Inhibition of CA-CypA interactions results in a virus strain specific effect and can cause an increase, decrease or no change in infectivity. Disruption of CA-CypA

interaction generally caused an increase in the susceptibility of HIV-1 strains to huTRIM5α restriction, but this effect is both strain specific and TRIM5α allele specific (310).

Although the presence of CypA in target cells is generally required for HIV-1 infection in human cells, its role is species specific. In many non-human primate cells the CypA-CA interaction actually enhances TRIM5α mediated restriction. This is possibly by CypA mediated isomerisation that may make the viral CA more efficiently bound by TRIM5α (or unknown restriction factors) (311, 312) or by stabilising the CA core and increasing the time for recognition by TRIM5α. However, even in the absence of CypA or when disrupting the CypA-CA interaction using the G89V mutation, TRIM5α is still able to restrict HIV-1 in Old World monkey cells to some extent (233).

Mutations in gag sequence and subsequent interactions with host factors, including TRIM5 and CypA, can cause significant effects in viral fitness and greater understanding of these interactions could allow exploitation of these systems in the future in antiviral therapies.

1.6.3 TRIMCyp fusion proteins

The species specific antiviral activity of TRIM5α confers resistance to HIV-1 in Old World monkeys and SIVmac in New World monkeys. Despite it being common amongst Old World monkeys for TRIM5α to confer resistance to HIV-1, most New World monkey cells are susceptible to infection by this virus (313). An exception is the New World owl monkey, of the genus *Aeotus*, due to expression of a fusion protein between TRIM5 and CypA. This has arisen from the LINE-1 (L1) mediated retrotransposition of a CypA cDNA into the TRIM5 intron 7 resulting in an in-frame fusion between exons 2 to 7 of TRIM5 and an entire CypA cDNA. CypA replaces the B30.2 domain that is encoded by exon 8, and is linked to the RBCC domains of TRIM5 via 11 amino acids encoded by the CypA 5' UTR. Owl monkeys are homozygous for this altered gene and do not have any other TRIM5 alleles. The owl

monkey TRIM5-CyclophilinA (omTRIM5Cyp) fusion protein is a strong inhibitor of HIV-1, due to the ability of CypA to bind HIV-1 CA and recruit the TRIM5 RBCC domains, and is responsible for the resistance of owl monkey cells to this virus. Inhibition can be overcome by treatment with CsA or the use of G89V HIV-1 mutants (314, 315). omTRIM5Cyp also restricts FIV and SIVagm, but leaves cells from this species susceptible to SIVmac (316).

There has been a second incident of retrotransposition of CypA into TRIM5 in Old World monkey macaques, including rhesus macaques (*Macaca mulatta*), pig-tailed macaques (*Macaca nemestrina*) and crab eating macaques (*Macaca fascicularis*) which, due to the different location of the Cyp DNA in the TRIM5 gene, is most likely to have occurred independently of the owl monkey event (231, 317-319). The resultant protein is encoded by exons 2 to 6 of TRIM5, with the CypA cDNA replacing exons 7 and 8, in contrast to the owl monkey fusion that is encoded by exons 2 to 7 of TRIM5 and the CypA cDNA. The antiviral specificity of this fusion protein is also distinct to the owl monkey, with the rhesus TRIM5Cyp being a strong inhibitor of HIV-2, HIV-1 group O and FIV, but not HIV-1 group M. This difference in restriction specificity is due to variations in the Cyp domain of rhTRIM5Cyp compared to that of the genomic Cyp, altering the configuration of the active site loop (320). Furthermore, TRIM5Cyp alleles from different macaque species also have diverse antiviral specificities due to additional mutations in their Cyp domains (321).

The proline-rich CypA binding loop is highly conserved amongst primate lentiviruses, as well as FIV and EIAV (316), suggesting that it plays an important role in a conserved function. This would reduce the probability of the development of TRIM5Cyp resistant escape mutants.

Despite the differences in specificity of the macaque and owl monkey TRIM5Cyp proteins, the mechanism of restriction is similar, causing a block in infection before reverse transcription which can be abrogated by CsA treatment or mutation of the TRIMCyp binding site on the viral CA. As with TRIM5α, the mechanism of

antiviral restriction has not been fully confirmed, but it is also likely to function through multiple actions. Restriction occurs rapidly after viral entry into the cell before reverse transcription, and does not require ubiquitin mediated proteasomal degradation or cytoplasmic body formation. TRIMCyp does form cytoplasmic bodies, although this is not essential for restriction (247). Deletion of the RING domain causes a reduction in restriction, and deletion of the RING and B-Box2 domains eliminates restriction (316) implying that they are important for efficient inhibition.

Like TRIM5, TRIM5Cyp forms multimers, and dimers, trimers and hexamers have all been identified. Multimerisation is mediated by the coiled coil domain, and this domain alone is sufficient to interact with full length TRIM5Cyp. This interaction means that mutated TRIM5Cyp proteins can elicit a dominant negative effect on native TRIM5Cyp in owl monkey cells (316)'(232). The L2 region, which is critical for the higher order multimerisation and therefore antiviral activity of TRIM5α (228), is present in all naturally occurring primate TRIM5Cyp proteins.

Like TRIM5α, TRIM5Cyp has been shown to disrupt CA cylinders *in vitro*, suggesting that at least one mechanism of antiviral activity is through interference with uncoating (243).

Human (322) and feline (323) TRIM5Cyp fusion proteins have been generated and are able to restrict HIV and both HIV and FIV respectively. Human TRIM5Cyp was able to restrict HIV-1 at levels comparable to the owl monkey fusion protein when expressed in cell lines and primary T cells and macrophages. It was also able to provide robust restriction of HIV-1 in a humanised mouse model of HIV-1 (322). Also TRIMCyp fusion proteins have been generated using alternative TRIM proteins to TRIM5, but that have the same domain structure (TRIM1, 18 and 19) (324) and Cyp has been fused to Fv1 to produce an inhibitor of HIV-1 (325).

These data suggest that fusion of CypA to the effector domain of restriction factors mediates recruitment to the virus through CA binding and is an effective method for the generation of HIV-1 restriction factors.

1.7 Targeting CCR5, an HIV-1 co-receptor

1.7.1 Identification of CCR5 as an HIV-1 co-receptor

In addition to the primary receptor CD4, HIV-1 requires the presence of a co-receptor to allow virus entry into a cell. Two co-receptors have been identified; CCR5 (5-9) and CXCR4 (10), which are used by R5 tropic and X4 tropics HIV-1 strains respectively. CCR5 and CXCR4 tropic strains were initially identified by the cell type which they were able to infect and consequently were named macrophage tropic and T cell tropic respectively. Dual tropic strains were identified as being able to replicate in both cell types.

Both CCR5 and CXCR4 are structurally similar chemokine receptors belonging to the superfamily of G protein coupled receptors and expressed on haematopoietic cells. Chemokines bind these receptors and activate intracellular signaling pathways involved in a variety of different cellular functions, particularly chemotaxis and cell migration and recruitment to sites of inflammation.

CCR5 is predominantly expressed on macrophages and particular subsets of $CD4^+$ T cells, typically those with a memory phenotype (326), and plays a role in haematopoiesis and inflammation. The natural ligands for CCR5 include RANTES, MIP-1α and MIP-1β and they can reduce infection by R5 tropic strains by competing with HIV-1 for the co-receptor and causing internalisation of the receptor, reducing the opportunity for viral binding (327). CCR5-tropic (R5) HIV-1 strains are the most common, are primarily responsible for viral transmission and are the predominant strain during initial infection (328).

CXCR4 is highly expressed on both $CD4^+$ and $CD8^+$ T cells, with around 90% of $CD4^+$ cells also expressing CXCR4. In addition, CXCR4 is also expressed on haematopoietic stem/progenitor cells and, with its ligand SDF-1α, plays an important role in their migration and haematopoiesis.

X4 HIV-1 strains become more prevalent throughout infection, typically at the onset of disease symptoms (111). It is debatable whether these strains are co-transmitted

with R5 viruses in the initial infection and then remain latent or suppressed until immunity wanes. Another theory is that R5 tropic strains evolve to use different co-receptors, from CCR5, through a dual tropic stage before developing a strong affinity to CXCR4 in later stage disease. After their appearance there is often rapid loss in T cells, particularly naïve cells, targeted due to their high levels of CXCR4 expression (329).

In vitro evidence suggests that some HIV-1 strains can use other seven transmembrane receptors in placement of CCR5 and CXCR4 alongside CD4, such as CCR2 (8), CCR3 (6), CCR8 (330), BOB and Bonzo (331). However, there is insufficient evidence to fully appreciate the role that these additional receptors play *in vivo*.

A small population of individuals who, despite repeated exposure to HIV-1, did not develop infection were found to be homozygous for a 32 base pair deletion (Δ32) in the CCR5 gene. This deletion results in a frame shift and expression of a truncated form of the receptor (332, 333). Absence of CCR5 from the cell surface means that R5 strains of HIV-1 are unable to infect target cells. Also the truncated form of the protein is thought to enhance HIV-1 resistance by sequestering CXCR4 from the cell surface, reducing infection by X4 virus in addition to there being no entry via CCR5 (334). Although it is rare, Δ32 homozygotes can still become infected with HIV-1, either by X4- or dual-tropic strains of virus.

CCR5 Δ32 heterozygotes express reduced levels of cell surface CCR5 (326), but this does not provide resistance to infection. However there is evidence to show that their loss of $CD4^+$ cells is slower and onset to AIDS is later compared to those with the wild type genotype (335, 336).

Importantly, individuals homozygous for the Δ32 mutation appear to have no defective phenotype due to this mutation, indicating redundancy in the role of CCR5. However, reports have suggested that CCR5 Δ32 homozygotes have an increased susceptibility to West Nile virus (337) and tickbourne encephalitis virus (338). Despite this, CCR5 remains a desirable target in anti-HIV therapy to mimic this naturally occurring protection against infection.

Recently substantial support for the theory of targeting CCR5 as an anti-HIV therapy has emerged from the allogenic transplant setting. An HIV-1 infected man developed acute myeloid leukaemia and received an allogenic transplantation of $CD34^+$ peripheral blood stem cells from an HLA-identical donor homozygous for the Δ32 CCR5 mutation (339). The patient suffered relapse of the leukaemia nearly one year later so received a second transplant from the same donor, resulting in complete remission of leukaemia. He also discontinued HAART at this time, which on previous occasions had led to a rapid viral rebound. However, following the second stem cell transplantation, HIV has been undetectable for more than 3 years without any administration of HAART (339, 340). Interestingly, although the patient had X4 tropic strains of virus before the transplant, these remained quiescent following the transplant. It is not known why these strains of virus did not continue replicating despite CXCR4 expressing target cells still remaining. It is possible that either the level of virus or number of target cells following transplantation were too low to facilitate viral rebound.

Allogenic transplant, which requires myeloblation and immune suppression, is not a viable option for wider treatment of HIV-1. In addition, the likelihood of obtaining an HLA matched, Δ32 homozygote donor is very low, with only around 1% of the Caucasian population being of this genotype.

Although only a single case, this successful treatment of an HIV-1 patient is the first documented functional cure and has confirmed the validity of CCR5 as a potential target in anti-HIV therapy. It also shows that delivery of an HIV-1 resistant population of cells to an infected individual can lead to repopulation of the immune system and apparent eradication of infection. There are many different groups working on various methods to disrupt CCR5 expression to prevent HIV-1 infection of cells. Amongst these, include the use of shRNA (341), ribozymes (342), intrabodies (343) and drugs, such as Maraviroc (344). One method that shows particular promise is the use of zinc finger nucleases (ZFNs) to specifically target and disrupt the CCR5 gene.

1.7.2 Generation of zinc finger nucleases (ZFNs)

ZFNs are chimeric nuclease proteins that are engineered to target a specific DNA sequence and induce a double stranded break (DSB). They are synthesised by assembling Cys_2His_2 zinc finger (ZF) DNA binding domains to the catalytic domain of FokI, a non-specific endonuclease (345). The ZFs are proteins motifs that consist of an α helix and an antiparallel β sheet folded around a zinc ion, which bind DNA by insertion of the α helix into the DNA major groove. Three or four ZFs, which each typically bind three base pairs of DNA, are combined in each ZFN resulting in a 9-12 nucleotide recognition site (346). This string of ZFs is fused to a FokI monomer, which must dimerise to function and cause a DSB (347). Therefore two ZFNs are required to bind on opposite strands of DNA and with correct spacing to allow FokI dimerisation and subsequent double strand DNA cleavage between the two ZFNs. Dimerisation means that the total nucleotide recognition site is 18-24 nucleotides long, providing high specificity. The FokI domains have been modified so that they can only function as heterodimers (348). Preventing homodimerisation further improves specificity of the ZFNs, as they will only cleave DNA when correctly paired. Adjusting the spacing between the two binding sites and linker length in the ZFN between ZF and FokI affects the efficiency of cleavage (349).

For this technology to be an effective therapy applicable to different diseases, ZFNs must be able to target a range of loci. The modular assembly of ZFNs has enabled them to be designed to target a large number of genomic sequences and theoretically, a ZFN site can be located approximately every 150bp within the genome.

ZF motifs recognise and bind a 3bp DNA sequence, and the most simple method of generating ZFNs with a particular target site is modular assembly, by which ZFs with a known target sequence are joined together. However, as the binding specificity of each ZF is not independent of its neighbours, the target site of a string of ZFs does not simply match the sequence of the individual motifs. Therefore there

is only a low success rate of approximately 6% in producing ZFN pairs using this method, and high levels of associated off target toxicity (350).

A more advanced method termed context dependent or sequential selection takes into account the influence of neighbouring ZFs and its position within the ZFN. The ZFs are selected one by one, allowing optimisation of binding of each ZF in the context of it neighbour (351). However, this method is labour intensive as it requires screening of each ZF motif and is therefore not practical for most laboratories, although it does generally result in highly specific ZFNs (351, 352).

Sangamo, a biopharmaceutical company which is a leader in the field of ZFN technology, uses a library of ZF protein pairs and combines these to produce a four fingered protein, using algorithms to predict the interaction between them and how this may influence binding specificity. Both the library and the algorithms used are exclusive to Sangamo and therefore cannot be utilised by other researchers to design novel ZFNs.

In contrast, a publically available source developed by the Zinc Finger Consortium, a collaboration between different academic laboratories, allows production of ZFNs using Oligomerised Pool Engineering (OPEN) technology (353). This consists of a library made up of different pools each containing fingers which bind to a particular DNA triplet depending on where in the string of ZFs they are found. OPEN requires 192 pools; 64 different possible DNA triplets, targeted by a ZF in three possible different positions within the ZFN. ZF motifs are then selected from these different pools and tested for specificity in a bacterial two-hybrid (B2H) system. Again, this method of ZFN assembly provides more specific and therefore less toxic proteins than modular assembly (353).

Whichever method is used to design ZFNs, it is important that the specificity is confirmed. The location and frequency of off target cleavage must be identified, and the subsequent toxicity on modified cells measured for the most efficient effect.

1.7.3 ZFNs as a tool for gene therapy

Once a genomic locus has been targeted by a pair of ZFNs and a DSB introduced, it can be repaired by two different mechanisms; non-homologous end joining (NHEJ) or homology directed repair (HDR), both of which are useful in different gene therapy scenarios.

NHEJ is the predominant repair mechanism and is more common at the G1 phase when sister chromatids are not present to provide a template for repair. After initiation of the DSB, the heterodimer Ku binds to the DNA ends and enhances recruitment of nucleases, polymerases and ligases required for NHEJ, including DNA-dependent protein kinase (DNA-PKcs) (354). Artemis forms a complex with DNA-PKcs, allowing its phosphorylation and subsequent activation. The Artemis-DNA-PKcs complex has a range of endonuclease activities, which allows cleavage of damaged DNA ends (355). Polymerases bind the Ku-DNA complex and are responsible for DNA extension, which is often required to create homology between the DNA ends. The XLF-XRCC4-DNA ligase IV complex is recruited by Ku and ligates DNA ends to restore the chromosome structure at a DSB (356).

The enzymes involved in NHEJ have a high degree of flexibility in their function, allowing repair of a diverse range of DNA ends, and resulting in a variety of different DNA sequences. NHEJ typically leads to the formation of mutations in the original wild type DNA sequence by insertions or deletions (InDels). These mutations frequently result in disruption of the reading frame and production of a truncated, non-functional protein.

This method would be effective in the case of HIV-1 treatment, where knockout of CCR5 would produce cells that are resistant to infection by R5 tropic HIV-1 strains. Only transient expression of the ZFN pair would be required to produce a permanent knockout of the gene, which would be passed on to daughter cells.

The second repair mechanism, HDR, is a less frequent, but more accurate method for repairing DSB and utilises a homologous DNA sequence as a template for repair. This mechanism is most common at the S and G2 phases, when sister chromatids are

available to act as a repair template, resulting in maintenance of the correct sequence.

When a DSB has occurred, the MRN (Mre11/Rad50/Nbs1) complex assembles at either end of the DNA keeping the two DNA ends in close contact (357). It also activates the protein kinase ATM (ataxia telangiectasia mutated), which in turn phosphorylates and activates other molecules involved in DNA repair (358). DNA resection of the 5' end forms a 3' ssDNA overhang to which the recombinase Rad51 is recruited by an array of accessory proteins, including direct interaction with BRCA2 (359). This results in a nucleoprotein filament of Rad51 multimers. Rad51 assembly, rearrangement and disassembly is responsible for the interaction of the ssDNA with a homologous dsDNA template; typically the sister chromatid or, if it is present, a donor DNA template.

Once a suitable template has been found, the Rad51 filament is able to insert into the dsDNA in a process called strand invasion. This strand invasion forms a displacement (D) loop intermediate and extension of the invading ssDNA is carried out by a DNA polymerase. In the synthesis dependent strand annealing model, after extension, the invading strand is displaced and anneals to the second end of the DSB. Alternatively, during extension, the D loop can anneal to the second ssDNA end of the DSB to provide another template for extension and leading to the formation of two Holliday junctions. Various endonucleases can resolve the Holliday junctions, and subsequent ligation of the DNA ends results in either crossover or non-crossover DNA products (reviewed in (360)) (Figure 1.2).

HDR can be exploited in therapy by providing a homologous DNA sequence termed the donor template to function as a template for repair. Provision of a high concentration of this donor template can increase the frequency of HDR over NHEJ (361). The template would consist of the sequence to be integrated flanked by DNA sequence homologous to that at the site of the break. This template can then be used to allow either correction of a mutation within the targeted gene or insertion of a novel transgene at this specific locus.

One particular example which has shown great promise for this technology is the treatment of a mouse model of haemophilia (362). Haemophilia is caused by

mutations in the blood coagulation factor IX, which is encoded by the F9 gene. ZFNs targeting intron 1 of F9 and a donor template encoding exons 2-8 of F9 were delivered using adeno-associated virus (AAV) vectors into a newborn haemophilia mouse model and resulted in a clear improvement in clotting.

Site specific integration is highly desirable in gene therapy, but using homologous recombination alone is far too inefficient to be used therapeutically. However, using ZFNs to introduce a DSB greatly increases the efficiency of homologous recombination to allow gene targeting. Proof of principle of this was first shown by introduction of a DSB using the endonuclease I-SceI, which has an 18bp recognition sequence and therefore cuts infrequently within a genome. Depending upon the cell type, introduction of a DSB increased the efficiency of homologous recombination 50-100 fold (363, 364).

Using this theory, ZFNs have been designed to target specific sequences and introduce a DSB at a desired location for integration by HDR. As well as ZFNs, other sequence specific nucleases, namely meganucleases and TALE nucleases (TALENs) have also been designed for this purpose. Transcription activator-like effectors (TALEs) are transcriptional activators derived from the plant bacterial pathogen, *Xanthomonas*. Each TALE specifically binds a single nucleotide via a central repeat domain of 30-35 amino acids, specifically a two amino acid motif named the repeat variable diresidue (365). As with ZFs, a series of TALEs combined to guide DNA binding specificity can be fused to the FokI endonuclease to form TALENs. These TALENs must also function in pairs with correct DNA binding and spacing to allow dimerisation of FokI and induction of a DSB (366, 367).

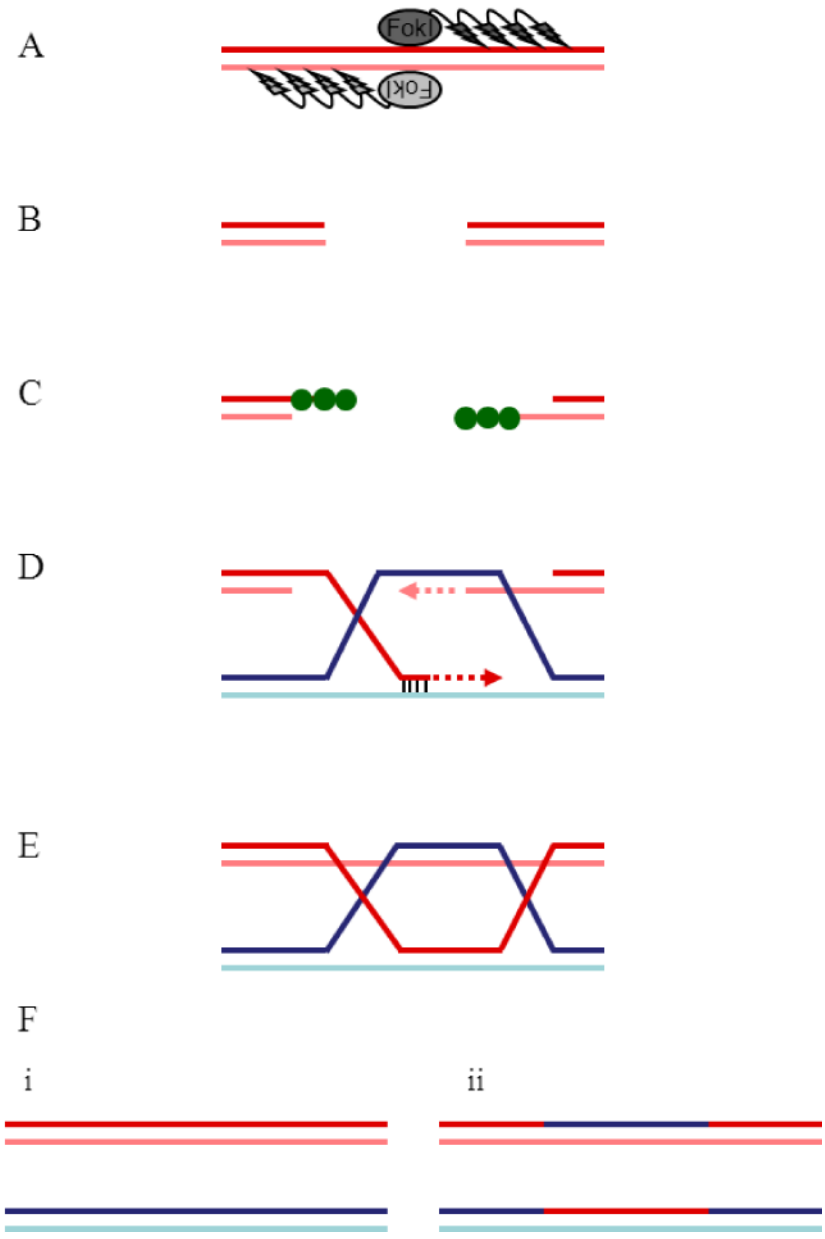


Figure 1.3 Mechanism of repair by homology directed repair after double strand break by zinc finger nucleases

Zinc finger nucleases bind opposing strands of DNA (A), allowing FokI heterodimerisation and induction of a double strand break (B). End resection results in 3' ssDNA overhangs on which filaments of Rad51 recombinase (green circles) accumulate (C). These filaments recognise and capture homologous duplex DNA, forming a D loop intermediate. The 3' end of the invading strand is extended by DNA polymerase using the duplex DNA as a template (D). The D loop can capture the second 3' end of the DSB, allowing DNA polymerisation and forming two Holliday junctions (E). The Holliday junctions can be resolved in two distinct ways, resulting in either non-crossover (i) or crossover (ii) products.

Site specific integration has advantages over random integration as transgenes could be inserted at a safe harbour site where they would not affect gene expression of neighbouring genes. Similarly, transgene expression would be protected from silencing or influence from surrounding genes which can be a problem with randomly integrating vectors causing issues with sustained treatment of a disease (368). Importantly, integration at a safe harbour would also avoid insertional mutagenesis, the problems of which have been demonstrated in clinical trials and discussed in section 1.3. Therefore, site specific integration using ZFNs is highly desirable in gene therapy. Although there has been significant development of lentiviral vectors to replace gamma retroviral vectors due to their safer integration profile, site specific integration using ZFNs is still highly desirable in gene therapy.

The CCR5 locus has attracted interest as a safe harbour because insertion at this site and consequent loss of expression does not cause serious side effects, as demonstrated with the naturally occurring Δ32 mutation. However, a significant benefit associated with this mutation is that Δ32 homozygotes have a strong resistance to HIV-1 infection, therefore making CCR5 specific ZFNs an attractive possibility for gene therapy against HIV-1.

Sangamo have developed a pair of obligate heterodimeric ZFNs to target and knockout CCR5 expression. In preclinical *in vitro* studies, delivery of these ZFNs into T cells using the Ad5/35 adenoviral vector led to CCR5 knockout in 40-60% of cells. These cells were transplanted into immunodeficient non-obese diabetic (NOD)/SCID/IL-2r γ chain (NSG) mice which were subsequently infected with R5 tropic HIV-1. ZFN treated mice had higher $CD4^+$ T cell counts and lower viral loads than control mice receiving wild type $CD4^+$ T cells (369). This data led to testing of the ZFNs in two phase I clinical trials, in which HIV patient $CD4^+$ T cells were modified with ZFNs. Preliminary data from these trials have so far been promising, showing no adverse effects in response to the procedure and an increased CD4 count (135). Alongside this, these ZFNs have been further tested by modification of HSCs. Modified haematopoietic progenitor cells were transplanted into NSG mice where they gave rise to multi-lineage progeny. Mice were infected with R5 tropic HIV-1,

and in those which received ZFN modified cells there was a lower viral load and an expansion of $CCR5^-$ cells. Mice engrafted with wild type HSCs suffered a significant loss of $CD4^+$ T cells after HIV infection (370).

This data further supports the theory of targeting HSCs through gene therapy to provide HIV-1 resistance in multiple cell types and repopulate the host immune system.

1.8 Project aims

Gene therapy offers potential for a single long term treatment of HIV-1, eliminating the requirement of intensive drug regimens. Numerous different anti-HIV-1 transgenes have been proposed for modification of T cells or HSCs to inhibit HIV-1 infection.

The aim of this project is to investigate possible gene therapy strategies against HIV as follows:

- Generate humanised TRIM5Cyp proteins based upon the naturally occurring TRIM5Cyp lentiviral restriction factors found in primates
- Compare TRIM5Cyp with the alternative TRIM21Cyp generated by replacement of the TRIM5 RBCC motif with the corresponding domains of TRIM21
- Characterise the effects of TRIMCyp expression on endogenous TRIM antiviral function
- Combine TRIMCyp proteins with CCR5 specific ZFNs using non-integrating lentiviral vectors to exploit this site as a safe harbour, and for CCR5 knockout
- Investigate the use of these strategies in anti-HIV gene therapy by expressing TRIMCyp proteins from lentiviral vectors in susceptible cell populations and measuring restriction of HIV-1

2 Materials and Methods

2.1 Materials

Unless otherwise stated, all cell culture reagents were supplied by Gibco BRL (Invitrogen) and all general chemicals by Sigma-Aldrich. Enzymes for molecular cloning were supplied by Promega and primers by Invitrogen. DNA sequencing reactions were performed by Eurofins MWG Operon.

2.1.1 General reagents

1kb Plus DNA ladder	Invitrogen
Agarose	Melford
Ampicillin	Sigma-Aldrich
Cyclosporine	Sandoz Pharmaceuticals
dNTPs	Applied Biosciences
Ficoll	GE Healthcare
FuGene-6	Roche
Interferon-α	SP Labo, Heist-op-den-Berg
Interferon-β	Merck Serono
Interleukin-2 (IL-2)	Proleukin, Chiron
Kanamycin	Sigma-Aldrich
LIVE/DEAD fixable blue dead cell stain	Invitrogen
MES SDS Running buffer (20x)	Invitrogen
NuPAGE 4-12% Bis-Tris gels	Invitrogen
NuPAGE Transfer buffer (20x)	Invitrogen
Proteinase K	Applied Biosciences
SeeBlue® Plus2 Protein standard	Invitrogen
T7 Endonuclease I	New England Biolabs

2.1.2 Buffers

10 x TAE: 400mM Tris-acetate, 10mM EDTA in H_2O

DNA loading buffer: 50% Glycerol, 0.4% Orange G

DNA lysis buffer: 1mg/ml Proteinase K, 0.5% NP40, 0.5% Tween 20 in TE

Laemmli buffer: 60mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue

Transformation buffer: 55mM $MnCl_2 \cdot 4H_2O$, 15mM $CaCl_2 \cdot 2H_2O$, 250mM KCl, 10mM PIPES in H_2O

2.1.3 Antibodies

AB1424	Anti-HA	Abcam
A1978	Anti-β-actin	Sigma-Aldrich
BML-SA296	Rabbit Anti-Cyclophilin A	Enzo Lifesciences
NXA931	Sheep HRP-linked Anti-Mouse	GE Healthcare
NA934	Donkey HRP-linked Anti-Rabbit	GE Healthcare
557755	APC-Cy7 Mouse Anti-Human CD195	BD Pharmingen
555349	APC Mouse Anti-Human CD4	BD Pharmingen
AB1056	Goat Anti-Adenovirus	Millipore
TC31-9C12.C9	Anti-Adenovirus type 5 hexon	Developmental studies hybridoma bank

2.1.4 Kits

Geneamp® RNA PCR core kit	Applied Biosciences
HIV-1 p24 antigen ELISA	ZeptoMetrix
In vitro toxicology assay kit (MTT based)	Sigma-Aldrich
Plasmid preparation (mini/maxi)	Qiagen
QiaQuik Gel Extraction	Qiagen

QiaQuik PCR Purification	Qiagen
TOPO TA cloning kit	Invitrogen

2.1.5 Cells

Bacteria

One Shot Stbl3 *E. coli* Invitrogen

Genotype: F- *mcr*B *mrr hs*dS20 (r_B^-, m_B^-) *rec*A13 *sup*E44 *ar*a-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20 *(Str) xyl-5* λ^- *leu mtl-1*r

One Shot TOP10 *E. coli* Invitrogen

Genotype: F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G λ-

Mammalian cell lines

CRFK Crandell-Reese feline kidney cells.

GHOST CD4 transformed human osteosarcoma cells (HOS-CD4) expressing chemokines, either CCR5 or CCR5 and CXCR4 (371). Obtained from NIBSC

HEK293T Human embryonic kidney cell line

HeLa Human cervix epithelial carcinoma cell line

Jurkat.CD4-CCR5 Human T cell line (CD4 positive) stably transfected with CCR5. Obtained from NIBSC

TE671 Human rhabdomyosarcoma cell line.

2.1.6 Media

Bacteria

LB Broth: 1% tryptone, 0.5% yeast extract, 1% NaCl

LB Agar: As above with the addition of 1.5% agar

Mammalian cells

All cell culture media were from Gibco BRL, Invitrogen unless otherwise stated.

Foetal calf serum (FCS)	Sigma-Aldrich
Human AB serum	Lonza
X-Vivo-10	Lonza
Dynabeads ® Anti-CD3/anti-CD28 beads	Invitrogen
G418 (500µg/ml)	Source Bioscience
Puromycin (1µg/ml)	Sigma-Aldrich
Hygromycin (100µg/ml)	Invitrogen

2.1.7 Quantitative real time PCR

Platinum qPCR SuperMix UDG with ROX	Invitrogen
Primers	Invitrogen
Probes	MWG

Human β actin forward	TCACCCACACTGTGCCCATCTACGA
Human β actin reverse	CAGCGGAACCGCTCATTGCCAATGG
Human β actin probe	FAM-ATGCCCTCCCCCATGCCATCCTGCGT-TAMRA
WPRE forward	TGGATTCTGCGCGGGA
WPRE reverse	GAAGGAAGGTCCGCTGGATT
WPRE probe	FAM-CTTCTGCTACGTCCCTTCGGCCCT-TAMRA

Table 2.1 Table of primers and probes used for quantitative polymerase chain reaction

Human TRIM5 (Hs01552552_g1)	Applied Biosystems
Human TRIM21 (Hs00989233_g1)	Applied Biosystems
Human GAPDH (Hs99999905_m1)	Applied Biosystems

2.1.8 PCR primers

Pfu DNA polymerase	Promega
Go Taq DNA polymerase	Promega
Primers	Invitrogen

Primer Name	**Primer sequence**	
TS141	GCATGCGGCCGCCATGGTCAACCCCACCGTGTTCTTCG	Fw CypA, adds NotI
TS142	GCATGTCGACTTATTCGAGTTGTCCACAGTCAGC	Rv CypA, adds SalI
TS58	ATGCCAATTGATGGCTTCTGGAATCCTGGTTAATGTAAAGG	Fw TRIM5, adds MfeI
TS201	AGCTGCGGCCGCGTTTGGAGCCACTGTCACATCAACCCAG	Rv TRIM5 aa306, adds NotI
TS202	AGCTGCGGCCGCCCAGTAGCGTCGGACATCTGTCAGCTC	Rv TRIM5 aa298, adds NotI
TS203	AGCTGCGGCCGCCACTTCTAGCATTCCTTTCAGATCAGG	Rv TRIM5 aa287, adds NotI
TS209	ATGCGGATCCACCATGGCTTCTGGAATCCTGGTTAATG	Fw TRIM5, adds BamHI
TS187	GCATGGATCCTTATTCGAGTTGTCCACAGTCAGCAATGGTG	Rv CypA, adds BamHI
TS154	AGCTGCGGCCGCTGTCCTCAGCATCTTCTTCAGCCCTGGCAC	Fw TRIM21, adds MfeI
TS43	CAGTGAATTCATGGCTTCAGCAGCACGCTTGACAATGATG	Rv TRIM21, adds NotI
ZFN1	CAGCTCGAGCCTGCAGGGTATGGACTACAA	Adds SbfI and XhoI
ZFN2	GCTGATCAGCGGGTTTAAACGGGCCCTCT	Adds an XhoI
T21Cfw	AGAAGATGCCACAGCAGCGCC	Fw TRIM21CypCO
T21Crv	GTTGGTGTTGGGTCCGGCGT	Rv TRIM21CypCO
PGKfw	TGAAGAATGTGCGAGACCCAGG	Fw PGK
8-CCR5	CCCCATAGCAAGACAAAGACCTGT	Rv CCR5
9-CCR5	CACTTTTTATTTATGCACAGGGTGGA	Fw CCR5 for T7 assay
10-CCR5	GATGATTCCTGGGAGAGACGC	Rv CCR5 for T7 assay

Table 2.2 Table of primers used in this study

2.1.9 Parental plasmids

pEXN: derivative of the Moloney MLV retroviral vector pLNCX2 containing a HA tag (372).

pLNT/SFFV-MCS-WPRE: self-inactivating (SIN) second generation lentiviral backbone derived from pHR plasmid. It includes a SFFV promoter followed by a multiple cloning site and Woodchuck hepatitis virus post transcriptional regulatory element (WPRE).

pLNT/SIEW: (SFFV-IRES-eGFP-WPRE) lentiviral vector with the same backbone as pLNT/SFFV-MCS-WPRE, with an IRES-eGFP inserted at the multiple cloning site (373).

pVax/CMV-ZFNEL: expression plasmid encoding the left CCR5 ZFN, ZFNEL, under control of the CMV promoter. Provided by Sangamo.

pVax/CMV-ZFNKK: expression plasmid encoding the right CCR5 ZFN, ZFNKK, under control of the CMV promoter. Provided by Sangamo.

pCR4/CCR5 BglI donor: TOPO plasmid with CCR5 genomic sequence containing BglI site for cloning ZFN donor template. Provided by Sangamo.

pCR4/CCR5 GFP donor: TOPO plasmid with CCR5 genomic sequence flanking GFP. Provided by Sangamo.

pMA/TRIM21CypCO: Codon optimised TRIM21Cyp synthesised by Geneart (Regensburg, Germany) using their in-house GeneOptimizer® software.

2.1.10 Generated plasmids

pEXN/Cyp

Human cyclophilin A was amplified from HeLa cell cDNA using primers TS141 and TS142. The PCR product was cloned into pEXN between NotI and SalI. Provided by Dr. Torsten Schaller

pEXN/TRIM5Cyp

Three human TRIM5 RBCC motif fragments were amplified by PCR using primer TS58 with TS201, TS202 or TS203. Human TRIM5 in pEXN was provided by Dr. Torsten Schaller and used as the template. The number of the final amino acid of the TRIM5 RBCC sequence is denoted in the name of the resultant TRIMCyp fusion protein construct i.e. 306, 298 and 287 (Figure 3.1).

The PCR product was ligated between EcoRI and NotI of pEXN/CypA (provided by Dr. Torsten Schaller). This produced three different TRIM5Cyp constructs of increasing lengths. The resulting plasmid was named pEXN-TRIM5Cyp (Figure 3.2).

pLNT/S-TRIMCyp-IEW

TRIM5Cyp constructs were amplified from the EXN vector by PCR using primers TS209 and TS187, and cloned into the BamHI site of pLNT/SIEW to produce the plasmid pLNT/S-TRIM5Cyp-IEW (Figure 3.5). pLNT/SIEW expressed just GFP and served as an empty vector control.

The TRIM21Cyp fusion construct was originally generated by fusing the first 284 amino acids of human TRIM21 amplified by primers TS154 and TS43 with human CypA with a NotI site in between the two genes. This construct was supplied by Dr. Torsten Schaller in the lentiviral plasmid, pSFXUC. The entire construct was removed by digestion with BamHI before ligation into pLNT/-SIEW at the BamHI site. The resulting plasmid was named pLNT/S-TRIM21Cyp-IEW (Figure 3.5).

pLNT/SFFV-TRIMCyp-WPRE: To produce lentiviral vectors without eGFP, TRIMCyp from pLNT/S-TRIM5Cyp-IEW was removed using BamHI and inserted into pLNT/SFFV-MCS-WPRE at BamHI. The resulting plasmid was named pLNT/SFFV-TRIMCyp-WPRE.

pLNT/SFFV-ZFNEL-WPRE

The ZFNEL gene was amplified from the Sangamo pVax plasmid by PCR using primers ZFN1 and ZFN2. The purified PCR product was ligated into the recipient

lentiviral backbone, pLNT/SFFV-MCS-WPRE at XhoI. The final plasmid was named pLNT/SFFV-ZFNEL-WPRE (Figure 6.1).

pLNT/SFFV-ZFNKK-WPRE

The pVax/CMV-ZFNKK plasmid was cut with EcoRI and the DNA overhangs filled in before digestion with XhoI. The recipient lentiviral backbone, pLNT-SFFV-MCS-WPRE was cut with BamHI and blunt ended using DNA polymerase I Large (Klenow) fragment and then cut with XhoI. The two DNA molecules were ligated together to produce pLNT/SFFV-ZFNKK-WPRE (Figure 6.1).

pLNT/CCR5-PGK-GFP

The CCR5 donor expression plasmid from Sangamo was digested with NsiI and NheI to remove a fragment consisting of PGK-GFP. The ends were filled in using DNA polymerase I Large (Klenow) fragment. The recipient lentiviral plasmid pLNT/SFFV-MCS-WPRE was digested with XhoI and EcoRI to remove SFFV and the ends filled in before 5' dephosphorylation using shrimp alkaline phosphatase (SAP). The PGK-GFP fragment was ligated into the lentiviral backbone. The final donor plasmid was named pLNT/CCR5-PGK-GFP (Figure 6.1).

pLNT/CCR5-PGK-TRIM21CypCO

A plasmid was synthesised by Geneart which consisted of PGK-driving expression of a codon optimised TRIM21Cyp transgene, T21CypCO. This plasmid was digested with BglI to yield the fragment PGK-T21CypCO. The Sangamo CCR5 donor TOPO plasmid, CCR5-BglI, was digested with BglI, dephosphorylated with SAP and was ligated to PGK-T21CypCO. The CCR5 flanked PGK-T21CypCO was removed from the plasmid by digestion with NsiI and NheI. The recipient plasmid, pLNT/SFFV-MCS-WPRE, was prepared by digestion with EcoRI and XhoI to remove SFFV, filling in the 5' DNA overhangs and dephosphorylation with SAP. The lentiviral backbone and CCR5 flanked PGK-T21CypCO were ligated together to produce the final plasmid, pLNT/CCR5-PGK-T21CypCO (Figure 6.1).

2.2 Methods

2.2.1 Growth and maintenance of E. coli

Escherichia coli (E. coli) were grown in LB broth at 37°C with agitation at 250 rpm or streaked out on LB agar plates (1.5% bacto agar). For selection of transformed *E. coli* the same media supplemented with 50µg/ml ampicillin or 50µg/ml kanamycin was used. Bacterial cultures were stored at -80°C in LB broth containing 15% (v/v) glycerol.

2.2.2 Production of chemically competent Stbl3 E. coli

Chemically competent *E. coli* were prepared using the Innoue method (374). Stbl3 *E. coli* were streaked out on a LB agar plate with no antibiotics and grown overnight. A single colony was used to inoculate 25ml of LB broth and grown for 8 hours at 37ºC with shaking. 2-10ml of this starter culture was used in inoculate 250ml LB and grown at room temperature until the OD_{600} reached 0.55. The culture was placed in ice for 10 minutes before harvesting the cells by centrifugation at 2500g for 10 minutes at 4ºC. The bacteria were resuspended in 80ml ice cold transformation buffer and then pelleted again at 2500g for 10 minutes at 4ºC. The cells were resuspended in 20ml ice cold transformation buffer and DMSO added to a final concentration of 7%. 100µl aliquots were frozen at -80ºC

2.2.3 Transformation of chemically competent E. coli

One vial of Stbl3 *E. coli* was thawed on ice and DNA was added, mixed gently and incubated on ice for 30 minutes. Cells were heat-shocked at 42°C for 45 seconds and cooled on ice for 2 minutes. 250µl of LB broth was added and the cells were incubated for 1 hour at 37°C with shaking. Cells were plated out on LB agar plates with the appropriate selection antibiotic and cultured overnight at 37ºC.

2.2.4 Plasmid DNA preparation

E. coli containing plasmid was grown overnight in LB broth with the appropriate antibiotic. Plasmid was extracted by alkaline lysis using Qiagen Miniprep or Maxiprep kits according to manufacturer's instructions. DNA concentration was measured using a Nanodrop ND-1000 spectrophotometer at a wavelength of 260nm.

2.2.5 Restriction endonuclease digests

DNA was digested in a final volume of 20µl containing 1x buffer (supplied by manufacturer), 0.1mg/ml BSA and one or two restriction enzymes (<10% final volume). The reaction was incubated for 4 hours at 37°C.

2.2.6 Filling in of 5' DNA overhangs

5' DNA overhangs generated by restriction enzyme digests were filled in using DNA polymerase I Large (Klenow) fragment. DNA was mixed with 1 unit (U) of enzyme per microgram of DNA, 1x buffer (supplied by Promega) and 40µM of each dNTP. The reaction was incubated at room temperature for 10 minutes, and stopped by heat inactivation of the enzyme for 10 minutes at 75°C.

2.2.7 Dephosphorylation of 5' phosphate groups from DNA

5' phosphate groups from DNA were removed prior to ligation to prevent re-ligation of linearised vector DNA with compatible ends. Thermosensitive alkaline phosphatase was added at 1U per microgram of DNA directly to restriction reactions. The reaction was incubated for 15 minutes at 37°C, and the enzyme inactivated at 75°C for 10 minutes.

2.2.8 DNA Ligation

Insert DNA was ligated into plasmid backbone in approximately 3:1 molar ratio using 1U of T4 DNA ligase and 1x T4 DNA ligase buffer (supplied by manufacturer) in a final reaction volume of 20µl. Reactions were incubated overnight at either 4°C or 16°C for sticky ends or blunt ends respectively and were then transformed into chemically competent Stbl3 *E. coli*.

2.2.9 Agarose gel electrophoresis and purification of DNA fragments

DNA fragments were resolved on a 1% agarose gel in 1x TAE buffer. Agarose was dissolved in TAE by heating and ethidium bromide was added to a final concentration of 0.5µg/ml before being allowed to set. Samples were mixed with DNA loading buffer before being loaded onto the gel alongside 1kb Plus DNA ladder. Fragments were separated by electrophoresis at 100-150V in 1x TAE and visualised under UV light using a UviDoc gel documentation system. DNA fragments were excised from the gel using a scalpel under UV light and purified using a Qiaquik gel extraction kit.

2.2.10 PCR cloning

100ng DNA was used as a template and mixed on ice with 1x PFU buffer supplied by the manufacturer, 1µM each primer, 250µM each dNTP, 2U Pfu DNA polymerase in 50µl volume. Initial denaturation was performed at 94ºC for 5 minutes, then 35 amplification cycles were carried out- denaturation at 94ºC for 30s, primer annealing at between 53 and 60ºC (depending upon primers used) for 30s, extension at 72ºC for 1 minute/kb, and a final extension step of 7 minutes at 72ºC.

2.2.11 Genomic DNA extraction

20µl of DNA lysis buffer was added to approximately $1x10^5$ cells and incubated at 56ºC for 2 hours, then 95ºC for 5minutes. 180µl H_2O was added and the sample

spun in a bench top centrifuge at 13 000rpm for 3minutes. The supernatant containing DNA was removed and stored at -20ºC.

2.2.12 Identification of integration of PGK-TRIMCyp at the CCR5 locus by PCR

Primers spanning the TRIM21 and Cyp junction (T21Cfw and T21Crv) were used to amplify the TRIM21CypCO transgene in GHOST clones. This reaction would detect the transgene after integration at any locus. 100ng template DNA was mixed with 1xTaq polymerase buffer, 1mM $MgCl_2$, 1µM each primer, 250µM each dNTP, 1.25U Taq polymerase in a total of 50µl. Denaturation was performed at 94ºC for 5 minutes, followed by 35 amplification cycles of denaturation at 94ºC for 1 minute, primer annealing at between at 50ºC for 30s, extension at 72ºC for 1 minute, and a final extension step of 10 minutes at 72ºC. The correct TRIM21Cyp product was 486bp.

PCR was used to amplify the junction between the TRIM21CypCO transgene and the CCR5 gene in GHOST clones. One primer bound within the PGK promoter of the insert (PGKfw), and the other to the endogenous CCR5 sequence (8-CCR5). Therefore, this PCR would only detect inserts at the correct site of integration within the CCR5 gene, producing a PCR product of 1507bp.
100ng template DNA was mixed with 1xTaq polymerase buffer, 3% DMSO, 1mM $MgCl_2$, 1µM each primer, 250µM each dNTP, 1.25U Taq polymerase in a total of 50µl. Samples were amplified using the following touchdown PCR conditions: 95ºC for 5 minutes, 17 cycles of 95ºC for 30s, 68ºC for 30s and 72ºC for 2 minutes 30s, then 25 cycles of 95ºC for 30s, 60ºC for 30s and 72ºC for 2 minutes 30s, then 6 cycles of 95ºC for 30s, 59ºC for 30s and 72ºC for 2 minutes 30s, then 6 cycles of 95ºC for 30s, 58ºC for 30s and 72ºC for 2 minutes 30s then a final extension of 72ºC for 10 minutes.

2.2.13 TOPO cloning

Gel extracted and purified PCR product was ligated into the pCR®4-TOPO® plasmid following manufacturers guidelines, which involved incubation of DNA with salt solution, water and vector for 5 minutes at room temperature. The resultant plasmid was used to transform One Shot TOP10 *E. coli* by heat shock, which were then plated onto ampicillin LB agar plates. DNA was extracted from colonies and the insert sequenced.

2.2.14 T7 endonuclease assay

The CCR5 ZFN target site was amplified by PCR using primers 9-CCR5 and 10-CCR5 and the 498bp product resolved by electrophoresis through an agarose gel. The relevant band was excised and purified by a QiaQuik gel extraction kit. Approximately 100ng DNA was denatured for 10 minutes at 98ºC in a total volume of 11.2µl containing 1x buffer 2 (supplied by manufacturer) and cooled to allow re-annealing. T7 endonuclease I recognises and cleaves mismatched double stranded DNA formed when NHEJ DNA containing mutations re-anneals with the native sequence. Therefore DNA was digested using 5U T7 endonuclease for 30 minutes at 37ºC to cleave mismatched DNA producing two products of approximately 200 and 300bp. DNA was visualised on a 1.5% agarose gel with 0.5µg/ml ethidium bromide under UV light.

2.2.15 Quantitative real time PCR (qPCR)

Approximately 100ng of genomic DNA was used as a template for each reaction. Reactions were performed in triplicate in a total volume of 25µl containing 0.9µM of both forward and reverse primers, 0.2µM fluorescently labeled probe and 1x master mix. WPRE primers and probes were used to detect integrated copies of lentiviral vector, and β-actin primers and probes were used to quantify cell number. For each reaction 1 cycle of 50ºC for 2 minutes, 1 cycle of 95ºC for 10 minutes, 40 cycles of 95°C for 15s and 60°C for 1 minute were performed using an ABI Prism

7000 sequence detection system (Applied Biosystems). Samples were compared to a standard curve generated by serially diluted plasmid stocks.

2.2.16 Total RNA isolation and reverse transcription

0.5ml Tri-reagent and 0.1ml chloroform were added to cell pellets, shaken and centrifuged at 13000rpm for 15 minutes. 0.2ml of the upper aqueous phase was added to 0.2ml isopropanol and incubated at -20ºC for 4 hours. Following this, RNA was pelleted by centrifugation at 13000rpm in a bench top centrifuge for 10 minutes. The supernatant was removed and pellet washed in 1ml 70% ethanol before air drying and resuspension in 10µl H_2O.

Reverse transcription was performed on RNA samples using the Geneamp® RNA PCR core kit. 2.85µl sample, 1x buffer, 5.5mM $MgCl_2$, 250µM each dNTP, 1.25µM random hexamers, 12.5U RT, and 4U RNAse inhibitor were incubated at 25ºC for 10 minutes, 48ºC for 30 minutes and 95ºC for 5 minutes. The resultant cDNA was used as a template for real time reverse transcription PCR (RT-PCR) using primer/probe sets for TRIM5 or TRIM21 normalised to the GAPDH housekeeping gene. For each reaction 1 cycle of 50ºC for 2 minutes, 1 cycle of 95ºC for 10 minutes, 40 cycles of 95°C for 15s and 60°C for 1 minute were performed using an ABI Prism 7000 sequence detection system (Applied Biosystems). The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method, assuming amplification efficiencies of TRIM and GAPDH were similar, using the formula:

$\Delta\Delta Ct = \Delta Ct_{sample} - \Delta Ct_{reference}$

2.2.17 Western blotting

$1x10^6$ cells were lysed by addition of 100µl Laemmli buffer and heating at 100ºC for 10 minutes. Samples were centrifuged in a bench top centrifuge for 3 minutes at 13000rpm. Samples and SeeBlue Plus2 Prestained Standard were separated by electrophoresis through a 4-12% Bis-Tris NuPAGE gel and 1x MES buffer at 200V for 45 minutes. Protein was then transferred onto Immobilon-P PVDF membrane

using an X-Cell II Blot module and 2x NuPAGE transfer buffer, 10% methanol at 18V for 45 minutes. The membrane was blocked in 4% milk powder in PBST (PBS containing 0.05% (v/v) Tween-20) for 1 hour at room temperature, and then primary antibody was added at 1:1000 for anti-HA tag or 1:5000 for anti-Cyclophilin and incubated at room temperature overnight. The membrane was washed three times in PBST before addition of horse-radish peroxidase conjugated secondary antibody diluted 1:2000 in 4% milk in PBST for 1 hour. The membrane was washed three times in PBST and bands visualised using Pierce ECL detection kit and UviChemi chemiluminescence detection system.

2.2.18 p24 ELISA

ELISA was used to measure the levels of HIV-1 p24 in culture supernatant after infection with HIV-1 or for physical titration of lentiviral vector. Samples were diluted appropriately in fresh complete medium. The assay was performed on these samples according to manufacturer's instructions. Briefly, samples were lysed and added to wells coated with anti-p24 antibody in duplicate alongside serial dilutions of p24 antigen standards supplied by the manufacturer. The plate was incubated at 4ºC overnight before washing and incubation with biotin conjugated anti-p24 antibody for 1 hour at 37ºC. After further washing, streptavidin-peroxidase solution was incubated on the plate for 30 minutes at 37ºC, colour developed by the addition of substrate at room temperature and the reaction stopped with stop solution. The absorbance was read at 450nm using a FLUOstar OPTIMA (BMG Labtech, Offenburg, Germany) and the concentration of p24 calculated using the p24 antigen standards.

2.2.19 MTT assay

In vitro cell viability was measured using the *In vitro* toxicology assay kit, based upon (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT, according to manufacturer's instructions. Briefly, MTT was added to cells in a 96

well plate in an amount equal to 10% of the culture medium volume. Cells were incubated at 37ºC for 2-4 hours to allow the formation of formazan crystals, which were then dissolved in MTT solubilisation solution provided with the kit. Absorbance was measured at 595nm using a FLUOstar OPTIMA (BMG Labtech, Offenburg, Germany).

2.2.20 Propagation and storage of mammalian cell lines

Adherent cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing GlutaMAX supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin (referred to as complete DMEM). Cells were passaged when 80-90% confluent by washing in Dulbecco's phosphate buffered saline (PBS) then incubating with trypsin/EDTA until cells became detached. Cells were diluted in fresh complete DMEM and plated at a suitable concentration into tissue culture flasks. GHOST cells were grown in selection media which consisted of complete DMEM supplemented with 500µg/ml G418, 100µg/ml hygromycin and 1µg/ml puromycin.

Non-adherent cell lines were maintained in RPMI 1640 media containing GlutaMAX supplemented with 10% (v/v) FCS and 1% (v/v) penicillin/streptomycin (referred to as complete RPMI). Cells were passaged when the media changed colour by transferring a proportion of the cells to fresh complete RPMI. All cells were incubated at 37°C in a 5% CO_2 atmosphere. Jurkat.CD4-CCR5 (Jurkat-CCR5) cells were grown in selection media which consisted of complete RPMI supplemented with 500µg/ml G418.

For long term storage of cell lines, 1-5x10^6 cells were centrifuged and the pellet was resuspended in FCS containing 10% (v/v) DMSO and frozen slowly overnight in an isopropanol freezing box at -80°C. Cells were then transferred into liquid nitrogen. Cells were rapidly thawed in a 37°C waterbath and slowly added to fresh complete media. Cells were centrifuged to remove DMSO and resuspended in fresh complete medium and transferred to a $T25^2$ or $T75^2$ tissue culture flask.

2.2.21 Isolation of peripheral blood mononuclear cells (PBMCs)

Heparinised blood was obtained from healthy donors and peripheral blood mononuclear cells (PBMCs) were separated from the blood by centrifugation over 20ml Ficoll at 800g, for 25 minutes with no brakes. PBMCs from the buffy coat were collected and washed twice in PBS. Cells were counted and seeded at $1x10^6$ cells per well in a 24 well plate in X Vivo-10 supplemented with 5% human AB serum and 100U/ml IL-2 with anti-CD3/anti-CD28 beads at a 1:1 ratio for activation. Half of the culture media was removed and replaced with fresh X Vivo-10 with supplements to replenish IL-2 levels every two days.

2.2.22 Production of retroviral vector

$2.2x10^6$ HEK293T were plated out in a 10cm dish and grown overnight to reach ~80% confluency. 1.5µg vector plasmid, 1µg MLV gag-pol packaging plasmid and 1µg vesicular stomatitis virus G protein expression plasmid (pMDG2) were mixed in a total volume of 15µl. This DNA was added to 200µl OptiMem containing 10µl FuGene-6, and incubated at room temperature for 15minutes. Cell medium was replaced with 8ml fresh complete DMEM and the DNA mix added. The following day the media was replaced with 8ml fresh complete DMEM. 48 and 72 hours after transfection the media was harvested, filtered at 0.45µm and stored at -80ºC.

2.2.23 Production of lentiviral vector

$1.5x10^7$ HEK293T were plated out in a T175cm^2 flask and grown overnight to reach ~80% confluency. DNA mixture for each flask was made by mixing 50µg vector plasmid, 32.5µg gag-pol packaging plasmid pCMV-dR8.74 (for integrating virus) or pCMV-dR8.74 D64V (for non-integrating virus) and 17.5µg pMDG2 in 5ml OptiMem and 0.22µm filtered to sterilise. 2µM PEI was added to 5ml OptiMem and sterilised by 0.22µm filtration and the two solutions mixed and incubated at room temperature for 20 minutes. Cells were washed in OptiMem before adding the DNA-PEI complex and incubating at 37°C 5% CO_2 for 4 hours, then replacing with

complete DMEM. After 24 hours the media was replaced with fresh complete DMEM. 48 and 72 hours after transfection the media was harvested and filtered at 0.22µm to remove cell debris, before centrifugation at 100 000g for 2 hours in a Sorvall Discovery SE ultracentrifuge. Viral pellets were resuspended in DMEM without supplements and stored at -80°C.

2.2.24 Production of replication competent HIV-1

$1x10^6$ HEK293T cells were plated out in a T25cm^2 flask and incubated overnight. 7.5µl of FuGene-6 was added to 90µl serum free DMEM and incubated at room temperature for 5minutes. 2.5µg HIV-1 plasmid was added and incubated at room temperature for 15minutes before adding directly to the media in the flask of cells. The following day media was replaced with 8ml fresh complete DMEM. 48 and 72 hours after transfection the media was collected, filtered at 0.45µm and stored at -80ºC. Two full length HIV-1 clones were used: R9 and NL4-3 (BaL), which has a BaL envelope. These clones have an X4 and R5 tropism respectively.

The amount of p24 in the harvested supernatant was quantified by p24 ELISA, as described above.

2.2.25 Titration of viral vector

2.2.25.1 GFP expression

HEK293T cells were plated at $1x10^5$ cells per well in 24 well plates the day before transduction. Lentiviral vector was diluted in five-fold serial dilutions and added to the cells in a final volume of 300µl complete DMEM. Cells were harvested at 72 hours after transduction and GFP expression measured by flow cytometry using a BD LSRII. Results were analysed using FlowJo software. The vector titre in infectious units per ml (IU/ml) was calculated from a well in which 1-10% of cells were GFP positive. The number of transduced cells was divided by the volume (in mls) of virus used to transduce the cells in this well.

MLV-YFP was titrated on $1x10^5$ CRFK cells to avoid restriction by human TRIM5α in the human derived HEK293T cells. Two-fold serial dilutions of MLV vector were added in a final volume of 1ml complete DMEM to cells, which were harvested and YFP expression measured by flow cytometry 72 hours later.

2.2.25.2 Quantification of WPRE copy number by qPCR

Cells were transduced as above and harvested for DNA extraction as outlined in methods. qPCR was performed as described. The total number of copies of WPRE in the whole well was divided by the volume of virus used to transduce them.

2.2.25.3 Quantification of physical titre by p24 ELISA

Concentrated lentiviral vector was diluted $1:1x10^6$ in DMEM. P24 levels were measured using a p24 ELISA kit according to manufacturer's guidelines as described above. Typically, there are 10-100 infectious units/pg of p24, which allowed calculation of viral titre.

2.2.26 Flow cytometry

Flow cytometry was performed on a BD LSR II (BD Biosciences) using FACSDiva software. Samples in 96 well plate format were captured on a BD FACSCalibur (BD Biosciences). All data were analysed using Flowjo software. Cells were sorted using a MoFlo XDP (Beckman Coulter).

For separation of GFP and YFP in restriction assays, a 525LP Dichroic mirror and optical filters were used on the BD LSRII. GFP was detected at 510/10 nm and YFP was detected at 550/30 nm

For measuring viability, cells were gated on forward/side scatter. Cell death was measured by a shift on the forward and side scatter axis as cells became smaller and more complex. The shift was confirmed as correlating with cell death by using LIVE/DEAD fixable stain according to manufacturer's guidelines. Briefly, cells were pelleted and stained for 30 minutes at room temperature in the dark using stain diluted in PBS. Cells were washed in PBS before fixing in 4% paraformaldehyde.

Viable cells negative for the LIVE/DEAD stain were backgated on forward/side scatter to confirm that they were included in the original viability gate.

For extracellular staining of CD4 and CCR5, cells were pelleted by centrifugation and incubated with antibody diluted in PBS for 20 minutes at room temperature in the dark. Cells were washed in PBS before resuspending for analysis by flow cytometry.

2.2.27 Viral transduction

For retroviral transduction with EXN, CRFK cells were plated at $1x10^5$ cells per well in a 6 well plate and 24 hours later media was replaced with 1ml virus and 5mg/ml polybrene (PB). The plate was centrifuged at 500rpm for 1 hour and then incubated for 6 hours before adding G418 for antibiotic selection. Cells were cultured for approximately 7-10 days for G418 selection of transduced cells.

Generally for lentiviral transduction, $1x10^5$ cells were seeded in a 24 well plate and incubated overnight. Lentiviral vector was added to the cells in a volume of 1ml media. Cells were cultured for at least 72 hours to allow gene expression before using for any further analysis or assays.

PBMCs were obtained from healthy donors by centrifugation through Ficoll and plated at $1x10^6$ per well in a 24 well plate (see section 2.2.21). After 48 hours activation, lentiviral vector was added, typically at an MOI of 30-50. Half of the culture medium was removed and replaced with fresh medium and 200U/ml IL-2 every two days throughout culture.

2.2.28 Retroviral restriction assays

Transduced cells were plated at $1x10^5$ cells per well in a 24 well plate and transduced with HIV-1 vector carrying either eGFP or YFP marker genes in a total volume of 1ml. The MOI used varied between experiments, but was typically between 1 and 10. HIV-1 reporter gene expression was measured in the cells by flow cytometry 72 hours post-transduction.

For measurement of endogenous TRIM5α restriction of retrovirus, TE671 cells were transduced with LNT/S-TRIMCyp-IEW. Cells were plated at $1x10^5$ per well in the presence or absence of 1000U IFNβ and the following day transduced with B- or N-MLV-YFP (MOI=1000), or HIV-1-YFP (MOI=5). Flow cytometry was used to quantify the percentage of the eGFP positive population that co-expressed YFP.
For restriction assays using full length HIV-1, cells were plated and incubated over night before infection with HIV-1. The day following infection, cells were washed with PBS to remove residual virus before replacement with fresh complete media.

2.2.29 Adenoviral restriction assay

TRIM21 restriction of adenovirus type 5 was measured following the protocol of Mallery et al (266). HeLa cells were transduced with LNT/SFFV-TRIMCyp-WPRE vectors without eGFP and seeded at $1x10^5$ cells per well with or without 1000U IFNα. Caesium chloride concentrated adenovirus expressing GFP (AdV-GFP) (a kind gift from Persis Amrolia) was incubated with increasing concentration of antibody for 30 minutes at room temperature, before infecting HeLa cells. GFP expression was measured after 48 hours. A polyclonal goat anti-hexon antibody (Millipore) was used in a total volume of 10µl in a concentration range of 200-1600ng/ml.
This neutralisation assay protocol was optimised by Choon Ping Tan, who also provided unconcentrated AdV-GFP preparations and an alternative antibody. The antibody 9C12 was used and incubated with AdV in a total volume of 500µl at a concentration range of 3.2-2000ng/ml. GFP expression was measured 48 hours following infection.

2.2.30 Statistics

Where stated, P values were obtained by performing two tailed, unpaired Student's T test.

3 Human TRIMCyp proteins restrict HIV-1

3.1 Aims

- To generate humanised TRIM5- and TRIM21-Cyclophilin A fusion constructs in retroviral and lentiviral vectors
- To produce high titre lentiviral vector stocks
- To test the restriction of HIV-1 derived vectors by TRIMCyp proteins in cell lines and primary T cells

3.2 Introduction

Gene therapy has the potential to provide a functional cure of HIV-1 infection after a single dose of treatment and eliminate the need for HAART. There are a wide variety of potential antiviral genes that could be introduced into cells to provide them with a resistance to HIV-1, and in addition, the target cell and delivery methods could also influence the effectiveness of any potential treatment. For a transgene to be successful and provide long term treatment of infection, it must promote robust restriction of HIV-1 whilst preventing mutagenic escape of the virus. One possibility is to use a naturally occurring restriction factor which is shown to restrict HIV-1, such as owl monkey TRIM5Cyp (314, 315). This fusion protein is formed from LINE-1-mediated retrotransposition of CypA cDNA in between exons 7 and 8 of the TRIM5 gene. This results in replacement of the B30.2 domain, which binds viral CA and determines the TRIM5α protein's restriction specificity, with CypA. As owl monkey CypA binds HIV-1, this moiety recruits the antiviral function of TRIM5 to HIV-1, providing potent restriction of the virus.

Retrotransposition occurs frequently, but does not often result in a functional protein, yet remarkably this event has occurred a second time in Old World monkeys (317-319, 375, 376). In this instance, CypA cDNA is found in the non-coding region of exon 8. Independently of the retrotransposition, a single nucleotide polymorphism has resulted in the inactivation of the splice acceptor in exon 7. This results in skipping of exons 7 and 8 and splicing of TRIM5 exon 6 to the CypA splice acceptor. Therefore owl monkey TRIM5Cyp encodes exons 2-7 of TRIM5 and in Old World monkeys exons 2-6 are fused to the Cyp domain. These different CypA insertion sites indicate that these proteins have evolved through entirely independent retrotransposition events. The restriction specificity of the two proteins also differs, with owl monkey TRIM5Cyp targeting HIV-1 and FIV, mirroring the binding capacity of the native Cyp protein. The Cyp domain in macaque TRIM5Cyp has acquired a point mutation, H69R, which alters the Cyp binding specificity from the genomic Cyp protein. Therefore, macaque TRIM5Cyp is able to restrict HIV-2, FIV and group O HIV-1 (320, 376).

Owl monkey TRIM5Cyp provides strong protection against HIV-1, but despite the high level of homology between human and owl monkey TRIM5 and CypA, there is a risk this protein would induce an immune response if expressed in humans in a gene therapy setting due to the variation between the human and primate sequences. However, production of a humanised TRIM5Cyp restriction factor mimicking the structure of the owl monkey protein may avoid this problem. A humanised TRIM5Cyp molecule was developed by Neagu et al independently of our efforts. The group has reported highly effective restriction of HIV-1 *in vitro* and in a humanised mouse model of HIV-1 infection (322).

The owl monkey TRIM5Cyp protein consists of a 299 amino acid fragment of TRIM5 encoding the RBCC domains, followed by an 11 amino acid linker derived from the 5' untranslated region of CypA, then amino acids 1-147 of CypA. The resultant protein has a weight of 54kDa (314, 315). Due to a lack of sequence homology between human and owl monkey, the 5'UTR cannot be used to generate a human fusion. Therefore Neagu et al generated several different TRIM5Cyp proteins

by fusion of CypA directly to TRIM5 at different amino acids along its length, varying from directly at the C terminal of the coiled coil domain, to over 100 amino acids within the B30.2 domain. These proteins were initially screened for restriction of an HIV-1 vector encoding GFP. There was significant variability in the restriction by different constructs, suggesting that the linker region between the TRIM5 and CypA fragments influences whether the protein is capable of restriction.

In addition to these human fusion proteins, a TRIMCyp protein using feline TRIM5 and CypA has been generated. Feline CypA is able to bind FIV and HIV-1 CA and accordingly this TRIMCyp can restrict these lentiviruses (323).

Using a similar strategy, the mouse restriction factor Fv1 has been fused to CypA from owl monkey TRIM5Cyp, to form a potent inhibitor of HIV-1 and FIV (325). Fusion of CypA to Fv1 alters the antiviral specificity of Fv1 to be determined by the CA binding specificity of CypA. However the restriction mechanism of Fv1 is maintained, in which restriction occurs later in infection than that by TRIM5 or TRIM5Cyp as shown by the presence of reverse transcripts. This demonstrates that Cyp is able to recruit these restriction factors to the CA, resulting in viral restriction.

Here, novel versions of human TRIM-Cyclophilin fusion molecules have been designed and tested for efficacy against HIV-1. The RBCC motifs of TRIM5 and its close phylogenetic relative, TRIM21, which shares the same domain structure, have been fused to human CypA. These constructs have been cloned into retroviral and lentiviral vectors to test their restriction of an HIV-1 vector to demonstrate their potential for anti-HIV-1 gene therapy.

Initial restriction assays have been performed in cell lines, but the ultimate target cell for gene therapy would be T cells or HSCs. Restriction was measured using a single round of transduction with a second generation SIN HIV-1 vector. This vector was generated in HEK293T cells using the same plasmids for the generation of gene therapy vectors. In these virus particles, CA is derived from a GagPol expression plasmid encoding wild type HIV-1 CA sequence, which includes the CypA binding site. In experiments described here, the HIV-1 vector carries a fluorescent marker gene allowing rapid quantification of infection by flow cytometry. Experimentally

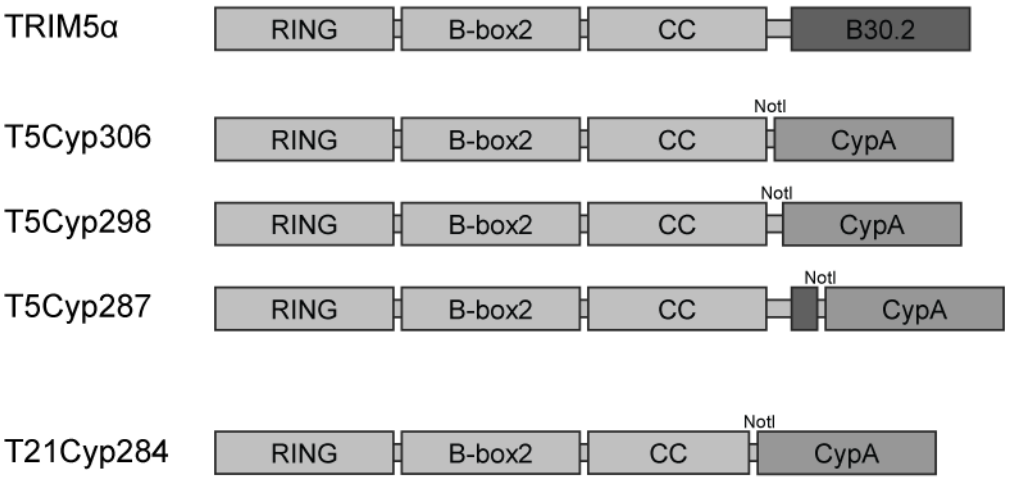
this is a convenient method to test restriction as work does not need to be performed at containment level 3 as for replication competent HIV-1.

3.3 Cloning TRIM5Cyp constructs into a gamma retroviral vector

Three novel TRIM5 and CypA fusion constructs were designed that consisted of TRIM5 sequence, a NotI restriction site that translated to three alanine residues (AAA) and then the Cyp cDNA sequence at the C terminal (Figure 3.1A). The TRIM5 fragments were generated by PCR amplification of the TRIM5 RBCC motif and ligation into pEXN containing the human CypA cDNA sequence between NotI and SalI (a gift from Dr. Torsten Schaller). The resulting plasmid expressed HA-tagged human TRIM5CypA under the control of the CMV promoter and was named pEXN/TRIM5Cyp. The constructs differed in the length of the linker region between the 3' end of the TRIM5 RBCC motif and the start codon at the 5' end of CypA. All constructs included the entire RBCC motif, with the longest protein including the first 8 amino acids of the B30.2 domain in the TRIM5 fragment 5' of the NotI site. The number of the final amino acid of the TRIM5 RBCC sequence 3' of the NotI restriction site and CypA sequence is denoted in the name of each construct i.e. 306, 298 and 287 (Figure 3.1B).

pEXN/T5Cyp was used to generate retroviral vector by transient transfection of HEK293T cells, in combination with an MLV gag-pol packaging plasmid and the VSV-G envelope plasmid, pMDG (Figure 3.2). Control vector was derived from the pEXN parent plasmid.

A



B

MASGILVNVKEEVTCPICLELLTQPLSLDCGHSFCQACLTANHKKSMLDKGESSCPVCRISY
RING

QPENIRPNRHVANIVEKLREVKLSPEGQKVDHCARHGEKLLLFCQEDGKVICWLCERSQEHR
B Box2

GHHTFLTEEVAREYQVKLQAALEMLRQKQQEAEELEADIREEKASWKTQIQYDKTNVLADFE
Coiled coil

QLRDILDWEESNELQNLEKEEEDILKSLTNSETEMVQQTQSLRELISDLEHRLQGSVMELLQ

287 298 306

GVDGVIKRTENVTLKKPETFPKNQRRVFRAPDLKGMLEVFRELTDVRRYWVDVTVAPNNISC

AVISEDKRQVSSPKPQIIYGARGTRYQTFVNFNYCTGILGSQSITSGKHYWEVDVSKKTAWI
B30.2

LGVCAGFQPDAMCNIEKNENYQPKYGYWVIGLEEGVKCSAFQDSSFHTPSVPFIVPLSVIIC

PDRVGVFLDYEACTVSFFNITNHGFLIYKFSHCSFSQPVFPYLNPRKCGVPMTLCSPSS

Figure 3.1 Design of TRIMCyp fusion constructs

A. Schematics of native TRIM5α and TRIMCyp fusion constructs. The figure shows RING, B-box2, coiled coil (CC) and B30.2 domains. Three TRIM5Cyp constructs were generated, which differed in the length of the linker region between the end of the RBCC and beginning of the CypA cDNA. The RBCC motif of TRIM21, which has a similar domain structure to TRIM5α, was also fused to CypA to create TRIM21Cyp.

B. Amino acid sequence of native TRIM5α. RING, B-box2, coiled coil and B30.2 domains are labelled. The horizontal red lines and numbers above indicate the TRIM5 amino acid at the C terminal of the TRIM5 RBCC fragment to which CypA is fused. This number is used in the naming of each construct.

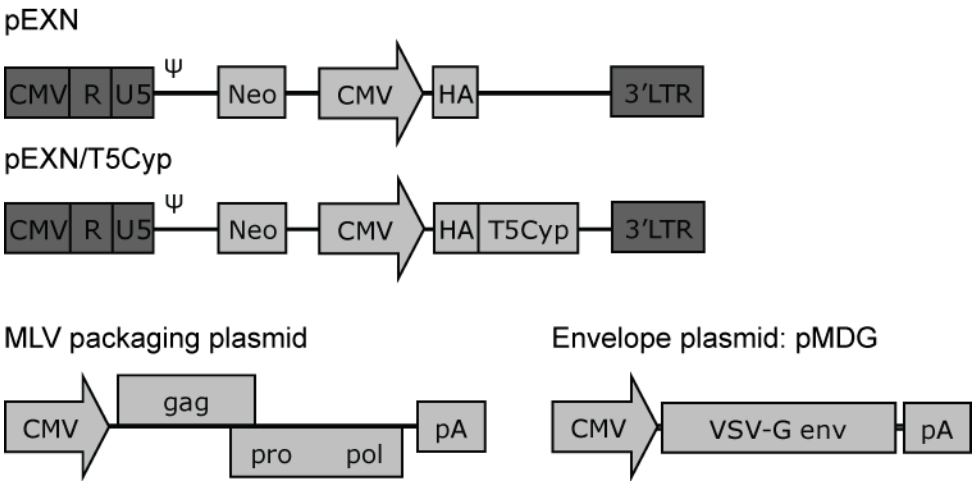


Figure 3.2 Plasmids for generation of retrovirus
Three TRIM5Cyp constructs were cloned into a murine leukaemia virus (MLV) retroviral vector, pEXN, allowing expression from the Cytomegalovirus (CMV) promoter and addition of a haemagglutinin (HA) tag at the N terminal. pEXN or pEXN/TCyp, MLV packaging plasmid and pMDG VSV-G env plasmid were used to transiently transfect HEK293T for generation of retroviral particles. LTR=long terminal repeat, Neo=neomycin resistance gene, pA=polyA

3.4 Expression of TRIM5Cyp protein in cell lines

CRFK cells were transduced with EXN/T5Cyp retrovirus carrying one of the three different TRIM5Cyp constructs, or EXN virus for control, and cultured in G418 selection media. CRFK cells were used because feline cells do not express TRIM5α, leaving them susceptible to retroviral infection. They only express the RBCC domains as there is a premature stop codon in exon 8, from which the B30.2 domain is normally expressed (377).

Western blotting was used to confirm the expression of the HA tagged TRIMCyp fusion protein from EXN using an anti-HA tag antibody (Figure 3.3). There were some non-specific bands present in all cell populations, but a highly specific band of the correct size present in TRIM5Cyp transduced populations, but not the controls. The molecular weights of the three different constructs are shown: T5Cyp306-55 kilodaltons (kDa), T5Cyp298-54kDa and T5Cyp287-52kDa.

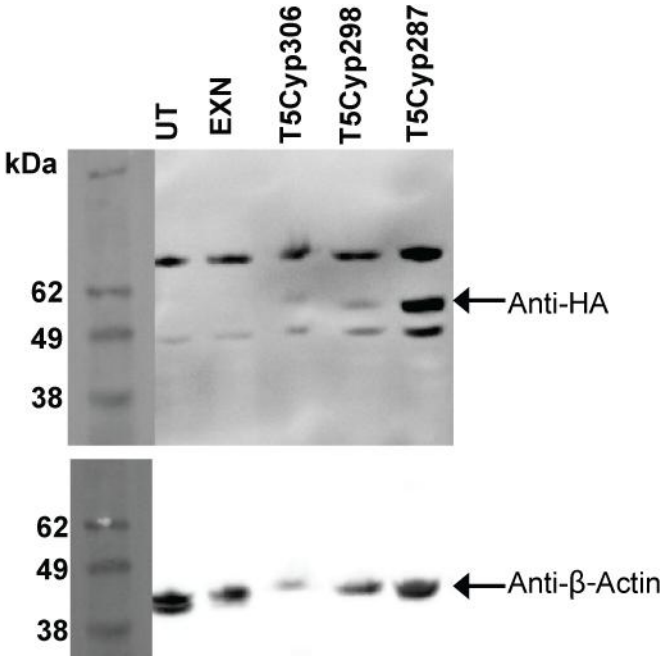


Figure 3.3 Expression of TRIM5Cyp from retroviral vector
Western blotting using anti- haemagglutinin (HA) tag antibody on transduced CRFK cells selected in puromycin. Untransduced (UT) or empty EXN vector transduced cells were used as controls. β-actin was used as a loading control.

3.5 CRFK cells transduced with TRIM5Cyp retroviral vectors are able to inhibit HIV-1 vectors

CRFK cells transduced with EXN/TRIM5Cyp virus carrying one of the three different TRIM5Cyp constructs, or EXN virus as a control, were selected in G418 media to produce a stably transduced polyclonal population of cells. In addition, limiting dilution was used to obtain individual clones for each of the three TRIM5Cyp constructs.

To test lentiviral restriction, the cells were challenged with an eGFP-expressing HIV-1 vector (HIV-1-GFP) in the presence or absence of CsA, a competitive inhibitor of the interaction between CypA and HIV CA. After 48 hours, cells were analysed by flow cytometry to measure the number of eGFP positive cells. Restriction in the polyclonal bulk population (Figure 3.4A) and three clones for each of the three TRIM5Cyp constructs (Figure 3.4B) was measured by flow cytometry.

All three TRIM5Cyp constructs were able to restrict HIV-1 entry into cells compared to the untransduced and the EXN transduced control cells, as shown by the reduction in eGFP positive cells. In particular, the inhibitory effect in the TRIM5Cyp306 and TRIM5Cyp298 bulk populations was most prominent, being able to reduce HIV-1 transduction approximately 10 fold. Addition of CsA at the point of HIV transduction prevented restriction by TRIM5Cyp in all instances. Minor variations between clonal populations were detected with approximately 10 fold decrease in HIV-1 transduction in almost all clones.

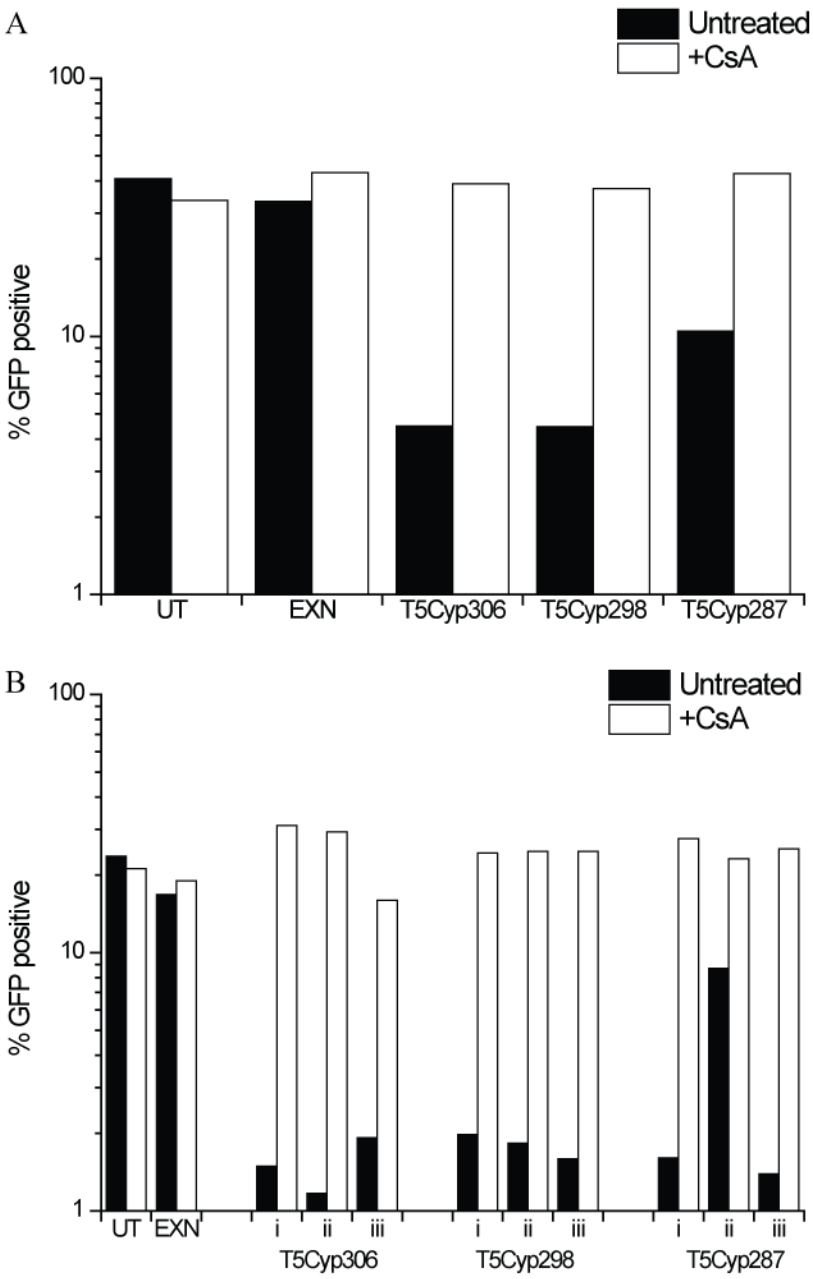


Figure 3.4 Restriction of HIV-1 by TRIM5Cyp retroviral vectors

A. CRFK cells transduced with the three different TRIM5Cyp vectors were transduced with an HIV-1 lentiviral vector expressing GFP in the presence or absence of 5μM Cyclosporin A (CsA). Untransduced (UT) cells or EXN transduced cells were used as controls. The percentage of GFP positive cells was measured by flow cytometry 48 hours post transduction. Results shown are representative of two independent experiments.

B. Bulk transduced populations of CRFK cells were plated out by limiting dilution to obtain clones. These cells were then transduced with an HIV-1-GFP lentiviral vector in the presence or absence of 5μM CsA. Untransduced cells and EXN transduced cells were used as controls. The percentage of GFP positive cells was measured by flow cytometry 48 hours following transduction. Results shown are representative of two independent experiments.

3.6 Cloning TRIMCyp constructs into a lentiviral vector

For use in gene therapy, lentiviral vectors may be advantageous to previously described gamma retroviral vectors because of their safer integration profile and their ability to transduce non-dividing cells. Therefore, the TRIM5Cyp constructs described above were cloned into an HIV-1 derived lentiviral vector backbone, pLNT/SIEW (373). This lentiviral backbone, abbreviated to SIEW, is a second generation HIV-1 derived vector, with a self-inactivating (SIN) 3' LTR and cPPT. It includes the spleen focus forming virus U3 promoter (SFFV), an internal ribosomal entry site (IRES) and a Woodchuck post transcriptional regulatory element (WPRE). All three TRIM5Cyp constructs were amplified from pEXN by PCR and ligated into pLNT/SIEW at the unique BamHI site to produce the plasmid pLNT/S-TRIM5Cyp-IEW (Figure 3.5). pLNT/SIEW expressed just eGFP and served as a vector backbone control.

Another TRIMCyp construct using the first 284 amino acids encoding the RBCC domains from TRIM21 (as discussed further in chapter 5) fused to CypA was also tested (Figure 3.1A). Similar to the TRIM5Cyp constructs, TRIM21Cyp was synthesised by PCR amplification of the TRIM21 RBCC domains and ligated to CypA at the 3' end via a NotI restriction site. The entire construct, TRIM21Cyp284, was provided in the lentiviral plasmid pSFXUC (a gift from Dr. Torsten Schaller). TRIM21Cyp284 was removed from pSFXUC by digestion with BamHI before ligation into pLNT/-SIEW. The resulting plasmid was named pLNT/S-TRIM21Cyp-IEW (Figure 3.5).

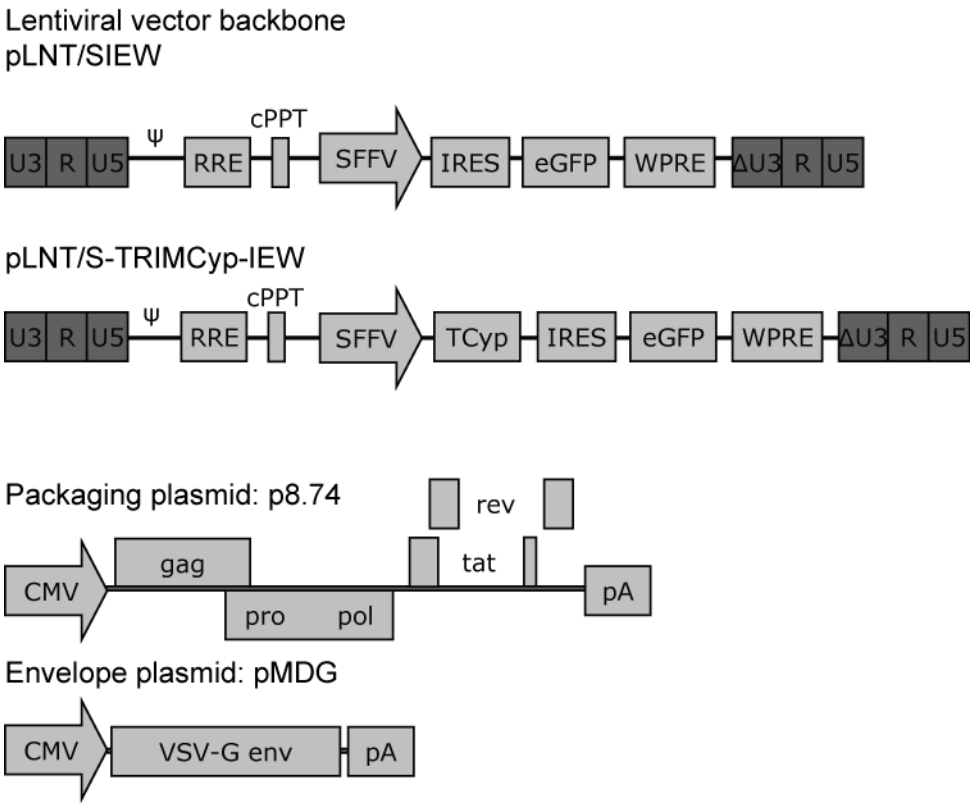


Figure 3.5 Plasmids for generation of lentivirus
A. TRIM5Cyp and TRIM21Cyp constructs were cloned into a self-inactivating lentiviral vector backbone, pLNT/SIEW, under control of the SFFV promoter and linked to eGFP expression with an IRES. Lentiviral vector particles were produced by transient transfection of HEK293T cells with this vector plasmid, the second generation lentiviral packaging plasmid p8.74 and pMDG. Ψ=packaging signal, RRE=Rev responsive element, cPPT=central polypurine tract, WPRE= Woodchuck hepatitis virus post transcriptional regulatory element, CMV=cytomegalovirus, pA=polyadenylation.

3.7 Expression of TRIMCyp protein in transduced cell lines

TRIMCyp expression in CRFK cells transduced with LNT/S-TRIM5Cyp306-IEW and LNT/TRIM21Cyp284-IEW was detected using an anti-Cyp antibody. Blots were re-probed using an anti-β-actin antibody as a protein loading control (Figure 3.6).

Without the HA tag, the TRIMCyp proteins are approximately 1kD smaller than those expressed from the EXN vector, giving the following final molecular weights: T5Cyp306-54kDa, T21Cyp284-51kDa.

Protein could be detected in TRIMCyp transduced populations, but not in the LNT/SIEW (GFP) transduced control. When using the anti-Cyp antibody, endogenous Cyp protein could also be detected. Although the antibody was raised against the human protein, human and feline CypA have over 95% amino acid sequence homology and both have a molecular weight of 18kDa. This band serves to act as an additional loading control.

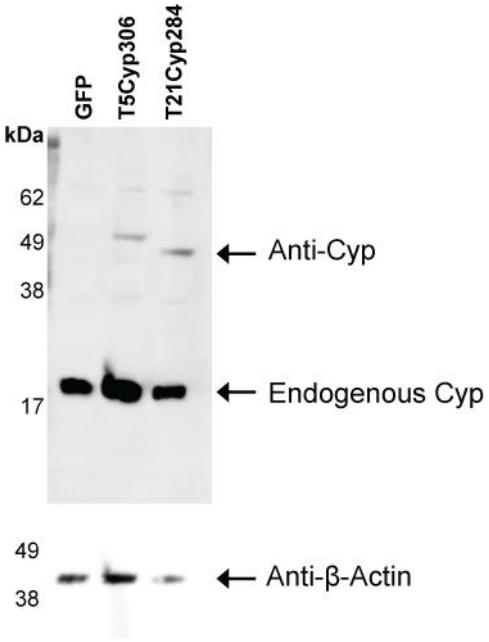


Figure 3.6 Expression of TRIMCyp from lentiviral vectors
Western blotting using anti-Cyp antibody was used to confirm TRIMCyp expression in transduced CRFK cells. SIEW (GFP) transduced cells were used as controls. Anti-β-actin and endogenous Cyp were used as loading controls.

3.8 Expression of TRIMCyp does not restrict lentiviral packaging during vector production

Lentiviral vector stocks were produced by transient transfection of HEK293T cells with the lentiviral vector plasmid, second generation lentiviral packaging plasmid p8.74 and VSV-G envelope plasmid, pMDG (Figure 3.5). The parental plasmid, pLNT/SIEW, was used to produce GFP virus for use as a control. Virus particles were harvested at 48 and 72 hours after transfection and concentrated by ultracentrifugation. The titre of each virus batch was quantified by transduction of HEK293T cells with serial dilutions of virus stock and enumeration of eGFP positive cells by flow cytometry 72 hours after transduction (Figure 3.7A). The number of infectious units per ml (IU/ml) was calculated. Values were only taken from samples where the percentage of transduced eGFP positive cells ranged between 1-10% to reduce the likelihood of multiple copies of integrated vector per cell.

Producing an HIV-1 derived lentiviral vector expressing an anti-HIV-1 restriction factor could potentially cause a decrease in titre through restriction during packaging, so initial vector stocks were generated in the presence or absence of CsA. CsA reversibly inhibits CypA binding of HIV-1 CA and could therefore prevent interaction of TRIMCyp with Gag in packaging cells. CsA was added to the medium after transfection and virus production was performed as previously. Vector was titrated on HEK293T by measuring eGFP expression as described above (Figure 3.7B).

There was no difference in titre for the different vectors either in the presence or absence of CsA, with all stocks generating titres of above $1x10^8$ IU/ml. This is within the expected range of titre using this protocol to produce lentiviral vector concentrated by ultracentrifugation, and other subsequent vector preparations have reached this titre.

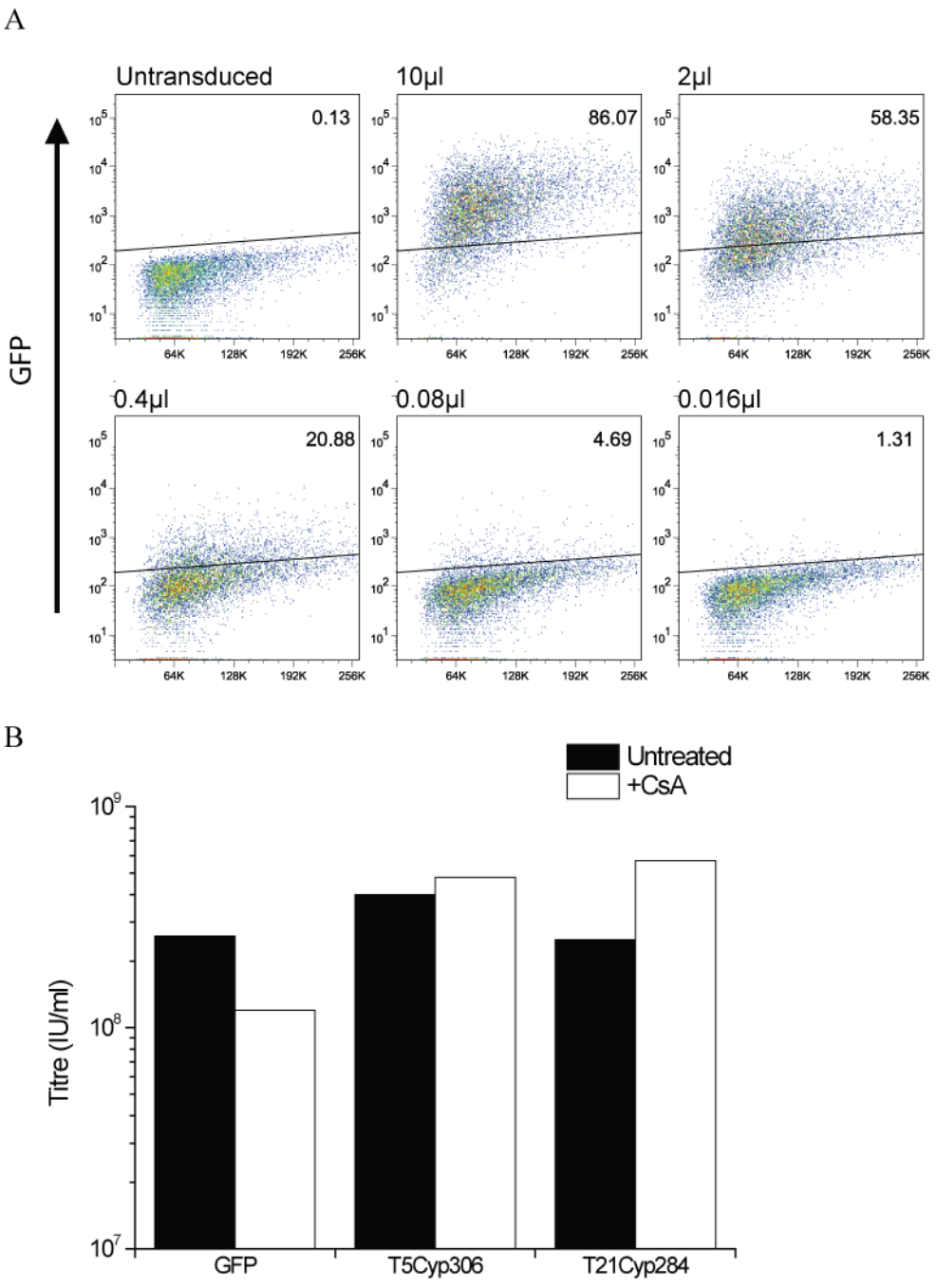


Figure 3.7 Titration of lentiviral vector

A. 1x10^5 HEK293T cells were transduced with serial dilutions of lentiviral vector stocks in a total volume of 300µl. eGFP positive cells were measured by flow cytometry 72 hours post transduction. The percentage of positive cells is displayed in the gate and was used to calculate titre in infectious units/millilitre (IU/ml).

B. Lentiviral vector stocks were produced in the presence or absence of 5µM Cyclosporin A (CsA) to inhibit potential interactions between TRIMCyp and HIV CA. The titre of viral stocks was measured by flow cytometry and compared between the two treatments.

3.9 TRIMCyp restricts HIV-1 vector in cell lines

Restriction of HIV-1 by TRIMCyp in CRFK cells

Following production of lentiviral vector carrying the TRIM5Cyp and TRIM21Cyp constructs and confirmation of protein expression by Western blotting, it was necessary to test their function as a restriction factor. CRFK cells were stably transduced with one of the three TRIM5Cyp or the TRIM21Cyp constructs in the SIEW lentiviral vector at an MOI of 3. Untransduced and SIEW (GFP) transduced cells were used as controls. The cells were cultured for at least 72 hours to allow protein expression of eGFP, which could be visualised by microscopy, before challenge with an HIV-1 vector expressing YFP at an MOI of 3 in the presence or absence of CsA. Cells were analysed by flow cytometry to measure eGFP and YFP expression following a further 72 hours (Figure 3.8).

The transduction efficiency with the SIEW vectors was high, with between 70 and 90% of cells expressing eGFP. Approximately 40% of untransduced or SIEW transduced control cells became infected by HIV-1 when challenged. In TRIMCyp-eGFP positive cells, there was a dramatic reduction in HIV-1-YFP transduction to less than 1% of cells as YFP positive.

CsA acts as a reversible inhibitor of CypA binding to HIV-1 CA, by interacting with the CypA active site. Treatment of cells with CsA at the time of HIV-1-YFP transduction abrogated TRIMCyp inhibition allowing HIV-1-YFP transduction levels comparable to that in the control cells.

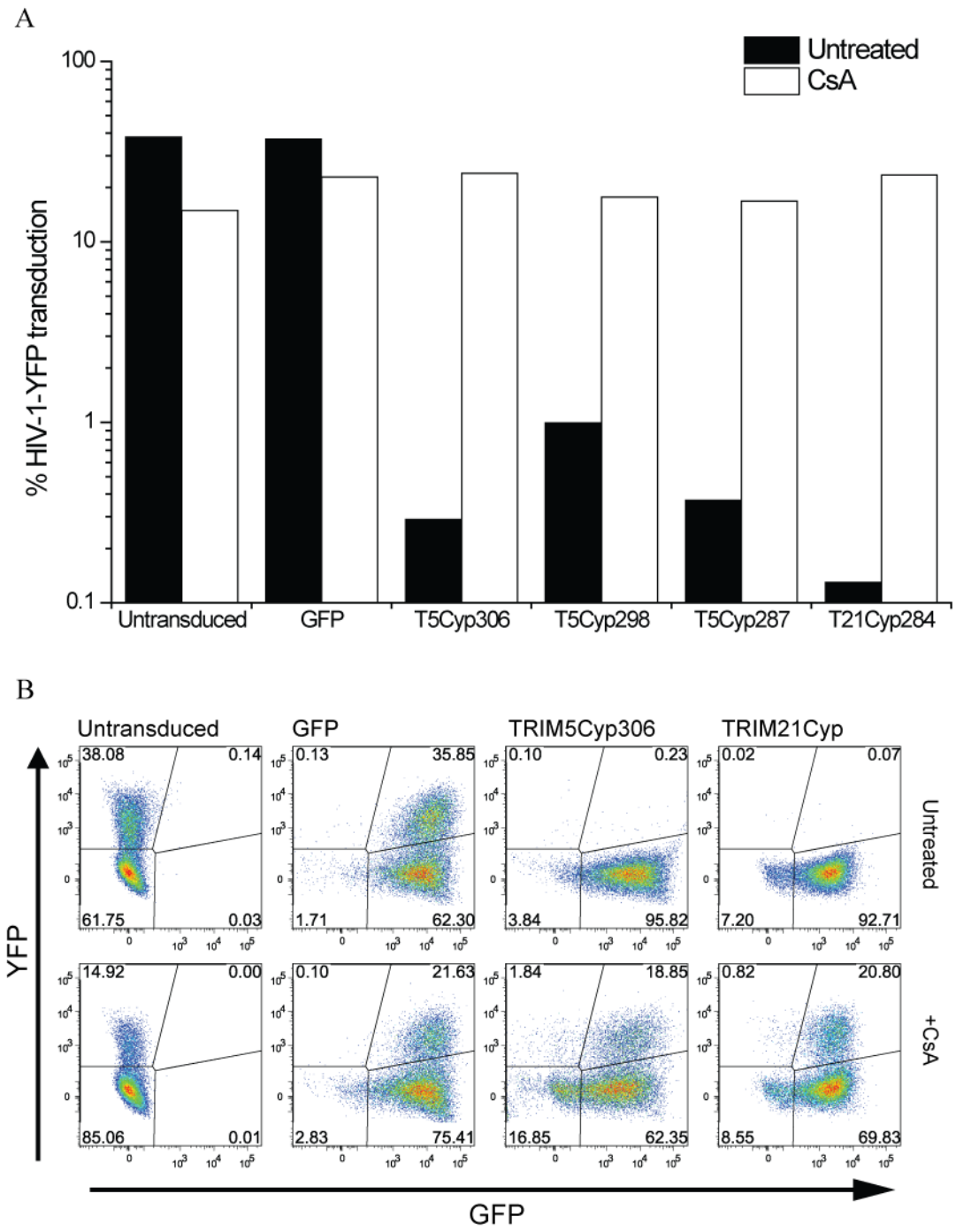


Figure 3.8 Restriction of HIV-1 by TRIMCyp proteins in CRFK cells

A. CRFK cells transduced with three different S-TRIM5Cyp-IEW or S-TRIM21Cyp-IEW lentiviral vectors were challenged with HIV-1-YFP vector in the presence or absence of 5μM Cyclosporin A (CsA). Untransduced and SIEW (GFP) transduced cells were used as controls. eGFP and YFP expression was measured by flow cytometry 72 hours after HIV transduction. After gating on eGFP positive cells, the percentage of YFP positive cells was measured.

B. Examples of flow cytometry plots of eGFP and YFP expression for the most potent inhibitors, TRIM5Cyp306 and TRIM21Cyp, are shown. Results shown are representative of two independent experiments.

Restriction of HIV-1 by codon optimised TRIM21Cyp

An additional variant based on TRIM21Cyp was generated using a codon optimised TRIM21Cyp gene (TRIM21CypCO). Codon optimisation of transgenes maintains the amino acid sequence of the original protein, but adjusts the cDNA sequence to improve mRNA stability and optimise translation for improved protein expression. Adjustments include alteration of codon usage for expression in human cells, removal of repeat sequences, RNA secondary structures, cryptic splice sites, intragenic polyA signals and internal ribosomal entry sites and optimisation of GC content. Codon optimisation was undertaken by Geneart using a proprietary algorithm. Direct comparison between the original vector, pLNT/S-T21Cyp-IEW, and the codon optimised variant pLNT/S-T21CypCO-IEW was undertaken.

Lentivirus was produced and the titre quantified by transduction of HEK293T cells and flow cytometry to measure eGFP expression. Both plasmids produced comparable titres of approximately $1x10^8$IU/ml (Figure 3.9A). Interestingly, the mean fluorescence intensity (MFI) of the eGFP expression from the vector carrying the codon optimised transgene was around 3 fold higher ($1.6x10^4$) compared to the original vector ($4.8x10^3$). Although all vector sequences, including the eGFP gene sequence, were identical in both plasmids, it is possible that codon optimisation of the TRIM21Cyp gene increased stability of the RNA transcripts and consequently allowed increased expression of eGFP.

CRFK cells were transduced with vector encoding either the original or the codon optimised TRIM21Cyp, at an MOI of 3 and cultured for three days. 85-95% of cells were transduced as shown by GFP expression measured by flow cytometry. These cells were analysed by Western blot and shown to express TRIM21Cyp protein by using an anti-CypA antibody (Figure 3.9B).

These cells were then transduced with HIV-1-YFP to measure viral restriction. Again, untransduced and GFP transduced cells were used as controls. eGFP and YFP expression were measured 72 hours after HIV-1-YFP transduction by flow cytometry (Figure 3.9C). Restriction by the codon optimised TRIM21Cyp was comparable to the original protein and suggests that simply increasing protein expression may not increase restriction.

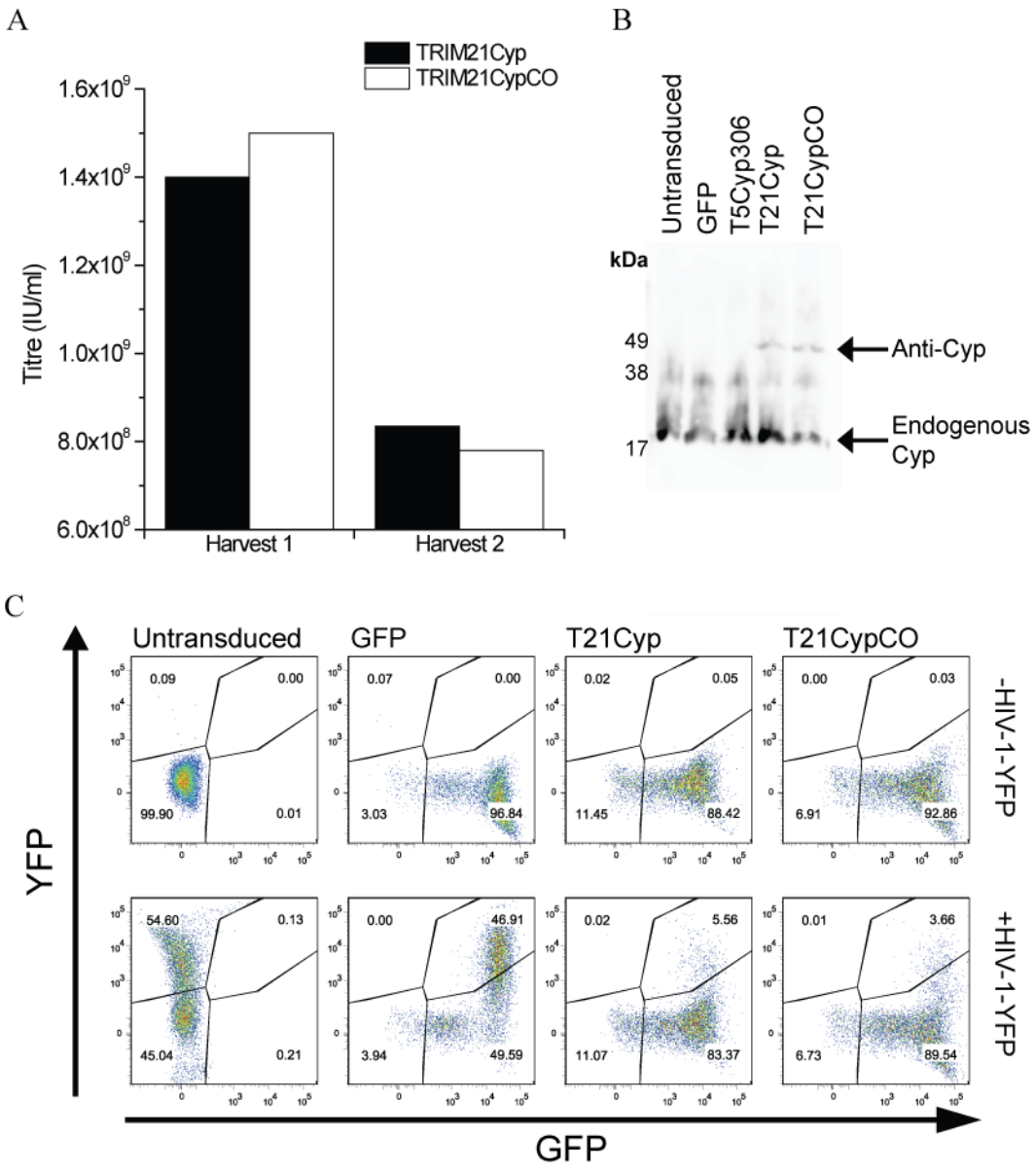


Figure 3.9 Analysis of the codon optimised TRIM21Cyp construct

The codon optimised TRIM21Cyp construct (TRIM21CypCO) was cloned into pLNT/SIEW for comparison with the original TRIM21Cyp transgene in terms of A) vector titre, B) protein expression and C) HIV-1-YFP restriction.

A. S-T21Cyp-IEW or S-T21CypCO-IEW virus was titrated on $1x10^5$ HEK293T cells by serial dilution and quantification of GFP expression by flow cytometry 72 hours post transduction. Titre was calculated in infectious units/millilitre (IU/ml).

B. Expression was measured by Western blotting of lysates from CRFK cells transduced with either of the TRIM21Cyp vectors, with untransduced or SIEW (GFP) transduced cells as control. Endogenous Cyp levels were used as a loading control.

C. CRFK cells transduced with S-T21Cyp-IEW or S-T21CypCO-IEW were challenged by transduction with HIV-1-YFP at an MOI of 10. GFP and YFP co-expression was quantified by flow cytometry 72 hours post transduction.

Saturation of TRIMCyp restriction of HIV-1

It is possible to saturate the restriction effect mediated by TRIM5α (183). To investigate the levels of restriction that TRIM5Cyp and TRIM21Cyp are able to provide against HIV-1, TRIMCyp expressing CRFK cells were subjected to increasing MOI of HIV-1-YFP, from 0.1 up to 1000. Levels of restriction were measured by flow cytometry for YFP expression at 72 hours after HIV-1-YFP transduction (Figure 3.10). At the lowest MOI of 0.1, HIV-1-YFP transduction was around background level in all populations. HIV-1-YFP transduction in untransduced and GFP control cells rapidly increased with increasing MOI, whereas the percentage of YFP positive cells in TRIMCyp expressing populations remained under 1% until an MOI of 10 was used. Control cells were fully transduced using an MOI of approximately 10, but an MOI of 1000 was required to saturate TRIMCyp restriction.

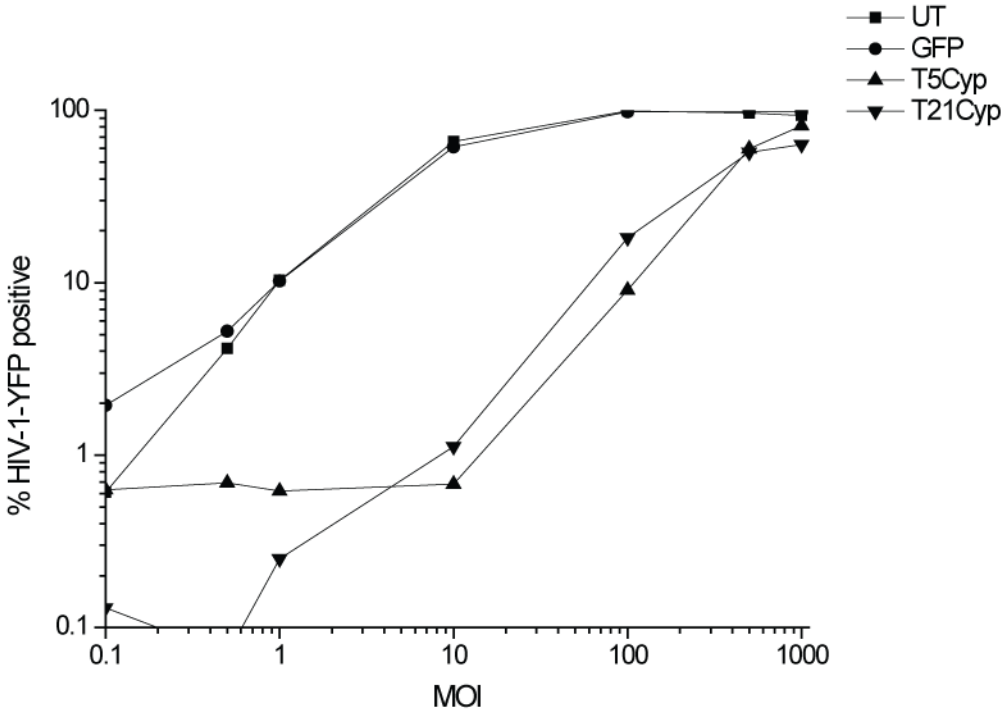


Figure 3.10 Saturation of TRIMCyp restriction of HIV-1 at high MOI
CRFK cells transduced with S-TRIM5Cyp-IEW or S-TRIM21Cyp-IEW lentiviral vectors were challenged with increasing multiplicities of infection (MOI) of HIV-1-YFP vector. Untransduced (UT) and SIEW (GFP) transduced cells were used as controls. eGFP and YFP expression was measured by flow cytometry 72 hours after HIV transduction. Gating on the eGFP positive population, the percentage of YFP positive cells was quantified. Data is representative of two separate experiments.

Comparable restriction of HIV-1 by TRIMCyp and published variants

The TRIMCyp constructs described in this thesis were generated independently of the published humanised TRIM5Cyp proteins, which also mimic the owl monkey restriction factor (322). This published TRIM5Cyp construct, which here is referred to as TRIM5Cyp322, was tested in a lentiviral vector, pscALPS (a gift from Jeremy Luban), and has been shown to inhibit HIV-1 at levels comparable to the naturally occurring owl monkey TRIM5Cyp restriction factor.

Although both the published construct and TRIM5Cyp306 are fusion proteins of human TRIM5 and full length CypA, there are differences in their design. Firstly, TRIM5Cyp322 has a longer linker region between the TRIM5 RBCC C terminal and beginning of the CypA domain. The TRIM5 fragment consists of the first 322 amino acids of full length TRIM5α, which includes the complete RBCC domain and the N terminal of the B30.2 domain. CypA, not including the ATG start codon, is then fused to the TRIM5α fragment at amino acid S322. Alternatively, TRIM5Cyp306 has a TRIM5α fragment truncated at amino acid N306, resulting in a linker region between RBCC and CypA that is 16 amino acids shorter than in TRIM5Cyp322. This linker incorporates 8 amino acids of the B30.2 domain N terminal.

Secondly, TRIM5Cyp306 includes a NotI restriction site between the TRIM5 C terminal and CypA N terminal, which was included in the construct during cloning. This restriction site encodes an additional three alanine residues. TRIM5Cyp322 was produced by overlapping PCR and therefore does not contain any extra sequences in addition to TRIM5 and CypA. DNA sequences of the TRIM5-Cyp junction of the two transgenes are aligned for comparison in Figure 3.11A.

The pLNT/SIEW and pscALPS vector backbones were of different configurations. pLNT/SIEW consisted of the SFFV promoter driving TRIMCyp expression followed by an IRES-eGFP, whereas pscALPS is a bicistronic vector which uses SFFV to drive TRIMCyp expression followed by the CypA promoter for expression of eGFP (Figure 3.11B).

To compare the restriction levels of this published TRIM5Cyp construct and the TRIMCyp constructs described in this project, lentivirus was produced using the

pLNT/SIEW and pscALPS plasmids carrying TRIM5Cyp306 and TRIM5Cyp322 respectively and the previously described packaging and envelope plasmids.

CRFK cells were transduced with LNT/S-TRIM5Cyp306-IEW, LNT/S-TRIM21Cyp284-IEW or scALPS/TRIM5Cyp322, using LNT/SIEW as a control, at an MOI of 10. The resultant populations had a high level of transduction, with between 75 and 85% GFP positive. These cells were transduced with HIV-1-YFP at an MOI of 3 or 30. After a further 72 hours, eGFP and YFP co-expression was measured by flow cytometry (Figure 3.11D).

By gating on eGFP positive cells, the percentage of cells co-expressing YFP was measured. All three TRIMCyp constructs were strong inhibitors of HIV-1-YFP, reducing YFP transduction by nearly 100 fold at the lower MOI. At the higher MOI of HIV-1-YFP of 30, TRIM5Cyp306 and TRIM21Cyp284 in the LNT/SIEW vector provided slightly more protection against HIV-1-YFP than scALPS-TRIM5Cyp322, with 2-3% of cells transduced compared to 9%, although this difference was not significant (P=0.15-0.16).

The copy number of integrated vector was compared between cells transduced with the different vectors by qPCR with primers targeting WPRE. The copy number was approximately 2 fold higher in the TRIM5Cyp306 transduced cells (20.9 copies) compared to TRIM5Cyp322 (9.4 copies); with TRIM21Cyp284 cells (14.6 copies) having an intermediate copy number. This may explain the slightly enhanced restriction in TRIM5Cyp306 cells, although the restriction by all three constructs is essentially comparable.

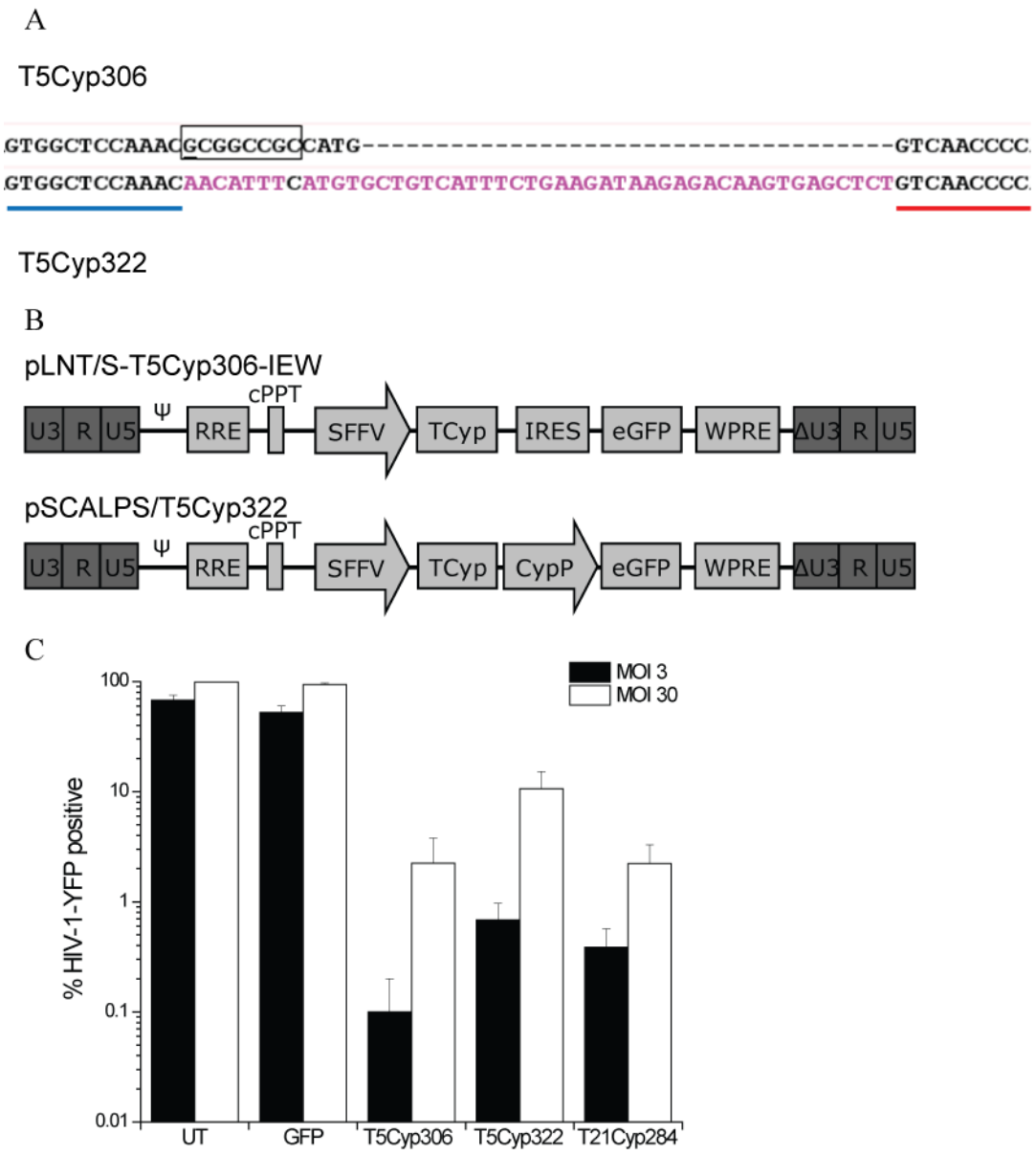


Figure 3.11 Comparison of TRIMCyp with published TRIM5Cyp

A. A humanised TRIM5Cyp construct has been independently designed and tested (322). This published variant, TRIM5Cyp322 (lower sequence) differs from the previously described TRIM5Cyp306 (upper sequence). The TRIM5 fragment is longer, terminating at amino acid 322 and does not include any sequences in addition to TRIM5 and Cyp, whereas TRIM5Cyp306 has a NotI restriction site (indicated by black box). The DNA sequences of the two TRIM5Cyp factors are aligned, showing the junction between TRIM5 (blue underlined) and Cyp sequences (red underlined). Nucleotides in pink differ between the two sequences and those in black are homologous.

B. TRIM5Cyp306 and TRIM21Cyp284 were in pLNT/SIEW, which uses an IRES-eGFP configuration. TRIM5Cyp322 was in a lentiviral vector under control of the viral SFFV promoter, followed by eGFP under control of the CypA promoter (CypP).

C. CRFK cells were transduced with S-TRIM5Cyp306-IEW, scALPS-TRIM5Cyp322 or S-TRIM21Cyp284-IEW at a multiplicity of infection (MOI) of 3. Untransduced (UT) and SIEW (GFP) transduced cells were used as controls. Cells were challenged with HIV-1-YFP at a MOI of 3 and 30, and eGFP and YFP co-expression was measured by flow cytometry 72 hours later. Samples were performed in triplicate, error bars show standard error of the mean.

3.10 TRIMCyp vectors transduce primary T cells and restrict HIV-1

For use in gene therapy, TRIMCyp vectors would be used to transduce patient cells *ex vivo*, producing a population that is resistant to HIV-1, before reintroducing them back into the patient. The primary target cells of HIV-1 are $CD4^+$ T cells, making them an obvious choice for vector modification. It is necessary to show that these vectors are capable of transducing human T cells and driving transgene expression.

PBMCs were harvested from healthy donors and activated with anti-CD3/anti-CD28 beads and IL-2 to enhance transduction. After 48 hours activation, cells were transduced with TRIMCyp vectors (either TRIM5Cyp306 or TRIM21Cyp284), with untransduced and (SIEW) GFP transduced cells as controls. After 72 hours, cells were transduced with an HIV-1-YFP, and eGFP and YFP expression measured by flow cytometry after a further 72 hours (Figure 3.12).

It was possible to efficiently transduce primary T cells with LNT/SIEW. However, there was great variation in efficiency. For TRIMCyp vectors the percentage of GFP positive cells after transduction ranged from 3-20%. In all experiments the transduction efficiency with the GFP vector was always higher than those also carrying the TRIMCyp transgene, reaching up to 40% in some experiments.

In TRIMCyp transduced populations there was very little co-expression of eGFP and YFP, indicating restriction of HIV-1-YFP in TRIMCyp expressing cells. The percentage of YFP transduced cells in the TRIMCyp-eGFP population is 10 fold lower than in control cells. Although there is co-expression of YFP in GFP positive cells transduced with LNT/SIEW, this level is reduced compared to untransduced control cells. It could be that the initial transduction with LNT/SIEW affects fitness of the T cells, leading to less efficient subsequent transduction with the HIV-1-YFP vector.

TRIMCyp expression could not be confirmed by Western blot in these cells. However, it has previously been shown difficult to obtain clear bands and therefore in the case of T cells, in which the percentage of transduced cells is much lower than

those seen in cell lines, it is not surprising that the protein could not be detected by Western blotting.

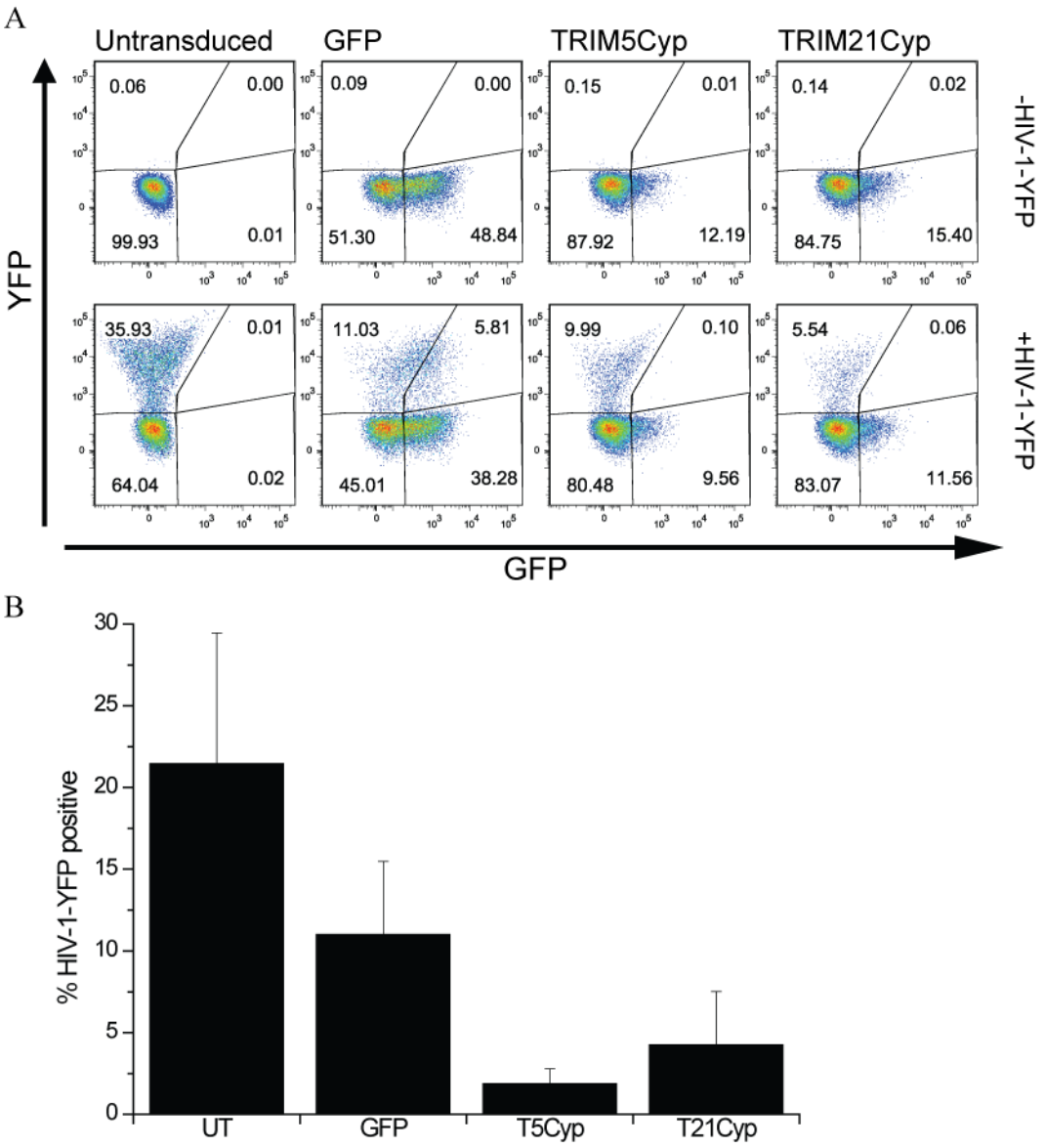


Figure 3.12 TRIMCyp restriction of HIV-1 in primary T cells
Peripheral blood mononuclear cells (PBMCs) from healthy volunteers were activated for 48 hours with anti-CD3/anti-CD28 beads, before transduction with S-TRIM5Cyp306-IEW or S-TRIM21Cyp284-IEW vectors. Untransduced (UT) and SIEW (GFP) transduced cells were used as controls. After 72 hours, cells were challenged with HIV-1-YFP and flow cytometry used to measure eGFP and YFP expression after a further 72 hours.
A. Example of flow cytometry plots showing eGFP and YFP expression.
B. Gating on the eGFP positive population, the percentage of YFP positive cells was measured and shown. N=3, error bars show the standard error of the mean.

3.11 Summary

Humanised TRIM5-Cyclophilin A fusions were designed mimicking the naturally occurring owl monkey restriction factor. They differed from previously published TRIM5Cyp restriction factors (322) in the length of the linker region between the end of the TRIM5 RBCC and the CypA cDNA and the presence of a NotI restriction site. Three different constructs were produced that varied in their linker lengths between the C terminal of the RBCC and start of the Cyp domain, with the longest linker in TRIM5Cyp306 including the first amino acids of the B30.2 domain.

Initially, the different TRIM5Cyp constructs were expressed from the retroviral vector EXN in CRFK cells and protein expression was confirmed by Western blot using an antibody against the N terminal HA tag on RING domain.

The function of the TRIM5Cyp proteins was then tested by restriction of an HIV-1 vector carrying an eGFP marker gene. All three TRIM5Cyp proteins were able to provide strong restriction of transduction of the HIV-1-GFP vector, as measured by flow cytometry. Restriction was comparable between the three different constructs; with each one causing between 10 and 100 fold restriction compared to untransduced cells or those transduced with the empty EXN vector. To confirm that the TRIM5Cyp protein was responsible for restriction, cells were treated with CsA, a reversible inhibitor of Cyp-CA binding, which rescued HIV-1-GFP transduction. The similar levels of potent restriction between the three constructs was unexpected considering the great variation in restriction seen by Neagu et al between TRIM5Cyp proteins of different lengths (322). Variation in viral inhibition is not simply explained by a correlation between length and restriction. Modeling by Neagu et al suggests that the point at which the CypA domain is fused to TRIM5 affects restrictive ability as fusion to TRIM5 at residues clustered around a variable region within the B30.2 domain generated the strongest HIV-1 inhibitors (322). This variable region has been shown to undergo strong selective pressure and be directly involved in interaction with CA of susceptible virus (239). It is proposed that positioning the CypA moiety at this point enables efficient interaction between CypA and CA and co-ordination with the TRIM5 RBCC domains for antiviral

function (322). The three TRIM5Cyp proteins designed in this project were all able to restrict HIV-1, despite CypA fusion in TRIM5Cyp287 and TRIM5Cyp298 occurring outside of the variable region in B30.2. This suggests that there is a degree of flexibility for the site on TRIM5 at which CypA is attached and that factors other than fusion within the B30.2 variable domain may influence whether TRIMCyp is capable of viral restriction.

In addition to the three TRIM5Cyp fusions, a humanised TRIM21-Cyclophilin A transgene, TRIM21Cyp284, was designed using the same principles (a gift from Dr. Torsten Schaller). TRIM21Cyp284 was also cloned into the lentiviral vector pLNT/SIEW under the SFFV promoter linked to IRES-eGFP. Western blots were used to confirm protein expression, but the blots were not very sensitive. Typically TRIM proteins are not detected efficiently by Western blot so tagged proteins are frequently used to look at protein levels. This was also seen by Neagu et al with both owl monkey and restrictive human TRIMCyp, which failed to be detected by Western blot. Conversely, some non-restrictive constructs gave rise to strong bands, indicating that there is no correlation between protein levels and restrictive capacity (322).

The unclear blots could be due to a problem with antibody recognition of the epitopes, although other anti-Cyp and anti-TRIM5 antibodies were tested, neither yielding clear protein bands through Western blotting. Therefore it could be indicative of low levels of expression or rapid protein degradation. In several instances, the TRIM21Cyp band was more prominent than the TRIM5Cyp, which was barely visible. TRIM5α and TRIM5Cyp are ubiquitinylated and degraded by the proteasome continuously and rapidly, with the TRIM5α half-life only 50-60 minutes. This degradation requires RING and B-Box domains. Substitution of the TRIM5α RING with that of TRIM21 increases the protein half-life to about 210 minutes (219). TRIM21 is a very stable protein (378), and presence of the TRIM21 RING domain in TRIM21Cyp probably influences its rate of degradation. Treatment of cells with proteasomal inhibitors, such as MG132, may result in more prominent bands on the Western blot for both TRIMCyp proteins. Alternatively proteins could

be tagged. Here, TRIMCyp with an HA tag expressed from EXN was more reliably detected by Western blot than the untagged protein, but this would be unsuitable in a clinical setting. Similar difficulties arose in quantifying protein expression of TRIM21Cyp after codon optimisation. The quality of the Western blots made it difficult to reliably quantify differences in protein levels between the original and codon optimised genes, but importantly, both were shown to produce high titre vector, detectable protein and mediate potent restriction.

Problems could arise when producing HIV-1 vectors carrying anti-HIV-1 transgenes as production of the vector could be inhibited following transgene expression in packaging cells, resulting in lower titres. In the case of the TRIMCyp transgene, there was no effect. Also, treatment of packaging cells with CsA during vector production did not result in any change in titre. The lack of interference is probably because Cyp only binds monomeric Gag weakly (241), with interactions occurring with high avidity to the mature processed Gag protein in a hexameric form (233, 379). This more complex CA structure is only found in virions that have undergone maturation after release from producer cells. Packaging cells during vector production only contain full length Gag protein which is not recognised by TRIMCyp. Similarly, TRIM5α hexameric viral CA after cell entry, restricting before reverse transcription (188). Although it has been proposed that TRIM5α targets full length Gag during packaging causing a reduction in virion production (380), this theory has largely been discredited (381). Therefore TRIMCyp only targets CA of incoming virions after uncoating, which does not interfere with the production of lentiviral vector expressing TRIMCyp.

CRFK cells transduced with both TRIM5Cyp and TRIM21Cyp lentiviral vectors were highly resistant to HIV-1. HIV-1 transduction was reduced between 10 and 100 fold compared to control cells. Again, CsA treatment abrogated this restriction. Transduction with increasing MOI of HIV-1-YFP led to increasing numbers of cells becoming YFP positive, even when cells are producing TRIMCyp. A characteristic of viral restriction factors, for instance TRIM5α, is that they can be saturated with

high levels of sensitive virus particles (183). It required an extremely high MOI of 1000 to saturate restriction of TRIMCyp protein. It is difficult to correlate this vector MOI with levels of HIV experienced by patients *in vivo* as there is both free virus in the blood and cell associated virus, and viral levels vary throughout the body, with higher concentrations at the lymph nodes for example. Restriction by TRIMCyp proteins here was similar to that mediated by the published humanised TRIM5Cyp, which has also been shown to restrict full length NL4-3 HIV in a humanised mouse model (322). The comparable levels of restriction suggest TRIM5Cyp306 and TRIM21Cyp284 are good candidates for further testing in clinical trials.

Restriction assays in primary T cells are important as these are the main HIV-1 target cell and are likely to be the first cells used for gene therapy in patients. Challenge of primary T cells with HIV-1-YFP following transduction with TRIMCyp or control vectors resulted in variable levels (5-40%) of infectivity. T cells are most efficiently transduced 48-72 hours after stimulation and in these experiments, challenge with HIV-1-YFP was undertaken 5 days after initial anti-CD3/anti-CD28 bead stimulation to ensure prior integration and expression of TRIMCyp vectors. Thus, the difference in HIV-1-YFP infection between control and TRIMCyp modified groups was less prominent than detected in cell lines.
In the future, these restriction experiments could use an HIV-1 vector carrying an alternative marker gene, such as red fluorescent protein. This would remove the requirement of separation of GFP and YFP fluorescence using alternative optical filters to the default filters of the flow cytometer. Although these filters and compensation allows GFP/YFP separation, use of an alternative to YFP would be more experimentally convenient.

The fact that retrotransposition has occurred twice independently in different primate species to produce functional restriction factors suggests that fusion of TRIM5 and CypA is a successful strategy to produce strong retrovirus inhibitors. Although the natural target of these TRIM5Cyp proteins is not known, their maintenance in the species throughout evolution, particularly in owl monkeys which express no other

TRIM5 allele (315), suggests that they have played an important role in these primates in the protection against pathogens.

Synthetic TRIMCyp fusion proteins have been generated using other TRIM RBCC motifs fused to CypA and have been shown to strongly restrict HIV-1 (324), but only restriction factors including the TRIM5 RBCC have been identified in nature. Therefore the TRIM5 RBCC must function particularly efficiently as a restriction factor effector domain, most likely due the roles of the native protein in innate immunity. Firstly, TRIM5α is a restriction factor and, although its specificity varies between species, provides potent inhibition of different retroviruses. Its C terminal B30.2 domain is involved in retroviral CA recognition, and the RBCC is involved in eliciting the downstream antiviral effect, including multimerisation and proteasomal degradation. Therefore after TRIM5Cyp recruitment via CypA recognition of viral CA, TRIM5 RBCC is able to function appropriately for retroviral restriction.

Secondly, TRIM5α has a role in innate immune signaling by acting as a pattern recognition receptor. Binding of viral CA by the B30.2 domain increases the E3 ubiquitin ligase activity of the TRIM5 RING domain, generating K63-linked ubiquitin chains. These in turn activate the TAK1 kinase complex causing upregulation of AP1 and NFκB signaling (252). This provides antiviral activity in addition to direct viral binding and degradation. Similarly with TRIM5Cyp, CA recognition by its Cyp domain allows downstream signaling via the RBCC domains as with TRIM5α (252). Of all of the members of the large TRIM family, this function as an effector in signaling has only been observed with TRIM5α. However, this role of activating innate immunity in response to retroviral infection is likely to be an important reason why there has been the evolution of efficient restriction factors by the fusion of the TRIM5 RBCC to Cyp on two separate occasions.

Here we have designed TRIM5Cyp and TRIM21Cyp fusion constructs and cloned them into lentiviral vectors. Restriction assays in cell lines show that these proteins are able to provide potent restriction of HIV-1 derived vectors. Using the RBCC domains from either TRIM5 or TRIM21 produce equally efficient restriction factors that show promise for further development as an anti-HIV-1 gene therapy transgene.

4 TRIMCyp proteins restrict replication competent HIV-1

4.1 Aims

- To demonstrate restriction of wild type HIV-1 by TRIMCyp proteins in cell lines and primary T cells
- To investigate whether TRIMCyp proteins provide cells with a survival advantage when infected with replication competent HIV-1

4.2 Introduction

Chapter 3 presents results in which TRIMCyp restriction of a single round of transduction by an HIV-1 derived vector is shown in different cell lines and primary T cells. These viral vectors include the CypA binding domain in their CA, enabling them to be targeted and efficiently inhibited by both TRIM5Cyp and TRIM21Cyp fusion proteins. In this chapter we have continued to investigate the restrictive abilities of one of the TRIM5Cyp proteins, TRIM5Cyp306, and TRIM21Cyp284, which are referred to as TRIM5Cyp and TRIM21Cyp respectively from this point forwards.

Although these restriction factors have been shown to mediate potent restriction of HIV-1 vectors it is also necessary to measure restriction of full length, replication competent HIV-1. Replication competent virus, in contrast to the SIN HIV-1 vector would be able to continually replicate in a population of susceptible cells, leading to cell death, mirroring the loss of T cells seen in HIV-1 infected patients. Ideally following gene therapy, TRIMCyp expressing cells would have a strong survival advantage over untransduced cells, allowing their continued growth and proliferation to repopulate the host's immune system. This would reduce the characteristic $CD4^+$ T cell decline seen in HIV-1 patients and alleviate the associated pathology. A

therapeutic transgene that promotes a strong survival advantage is highly desirable in any gene therapy situation as it means that a smaller population of cells needs to be transduced to see a clinical benefit.

To test the restriction of full length HIV-1 it is necessary to use cells that express the required receptors to allow HIV-1 entry, namely CD4 and either of the co-receptors CCR5 or CXCR4, depending upon the tropism of the virus. CCR5 is rarely expressed at high levels in cell lines and thus experiments described here have used Jurkats and GHOST cells which have been either been stably transfected or transduced with retroviruses to express CCR5/CXCR4. These lines allowed ready and reproducible quantification of HIV-1 restriction.

4.3 TRIMCyp proteins restrict HIV-1 in cell lines

Restriction of replication competent HIV-1 was first measured in the GHOST cell line. GHOST-CCR5 cells are derived from human osteosarcoma cells and have been transduced with a retrovirus to express high levels of CD4 and CCR5 to allow infection with R5 tropic HIV-1 (371). These cells were transduced with LNT/SIEW expressing GFP alone as a control or the S-TRIMCyp-IEW vectors. Cells were cultured for several days to allow expansion and stable expression of TRIMCyp before staining with an anti-CCR5 antibody and FACS sorting for eGFP/CCR5 double positive cells (Figure 4.1). These cells were infected with a single round of 4.8ng p24 of the HIV-1 clone NL4-3 (BaL), which was derived from NL4-3 but with a BaL envelope to confer a CCR5 tropism. Seven days after infection, supernatant was harvested from cells. Levels of Gag protein within the media were measured by p24 ELISA and comparison to a standard curve of serially diluted p24 antigen (Figure 4.2A). Both untransduced and GFP transduced cells supported HIV-1 replication leading to high levels of p24 being released into the culture medium (Figure 4.1B). However, in the cells expressing either TRIM5Cyp or TRIM21Cyp levels of p24 were highly significantly reduced compared to untransduced cells ($P=0.001$) and there was virtually no p24 detectable in the media. TRIM5Cyp and TRIM21Cyp appeared to restrict at a similar level with no significant difference between the levels of p24 in these populations ($P=0.38$).

Recombinant replication competent lentivirus is not detected in TRIMCyp expressing cultures

One concern associated with gene therapy is recombination events leading to the development of RCL. This could result in novel, pathogenic strains of virus and uncontrolled infection. This must be thoroughly tested before use in clinic, but here preliminary investigations have carried out to see whether recombinants could be detected.

Culture supernatant was harvested from the transduced GHOST cells infected with HIV-1 in the above experiment. Supernatant was added to HIV-1 permissive Jurkat-

CCR5 cells, which were grown for two weeks. Cell samples were taken at days 7 and 14 and for WPRE copy number quantification by qPCR (Figure 4.2C).

If RCL containing WPRE had formed in the GHOST-CCR5 cells after co-infection with lentiviral vector and full length HIV-1, this virus would be able to infect the permissive Jurkat cells. Therefore integrated WPRE would be detectable in these cells which have had no other exposure to vector. Once this occurred, further replication of RCL would lead to an increase in WPRE copy number over time. HEK293T transduced with LNT/S-T5Cyp-IEW DNA, and therefore containing integrated WPRE, was used as a positive control for the qPCR. In all Jurkat samples, the copy number of WPRE was never above the untransduced Jurkat negative control.

This was a very preliminary experiment to detect mobilisation of WPRE from the vector construct in RCL, but thorough testing must be performed before any vectors can be used in patients, particularly for HIV-1 therapy as there is theoretically more chance of recombination occurring between both the HIV-1 derived vector and the infectious virus. Also, for use in a clinical setting, third generation packaging plasmids would be used rather than this second generation system. This would reduce the likelihood of RCL development by separation of the required genes over four plasmids and by deletion of the tat gene.

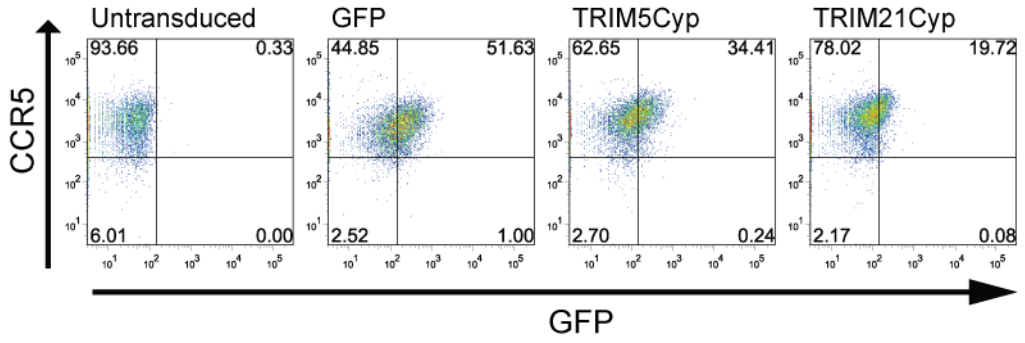
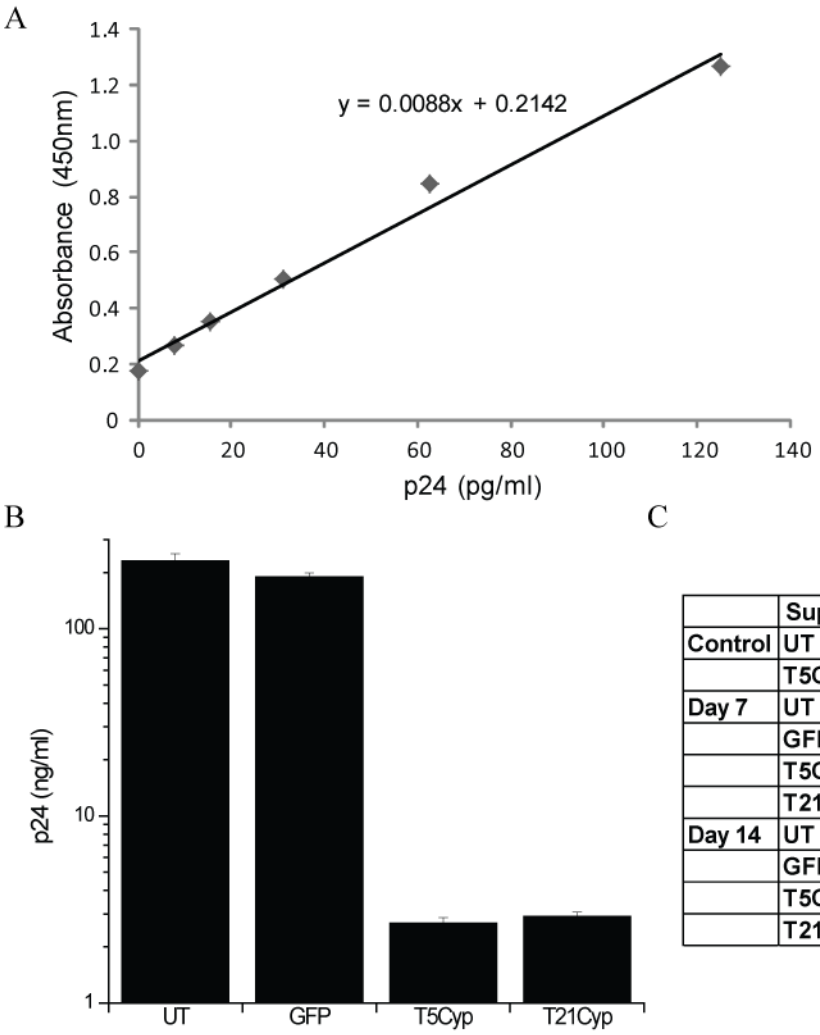


Figure 4.1 Flow cytometric sorting of GFP$^+$ CCR5$^+$ GHOST cells
GHOST-CCR5 cells were transduced with SIEW (GFP), S-TRIM5Cyp-IEW or S-TRIM21Cyp-IEW vectors and stained with an anti-CCR5 antibody. GFP and CCR5 expression was measured by flow cytometry as shown in the plots. CCR5 GFP double positive cells were sorted using a MoFlo XDP sorter and expanded for further experiments.



	Sup. Source	Copy number
Control	UT 293T	0.027
	T5Cyp 293T	0.168
Day 7	UT	0.002
	GFP	0.001
	T5Cyp	0.013
	T21Cyp	0.003
Day 14	UT	0.006
	GFP	0.005
	T5Cyp	0.006
	T21Cyp	0.004

Figure 4.2 Restriction of replication competent HIV-1 in cell lines

A. Representative graph of the protein standard curve used in p24 ELISAs. P24 antigen standard provided by the manufacturer was serially diluted to generate a standard curve, which was used to quantify p24 in culture supernatants.

B. GFP/CCR5 double positive cells sorted from Figure 4.1 and untransduced (UT) control cells were infected with the HIV-1 clone NL4-3 (BaL). Culture supernatant was harvested 7 days after infection and levels of HIV-1 p24 in the medium measured by ELISA. Samples were performed in triplicate, error bars show the standard error of the mean.

C. Culture supernatant was collected from the four cell lines in the experiment described in B and added to Jurkat-CCR5 cells. The cell population providing the supernatant (sup.) sample is indicated in the second column. Jurkat cells were cultured for 14 days with cell samples taken at days 7 and 14. DNA was extracted and WPRE copy number measured by quantitative PCR. Untransduced HEK293T cell DNA (UT 293T) was used as a negative control (top row). HEK293T cells transduced with S-T5Cyp-IEW (T5Cyp 293T) resulting in 8% eGFP positive were used as a positive control.

4.4 TRIMCyp confers a survival advantage to Jurkat cells infected with HIV-1

Jurkat-CCR5 cells were transduced with LNT/SIEW, LNT/S-T5Cyp-IEW or LNT/S-T21Cyp-IEW at an MOI of 50 to produce a mixed population of partially transduced cells. Approximately 20-25% of cells were eGFP positive, as measured by flow cytometry (Figure 4.4A). This mixed population of cells was infected with 0.32ng of replication competent HIV-1 R9, which is X4 tropic. The cells were grown in culture for one month, with regular passaging and flow cytometry to measure the number of viable cells.

In preliminary experiments, R9 infected Jurkats were stained with fixable viability dye to measure the percentage of viable cells. The viable cells, which were negative for the stain, were backgated and a viable gate drawn on the forward/side scatter plot (Figure 4.3). There was a strong correlation between cell viability measured by live/dead staining or by forward/side scatter spread. Therefore for convenience in subsequent experiments, measurements of viability were taken simply by looking at the shift on forward/side scatter.

Cells uninfected with HIV-1 maintained high viability of 80-90% throughout the experiment. In contrast after HIV-1 infection, viability in populations of partially transduced cells (containing 20-25% transduced cells) dropped dramatically in all samples to between 1 and 20% viable (Figure 4.4B). However, by day 17 post infection the TRIM5Cyp and TRIM21Cyp partially transduced cultures started to recover and there was an increase in the percentage of viable cells in these samples as protected cells replicated. By day 29 post infection, there were similar numbers of viable cells, approximately 70-80% of total cells, in the TRIMCyp transduced cultures as in the samples uninfected with HIV-1.

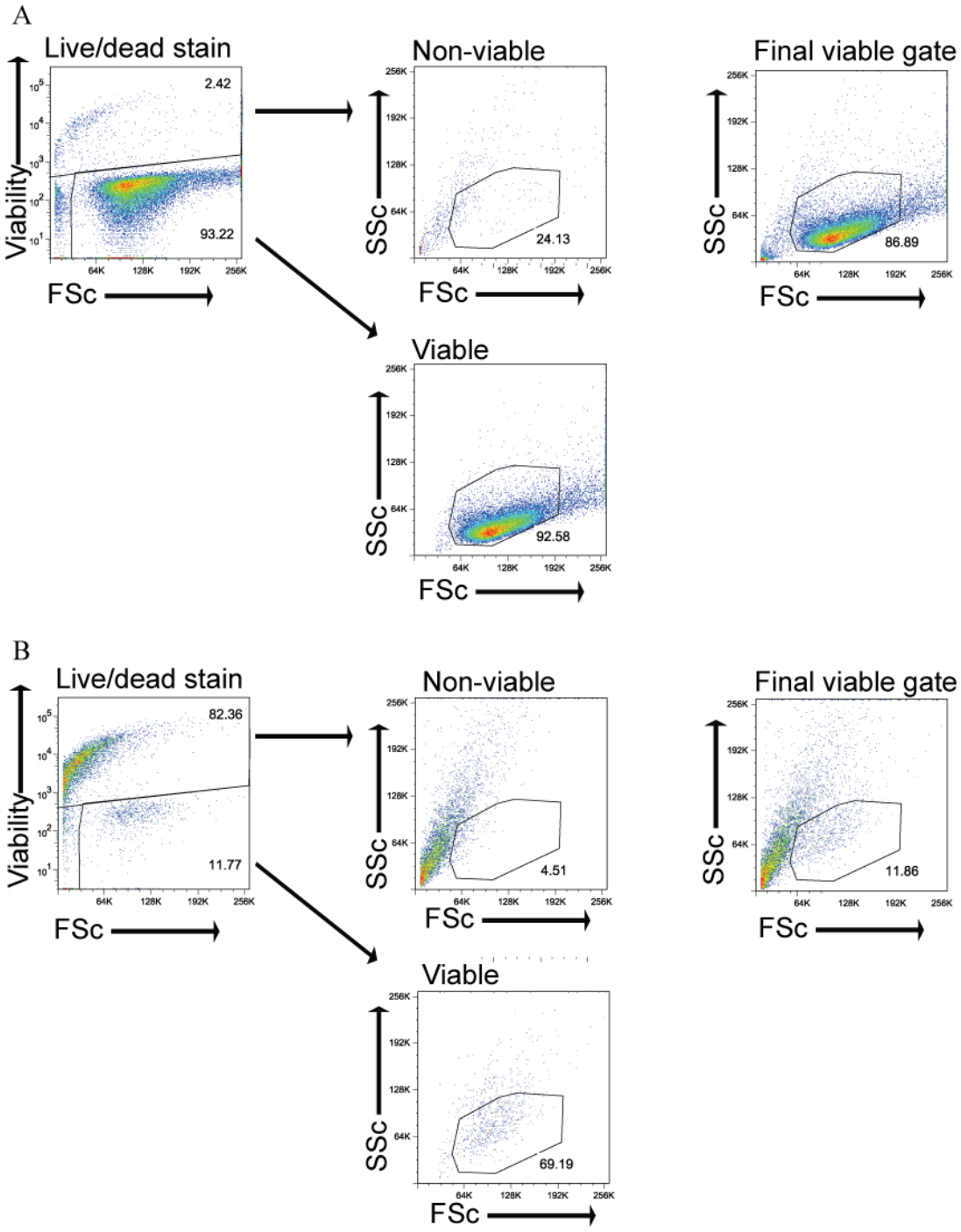


Figure 4.3 Confirmation of cell death using viability dye
Cells were stained with viability dye and a viable gate drawn around viable cells, which were viability dye negative, in the total population (left hand panels). These cells were then backgated onto a forward/side scatter (FSc/SSc) plot and a gate drawn around the cells (central panels). This gate was then used to determine viable cells in the total cell population (right hand panels) and change in viability was measured as a shift on the FSc/SSc from this gate. Panels in A are from uninfected cells with high viability, in B cells are infected with HIV-1 and have a low viability.

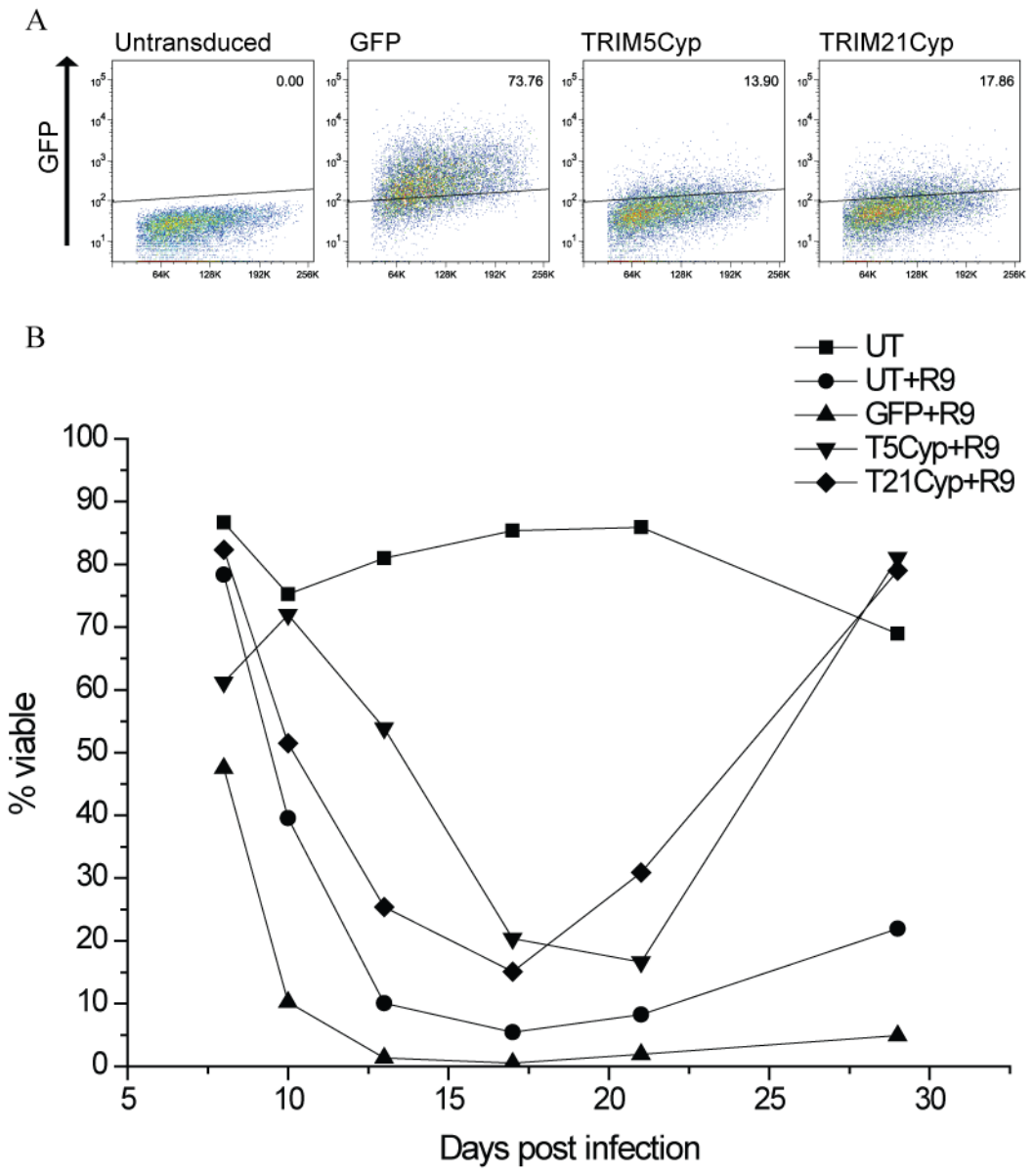


Figure 4.4 TRIMCyp proteins confer a survival advantage to cells cultured with HIV-1
A. Jurkat-CCR5 cells were transduced with TRIM5Cyp or TRIM21Cyp vectors to reach approximately 20% of total cells transduced. Untransduced (UT) and SIEW (GFP) transduced cells were used as controls.
B. Cells were infected with a single dose of R9 HIV-1. Cell samples were taken regularly to measure cell viability by flow cytometry using the gating described in 4.3.

4.5 Restriction of HIV-1 by TRIMCyp in primary T cells

As the predominant cell type infected by HIV-1, the most likely target cell for gene therapy in the first instance would be $CD4^+$ T cells. It is important that TRIMCyp vectors are able to transduce and express in this cell population and to provide potent inhibition of HIV-1 as seen in the experiments in cell lines.

Healthy volunteer donor PBMCs were separated through Ficoll gradient centrifugation of whole blood. These cells were activated using anti-CD3/anti-CD28 beads and 100U/ml IL-2 for 48 hours before transduction with LNT/SIEW or TRIMCyp vectors at an MOI of between 10 and 30. Cells were expanded for 7 days to allow transgene expression, before staining for CD4 and FACS sorting for CD4/eGFP double positive cells (Figure 4.5).

$5x10^4$ sorted cells were seeded in a 96 well U bottomed plate and infected with 0.45ng p24 NL4-3 (BaL) HIV-1. Five days post infection, p24 levels were measured in the media by ELISA (Figure 4.6A). Levels of p24 released into the media from TRIMCyp transduced cells was highly reduced compared to control samples.

An MTT assay was performed on the cell pellets at the end of the experiment, but there was little cell death in HIV-1 infected cells compared to uninfected cells. Cells transduced with all vectors, including the SIEW (GFP) control, had increased survival compared to untransduced HIV-1 infected cells (Figure 4.6B). It is likely that as the cells were only cultured for five days after infection, this time period was not sufficient for cell death to occur, as with HIV-1 infected Jurkat cells, a decrease in viability was not seen until at least 10 days post infection. Ideally cells would be cultured for a longer time period to confirm whether a survival advantage is also seen in primary T cells, as for Jurkats, but it is difficult to culture these primary cells for such an extended period.

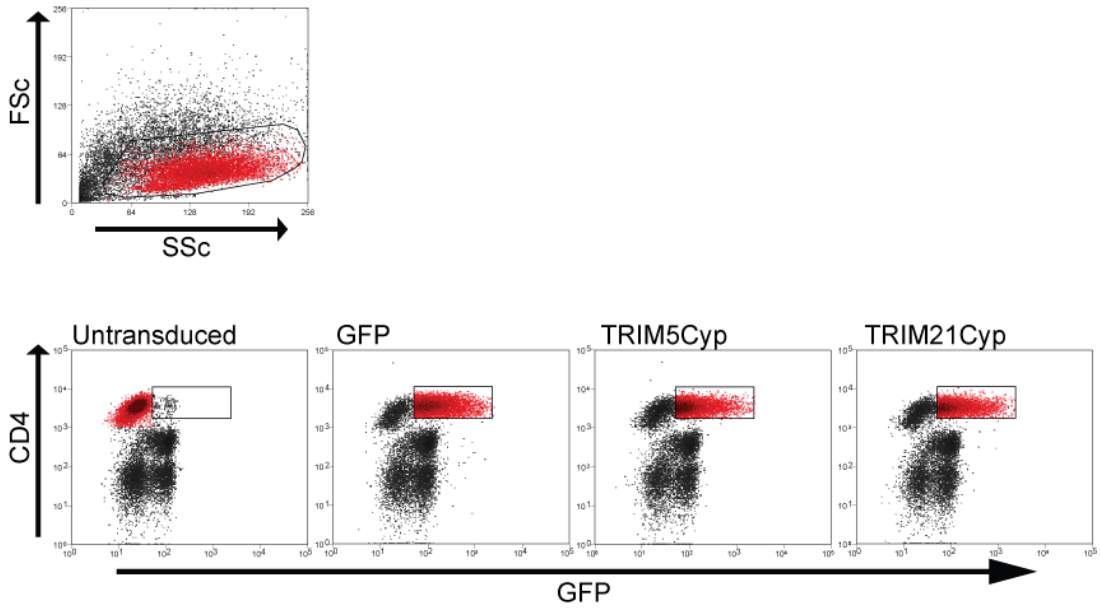


Figure 4.5 Flow cytometric sorting of $CD4^+$ GFP^+ T cells
Peripheral blood mononuclear cells activated with anti-CD3/anti-CD28 beads and IL-2 were transduced with SIEW (GFP) or TRIMCyp vectors. Cells were stained with anti-CD4 antibody before sorting for $CD4^+$ GFP^+ double positive cells. The top panel shows a representative forward and side scatter plot (FSc and SSc respectively) and the gate used for viable cells. The bottom four panels show each sorted sample, and the gate used for double positive cells. Untransduced cells were sorted for $CD4^+$ only. The sorted cells, shown in red, were then used for HIV-1 restriction experiments.

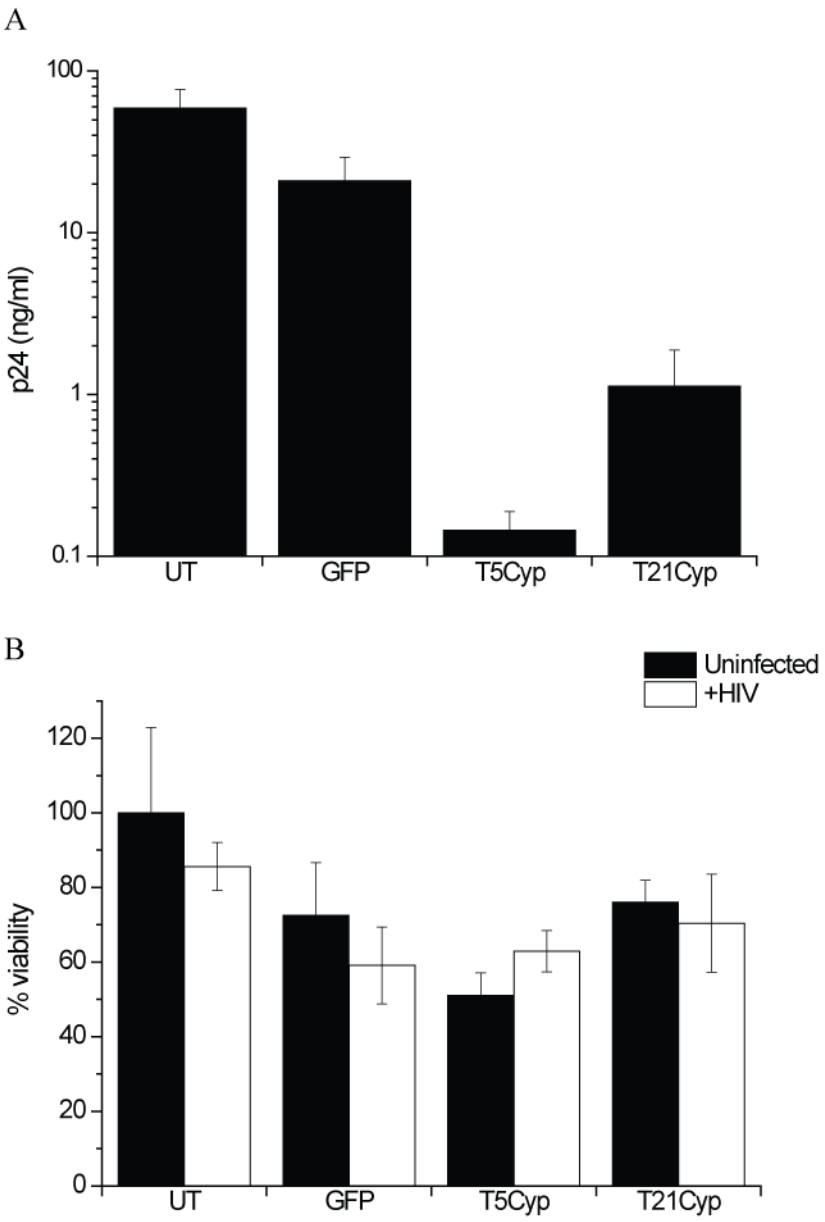


Figure 4.6 TRIMCyp restriction of HIV-1 in primary T cells
CD4/GFP double positive primary T cells obtained by flow cytometric sorting (shown in Figure 4.5) were infected with replication competent HIV-1 NL4-3 (BaL). At day 5 post infection, p24 was measured in the media by ELISA (A) and cell pellets were used for MTT assay (B). n=6 from two different donors, error bars show standard error of the mean.

Similarly, experiments in which a small proportion of TRIMCyp modified cells within an a bulk, unsorted T cell population were not extended sufficiently to detect a survival advantage. CD3/CD28 activated primary T cells were transduced with 50µl LNT/SIEW or TRIMCyp vectors (MOI of 5-10), which resulted in 2-3% of TRIMCyp transduced cells as eGFP positive at the time of challenge with NL4-3 (BaL) (Figure 4.7A). The proportion of eGFP positive cells remained constant over a period of 7 days after which cells were harvested and stained for viability (Figure 4.7B). DNA was also extracted and WPRE copy number was measured by qPCR (Figure 4.7C). There was an increase in copy number per cell in TRIMCyp populations after infection with HIV-1. This suggests that transduced cells do have a survival advantage, although it is not large enough to be detected by flow cytometry with this experimental protocol.

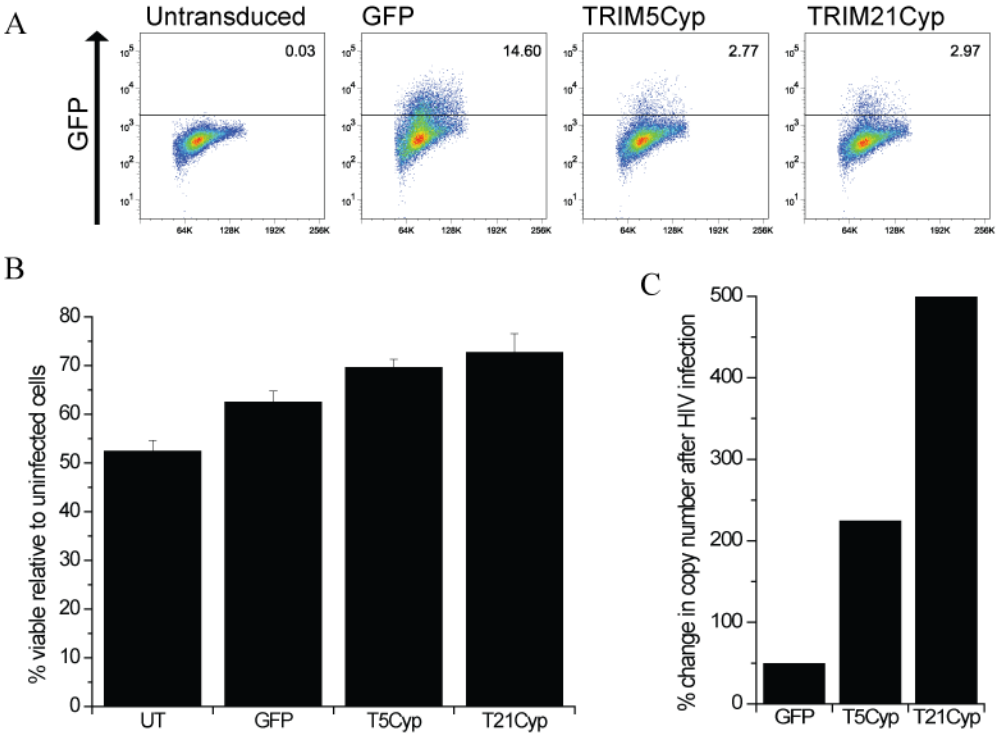


Figure 4.7 HIV-1 infection of primary T cells expressing TRIMCyp
A. Primary T cells were transduced with LNT/SIEW (GFP) or TRIMCyp vectors and eGFP expression measured by flow cytometry. B. Seven days post infection with NL4-3 (BaL), cells were stained with viability dye and measured by flow cytometry. The percentage of viable cells in each population was calculated relative to uninfected cells of the same transduction. Samples were performed in triplicate, error bars represent standard error of the mean. C. DNA was extracted from cell seven days after infection. WPRE copy number was measured by quantitative PCR in uninfected populations and those infected by HIV-1.

4.6 Summary

This chapter has shown that both TRIM5Cyp and TRIM21Cyp elicit strong inhibition of full length, replication HIV-1 clones. This has been shown in different permissive cell lines engineered to express HIV-1 receptors and also in primary T cells.

In pure populations of GHOST cells expressing TRIMCyp, HIV-1 replication is effectively terminated, with very low levels of p24 present in the medium after infection. Similarly, in sorted T cell populations there was a significant decrease in p24 levels in the media of TRIMCyp expressing cells.

Importantly, in experiments that are relevant to a clinical gene therapy situation, TRIMCyp expressing Jurkat cells have a survival advantage in mixed populations of partially transduced cells infected with replication competent HIV-1. Initially there was a drastic decrease in cell viability and the majority of cells died, but the TRIMCyp transduced populations recovered, with a large increase in viability. The percentage of cells that remain viable after the initial HIV-1 infection are around 20%, which correlates with the percentage of transduced cells in the initial culture, indicating that it is TRIMCyp expressing cells that survive. This survival advantage is an appealing situation for gene therapy as it would mean that a smaller percentage of cells could be modified *ex vivo* prior to re-infusion back into a patient, and that these cells would survive and reconstitute a patient's immune system with HIV-1 resistant cells. The requirement of only a low percentage of transduced cells means that the transduction efficiency does not need to be as high and reduces the likelihood of multiple copies per cell, and therefore lowers the risk of insertional mutagenesis.

Sufficient data was not collected to demonstrate this survival advantage in primary T cells. In a population of primary T cells with 2-3% of cells expressing TRIMCyp there was no significant difference in viability in TRIMCyp populations compared to untransduced cells. Longer culture periods may lead to an expansion of TRIMCyp expressing cells and loss of unmodified cells so that a difference between the two

populations could be detected. Prolonged culture of primary T cells *in vitro* will also have a negative effect on cell viability, making this protocol more problematic. However, it is important to be able to show that these TRIMCyp vectors can be used to transduce T cells, providing restriction of HIV-1, and that these cells have a survival advantage in infected populations.

The survival and expansion of modified, resistant cells has been demonstrated *in vivo* in some clinical trials. For instance, $CD34^+$ HSCs transduced to express a dominant negative Rev protein (130) and $CD4^+$ T cells expressing antisense trans-activation response (TAR) element and/or trans-dominant Rev (132) exhibited a survival advantage when under the selective pressure of HIV in patients. Therefore, to fully benefit from the survival advantage of transduced cells and promote their expansion, it may be necessary to interrupt HAART to allow an increase in viral load to drive selection and expansion of transduced cells. There are obvious risks with this method and viral load in these patients must be carefully monitored during this procedure.

However, it would still be necessary for sufficient numbers of cells to be initially modified and expressing high enough levels of protein. Modeling and preclinical trials show that it is preferential to start with as large a number of modified cells as possible for a more successful therapeutic outcome (126). The actual percentage of cells that would need to be transduced to confer a therapeutic benefit to a patient is not known and would best be tested in patients in clinical trials.

Although it has not been observed, gene therapy presents a theoretical risk of mobilisation of the vector and the development of RCL. This is predicted to be most probable during vector production due to recombination between the transfer vector and packaging plasmid. Recombination of the viral protein encoding genes with the cis-acting elements of the transfer vector could result in RCL. In HIV-1 patients, use of an HIV-1 derived vector for gene therapy increases this potential risk due to the additional presence of HIV-1 genes from the infectious virus, which includes the virulent accessory genes not present during vector production.

In preliminary experiments described here we were unable to detect by qPCR any mobilised vector in cells exposed to both lentiviral vector and full length HIV-1. This method would only detect RCL that includes WPRE. As RCL have never been observed, their genome can only be predicted so it is not known whether there would be inclusion of this element and therefore if this assay would detect their presence.

This qPCR was only a preliminary measurement, and biosafety must be more thoroughly tested once clinically useable vector stocks are produced. This can be performed by companies such as Bioreliance, which use a highly sensitive assay to detect RCL RT activity. Briefly, vector stocks are used to transduce the C8166 T cell line, which is highly permissive and supports efficient viral replication. These cells are cultured for 8 passages to allow amplification of RCL and removal of vector RT. Supernatant from the later passages is harvested and RT activity is quantified using a highly sensitive PCR based assay. This involves reverse transcription of an RNA template and PCR amplification of the resultant cDNA.

To detect vector mobilisation *in vivo*, plasma of treated patients can be screened by qRT-PCR for the presence of vector genome RNA that is unique to the vector and absent from wild type HIV-1. This procedure was performed on patients who received T cells transduced with a conditionally replicating lentiviral vector with intact LTRs to detect mobilised vector (120).

A major problem with the development of anti-HIV-1 therapies is that the virus has a high mutation rate and is frequently able to escape restriction. For therapy to be successful, preventing this phenomenon is crucial. During the month long culture of TRIMCyp expressing cells infected with HIV-1 clones, resistance to virus was maintained as demonstrated by the high viability of cells expressing TRIMCyp. This suggests that viral escape mutants did not develop over this time course. Similarly, TRIMCyp resistant HIV-1 strains could not be identified by Neagu et al (322). Conversely, *in vivo* experiments have shown that despite initial restriction of susceptible SIVsm strains, CA mutations developed allowing escape from both TRIM5α and TRIM5Cyp restriction, and rescue of infection after forced passage through rhesus macaques. However, in these experiments, there was forced viral

transmission primarily through intravenous injection using high levels of virus. These large MOI may saturate TRIM5α and TRIM5Cyp restriction and not be truly representative of naturally occurring infection, conferring an advantage on the virus over the restriction factors (382).

In addition *in vitro* experiments using HIV-1 clones with mutated CA sequences that do not bind CypA avoid TRIMCyp restriction (311). However, these CA mutations come at a cost to viral fitness, as resultant strains have reduced infectivity probably due to the requirement of CypA for proper uncoating and the viral lifecycle (306). Therefore the maintenance of the CypA binding region of HIV-1 CA is thought to be critical for productive infection.

One possible explanation for the requirement of CypA binding is that it is needed for correct nuclear import and influences integration site. Several Nup proteins, such as Nup358, have been identified as important for the HIV lifecycle by an RNAi screen of cellular proteins (383). Nup358 includes a cyclophilin-like domain, which has been shown to interact directly with the HIV-1 CA via its Cyp binding loop (45). In human cells, CypA binding to HIV-1 CA promotes nuclear entry in a Nup358/Nup153 dependent pathway. This results in integration in the host genome in regions of optimal gene density, allowing efficient transcription of viral genes.

Some CA mutants, for instance the G89V mutant, have an altered integration site profile that does not target gene dense regions (270). CA mutations that disrupt the Cyp binding loop disrupt interactions with CypA and Nup358. Therefore Nup358/Nup153 independent pathways must be used, resulting in integration in suboptimal locations that do not support proviral transcription, leading to a termination of the viral lifecycle in primary macrophages (45).

This suggests that conservation of CA interaction with CypA and Nup358 is essential for utilisation of the optimal nuclear import mechanism and integration site targeting. If TRIMCyp resistant viral strains develop by mutation of their Cyp binding loop, interactions with both CypA and Nup358 would also be disrupted. This would force these HIV-1 strains to utilise alternative pathways of nuclear entry and possibly uncoating, altering the site of integration and affecting transcription and viral replication. Therefore, maintaining the HIV-1 CypA binding loop appears to be

crucial for productive viral infection *in vivo* and could explain the conservation of CypA binding.

This suggests that HIV-1 will be unlikely to develop escape mutants that are resistant to TRIMCyp binding and restriction. If any escape mutants evolve, their reduced fitness will limit their infection and replication, even in unmodified cells, preventing expansion of these resistant strains. Evidence suggests that HIV-1 is unable to mutate to avoid binding to CypA, and therefore avoiding restriction by TRIMCyp restriction factors, making them a highly attractive protein for use in gene therapy against HIV-1. However, it will be important to test whether any CA mutants emerge and whether they are capable of productive infection.

Results presented here have shown TRIMCyp restriction of both R5 and X4 tropic HIV-1 in different assays. In the future it will be important to also measure restriction of primary isolates of HIV, including drug resistant strains.

5 Interaction of TRIMCyp with endogenous TRIM proteins

5.1 Aims

- To assess whether TRIMCyp expression interferes with the endogenous TRIM5 or TRIM21 activity

5.2 Introduction

Chapter 3 assesses restriction by two engineered anti-HIV-1 factors based upon the naturally occurring primate TRIM5CypA fusion proteins that provide potent inhibition of lentiviruses. In all of the assays performed, there was no significant difference in the level of restriction mediated by TRIM5Cyp or TRIM21Cyp. Although it is the TRIM5 RBCC domains that are linked to CypA in both the natural fusions proteins and the published restriction factor (322), these data show that fusion of CypA to the RBCC domains of other TRIM molecules can provide equally potent restriction. For future development as a gene therapy treatment, it is important to decide which of the proteins should be developed further and would be most effective in a clinical setting. As the level of restriction against HIV-1 that they provide is comparable, investigating whether they interfere with the normal function of cells can be used to determine if one has an advantage over the other.

Both TRIM5 and TRIM21 have been demonstrated to play an important function in human immune responses. TRIM5α has been identified as an antiretroviral molecule, which is important in innate immunity via two different mechanisms. Firstly, TRIM5α functions as a restriction factor capable of recognising and binding incoming retroviruses in cells (188). The antiviral specificity of TRIM5α is species specific, with the human protein being a strong inhibitor of N tropic MLV but only a

weak inhibitor of HIV-1 [179, 180]. As well as a restriction factor, TRIM5α has recently been identified as a PRR and affects gene expression. This is also true for the naturally occurring owl monkey TRIMCyp restriction factor (252).

TRIM21 functions by a different mechanism, bridging innate and humoral immunity. Unlike TRIM5α, whose B30.2 domain binds retroviral capsid and determines the specificity of its antiviral activity, in TRIM21 this domain acts as an IgG receptor (259, 265). It plays an antiviral role by binding antibody coated virus in the cytosol and targeting it for proteasomal degradation (266). TRIM21 also plays a role in innate immune signaling and is involved in IFN signaling through interaction with various IRFs.

As both of these TRIM proteins may play critical roles in normal antiviral immune responses, it is important that expression of the TRIMCyp fusion proteins does not cause any interference in their function, leaving transduced cells susceptible to infection from other pathogens or negatively affecting immune function.

TRIM proteins are known to form multimers, and that this is necessary for their normal function (223, 224, 228). There are several different splice variants of TRIM5, but only TRIM5α functions as an antiviral restriction factor, as it is the only full length protein that includes the B30.2 domain that is required for CA binding. However, the truncated transcripts, TRIM5δ and TRIM5γ, that terminate before the B30.2 can act as dominant negative proteins and downregulate TRIM5α antiviral activity by interaction via their coiled coil domains. A similar mechanism could occur between different splice variants of TRIM5 and TRIM5Cyp as all would include compatible coiled coil domains.

In addition to a possible physical interaction with endogenous TRIM proteins, as both TRIM5 and TRIM21 are involved in innate immune signaling, it is possible that expression of TRIMCyp proteins may affect normal signaling mediated by TRIM proteins within the cell.

In this chapter antiviral restriction mediated by TRIM5 and TRIM21 is measured in cells expressing the TRIMCyp transgenes to identify any interference that they may have with the endogenous proteins.

5.3 Production and titration of MLV

B and N tropic MLV vector carrying a YFP marker gene was made using the plasmids depicted in Figure 5.1A. Vector particles were titrated on CRFK cells. Titration of other vectors was performed on HEK293T cells, but because these cells are human derived they express TRIM5α and therefore restrict N-MLV, leading to a much lower titre than B-MLV. CRFK cells were transduced with serial dilutions of virus and YFP expression measured by flow cytometry 3 days later (Figure 5.1B). Titres were $7.9x10^5$ and $4.4x10^5$ IU/ml for B and N tropic MLV respectively.

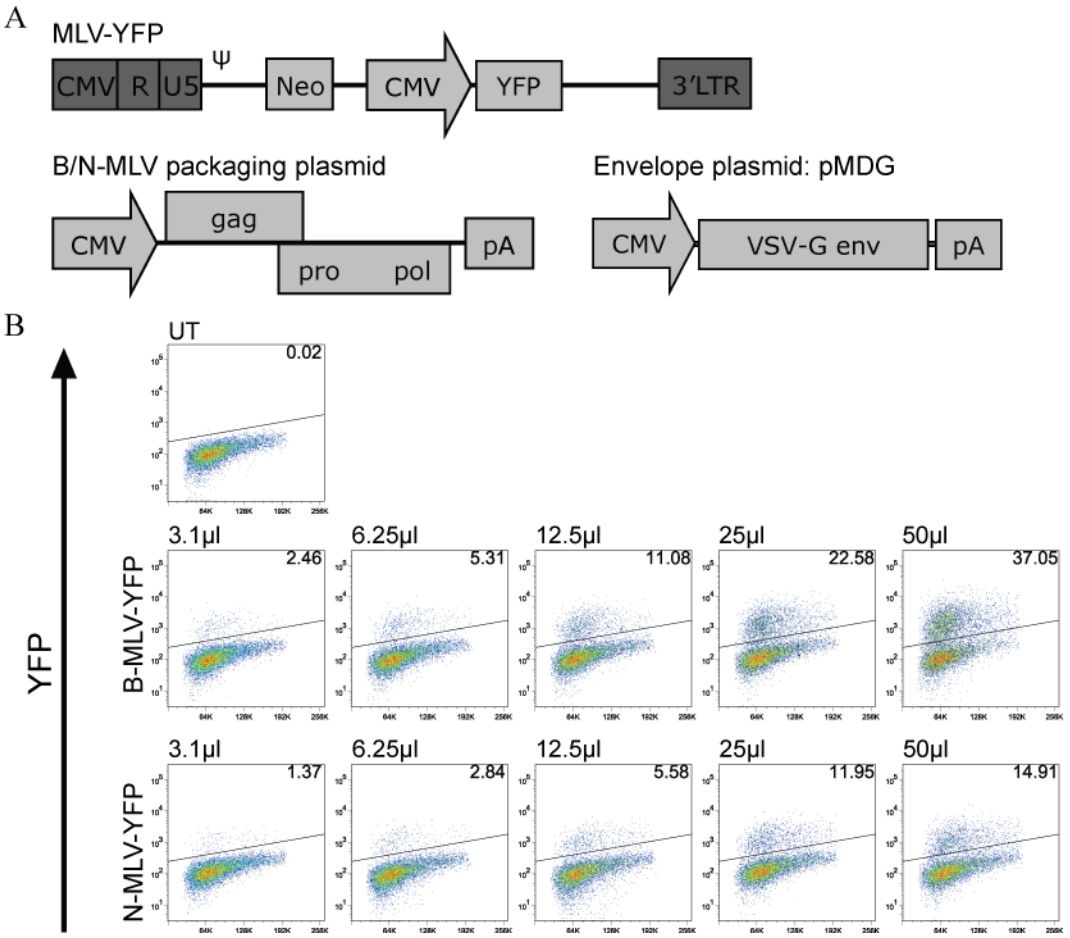


Figure 5.1 Production and titration of MLV
A. Plasmids used to produce murine leukaemia virus (MLV). The transfer plasmid contained a YFP marker gene under control of the Cytomegalovirus (CMV) promoter. The gag-pol packaging plasmid was either B or N tropic MLV. Virus was pseudotyped with VSV-G using envelope plasmid pMDG.
B. MLV-YFP titre was measured by transduction of CRFK cells with serial dilutions of MLV stocks. YFP positive cells were measured by flow cytometry 72 hours after transduction and titre in infectious units/millilitre calculated.

5.4 TRIM5α activity is disrupted by TRIM5Cyp, but not TRIM21Cyp

TRIM5α from different species restricts a distinct range of retroviruses. Often TRIM5α is unable to restrict viruses native to the same species, but is effective against viruses from other species. Therefore, although it does not significantly inhibit HIV-1 or -2, human TRIM5α is a strong inhibitor of N-MLV [179, 180]. It may be important that this function of TRIM5 is not disrupted by introduction of the TRIM5Cyp transgene in modified cells. To investigate whether there is an interaction between TRIMCyp and endogenous TRIM5α, the ability of TRIM5α to restrict N-MLV in TRIMCyp expressing cells was quantified. N-MLV restriction was used as a measure of TRIM5α function.

TE671 cells, a human cell line which expresses TRIM5α and can therefore restrict N tropic, but not B-MLV, were transduced with SIEW (GFP), TRIM5Cyp or TRIM21Cyp vectors to obtain over 95% of cells eGFP positive. These cells were then challenged with HIV-1 (MOI of 5), B-MLV or N-MLV (MOI of 2), all carrying a YFP marker gene. Cells were treated with 1000U/ml IFNβ at the time of seeding. 72 hours following transduction, YFP positive cells were quantified by flow cytometry after gating on eGFP positive cells (Figure 5.2).

Similar to results in CRFK cells in chapter 3, HIV-1 was strongly restricted approximately 100 fold in TRIM5Cyp and TRIM21Cyp expressing cells, compared to untransduced and GFP transduced control cells (Figure 5.2A). There is slightly stronger restriction conferred by TRIM21Cyp than TRIM5Cyp ($P=<0.05$) in this experiment.

B-MLV was able to transduce all cell populations at an equal level (Figure 5.2C). Treatment with IFNβ caused a minor reduction in transduction across groups, though the percentage of transduced cells remained high.

N-MLV was restricted in untransduced and GFP transduced cells, with nearly 100 fold lower transduction of this virus compared to unrestricted B-MLV (Figure 5.2B). The same decrease in transduction was seen in TRIM21Cyp expressing cells. However, in cells expressing TRIM5Cyp the level of transduction by N-MLV was

similar to that of unrestricted B-MLV (17% for N-MLV and 20% for B-MLV) suggesting that there is no N-MLV restriction, and was significantly higher than in the other three cell populations (P=<0.0001). This suggests that restriction of N-MLV by endogenous TRIM5α is abrogated by co-expression of TRIM5Cyp.
Even after treatment with IFNβ to upregulate endogenous TRIM5α expression, TRIM5α restriction of N-MLV could not be seen in TRIM5Cyp cells. In other cell types, IFNβ treatment led to complete restriction of N-MLV with the percentage of YFP positive cells not reaching above background levels.

Recently, TRIM5α and primate TRIM5Cyp were shown to be involved in activating AP-1 and NFκB signaling (252) which may be involved in a negative feedback loop, leading to downregulation of TRIM5α expression. Therefore, qRT-PCR was performed to assess whether the increase in N-MLV transduction in TRIM5Cyp expressing cells was due to differences in TRIM5 expression levels. RNA was extracted from TE671 transduced cells and reverse transcribed. qRT-PCR was performed to measure the relative levels of expression of both TRIM5 and TRIM21 in these cells (Figure 5.3). The primer sets used for these reactions bound to either the TRIM5 or TRIM21 cDNA at the 3' end in a region that is not present in the TRIMCyp fusion proteins to avoid detection of the transgene.
In the two experiments performed, co-expression of TRIM5Cyp in cells caused a very slight increase in TRIM5 expression, but additional repeats would be required to confirm that this increase is consistent upon TRIM5Cyp expression. There did not appear to be a noticeable decrease in TRIM5 expression, which could have explained the loss of N-MLV restriction seen upon TRIM5Cyp expression. Therefore the increase in N-MLV infection in TRIM5Cyp expressing cells is unlikely to be due to a loss of TRIM5α expression, but through titration of active homodimers into inactive TRIM5α/TRIM5Cyp heterodimers.

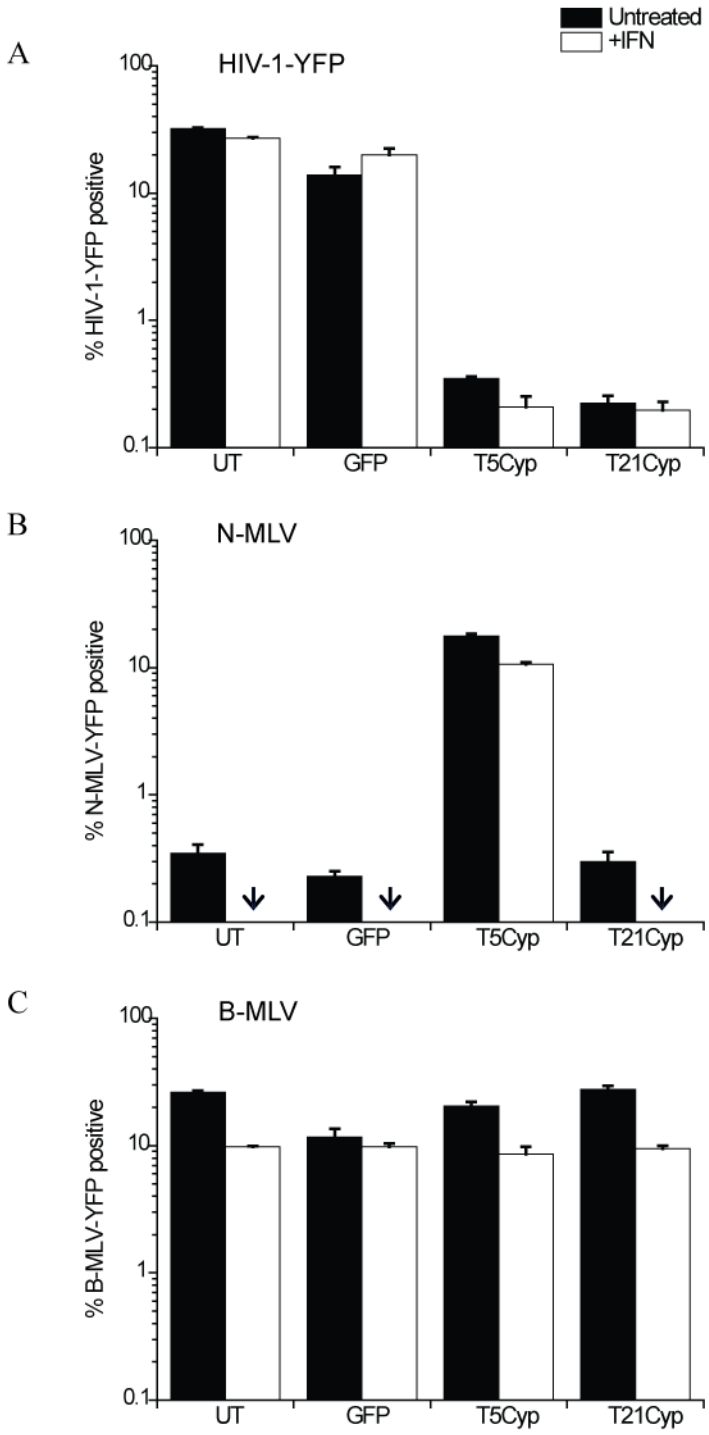


Figure 5.2 Restriction of retrovirus in cell lines
TE671 cells transduced with SIEW (GFP), TRIM5Cyp or TRIM21Cyp vectors and untransduced (UT) control cells were cultured overnight in the presence or absence of interferon-β (IFN). Cells were then challenged with HIV-1 (A), N-MLV (B) or B-MLV (C) vectors encoding YFP. eGFP and YFP co-expression was measured by flow cytometry 72 hours after transduction. Arrows in B indicate samples with YFP positive percentages below background levels. Samples were performed in triplicate, error bars show standard error of the mean.

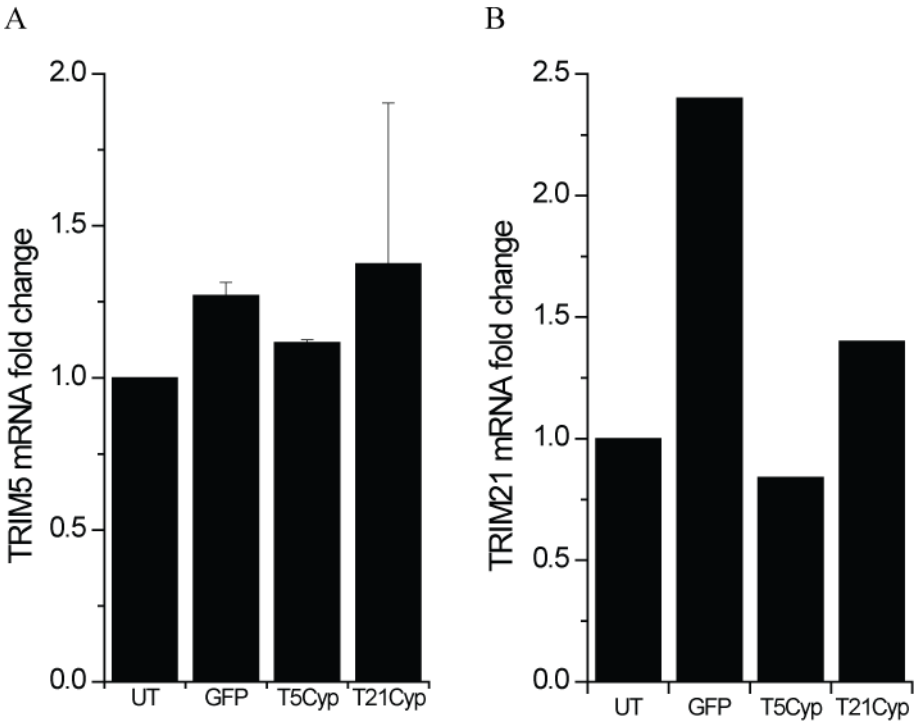


Figure 5.3 Endogenous TRIM expression in TE671 cells
TE671 cells transduced with SIEW (GFP) or TRIMCyp vectors and untransduced (UT) control cells were harvested and RNA extracted using Trizol before reverse transcription. Quantitative PCR was performed to measure TRIM5 (A) and TRIM21 (B) expression. TRIM5 data is from two experiments and error bars show standard deviation, TRIM21 data from one experiment.

5.5 TRIM21 activity is maintained in TRIMCyp expressing cells

Recently, TRIM21 has been shown to act as an intracellular receptor for opsonised pathogens by binding the IgG Fc region with very high affinity and then targeting them for proteasomal degradation via its RING domain (266). The importance of TRIM21 mediated restriction *in vivo* is not known and interfering with the native function of TRIM21 could leave an individual susceptible to viral infections.

Therefore, the ability of TRIM21 to restrict antibody coated adenovirus particles in cells transduced to express TRIMCyp was assessed. Following the protocol described by Mallery et al, adenovirus encoding GFP (AdV-GFP) was incubated with increasing concentrations of a polyclonal anti-adenoviral hexon antibody. This opsonised virus was then used to infect HeLa cells which had been seeded the previous day with or without IFNα. Interferon treatment has been shown to cause an increase in TRIM21 expression (262). After 48 hours, GFP expression was measured by flow cytometry. Untransduced (UT) HeLa cells were compared to cells transduced with TRIM5Cyp and TRIM21Cyp vectors without GFP (Figure 5.4).

Incubation of adenovirus with anti-hexon IgG prior to infection caused a decrease in AdV-GFP infection, although even at the highest concentration, GFP expression was only reduced to 20% of the no antibody control in which there was no viral restriction. Treatment with IFNα caused no significant change to adenoviral restriction. In published work, HeLa cells were transduced to express high levels of TRIM21, which resulted in potent restriction of adenoviral infection. In experiments described here, adenoviral restriction was reliant on endogenous TRIM21 which, even after IFNα treatment, was not sufficient to mediate very strong restriction. As restriction was mild, it was more difficult to identify any potential differences in restriction between untransduced and TRIM21Cyp transduced cells.

Following this, a protocol optimised by a post-doctoral researcher in the lab, Dr. Choon Ping Tan, was tested. A different antibody was used and incubated with adenovirus in a greater total volume before infection. This led to a stronger restriction of adenoviral infection. AdV-GFP incubated with this alternate antibody

was specifically restricted by TRIM21, as shown by the rescue of infection after TRIM21 depletion by TRIM21 shRNA expression (Figure 5.5A). Figure 5.5B shows the specificity of shRNA causing a decrease in TRIM21 levels. Figures 5.5A and B were kindly provided by Dr. Choon Ping Tan.

Using this modified protocol, the experiment was repeated and resulted in a greater decrease in infection with increasing antibody concentration (Figure 5.5C). In all cells, IFNα treatment enhanced adenoviral restriction, presumably by upregulating TRIM21 expression. At the highest antibody concentration all cells incubated with IFNα reduced GFP expression to around 5% of the no antibody control, compared to 20% with the original method. There was no significant difference in restriction of adenovirus in TRIM21Cyp expressing cells compared to control cells.

TRIM21 expression measured by qRT-PCR was shown to be maintained at a similar level in cells expressing either of the TRIMCyp proteins (Figure 5.6). This reflects the results that show TRIM21 mediated antiviral activity is maintained upon TRIMCyp expression. There appeared to be some upregulation of TRIM5 expression in cells co-expressing TRIM5Cyp. Again, experiments were performed only once, and must be repeated for more accurate measurements.

Therefore, cells expressing either TRIM5Cyp or TRIM21Cyp are capable of efficient restriction of IgG coated adenovirus at levels equal to untransduced control cells suggesting that endogenous TRIM21 function has remained intact.

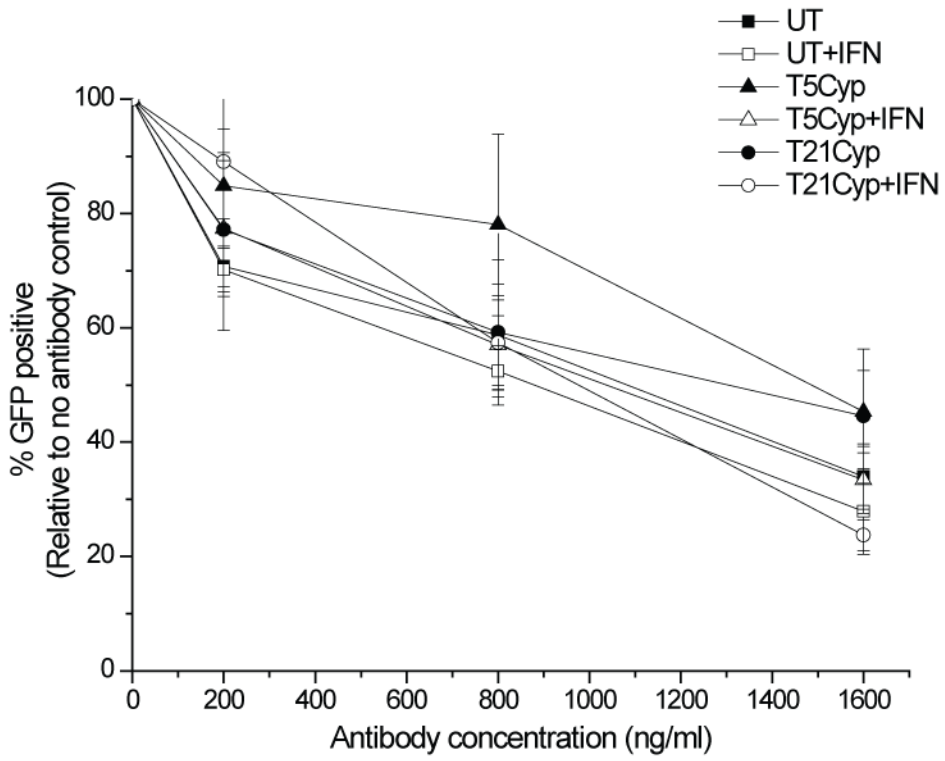


Figure 5.4 Adenoviral neutralisation by TRIM21
Adenovirus-GFP was incubated with anti-Adenovirus antibody at increasing concentrations and then used to infect untransduced (UT) HeLa cells or those expressing TRIMCyp. GFP expression was measured by flow cytometry 48 hours later. All samples were performed in triplicate, error bars represent the standard error of the mean.

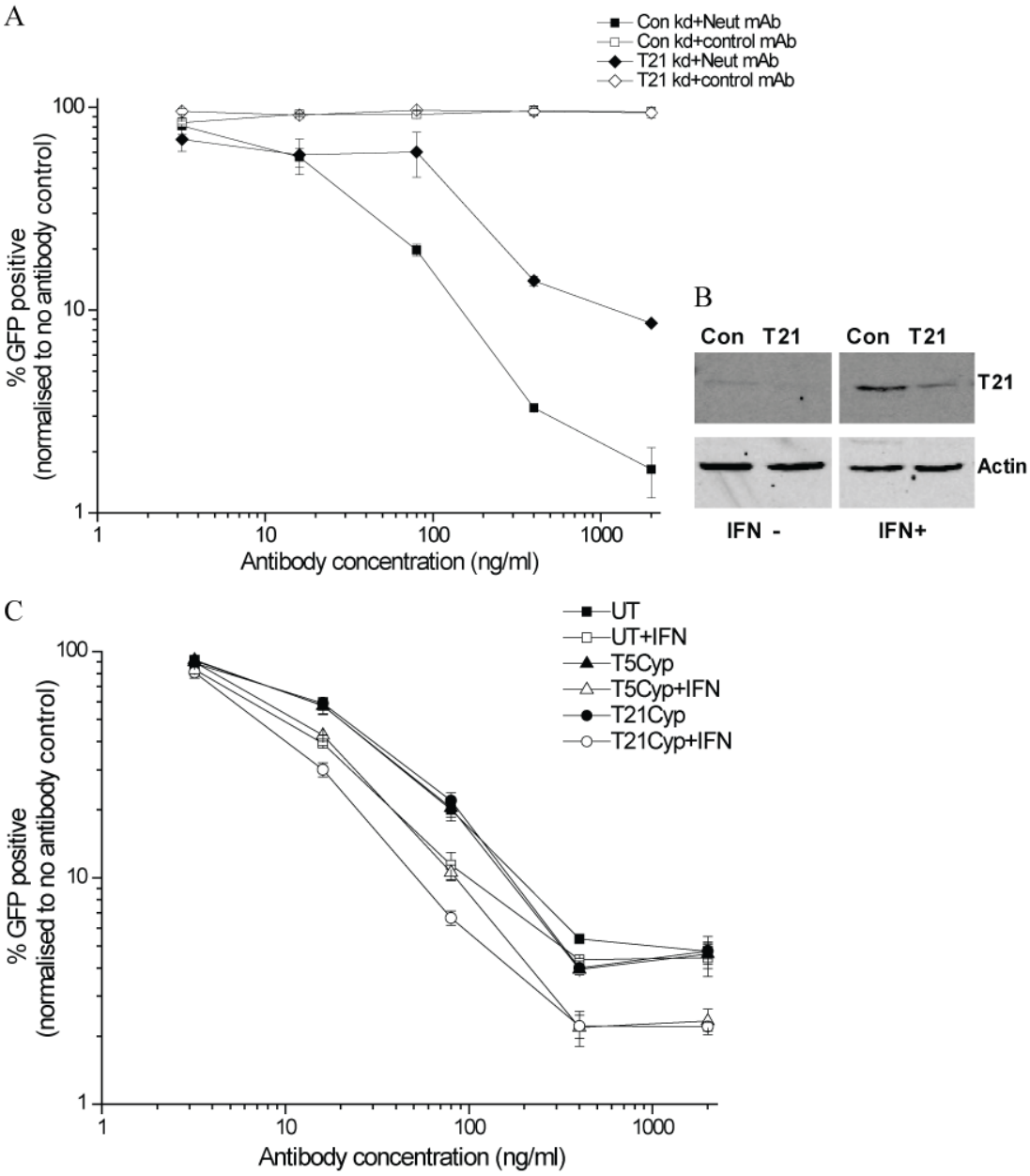


Figure 5.5 TRIM21 mediated neutralisation of adenovirus using an optimised protocol
The adenoviral neutralisation assay was repeated using a modified protocol and an alternative anti-adenoviral antibody both provided by collaborators at UCL. A. HeLa cells with stable knockdown of TRIM21 (T21 kd) or control cells with non-targeting shRNA (Con kd) were infected with adenovirus-GFP incubated with neutralising antibody (Neut mAb) or isotype matched control (control mAb). Infection was quantified by GFP expression 48 hours later by flow cytometry.
B. Western blot showing TRIM21 (T21) expression following interferon-α (IFN) stimulation and expression of either control (Con) or T21 shRNA.
C. HeLa cells transduced with TRIM5Cyp or TRIM21Cyp vectors and untransduced (UT) control cells were cultured overnight in the presence or absence of interferon-α (IFN). Adenovirus-GFP was incubated with increasing concentrations of the neutralising antibody tested in A and used to challenge HeLa cells. GFP expression was measured by flow cytometry 48 hours later. Samples were performed in triplicate, error bars represent the standard error of the mean.
Data in panels A and B provided by Dr. Choon Ping Tan.

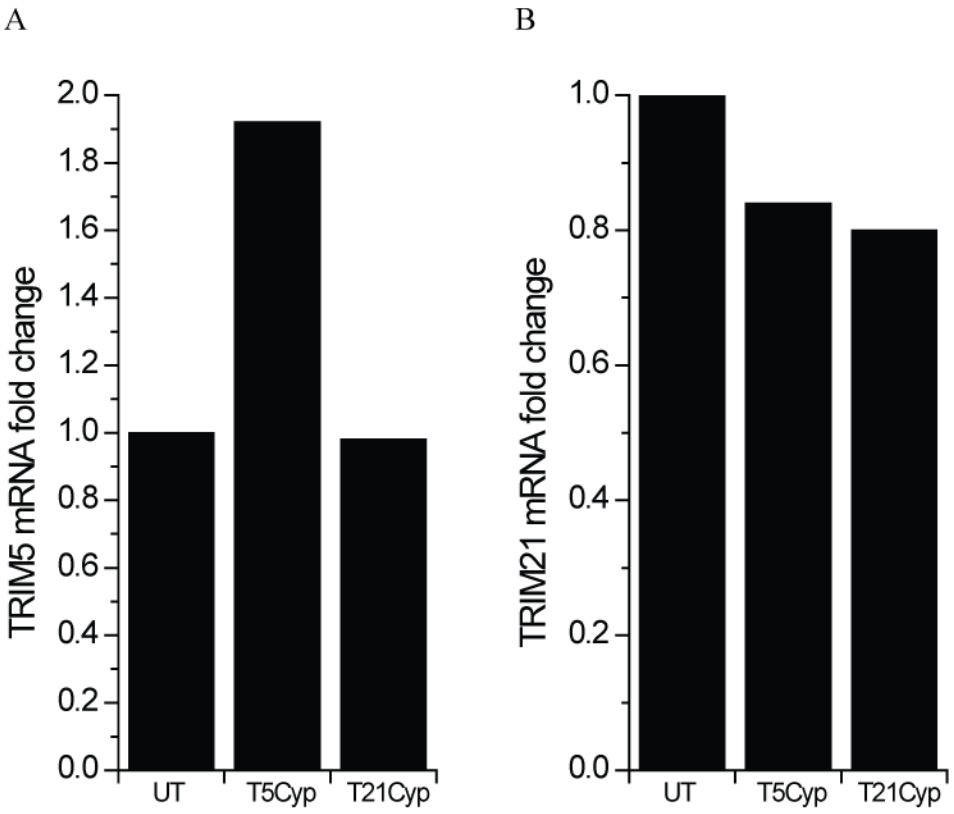


Figure 5.6 Endogenous TRIM expression in HeLa cells
HeLa cells transduced with TRIMCyp vectors devoid of GFP or untransduced (UT) control cells were harvested and RNA extracted using Trizol before reverse transcription. Quantitative PCR was performed to measure TRIM5 (A) and TRIM21 (B) expression. Data from one experiment only.

5.6 Summary

Although *in vitro* experiments presented in chapters 3 and 4 have shown that both TRIM5Cyp and TRIM21Cyp are strong inhibitors of HIV-1, it is important that their expression does not affect normal functioning of transduced cells. In this chapter experiments have been carried out to test whether there is any interaction between TRIMCyp and the endogenous TRIM proteins that interferes with their function. Both TRIM5 and TRIM21 play complex roles in the immune system, the full importance of which are still emerging. Therefore there could potentially be problems with therapy if TRIMCyp proteins interact with endogenous TRIM proteins and disturb their function.

TRIM5α restricts retrovirus in a species specific pattern, with the human protein potently restricting N-MLV, but not HIV-1 or B-MLV (180). This is seen here in the human TE671 cell line; untransduced and GFP transduced control cells are easily transduced with both B-MLV and HIV-1, but N-MLV is strongly restricted. Despite typically low basal levels of TRIM5α expression and expression being IFN inducible (211), untreated cells were still able to mediate effective restriction of N-MLV. Restriction was further enhanced upon IFNβ treatment of cells.

In contrast, N-MLV infection is rescued in TRIM5Cyp expressing cells. RT-PCR shows that there is no difference in TRIM5 expression levels in these transduced cells compared to controls. Another possible explanation for the loss of restriction is the dimerisation of TRIM5Cyp with TRIM5α resulting in a non-functional protein complex. TRIM5δ and γ, the splice variants that lack the B30.2 domain, are able to form multimers with TRIM5α which are non-restrictive, acting as a dominant negative protein (182, 192). Similarly, expression of rhTRIM5α or omTRIMCyp in human cells interferes with the restrictive abilities of huTRIM5α against N-MLV, due to the different C termini, which confers the antiviral specificities of the protein (384). Presumably expression of humanised TRIM5Cyp facilitates the formation of non-functional dimers with endogenous TRIM5 as both proteins include compatible coiled coil domains, which are required for dimerisation (205). In cells expressing

TRIM21Cyp, N-MLV restriction is equal to the control cells, indicating that TRIM21Cyp does not cause any interference with TRIM5α mediated restriction. This is supported by the fact that different TRIM proteins rarely heterodimerise with each other (205).

Conversely, restriction of HIV-1 by TRIMCyp was also measured in these cells to see if there was any rescue of HIV-1 infection by endogenous TRIM5α. However, even with TRIM5α upregulation by IFN treatment, there was no detrimental effect to TRIM5Cyp or TRIM21Cyp restriction of HIV-1. It is likely that high levels of TRIMCyp were expressed from the SFFV promoter possibly from multiple copies per cell, so could not be saturated by heterodimerising with endogenous TRIM5α.

To confirm this multimerisation of TRIM and TRIMCyp proteins, transduced cells could be treated with glutaraldehyde which crosslinks and stabilise multimers (232). Multimers could be studied by Western blotting to look for complexes of TRIM and TRIMCyp. However, using the anti-Cyp antibody in previous Western blots here did not produce very clear bands, so this method would probably be more effective using tagged proteins.

Rhesus macaques express different TRIM5α alleles, including a TRIM5Cyp fusion protein. If these different alleles are co-expressed within a cell they illicit a dominant negative effect on each other. It is speculated that high expression levels of protein in this *in vitro* system over exaggerates these dominant negative effects caused by heteromultimerisation. In TRIM5α heterozygotes, when there are more equal levels of expression of different TRIM5α alleles, they may act in a co-dominant way (193). This co-dominance is seen with different Fv1 alleles in mice and heterozygote cell lines, and the dominant negative effect of certain Fv1 alleles only occurs upon overexpression (180). Therefore if heterodimerisation is the cause of the dominant negative effect of TRIM5Cyp on endogenous TRIM5α, it could possibly be avoided or reduced by lower expression levels more equal to that of endogenous TRIM5. The promoter used in the vectors here was the highly active and constitutive SFFV promoter, but expression of the transgene from the endogenous TRIM5 promoter would produce more physiological levels of expression. As the TRIM5 promoter is IFN stimulated, both endogenous TRIM5 and transgenic TRIMCyp expression

would be high upon viral infection. Limiting upregulation of expression to respond to IFN stimulation would prevent high levels of background expression.

Restriction factors are important in preventing cross-species transmission of retroviruses. HuTRIM5α has been shown to restrict N-MLV *in vitro*, and so it may be involved in protecting humans from retroviral infection. Although viruses such as the recently described xenotropic MLV-related virus (XMRV) have a CA sequence highly homologous to B-MLV and are not restricted by huTRIM5α (385), until the role of this restriction factor is more clearly understood, the consequences of its disruption by expression of TRIM5Cyp are not known.

As well as binding and restricting retroviruses, huTRIM5α, and TRIM5Cyp, are proving to be important in immune signaling (252). TRIM5α and TRIM5Cyp promote NFκB signaling via TAK1 which may lead to negative feedback and downregulation of TRIM5α expression, consequently abrogating N-MLV restriction. Results shown here suggest that TRIMCyp does not affect the expression levels of endogenous TRIM5 thereby explaining the loss of N-MLV restriction.

Throughout any gene therapy treatment, it is important that the role of TRIM5α in innate immune signaling is also maintained. This could be investigated by following protocols from Pertel et al (252) in which NFκB and AP-1 signaling is monitored by using luciferase reporter plasmids. These reporters could be transfected into cells expressing TRIM and TRIMCyp, either together or individually, and luciferase activity quantified. This will determine whether the artificial humanised TRIM5Cyp, like owl monkey TRIM5Cyp, can mediate NFκB signaling and whether signaling is further enhanced by retroviral CA recognition, as is the case with TRIM5α. It will also be interesting to investigate whether TRIM21Cyp mediates NFκB immune signaling in response to viral recognition. TRIMCyp mediated activation of innate immune responses upon viral infection could be beneficial and influence infection and disease progression.

Owl monkeys only have the TRIM5Cyp allele and no other TRIM5 alleles (315), suggesting that primates can function normally in the absence of a TRIM5 protein. Rhesus macaques may also be heterozygous for TRIMCyp, having one TRIMCyp

and one TRIM5 allele. Therefore, interference of TRIM5Cyp with endogenous TRIM5 function may not actually prevent normal functionality of the cell.

The TRIM21 mediated restriction of antibody coated adenovirus was tested using the experimental protocol from Mallery et al (266). However, this group used HeLa cells that were transduced to express high levels of TRIM21 and therefore experienced much higher levels of viral restriction. However, when just using endogenously expressed TRIM21 and the published protocol, adenoviral restriction was low. Therefore a modified protocol provided by collaborators at UCL was used, in which larger volumes of a different antibody were used. This resulted in much higher levels of restriction, which increased with increasing concentrations of antibody. Also in this final assay, crude preparations, rather than caesium chloride purified adenovirus, were used. It is possible that the purification method may affect epitopes recognised by the restricting antibody resulting in less efficient antibody recognition and therefore restriction.

In both experimental setups there was no difference in restriction of opsonised adenovirus between untransduced and TRIMCyp expressing cells. Similarly there was no difference in endogenous TRIM expression in the different cells. IFNα treatment significantly enhanced adenovirus restriction in all cells using the modified protocol.

TRIM21 is also known to be intricately involved in IFN signaling through interaction with several members of the IRF family. It is important that both the direct antiviral activity of TRIM21 against adenovirus and its role in signaling are maintained in modified cells. Although expression of TRIMCyp did not interfere with restriction of opsonised adenovirus, effects on IFN signaling were not investigated. IFN responses measured by using an IFNβ luciferase reporter or qRT-PCR of IFNβ responsive genes, such as IFNβ, ISG56 and RANTES, could be compared in untransduced and TRIM21Cyp expressing cells.

As well as interference with IFN signaling, another potential problem with the use of TRIM21Cyp is that TRIM21 is the autoantigen in the autoimmune disease Sjogren's disease. The antibody target in this disease is found in the B30.2 domain, which has

been removed in the TRIM21Cyp construct. However, all healthy individuals possess auto-antibodies against regions throughout the TRIM21 protein, the most antigenic being the B30.2 domain, but also the coiled coil domain, which remains in the TRIM21Cyp fusion protein. In contrast, TRIM5 is not associated with autoimmunity (386). It is important that overexpression of this domain in the TRIM21Cyp protein does not lead to autoimmune pathology.

The reason for the detrimental interaction of TRIM5Cyp with TRIM5α, but for no detectable effect on TRIM21 restriction may be due to differences in the stoichiometry of the binding of each TRIM B30.2 domain with its target. TRIM21 binds IgG Fc with high affinity at a stoichiometry of one TRIM21 molecule to one Fc fragment (266). In contrast, TRIM5α binds N-MLV CA in multimers, the formation of which is likely to be interrupted by the presence of TRIM5Cyp, which maintains the TRIM5 domains required for dimerisation and high order multimerisation, namely the coiled coil and B-Box2 respectively, but differs at the C terminus with the presence of a CypA moiety.

In summary, there is no significant difference between restriction of HIV-1 provided by TRIM5Cyp or TRIM21Cyp. Therefore study of the interaction and interference with endogenous TRIM proteins may provide reasons to use one restriction factor over another. Results shown here suggest that TRIM21Cyp may have an advantage as a therapeutic gene as its expression does not interfere with endogenous TRIM function whilst still providing potent restriction of HIV-1.

6 Site specific integration of TRIMCyp and disruption of CCR5 using zinc finger nucleases

6.1 Aims

- To produce CCR5 specific ZFNs and donor templates in a non-integrating lentiviral vector (NILV)
- To modify the CCR5 gene using NILV-ZFNs in cell lines
- To introduce TRIMCyp into the CCR5 locus in cell lines by homology directed repair
- To compare restriction of HIV-1 in cell lines modified with ZFNs and expressing TRIMCyp constructs compared to either method alone

6.2 Introduction

CCR5 is the primary co-receptor used for entry of HIV-1 alongside CD4 (5-9). Homozygotes for the CCR5 Δ32 mutation are highly resistant to HIV-1 infection, even after repeated exposure to virus (332, 333). This has resulted in substantial interest in targeting CCR5 expression as a means of anti-HIV-1 therapy. In addition, CCR5 Δ32 homozygotes do not have any significant adverse phenotypes, suggesting that this locus is ideal as a safe harbour for site specific integration as there is no pathology associated with its loss. The potential of CCR5 as an HIV therapeutic target, and the feasibility of reconstituting a patient's immune system with resistant cells has been demonstrated by a single case in which an HSC transplant of an HIV-1 patient with cells from a CCR5 Δ32 homozygous donor led to the eradication of the virus (339). Despite the success of this first example of a functional cure of HIV-1, it would not be possible to routinely treat infected individuals with HSC

transplant. CCR5 Δ32 donors are rare with only approximately 1% of the Caucasian population being homozygous for the allele, and below this in Western and central Africa (333). The risks and high mortality rate associated with allogenic transplant reduce wider applicability. However, it could be possible to replicate the phenotype seen in Δ32 individuals by knockout of CCR5 using ZFNs in a patient's own cells.

The biopharmaceutical company Sangamo has produced ZFNs that specifically target and disrupt CCR5. Each ZFN of the pair contain four ZF binding motifs recognising a 12bp sequence. In total, the ZFN pair recognises a 24bp sequence in the first transmembrane region of CCR5 (369). These ZFNs have been tested in a variety of cell types and animal models and have been shown to knockout CCR5 expression and consequently restrict R5 tropic HIV-1 infection (369, 370). These ZFNs are also currently in a phase I/II clinical trial in which autologous T cells have been modified *ex vivo* by CCR5 specific ZFNs delivered via an adenoviral vector. So far, preliminary results from this trial have been promising; the procedure has been well tolerated by patients and modified cells function normally. In addition they show some cases of successful engraftment of modified cells, and an increase in T cell numbers (135). In this trial, adenoviral vectors were used to deliver the ZFNs as they do not integrate into the genome, thereby providing only transient expression of the two ZFN genes. Continuous expression of the genes and presence of the ZFNs would promote toxicity by increasing the likelihood of off target cleavage. Adenoviral vectors are used in both research and clinical trials. However, a major problem with this delivery method is that most people have pre-existing immunity against adenovirus and vectors induce both innate and adaptive immune responses (387). An alternative delivery method to adenoviral vectors to provide transient ZFN expression is to use non-integrating lentivirus (NILV).

After cell entry, lentiviruses and their derived vectors undergo uncoating and reverse transcription within the cytoplasm. Reverse transcription of the viral RNA genome results in a double stranded DNA molecule capable of integration into the host

genome. Normally, this linear strand of DNA is transported to the nucleus where the viral IN protein mediates integration.

However not all DNA is integrated and there is accumulation of circularised viral DNA in two different forms: 1-LTR circles formed by homologous recombination (388) and 2-LTR circles due to NHEJ (389). This occurs in normal lentiviral infection, but can be enhanced by introducing mutations in the IN gene. As well as integration, IN also plays a role in reverse transcription and transport of the PIC into the nucleus. IN mutations can be grouped into two categories depending upon the effect of the mutation. Class I mutations solely affect the ability of IN to promote viral DNA integration, whereas class II mutations affect all IN functions including reverse transcription and nuclear import and therefore would be of no use in the development of vectors. The catalytic core of IN contains a highly conserved amino acid motif, D,D-35-E, which consists of two aspartic acids and one glutamic acid with a 35 amino acid region between the second and third residues. Alteration of any of these three residues disrupts integration and significantly reduces provirus formation. The D64V mutation is commonly used to produce NILV as it reduces integration by about four logs, whilst maintaining a high viral titre (390, 391).

The use of NILV greatly improves the safety of gene therapy; integration, and consequently the likelihood of insertional mutagenesis, is greatly reduced. NILV are useful tools for transient gene expression in dividing cells, as episomal DNA is progressively diluted out through cell division. Alternatively, NILVs are able to transduce similar post-mitotic target cells as integrating lentivirus, for example in the brain and retinal tissue, where they can support continued gene expression (392, 393).

Despite this, there is evidence to suggest that expression from NILV is not as high as that from integrating vectors, which could be a problem in particular therapeutic circumstances (394). However, they could still be appropriate to deliver ZFNs, as only transient expression is sufficient to cause a permanent disruption of the target gene, which is passed on to the cell's progeny.

Site specific integration of a transgene is the ideal in gene therapy. However, performing this by homologous recombination is too inefficient to be of therapeutic benefit. Introduction of a DSB within the genome increases the likelihood of HDR of a DSB three or four fold, and this can be further enhanced by the presence of a large amount of donor template. This is the rationale behind ZFN technology; transient expression of ZFNs causes a DSB which is repaired by HDR using a donor template delivered to the cells in a high concentration.

CCR5 Δ32 homozygotes do not express any functional protein but do not suffer any detrimental consequences. Therefore CCR5 specific ZFNs could be used to insert an array of different transgenes at the CCR5 locus, utilising it as a safe harbour site to avoid insertional mutagenesis. It could be possible to use this technology to insert a gene encoding an anti-HIV restriction factor at this specific locus in the host genome. This would be additionally beneficial as not only would the restriction factor gene be integrated at a safe location to reduce insertional mutagenesis, but by knockout of CCR5 expression, the modified cells would be protected from cell entry by R5 tropic HIV-1. This would provide a dual mode of protection for modified cells and confer resistance to both R5 and X4 tropic strains of HIV-1.

Here CCR5 specific ZFNs have been delivered to cells using NILV alongside two donor templates, one encoding GFP as a marker and the other TRIM21Cyp to provide dual protection against HIV infection.

6.3 Production of ZFNs and CCR5 donor lentivirus

Sangamo provided heterodimerising ZFNs targeting the CCR5 locus and two CCR5 donor template plasmids; one consisting of PGK promoter and GFP transgene flanked by CCR5 homology arms (CCR5-PGK-GFP) and the second a BglI restriction site flanked by CCR5 homology arms (CCR5-BglI). This second donor plasmid allows the construction of alternative donor templates by cloning genes into the BglI site, which can then be integrated into the CCR5 locus by HDR. Here the codon optimised TRIM21Cyp under control of the PGK promoter was cloned into the BglI site (PGK-TRIM21CypCO). To assist in cloning of this second donor, a plasmid was generated consisting of the PGK promoter driving TRIM21Cyp expression, with appropriate restriction sites. This was performed by Geneart, who used their in house GeneOptimizer software to produce a codon optimised TRIM21Cyp (TRIM21CypCO) transgene (see Chapter 3.9).

The two ZFNs, EL and KK, were cloned separately into lentiviral backbones under control of the SFFV promoter (Figure 6.1A). The CCR5 flanked PGK-GFP donor and PGK-TRIM21CypCO donor were also cloned into a lentiviral backbone (Figure 6.1B and C). As there was a polyA signal at the end of the TRIM21CypCO transgene, inserts were cloned between the CCR5 homology arms in the reverse orientation. This was to avoid premature termination of transcription at the polyA, allowing production of genomic viral RNA. All four plasmids were used to make non-integrating lentivirus using the IN mutant D64V and pMDG.

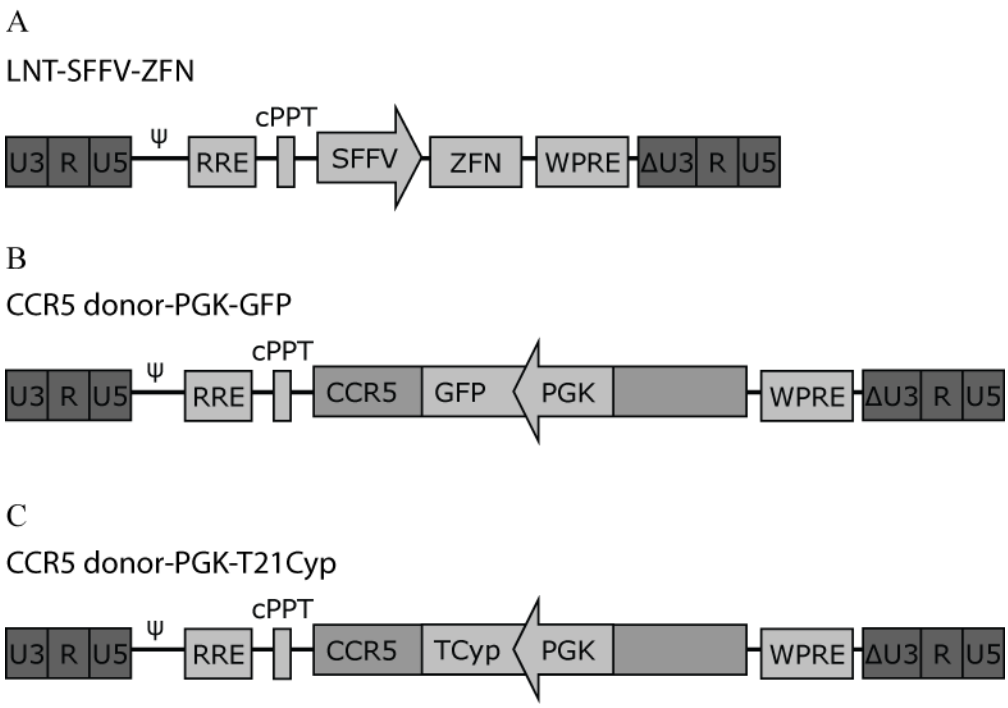


Figure 6.1 Plasmids used to generate ZFN and donor template non-integrating lentiviral vector (NILV)

A. CCR5 specific zinc finger nucleases (ZFNs) were cloned into pLNT/SFFV-MCS, with each member of the pair in a separate plasmid.

Donor templates for homology directed repair expressing a green fluorescent protein (GFP) gene (B) or a codon optimised TRIM21Cyp transgene (TCyp) (C) under control of the human phosphoglycerate kinase (PGK) promoter, flanked by CCR5 sequence homologous to the ZFN target site. These plasmids were used to generate non-integrating lentiviral vector using the integrase deficient gag-pol packaging plasmid (D64V) and pseudotyped with VSV-G. SFFV=spleen focus-forming virus, WPRE=Woodchuck hepatitis virus post transcriptional regulatory element, RRE=rev response element, cPPT=central polypurine tract

6.4 ZFNs knockout CCR5 in GHOST cells

The two different ZFNs have distinct DNA recognition sites and they bind opposing DNA strands, allowing their FokI nuclease domains to dimerise and cause a DSB in the DNA.

GHOST-CCR5 is a human osteosarcoma cell line that has been transduced with MLV vectors to express high levels of CD4 and CCR5 to allow infection by R5 tropic HIV-1. These GHOST cells contain on average four copies of CCR5 cDNA so for cells to become CCR5 negative it requires targeting of all copies within the cell.

Transduction of GHOST-CCR5 cells with either of the individual ZFN NILV does not cause any loss of CCR5 expression (Figure 6.2A), which supports the obligate heterodimeric nature of these ZFNs. Transduction of GHOST cells with both ZFNs resulted in a loss of CCR5 over time and five days after transduction the level of CCR5 expression reached a stable low point of approximately 20% of cells (Figure 6.2B). CCR5 was measured by staining with an anti-CCR5 antibody conjugated to APC-Cy7 and flow cytometry

The percentage of cells in which CCR5 was knocked out could be increased by the addition of increasing volumes of ZFN NILV. CCR5 expression was measured seven days post transduction (Figure 6.2C and D). Therefore, in all subsequent experiments, 10µl of each ZFN NILV were used unless otherwise stated.

The physical titre of the vector preparations was subsequently determined by p24 ELISA. The two ZFN vectors EL and KK had titres of $5.7x10^7$ and $1.3x10^7$ pg/ml respectively. Therefore as equal volumes of the two ZFNs had been added, the total number of virus particles was different. Addition of equal titres of each ZFN may improve the efficiency of targeting.

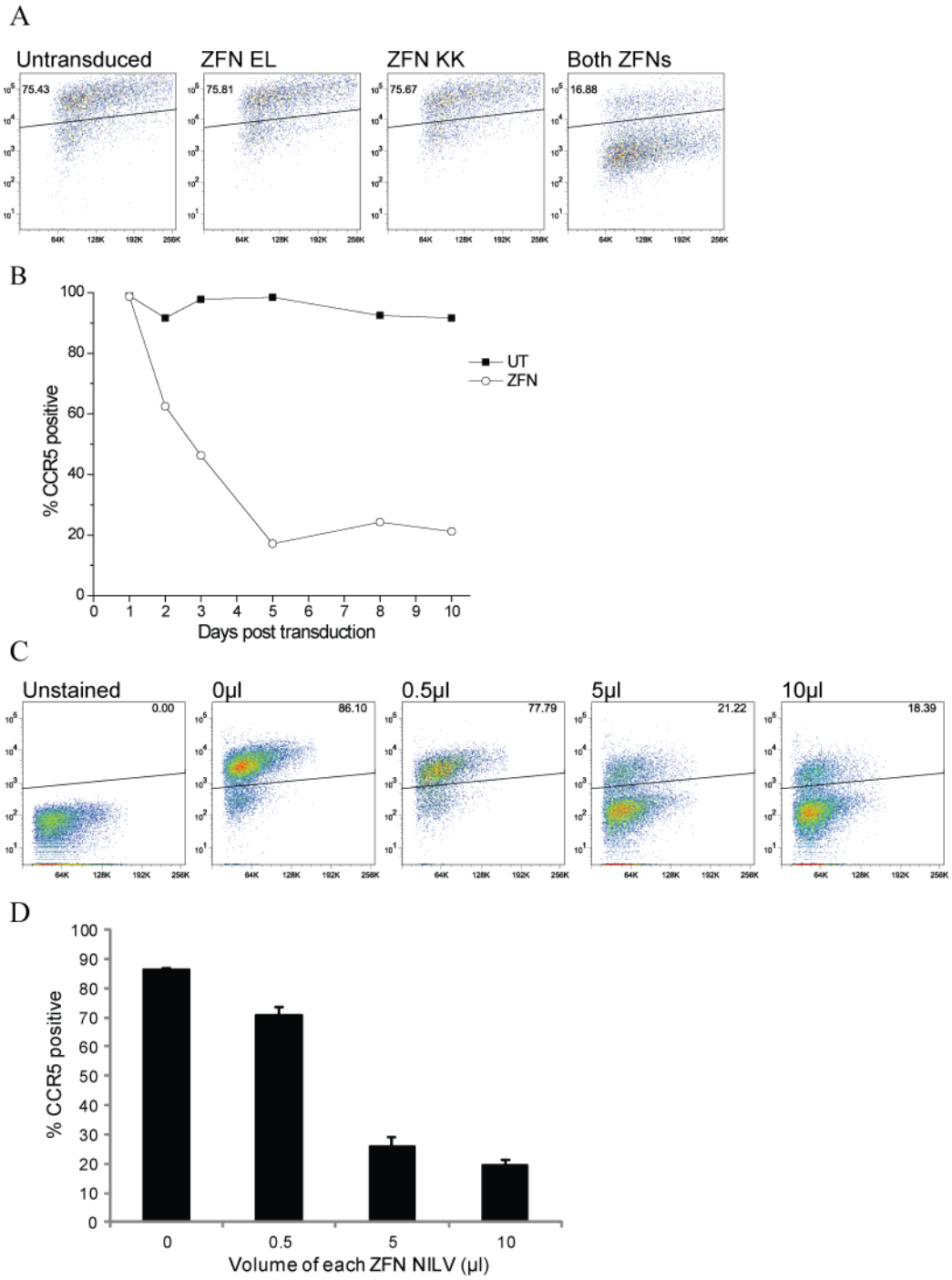


Figure 6.2 ZFNs lead to knock down of CCR5 in cell lines

A. GHOST-CCR5 cells transduced with individual ZFNs and stained for CCR5, which was measured by flow cytometry.

B. GHOST-CCR5 cells were transduced with ZFNs and measured for cell surface CCR5 at regular intervals for 10 days post transduction. Data is representative of two experiments.

GHOST-CCR5 cells were transduced with increasing volumes of the pair of ZFNs. Cells were cultured for 14 days, before staining for CCR5 and measuring by flow cytometry. Examples plots are shown in C. The experiment was performed on four independent samples shown in D. Error bars represent the standard error of the mean.

6.5 Site specific integration of GFP in GHOST cells

LNT/CCR5-PGK-GFP NILV was generated and titrated by transducing HEK293T cells and measurement of GFP expression by flow cytometry. GHOST-CCR5 cells were then transduced with ZFN NILVs and increasing MOI of CCR5-GFP donor NILV. GFP expression was measured in the cells by flow cytometry for two weeks following transduction (Figure 6.3A). Initially, between 30 and 40% of cells were GFP positive, with the higher MOI leading to a greater percentage of transduced cells. However, over the two week period, the percentage of GFP positive cells rapidly decreased, finally reaching stable expression in about 1-3% of cells. There was a slight difference in the final percentage of GFP positive cells depending upon the MOI of CCR5-GFP NILV used. Similarly, higher ZFN volume also resulted in increased levels of stable GFP expression at day 14 at all CCR5-GFP MOI tested.

Staining of transduced cells for CCR5 revealed that there were low levels of GFP expression even in cells that remained positive to CCR5 (0.58% compared to 0.14% in untransduced cells) (Figure 6.3B). As GHOST-CCR5 cells contain multiple copies of the CCR5 gene, it is also possible that GFP was integrated at the correct locus but not all of the CCR5 genes were disrupted by ZFNs. Alternatively, GFP expression could be a result of background integration of the donor DNA, which is observed at low levels (approximately 1/10 000) even when using NILV.

To try to optimise the efficiency of HDR and GFP site specific integration, following ZFN transduction, GHOST-CCR5 cells were transduced with the CCR5-GFP donor at different time points. GFP expression was measured by flow cytometry 14 days after ZFN transduction (Figure 6.3C). However, there was little difference between the samples that were transduced with the GFP donor template at various time points.

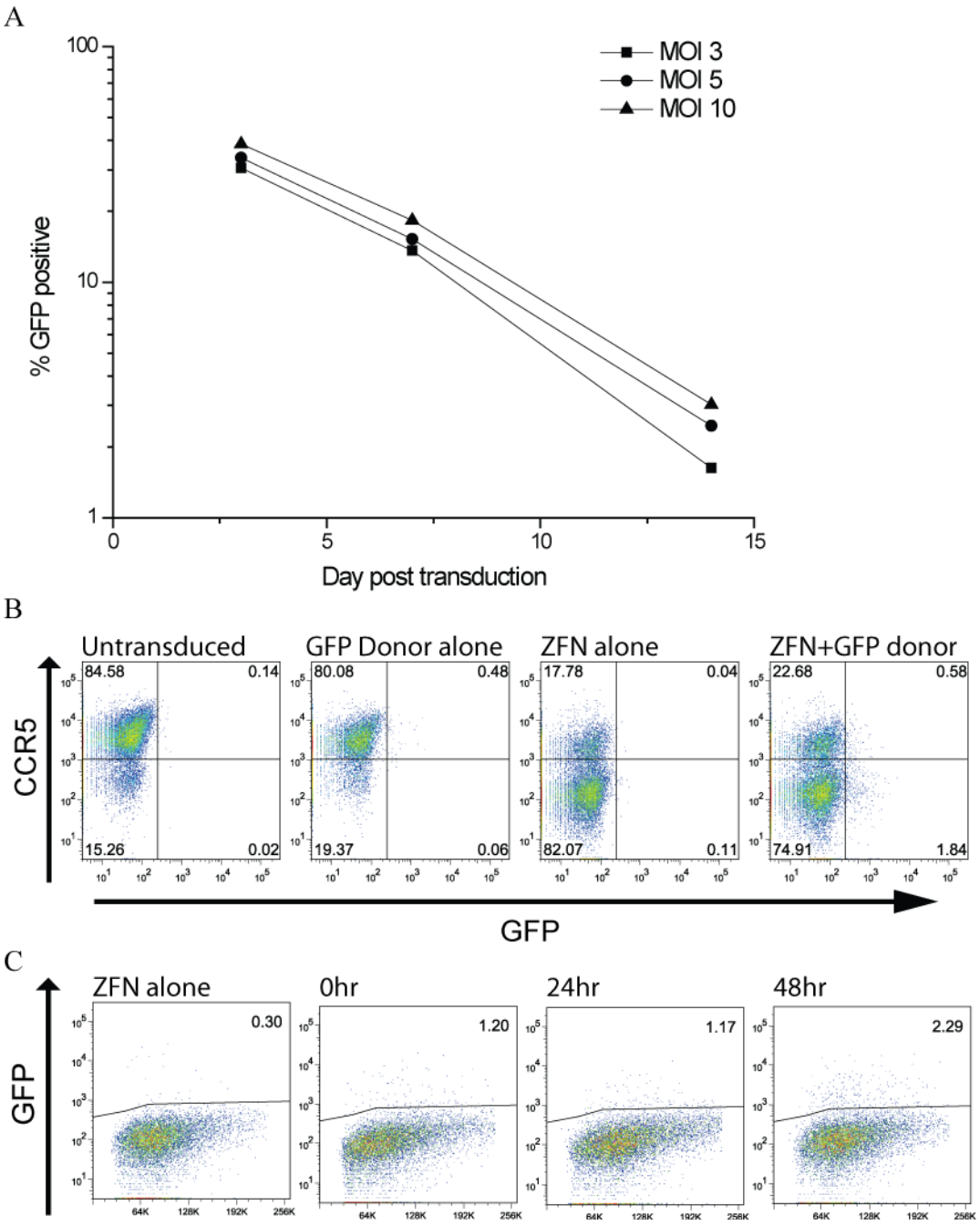


Figure 6.3 Site specific integration of GFP using zinc finger nucleases

A. Increasing multiplicities of infection (MOI) of CCR5-GFP donor non-integrating lentivirus (NILV) were used to transduce GHOST-CCR5 cells simultaneously with CCR5 ZFNs. GFP expression was measured over time by flow cytometry until 14 days post transduction.

B. GHOST-CCR5 cells were transduced with CCR5 ZFN and GFP donor NILV. 19 days post transduction cells were stained with an anti-CCR5 antibody and CCR5 and GFP expression measured by flow cytometry.

C. GHOST-CCR5 cells were transduced with CCR5 ZFN at time 0. CCR5-GFP donor NILV was used to transduce the cells at the following different time points after ZFN transduction: 0, 24 and 48 hours. GFP expression was measured 14 days after ZFN transduction.

6.6 CCR5 site specific integration of TRIM21Cyp in GHOST cell line

To provide a second method of protection against HIV-1, it is possible to integrate an anti-HIV transgene in the CCR5 locus by HDR. In addition to the CCR5-GFP donor template, a donor consisting of PGK-TRIM21CypCO flanked by CCR5 was also cloned into the lentiviral backbone pLNT/SFFV-MCS-WPRE and used to make NILV. The titre of this virus was determined by measuring p24 levels in the viral preparation by ELISA ($2.7x10^7$ pg/ml).

GHOST-CCR5 cells were first transduced with the two ZFN NILV, and then transduced with the CCR5-TRIM21CypCO NILV at different time points after the ZFNs. Cells were cultured for two weeks after transduction, by which time non-integrated episomal DNA would be diluted out by cell division. Then cells were challenged with HIV-1-YFP at an MOI of 150 to obtain very high levels of YFP expression in cells untransduced by ZFNs (referred to as UT in Figure 6.4). Cells were stained with an anti-CCR5-APC-Cy7 antibody, and CCR5 and YFP expression was measured by flow cytometry (Figure 6.4). HIV-1-YFP restriction was taken as a marker of TRIM21CypCO integration and expression.

The high MOI of HIV-1-YFP used resulted in nearly all cells of the ZFN/donor untransduced population becoming YFP positive. In the populations transduced with both ZFNs and TRIM21CypCO donor NILV, there were a small proportion of cells, between 17 and 24%, that were CCR5 and YFP double negative (Figure 6.4). This suggested that they were successfully modified to knockout CCR5 expression and were expressing TRIM21Cyp, which caused the restriction of HIV-1-YFP. This population of CCR5 and YFP double negative cells were sorted by flow cytometry and plated as single cells in a 96 well plate and cultured to obtain clones.

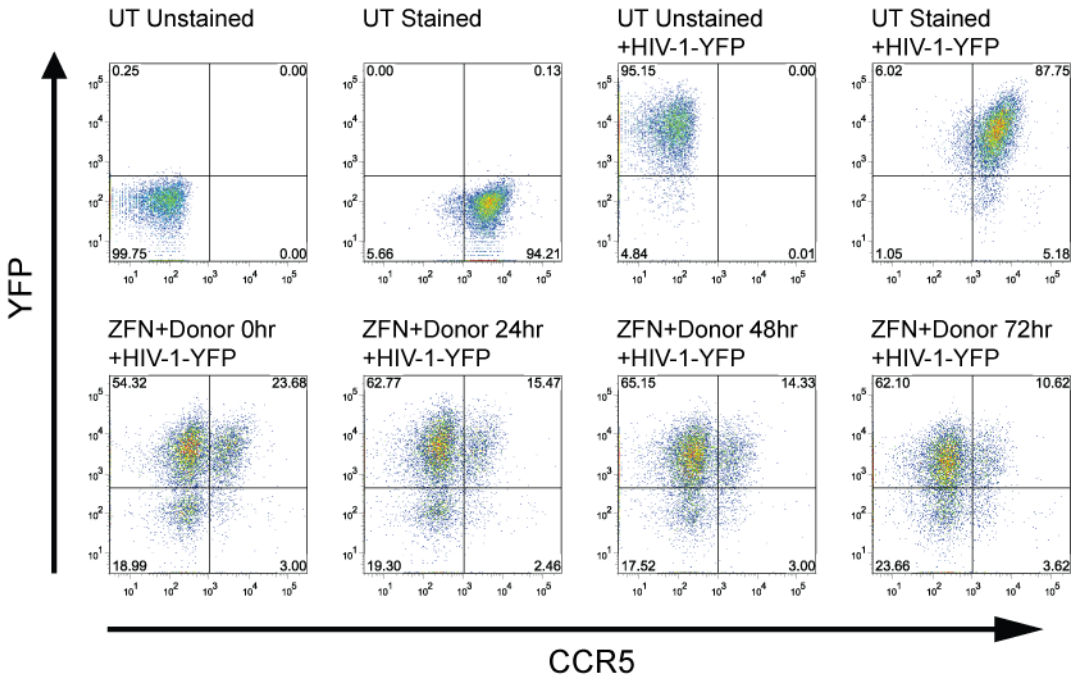


Figure 6.4 Site specific integration of TRIM21CypCO using zinc finger nucleases (ZFNs)
GHOST-CCR5 cells were transduced with ZFN NILV at time 0. CCR5-TRIM21CypCO donor NILV was used to transduce the cells at the following different time points after ZFN transduction: 0, 24, 48 and 72 hours. 14 days post transduction, cells were replated and transduced with HIV-1-YFP. After 72 hours, cells were stained using an anti-CCR5-APCCy7 antibody and CCR5 and YFP expression measured by flow cytometry. Restriction of YFP expression was taken as an indicator of TRIM21Cyp expression. Top panels show control cells untransduced (UT) with ZFN or TRIM21CypCO donor, bottom panels are transduced with ZFNs and then TRIM21CypCO donor at different time points.

Sorted colonies were expanded and when sufficient cells were acquired, the cells were transduced with HIV-1-YFP to confirm restriction shown by inhibition of YFP expression (Figure 6.5). The level of restriction varied between clones and in some cases, clones were fully permissive to HIV-1-YFP. As HIV-1-YFP expression in ZFN untransduced cells was not quite 100% efficient before sorting (Figure 6.4), it is likely that these non-restricting clones were within this small population, but do not express TRIM21CypCO.

Protein and DNA was extracted from the colonies able to restrict HIV-1-YFP for further analysis. Western blots were performed using an anti-CypA antibody to look for production of TRIM21Cyp protein (Figure 6.6A). In clones 14 and 16, appropriate sized bands were visualised. However, despite certain cell populations appearing to restrict HIV-1-YFP, TRIM21Cyp protein could not be detected clearly by Western blot in most of the clones. As previously mentioned, the anti-Cyp antibody for Western blot has not been very effective at detecting protein expression and even positive controls transduced at high levels with LNT/S-TRIM21Cyp-IEW did not produce prominent bands in Western blots of this cell line.

PCR was carried out on the clones using primer sets binding in the integrant within the PGK promoter and within the CCR5 locus outside of the homology region of the donor template to produce a product of 1507bp. This would amplify the junction between integrant and genomic DNA at the 3' end (Figure 6.6B). Therefore, a PCR product would only be produced if the TRIM21CypCO gene was present at the correct locus in the CCR5 gene. However, despite there being some HIV-1-YFP restriction in several of the clones, the correct sized PCR product could only be detected in one of the clones tested (Figure 6.6D). This PCR product was purified, cloned into a TOPO plasmid and then sequenced. Sequencing results confirmed that TRIM21CypCO had integrated at the correct CCR5 locus.

PCR using primers to amplify the junction between the integrant and genomic DNA at the 5' end of the integrant could not detect bands of the correct size for any of the

clones tested, including clone 14 which tested positive for the amplification of the other junction.

Another PCR was carried out to amplify the TRIM21CypCO gene using primers that both bind within the transgene. As the Western blot results were not very reliable, this PCR enabled more conclusive evidence that particular clones contained an integrated copy of the transgene, although not necessarily integrated at the CCR5 locus. As shown in the schematic (Figure 6.6B) one primer bound within TRIM21 and the other in Cyp to avoid amplification of either of the individual genes and produce a 486bp product that spans the TRIM21-Cyp junction. The lentiviral plasmid pLNT/CCR5-PGK-TRIM21CypCO was used as a positive control. Both clone 14 and 16, and other clones, produced a band of the correct size (Figure 6.6C). Some clones produced a PCR product for this TRIM21Cyp reaction but did not produce bands on the Western blot. This could be due to problems with the Western blot, or could also indicate that the transgene has been silenced in these clones.

Clones in which the TRIM21CypCO transgene itself was detected, but not the expected integrant/CCR5 junction, could contain randomly integrated copies of the entire donor NILV genome. In this case, this would also include integration of WPRE. Therefore qPCR was performed using primer and probe sets for WPRE and β-actin to calculate the average WPRE copy number in the TRIM21CypCO positive clones. Clones 2, 12 and 16 had approximately 1-1.5 copies of WPRE per cell (Table 6.1). This suggests that these clones contain an integrated copy of the entire lentiviral genome, including WPRE. If site specific integration had occurred by HDR, PGK-TRIM21CypCO flanked by CCR5 homology arms would be integrated, but WPRE would be absent from the cells. Clone 14 had a lower average copy number of 0.36 per cell, which could be due to contamination in the sample, rather than representing actual integrated copies.

Collectively these results suggest that only clone 14 contains a correctly integrated TRIM21CypCO transgene as it tested positive for the TRIM21CypCO PCR and the junction PCR, as well as detection of protein by Western blot. The very low WPRE copy number indicates that there is no background integration of the vector genome. Of the other clones, some did not appear to contain TRIM21CypCO at all, whereas

in others, the transgene could be detected by PCR, but is likely to be from background integration of the transgene as indicated by the inability to amplify the integrant/CCR5 junction and the high WPRE copy number.
Results of molecular analysis are summarised in Table 6.1.

Of the clones that grew after the single cell sort, there were very few that restricted HIV-1-YFP in subsequent restriction assays. Approximately 200 clones were grown from two separate single cell sorts for CCR5/YFP double negative cells. Of these, about 10 were able to restrict HIV-1-YFP when tested after sorting. This implies that this method for selecting modified cells is not efficient. It is also difficult to test for integration as there is only one set of PCR conditions that have been optimised to detect TRIM21CypCO integration. PCRs were attempted to amplify the 5' end of the expected integrant, with one primer binding in the CCR5 gene and the other in the polyA tail at the end of the TRIM21Cyp transgene. However, this product could not be amplified in any of the reactions conditions tested. There was no positive control for this reaction, so the absence of a product could have been an experimental problem or it could be because the integrant is not actually at the correct site. It is possible that in clone 14 TRIM21Cyp is integrated at one end by HDR giving the expected junction between integrant and CCR5, with the other end being repaired by NHEJ. In this case, the PCR used to detect the junction would not produce a product.

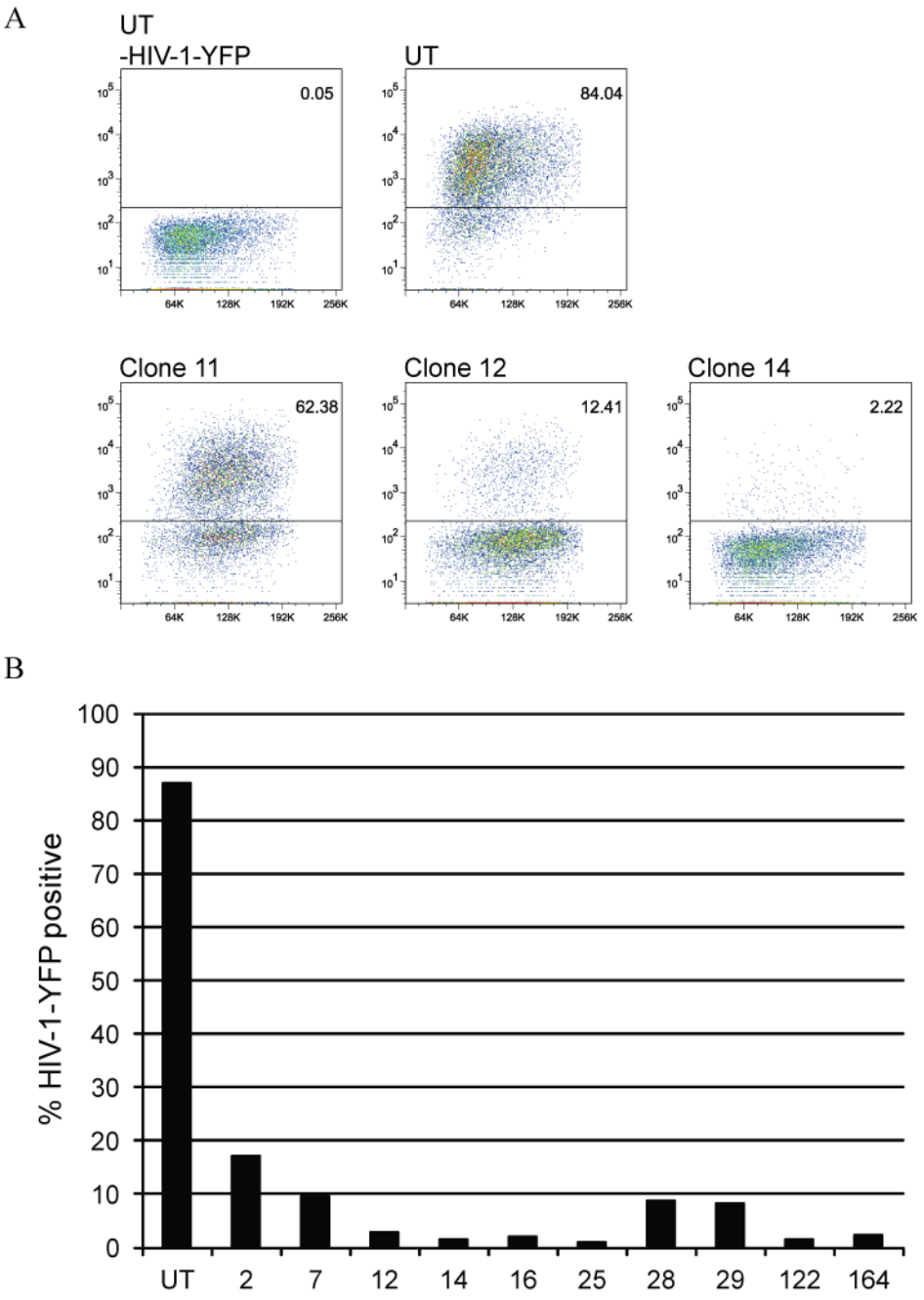


Figure 6.5 Restriction of HIV-1 by CCR5-TRIM21Cyp clones

YFP and CCR5 double negative single cell clones were flow cytometrically sorted from the experiment in Figure 6.4 and expanded. Each colony was challenged by transduction with HIV-1-YFP. 72 hours later, HIV-1-YFP expression was measured by flow cytometry to quantify restriction.

A. FACS plots of untransduced (UT) cells (top panels) and examples of some clones tested (bottom panels).

B. Selection of clones that restricted HIV-1-YFP as measured by flow cytometry. Clones 2-29 and clones 122 and 164 were from two separate transductions and sorts.

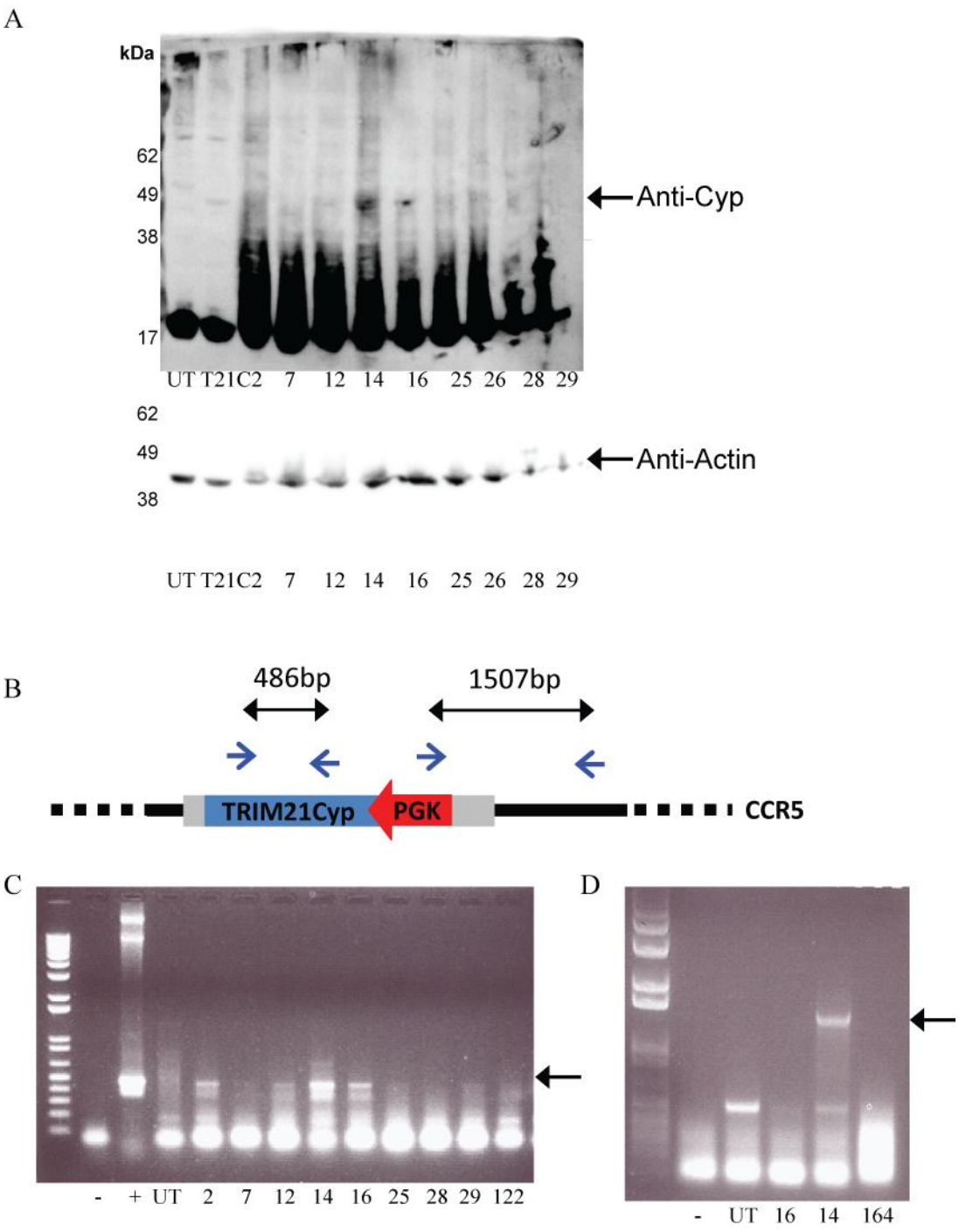


Figure 6.6 Molecular analysis of GHOST CCR5-TRIM21Cyp clones
A. Western blot of GHOST clones that restricted HIV-1-YFP using an anti-Cyp antibody to identify TRIM21Cyp protein expression. Loading control with anti-β-actin antibody is shown in the bottom panel. UT-untransduced, T21C-sample from cells transduced with LNT/S-TRIM21Cyp-IEW.
B. Schematic diagram to show the primer binding sites (blue arrows) used in the two PCRs to identify clones that had an integrated TRIM21CypCO transgene (486 base pair (bp) product) and integration at the correct locus enabling amplification of the integrant-CCR5 junction (1507bp product). DNA was extracted from GHOST clones that restricted HIV-1-YFP and subject to PCR analysis.

C. PCR using primers that bind within the TRIM21CypCO transgene, allowing identification of clones with an integrated copy irrespective of integration site. One primer binds within TRIM21 and the other in Cyp to avoid detection of either individual endogenous gene. A 486bp product of the correct size was identified in several clones and is indicated with the black arrow. CCR5-PGK-TRIM21CypCO donor template plasmid (+) was used as a positive control.
D. PCR was performed using primers that bind in the TRIM21Cyp insert and in the genomic CCR5 DNA to amplify the junction between insert and endogenous CCR5 sequence if integration occurred at the correct locus. For the one clone which had a band of the correct size (clone 14), the PCR product was sequenced to confirm the integration site in the CCR5 gene. The correct band is indicated with a black arrow. Examples of some of the other clones without the correct sized bands are shown.

Clone	TRIM21Cyp PCR	Western blot (anti-Cyp)	Junction PCR	WPRE qPCR (copy number)
2	✓	X	X	1.5
7	X	X	X	-
12	✓	X	X	1.18
14	✓	✓	✓	0.36
16	✓	✓	X	1.06
25	X	X	X	-
28	X	X	X	-
29	✓	X	X	1.38
122	X	-	X	-
164	X	-	X	-

Table 6.1 Molecular analysis of GHOST clones
Summary of PCR and Western blot analysis of GHOST clones derived from cell sorting.

6.7 HIV-1 restriction in GHOST cells transduced with ZFNs and integrating TRIMCyp vectors

The use of CCR5 specific ZFNs to site specifically integrate TRIMCyp would provide cells with two modes of protection against HIV infection. CCR5 would be absent from the cell surface and TRIMCyp would be expressed, providing a second level of defence against any virus that was able to enter cells, in particular X4 or dual tropic strains. Due to the inefficiency of HDR and time restrictions, these two levels of defence could not be tested through site specific integration of TRIMCyp at the CCR5 locus. As a proof of principle experiment, GHOST-R5X4 cells were transduced with CCR5 ZFN NILV and after several days in culture, also with LNT/S-TRIMCyp-IEW. This GHOST cell line expresses both CCR5 and CXCR4 co-receptors, as well as CD4 to allow infection with R5, X4 and dual tropic HIV-1 strains.

After the two rounds of transduction, cells were stained with anti-CCR5 antibody before FACS sorting to obtain GFP positive populations, either positive or negative for CCR5. These cells had knockout of CCR5 by ZFNs and expression of TRIMCyp from an integrating vector, rather than a transgene specifically integrated at the CCR5 locus.

After sorting cells, eGFP expression remained low in all TRIMCyp populations (Figure 6.7A). To confirm high levels of TRIMCyp in all cells in the sorted population, cells were transduced with HIV-1-YFP, and YFP expression measured by flow cytometry 72 hours later (Figure 6.7C). Despite inefficient eGFP expression in the sorted cell populations nearly all TRIMCyp transduced cells were are able to restrict HIV-1-YFP, compared to 80% of cells untransduced with TRIMCyp vectors (UT in Figure 6.7). As HIV-1-YFP was pseudotyped with VSV-G, giving it a broad tropism, the presence or absence of CCR5 on the cell surface did not affect levels of HIV-1-YFP transduction. DNA samples were extracted from cells and the ZFN site amplified by PCR. The PCR product was then used for a T7 endonuclease assay to detect the presence of mismatched DNA binding characteristic of NHEJ and CCR5 knockout (Figure 6.7B).

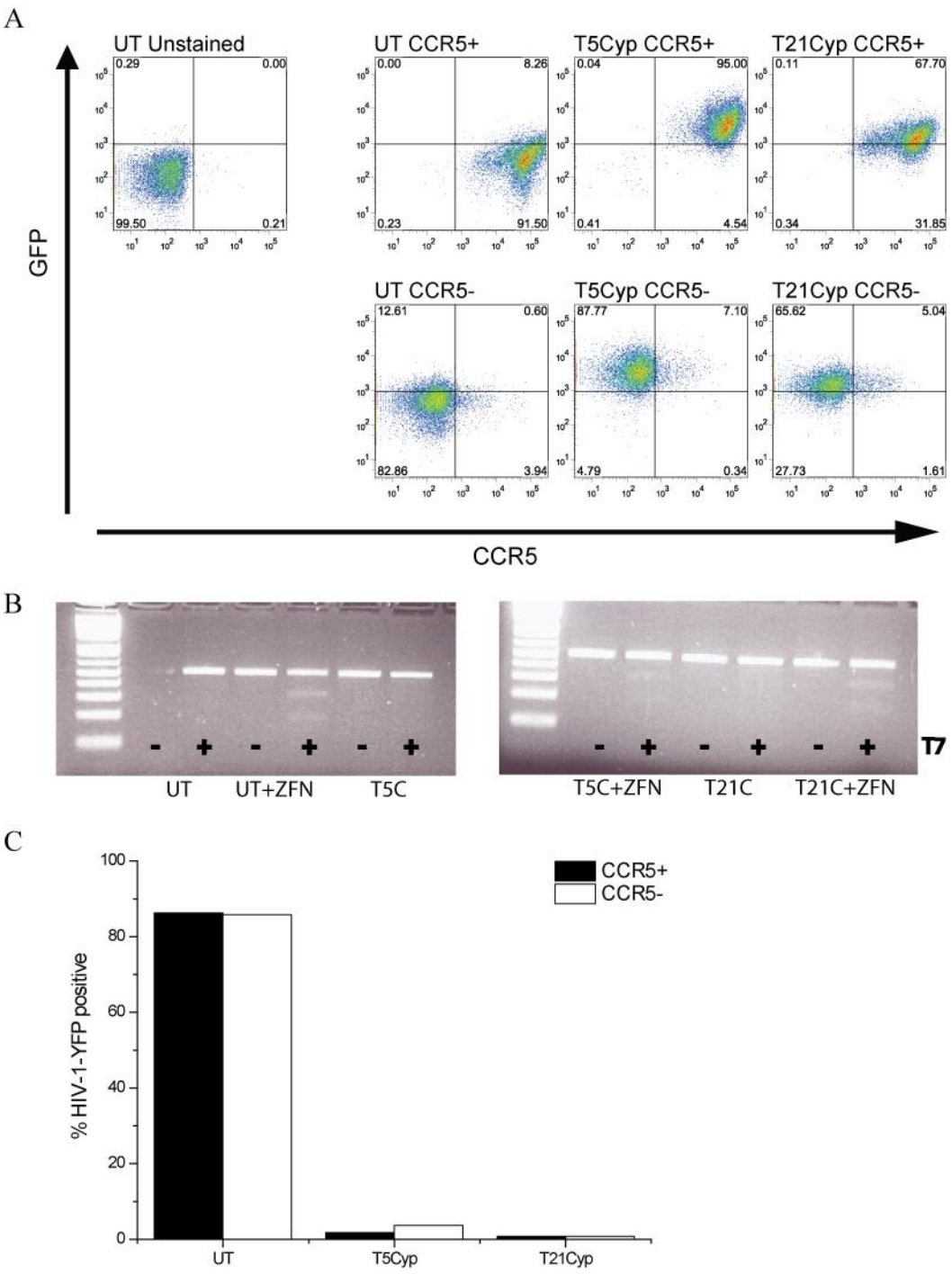


Figure 6.7 Restriction of HIV-1-YFP by TRIMCyp and CCR5 specific ZFN

A. GHOST-R5X4 cells were transduced with CCR5 specific ZFNs. Cells were subsequently transduced with LNT/S-TRIMCyp-IEW vectors. Cells were stained with an anti-CCR5-APCCy7 antibody and were then flow cytometrically sorted for GFP expression and the presence or absence of CCR5. Flow cytometry of cells after sorting are shown.

B. DNA was extracted after sorting and subject to a T7 endonuclease assay. Each sample also had a no enzyme (-) control.

C. After sorting, cells were transduced with HIV-1-YFP. 72 hours later restriction was measured by YFP expression by flow cytometry to measure restriction in the whole population independently of GFP expression.

These sorted cells were then infected with R5 tropic replication competent HIV-1 NL4-3 (BaL) to investigate whether there is an added benefit to have both CCR5-ZFN and TRIMCyp mechanisms of restriction.

Culture supernatant was harvested 7 days after infection to measure p24 levels by ELISA (Figure 6.8A). Cells were passaged every 3 to 4 days to prevent cell overcrowding. At day 18 cell viability was measured by flow cytometry to gauge the level of cytotoxic effects from HIV-1 replication (Figure 6.8B).

Untransduced cells expressing CCR5 supported replication of R5 tropic HIV-1 and high levels of p24 were detected in the culture medium by ELISA. Knockout of CCR5 expression by ZFNs led to an approximately 50 fold decrease in p24 levels in the media compared to this control. Even after sorting there was a small percentage of cells that was CCR5 positive, which supported viral replication and lead to increased p24 levels detectable by ELISA. TRIMCyp expressing cells produced levels of p24 that were undetectable above background levels in cells both with and without CCR5.

This inhibition of HIV-1 replication was mirrored in the cell viability. By around day 14 there was a noticeable loss of cell density when passaging $CCR5^+$ cells not expressing TRIMCyp. $CCR5^-$ cells continued to grow rapidly throughout the experiment, whether expressing TRIMCyp or not. Cell death was quantified by staining cells for viability of day 18. Cells expressing CCR5 had a low viability of only 9% compared to 59% for uninfected control cells.

All other cell populations had similar viabilities to the uninfected control. However, there was a 12% increase in viability in TRIMCyp $CCR5^-$ cells compared to TRIMCyp $CCR5^+$ cells, despite neither population supporting viral replication.

These results suggest that TRIMCyp factors confer such a strong level of restriction that there is little additional benefit, in terms of viral replication, in disrupting CCR5. However, using ZFNs for site specific integration has safety benefits in drastically reducing the likelihood of insertional mutagenesis.

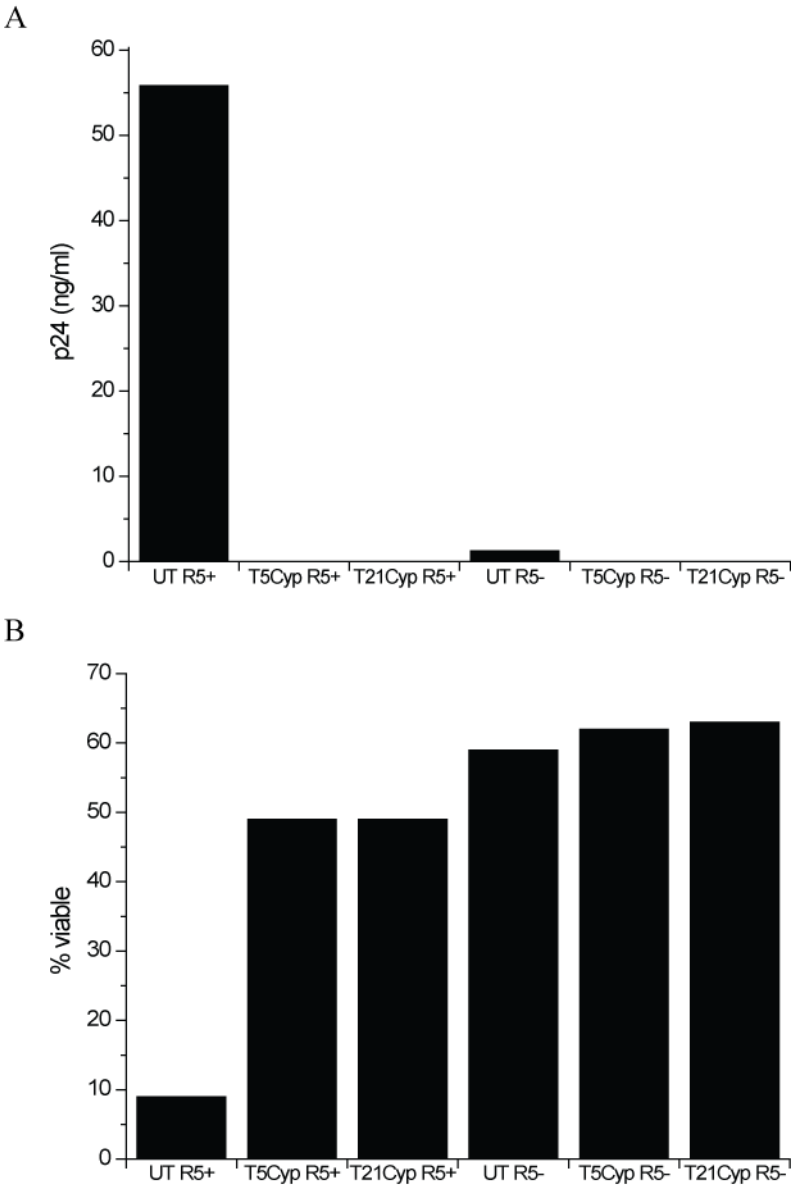


Figure 6.8 Restriction of HIV-1 by TRIMCyp and CCR5 zinc finger nucleases
GHOST cells with or without CCR5 ($R5^+$ and $R5^-$ respectively) and either untransduced (UT), T5Cyp or T21Cyp transduced (from Figure 6.7) were infected with R5 tropic HIV-1 NL4-3 (BaL).
A. Culture supernatant was harvested 7 days post infection and p24 levels measured by ELISA.
B. At day 18 post infection cells were harvested and stained with live/dead fixable stain to measure viability by flow cytometry.

6.8 Summary

CCR5 has been a target in anti-HIV-1 therapy since the identification of the Δ32 mutation in the CCR5 gene that provides protection against infection (332, 333). One possible way to disrupt CCR5 expression is ZFNs.

ZFNs are advantageous compared some other techniques as they only require transient expression to provide permanent knockout in cells and all of their progeny, thereby increasing safety by eliminating the need for integrating genes and reducing the risk of an immune response to these artificial proteins.

Knockout of both CCR5 alleles is required to provide full resistance to R5 tropic HIV-1. In alternative approaches using RNAi and ribozymes there is often only partial knockdown and there can be problems with maintaining protection due to shut down of gene expression. Low levels of cell surface CCR5 would be sufficient to allow some HIV-1 infection.

There are several different methods that can be used for delivery of ZFN genes. Here, NILV have been used, but it is also possible to use nucleofection or adenoviral and AAV vectors. Different methods may affect the efficiency of gene delivery, and this may occur in a cell type dependent manner. Combination of both ZFN genes on one NILV by using a bicistronic vector, IRES or P2A (369, 370) construct may increase efficiency as cells would only need to be transduced with two viral vectors; one encoding the ZFNs and the other as the donor template, rather than three as used in these experiments.

ZFNs were initially tested in the GHOST cell line, which have been transduced with a gamma retroviral vector to express high levels of CCR5. Therefore, each cell contains multiple copies of CCR5 cDNA and all copies would need to be targeted by ZFNs for the cell to become CCR5 negative. Despite this, CCR5 knockout was quite efficient, with nearly 80% of cells becoming CCR5 negative. Incomplete knockout targeting just some of the multiple copies per cell may lead to reduced levels of CCR5. *In vivo* this has been suggested to result in a slower disease progression and onset of AIDS, as seen in Δ32 heterozygotes which only have one functional copy of

the CCR5 gene (335, 336). However, these cells would not be resistant to R5 tropic HIV-1.

In addition to using ZFNs to disrupt gene expression, introduction of a DSB increases the efficiency of HDR which can allow site specific integration of a transgene. The aim here was to use a donor template encoding TRIMCyp to promote HDR and integration of this transgene at the CCR5 locus. This strategy would provide dual protection against HIV-1; firstly by knockout of CCR5, and secondly by expressing TRIMCyp, which would target virus that is able to enter the cell, presumably a strain that utilises CXCR4 for cell entry. Additionally, site specific integration would reduce the risk of insertional mutagenesis. In situations where ZFNs are aimed at replacing a mutated gene with the correct, functional sequence by HDR, repair of DSB by NHEJ would be non-beneficial. However, in anti-HIV-1 gene therapy aiming to integrate TRIMCyp by HDR, knockout of the CCR5 gene by NHEJ will still provide some protection by restricting R5 tropic HIV-1. Targeting two different points in the HIV lifecycle is an attractive option as it reduces the possibility of mutagenic escape by the virus.

CCR5 is the predominant tropism for HIV-1 responsible for initial infection (328), but even with complete CCR5 knockout, cells are still susceptible to X4 tropic virus. Individuals may harbour X4 or dual tropic viral strains, and although rare, there have been several cases of Δ32 CCR5 homozygotes becoming infected with HIV-1 (395). Restricting just R5 tropic virus could result in selection and expansion of X4 tropic strains that are often associated with $CD4^+$ T cell loss and more rapid disease progression.

Targeting these strains of virus in addition to R5 tropic strains would provide a more comprehensive and effective therapy. CXCR4 specific ZFNs have been designed and shown to specifically disrupt CXCR4 in human T cells, resulting in HIV resistance and a survival advantage for modified cells (396). However, in a NSG mouse model only a transient restriction of HIV-1 was seen. This was hypothesised to be due to the evolution and emergence of either dual tropic or R5 tropic strains.

Another problem with this strategy is that unlike CCR5, CXCR4 does not have such a redundant role in the immune system. Although no effect on cell growth or function was reported, CXCR4 knockout mice have severe problems in haematopoiesis and cerebellar and cardiac development (397). Therefore, using TRIMCyp restriction factors could be a more applicable mode of protection to target X4 and dual tropic strains of HIV-1. This could be carried out either by using ZFNs and HDR, or using ZFNs and integrating lentivirus expressing TRIMCyp if the efficiency of HDR is too low.

In preliminary experiments, CCR5 specific ZFNs and a PGK-eGFP donor template NILV were used. This allowed easy quantification of integration by flow cytometry. After transduction with ZFN and GFP donor NILV, cells were cultured for two weeks to allow dilution of episomal DNA. The final percentage of GFP positive cells increased with higher quantities of both ZFNs and donor virus. However, even at the highest concentrations, only 2-4% of cells stably expressed GFP.

Published data has already shown that efficiency of HDR varies greatly between cell types. When using NILV to deliver ZFNs and a GFP donor template, the rate of site specific integration of GFP ranged from 39% in Jurkats to 3.5% in human embryonic stem cells to 0.11% in $CD34^+$ haematopoietic stem cells (361). In the published data, rates of integration were much higher than seen here in GHOST cells. One problem in initial experiments was the use of unequal quantities of ZFNs. ZFN EL had approximately 4 times higher p24 levels than ZFN KK (0.05µg/µl compared to 0.013µg/µl) and this only became apparent on retrospective p24 based titre analysis. The low efficiency of integration by HDR is an obstacle that must be overcome before this technology could become a feasible therapeutic option.

Some possible methods to increase efficiency include targeting cells during the G2 phase of the cell cycle, a variable that was not controlled for in these experiments. At G2, the likelihood of cells repairing DSB by HDR rather than NHEJ increases, so treatment of cells with vinblastine to arrest the cell cycle could increase donor integration (398). Providing the cells with a cold snap at 30ºC after delivery has also

been shown to improve efficiency of ZFN action, at least in part due to accumulation of ZFN protein (399).

A second donor template was generated that encoded codon optimised TRIM21Cyp (TRIM21CypCO) driven by the PGK promoter. This would allow integration of the TRIMCyp transgene into the safe harbour CCR5 locus. Targeting different points in the HIV lifecycle is an attractive option as it should reduce the possibility of mutagenic escape by the virus. Again, NILV was used to deliver ZFNs and the TRIM21CypCO donor into GHOST-CCR5 cells. To distinguish which cells were expressing TRIM21Cyp, cells were then transduced with HIV-1-YFP. HIV-1-YFP restriction was taken as a marker of TRIM21Cyp expression and cells were subsequently sorted by flow cytometry for YFP and CCR5 double negative cells to produce clones predicted to have TRIM21Cyp integrated at the CCR5 locus.

This method produced lots of clones, and after two individual transductions of GHOST cells with ZFNs and TRIM21CypCO donor, approximately 200 colonies were grown and tested for restriction of HIV-1-YFP. However, the majority of these clones were not resistant to HIV-1, and molecular analysis did not confirm correct integration at the CCR5 locus. The high number of false positive clones is probably a result of incomplete transduction of the entire population of cells prior to sorting despite the high MOI of HIV-1-YFP used.

As discussed previously, TRIMCyp protein is not reliably detected by Western blot. This caused difficulties in confirming TRIM21Cyp protein in the clones by this method. Therefore further analysis was performed by PCR. Of the clones tested, only one clone produced the correct amplicon of the TRIMCyp/CCR5 junction.

If this PCR product, or that of the other TRIMCyp/CCR5 junction, cannot be amplified, it could be because this end was repaired not by HDR as expected, but by NHEJ, resulting in the insertion of the remainder of the vector DNA. This resulting incorrect junction could be too long to amplify by the PCR using the tested conditions.

Other clones that did not contain this expected junction, but tested positive in the TRIM21Cyp PCR, had approximately one copy of WPRE per cell suggesting

integration of the whole vector genome. This could either be random integration of the vector DNA or off target integration mediated by ZFNs. Random background integration is likely to be a result of recombination between the vector and genomic DNA rather than IN activity (400). Alternatively, if the ZFNs cause off target DSBs, linear vector DNA can be integrated at this site by NHEJ. In fact this phenomenon has even been exploited for the identification of off target ZFN sites (401). These off target events can be identified by using ligation-mediated PCR and high throughput sequencing. Briefly, this involves digestion of genomic DNA from transduced cells, and ligation of linkers to the overhanging DNA ends. The genomic-viral DNA junction is amplified by PCR using primers that bind within the viral DNA and to the linker.

Although background integration could still provide protection to the cell if it allows expression of TRIMCyp, there would be no additional protection by CCR5 knockout and the risk of insertional mutagenesis will be similar to using integrating vector.

Bioinformatics can be used to predict ZFN off target sites. The simplest method locates sites with high homology to the binding sites of the two ZFN monomers and the top 15 predicted sites for the CCR5 ZFNs have been identified and further analysed by high throughput sequencing (369). However, calculating putative off target sites by sequence homology with the binding site of each individual ZFN may not identify genuine target sites of the dimeric pair of ZFNs. Binding by a ZFN pair may tolerate a greater number of base pair mismatches than the two individual ZFN binding sites. Using alternative methods, more recent investigations have confirmed previously identified off target sites and also determined additional sites for CCR5 ZFNs (401, 402). Off target sites shown to be cleaved by CCR5 ZFNs include CCR2, BTBD10 and ABLIM2 (369, 401, 402).

CCR2 has a high level of sequence homology to CCR5 and each CCR5 ZFN DNA binding site is only one base pair different to a homologous sequence of the CCR2 gene. Like CCR5, CCR2 is a G protein coupled chemokine receptor and knockout of the gene results in mice that develop normally, but have impaired recruitment of monocytes and macrophages to sites of inflammation (403-405). Despite this, off

target knockout of CCR2 is likely to be tolerated, and as CCR2 mutations have been shown to delay the onset of AIDS, disruption of CCR2 may provide some protection in HIV patients (406). However, CCR2 is located adjacent to CCR5 on chromosome 3 and it has been shown that simultaneous cleavage at CCR5 and CCR2 can lead to deletion of large 15kb region of DNA (407). This could even potentially result in a CCR5-CCR2 fusion protein, which may trigger an immune response against transduced cells expressing this novel epitope.

As well as background integration, the cytotoxic effects of ZFNs must also be assessed. Cytotoxicity is primarily is caused by off target DSBs, which can be visualised within a cell by staining for 53BP1, which is recruited to the site of DSB (408).

If cells have a strong survival advantage, modification of only a small number of cells by HDR to insert TRIMCyp may be sufficient to observe a therapeutic benefit, especially if targeting HSCs that are able to produce resistant cells of all haematopoietic lineages. However, whilst the current methods to induce site specific integration through HDR are inefficient, a more feasible method may be to transduce cells separately with integrating TRIMCyp vector and with CCR5 ZFN NILV.

Adenoviral vectors for ZFN delivery, especially with T cells, have been used by other groups. This vector system is being used in the Sangamo clinical trial, where preliminary data show that this vector is likely to be able to mediate CCR5 knockout at a therapeutically beneficial efficiency. However, a major drawback with this delivery method is that most people initiate an immune response to adenovirus, which could result in immune pathologies.

In the future, alternative nucleases called TALENs, which use a similar principle to ZFNs, could be used (366, 367). These proteins are proposed to be more efficient, but with lower off target cytotoxicity causing less cell death, and less cleavage at CCR2. If the efficiency and toxicity of these nucleases is more suitable, they may be preferential to use as a potential gene therapy method. An additional benefit of using TALENs is that TALEs bind DNA in a context independent fashion. This makes assembling TALEs to bind a specified sequence simpler than when using ZFs, which

are influenced by their neighbouring ZFs. Therefore, designing ZFNs also requires time consuming screening of potential proteins, a step which could be eliminated if using TALENs.

Data presented here has shown that TRIMCyp expressing cells provide such strong inhibition of HIV-1 that the additional knockout of CCR5 using ZFNs does not provide any further viral restriction. Despite there being no enhancement of restriction, using ZFNs to site specifically integrate TRIMCyp at the CCR5 locus would improve the safety profile of gene therapy. However for this to become a viable therapeutic mechanism, the efficiency of the technology must be greatly increased. Here, modification of the GHOST cell line by HDR was highly inefficient and targeting T cells or HSC, as would be required in a patient, is likely to be even more inefficient. However, the principle of site specific integration of an anti-HIV-1 transgene at the CCR5 locus to provide a dual mechanism of protection against HIV-1 is highly desirable, and optimisation of ZFN technology, for HIV-1 and other diseases, is being continued to improve this possibility.

7 Discussion

Here we have designed TRIMCyp constructs using both human TRIM5 and TRIM21 RBCC domains fused to human Cyclophilin A (Cyp) based upon the naturally occurring owl monkey TRIM5Cyp protein. Both of these human proteins were found to be potent inhibitors of HIV-1. However, TRIM5Cyp, but not TRIM21Cyp, was shown to interfere with the antiretroviral function of endogenous TRIM5α. The endogenous function of TRIM21, quantified by adenoviral restriction, was not disrupted by expression of either TRIMCyp variant. Thus, although the role of endogenous TRIM5 in human immunity is poorly defined, TRIM21Cyp may be a more suitable choice for clinical therapy than TRIM5Cyp.

These transgenes could form the basis of a new gene therapy treatment against HIV and this discussion illustrates some of the aspects that must be further considered before application of this technology.

7.1 Vector modification for clinical use

In anticipation of further testing of these restriction factors, the TRIM5Cyp and TRIM21Cyp vectors have been modified to increase their suitably for clinical use. Important alterations include the removal of the eGFP reporter gene and replacement of the strong viral SFFV promoter with the clinically approved human PGK promoter. This promoter also provides high, constitutive expression, but as it is human, rather than viral, in origin, it is less likely to be silenced or induce an immune response. For these changes, the TRIMCyp constructs have been cloned into the third generation lentiviral plasmid, pCCL, which has been approved for clinical use and is currently in clinical trial.

The third generation packaging system uses a constitutive viral promoter in the 3'LTR of the transfer plasmid, rather than U3. This eliminates the requirement of Tat, which is removed from the packaging plasmid. The rev gene is also removed from the packaging plasmid, and is supplied in trans on a separate plasmid to the

packaging cells upon transfection (107). Vector must also be treated with benzonase for the removal of contaminating DNA to improve purity before use.

Although the human PGK promoter has been used in clinical trials and promotes strong constitutive expression, preliminary work has shown that the TRIM5 promoter may drive expression comparable to human PGK. Use of physiological promoters reduces the risk of cytotoxicity in transduced cells (102) and using the TRIM5 promoter would allow more cell specific expression at a physiological level. The TRIM5 promoter is also IFN inducible (211), which would allow upregulation of TRIMCyp expression upon viral infection. This would relieve the requirement of high, constitutive expression of TRIMCyp and any associated toxic side effects. The full length TRIM5 promoter is just over 1kb in length, but for use in a vector could possibly be reduced in length whilst still maintaining high levels of expression with the IFN inducible elements.

Another possible scenario to use the native TRIM promoter would be to use ZFNs to insert CypA cDNA into the TRIM5 locus to produce a TRIMCyp fusion protein under control of the endogenous TRIM5 promoter. This would result in physiological levels of expression and IFN induced upregulation upon viral infection. However, ZFNs currently have problems of low efficiency, particularly for HDR. This strategy would also reduce expression of TRIM5, which is now known to be important in innate signaling (252).

In patients it is possible that lentiviral therapeutic vectors could come into contact with replication competent wild type HIV-1, from which the vector was originally derived. This means that HIV-1 gene therapy has a higher possibility of generating RCL through recombination between the vector and the wild type virus. Although this phenomenon has yet to be observed in any setting, it is important to use all possibilities to limit the likelihood of these events occurring. The third generation system includes fewer HIV-1 sequences, so reduces the likelihood of recombination. Clinical grade vector must be subject to stringent testing to detect the presence of any RCL. In addition, bespoke RCL assays will be required to screen patients for

replication competent recombinants formed by recombination between HIV-1 vector and wild type virus.

7.2 Target cell populations for HIV-1 gene therapy

If TRIMCyp is considered for clinical trials, it would be necessary to decide whether to target T cells or HSC for *ex vivo* modification using third generation vectors. Both populations have already been targeted in different HIV-1 gene therapy trials, and both are associated with different benefits which are summarised below.

A key factor with targeting T cells is that they are less likely than HSCs to be transformed and cause leukaemia, which has been shown to be a problem in HSCs gene therapy (100, 101). In various gene therapy trials targeting T cells that have been performed to date, there has been no evidence of mutagenesis, including in anti-HIV-1 trials (120, 125, 409-412). Analysis of T cells transduced with a retroviral vector show that even with some gene upregulation, T cell function is not disrupted and there is no evidence of clonal expansion (413), suggesting that T cell transduction will be safely tolerated in patients. It would be necessary to check that modified T cells expressing TRIMCyp are still able to function and replicate as normal to restore the immune system of the patient. Functional assays to assess this could include measuring proliferation, cytokine expression, chromium release assays and response to infections that are common problems in HIV-1 patients, such as CMV.

Transduced T cells may survive for many years, enabling continued protection and maintenance of a functional T cell count, particularly if both naïve and memory cells are targeted. Ideally, the ratio of naïve to memory T cells would be maintained after transduction, but stimulation of the cells prior to transduction may skew the cells towards a memory phenotype. Stimulation with cytokines alone may cause less of an effect (414, 415) than via the TCR, but it is important that transduction efficiencies remain high. As T cells are already differentiated, they have a reduced life span compared to HSCs, which improves their safety profile, but reduces the longevity of

the treatment. T cell gene therapy is highly appealing for adult patients for whom the ability to reconstitute the immune system from HSCs is limited.

As $CD4^+$ T cells are the prime target cell of HIV-1 and numbers are depleted throughout infection, it may be difficult to acquire sufficient cell numbers for transduction in patients with low $CD4^+$ counts. Trials would need to be performed on patients with a sufficient T cell counts, or cells could be expanded *ex vivo* to increase the initial numbers of cells provided to a patient. However, there are associated problems with *ex vivo* expansion of cells in terms of negatively affecting their naivety and fitness. Also, using a cell population that could potentially harbour HIV-1 adds logistical complications in terms of manipulating cells, and sufficient safety measures must be taken during *ex vivo* transduction and culture protocols. Any cells that are already infected with HIV-1 will receive no protection by subsequent TRIMCyp transduction.

In vitro experiments can be performed to test transduction and expression in HSCs using the TRIMCyp lentiviral vector. Transduced HSCs can be cultured and differentiated into CD4/CD8 double positive cells *in vitro* (416) and then subsequently challenged with HIV-1. Alternatively, transduced HSCs can be transplanted into NSG mice and allowed to differentiate *in vivo* before infection with HIV-1. T cell numbers and viral load can be measured to test the efficiency of engraftment and TRIMCyp expression.

It is generally considered that HSCs are not infected by HIV-1, eliminating the concern with T cells of transducing already infected cells. However, there has been some evidence that shows latent infections of haematopoietic progenitor cells (417), which must be considered if this cell population is to be targeted.

An important safety concern with HSCs is that they are more susceptible to insertional mutagenesis than T cells as they have prolonged and increased replicative capacity. This involves higher expression of genes involved in proliferation, which are likely to be frequently targeted as an integration site, potentially disrupting normal expression. There are already examples of gene therapy clinical trials for SCID-X1 targeting HSCs in which insertional mutagenesis led to T cell clonal

expansion and the development of leukaemia (100, 101). However, these trials used gamma retroviral vectors with intact LTRs to drive IL γ chain expression. Subsequent vector design was improved by using SIN LTRs with weaker internal promoters driving expression, which significantly increased safety in *in vitro* toxicity assays. Furthermore, replacement of retroviral vectors with lentiviral vectors further improves safety, primarily due to the differences in their respective integration profiles (104, 418). Although current SIN lentiviral vectors have a significantly higher safety profile than retroviral vectors used in the SCID-X1 trial, this trial has highlighted the risks associated with HSC modification. Further observations and longer follow-up periods in current trials are required to determine the *in vivo* toxicity of these latest vectors.

If HSCs are used as target cells, it is important to ensure that the potency of the HSCs is not affected. *Ex vivo* culture of HSCs requires a cocktail of cytokines to promote expansion and maintain an undifferentiated state, but this has been associated with loss of potency after transplant. These culture methods are being optimised to reduce loss of potency whilst maximising transduction efficiency (419).

Despite these concerns, modification of HSCs would be the ideal ultimate target cells as this would result in transfer of the transgene to all haematopoietic lineages that are susceptible to HIV-1 infection through differentiation. A supply of protected T cells could be provided by HSCs to prevent the characteristic HIV-1 induced T cell loss. Similarly, macrophages, dendritic cells and other haematopoietic cells derived from the modified HSCs would be protected. There are various pathologies associated with HIV-1 infection that do not appear to be simply due to depletion of T cells, and these may be prevented or reduced with HSC gene therapy, rather than modification of only T cells.

HSC gene therapy is particularly appealing in a paediatric setting due to the higher thymic activity of these patients, which would support development of T cells to replace those lost by HIV-1 infection. Also, as these patients are likely to have been on HAART for many years since birth, giving them a high risk of drug resistant escape mutants, they would be ideal candidates for clinical trials.

For initial trials, patients who have developed AIDS lymphoma and are receiving HSC transplant offer ideal opportunities to test this method as autologous cells that have been mobilised and harvested could be transduced with the therapeutic vector prior to infusion. This setting has already been used to transduce $CD34^+$ HPCs with RNA based anti-HIV genes (129). Four patients with lymphoma were transplanted with both unmodified and lentiviral vector transduced cells. Although there was limited therapeutic benefit from the transduced cells, the procedure was tolerated and gene marking was detected in cells of all lineages for up to 24 months, indicating that the procedure did not adversely affect cell viability or potency.

Patients receiving HSCs as part of treatment for lymphoma will already be receiving chemotherapy and/or irradiation prior to transplant to eradicate leukaemic cells and to ablate HSCs to enhance engraftment of transplanted cells. This resolves any ethical issues associated with the provision of potentially dangerous treatments prior to HSC transplant being performed solely for gene therapy.

Conditioning prior to transplant, for instance using busulfan, may have additional advantages of reducing latent cellular reservoirs of HIV and improving engraftment of transduced cells. Although myeloblation and HSC transplant from a healthy donor is not sufficient to eradicate the viral latent reservoir, it is thought that the combination of chemotherapy and total body irradiation received by the 'Berlin' patient may have had a beneficial effect in eradicating latent reservoirs of HIV. This could in part be responsible for the absence of X4 HIV-1 after transplant, despite their presence before the procedure.

It is likely than any initial trials performed in patients will involve transduction of T cells. This will allow observation of any adverse effects associated with TRIMCyp expression and also whether there is a detectable therapeutic benefit. If results from preliminary T cell trials are positive, further work can be carried out by treating patients with modified HSCs.

7.3 Host responses in gene therapy

Using viral vectors expressing TRIMCyp proteins for the treatment of HIV-1 infection has potential issues with inducing a host immune response, against both the virus and transgene product.

Ex vivo transduction of cells would be the most likely method used for anti-HIV-1 therapy. This would reduce the likelihood of developing an immune response against viral proteins as long as there is no residual lentivirus remaining associated with cells during transduction. Even with *ex vivo* transduction, retroviral vectors may induce expression of immunogens, leaving transduced cells susceptible to cytotoxic immune responses (420).

Both TRIM5 and CypA are naturally expressed in humans, reducing the likelihood of TRIMCyp inducing an immune response. However, the junction formed at the site of fusion between the two proteins could result in novel immunogenic epitopes. Also, as mentioned previously, TRIM21 is a known autoantigen associated with autoimmune diseases such as Sjogren's disease, so TRIM21Cyp may be more likely to induce an immune response. However, removal of the highly immunogenic B30.2 domain will hopefully eliminate this problem. Whether TRIMCyp induces an immune response and whether host mediated rejection of gene modified cells arises will not be determined until the strategy is tested in humans.

7.4 The HIV latent reservoir

A major hurdle to the complete eradication of HIV-1 infection by gene therapy, or any other treatment, is the extensive latent viral reservoir. Even with intense HAART and undetectable circulating levels of HIV-1, latent reservoirs are unaffected, and once HAART is discontinued, there is a rapid increase in viral load from these reservoirs. These cell reservoirs are one of the reasons that HAART will not be able to cure a patient of HIV.

The most well characterised reservoir is the resting memory $CD4^+$ T cells, but various other cell types have been proposed to harbour latent provirus, including

naïve T cells, macrophages, microglia, astrocytes and recently, HSCs (417). These cells can survive for many years, resulting in a stable reservoir that decays so slowly that without intervention it would persist for far longer than the survival of an infected individual (421). It has not been fully determined how normal viral replication is prevented in latency. Different mechanisms have been proposed including chromatin effects, an absence of proteins required for transcription such as P-TEFb, NFκB and NFAT, disruption of Tat levels and inhibition by microRNAs present in resting $CD4^+$ T cells (422).

TRIMCyp gene therapy would only provide resistance to T cells from new infections, but would not eradicate any latent virus. However, if there are significant numbers of resistant T cells this could be sufficient to relieve symptoms and pathogenesis of HIV-1 infection. Importantly, as TRIMCyp acts to restrict HIV-1 before integration, and typically before reverse transcription, it will prevent the development of further latent reservoirs in modified cells. If HSCs are the gene therapy target, daughter cells of all lineages susceptible to infection will be protected and prevented from becoming host to latent virus, including macrophages and cells of the nervous system.

Proposed strategies to eliminate the reservoir include intensification of HAART, or its earlier initiation during acute rather than chronic infection to prevent such extensive establishment of the reservoir. Alternative methods aim to use drugs to drive cells out of latency by activating them, leading to the initiation of viral replication. One possible example is to use valproic acid to inhibit histone deacetylases, promoting proviral transcription without the activation and expansion of T cells (423). If this is used alongside an effective HAART regimen or, if it proves successful, TRIMCyp gene therapy, further infection should be prevented and the cells drawn out of latency should be killed by productive infection.

7.5 Combining TRIMCyp restriction factors with CCR5 knockout

The only recorded functional cure of HIV was shown in a single patient who received a bone marrow transplant from a CCR5 Δ32 homozygous donor (339). Clinical trials are already underway to test knockout of CCR5 using ZFNs in T cells to mimic the Δ32 resistant cell phenotype (135). So far results are promising, both in terms of safety and efficacy. This, in addition to the case of the 'Berlin' patient, underlines the importance that an absence of CCR5 expression can have on treating HIV-1 infection.

However, all of the aspects of the Δ32 transplant that contributed to the apparent cure are not fully understood. As well as provision of R5 tropic resistant Δ32 donor cells, the patient received intensive chemotherapy and irradiation before the transplant. This was required for destruction of leukaemic cells, and to assist engraftment of transplanted progenitor cells. In addition, it could have played a role in destruction of latent reservoirs leading to the eradication of X4 tropic virus which, despite being able to infect CCR5 negative cells, was undetectable after transplant. This conditioning could play an important part in any gene therapy treatment before delivery of *ex vivo* modified cells, but cannot be relied on to eliminate X4 tropic virus.

Therefore a major drawback with the knockout of CCR5 is that cells remain susceptible to X4 and dual tropic virus strains, requiring an additional strategy to provide protection from all viral tropisms. In contrast, TRIMCyp proteins are able to restrict HIV-1 using any co-receptor after cell entry. Restriction mediated by TRIMCyp proteins has shown to be highly potent and additional knockout of CCR5 using ZFNs may not provide any further protection. However, the benefit of combining CCR5 specific ZFNs with TRIMCyp is not just enhancing protection of the cells, but in greatly reducing the likelihood of insertional mutagenesis, by exploiting the CCR5 locus as a safe harbour site using only NILVs. Although knockout of CCR5 in TRIMCyp expressing cells may not further restrict viral replication in *in vitro* experiments using a single round of infection, use of two

antiviral strategies *in vivo* may reduce the emergence of HIV-1 escape mutants as modeling suggests that the use of multiple transgenes will enhance the antiviral effect and prevent viral escape (126).

Both of these restriction methods act upon HIV-1 early in its life cycle, at cell entry via CCR5 ZFNs and before reverse transcription by TRIMCyp. Any X4 or dual tropic virus that is able to enter cells not expressing CCR5 would then be susceptible to restriction by TRIMCyp. Restriction early in the viral lifecycle is ideal for gene therapy as it will avoid the pathogenic effects associated with viral protein expression, reducing T cell death and subsequent associated pathology (424). It will prevent the establishment of latent reservoirs and reduce the likelihood of mutations conferring resistance occurring during reverse transcription.

7.6 Conclusion

TRIMCyp shows great potential as a possible transgene for anti-HIV therapy, as *in vitro* they have been shown to provide cells with a strong resistance to infection that so far has been not been susceptible to viral escape. This suggests that they may be able to form the basis of a novel mode of treatment in infected patients based around the intracellular immunisation of susceptible cell populations. The next step of development is to produce clinical grade vector for evaluation in T cells, in the first instance, to determine safety and evidence of a therapeutic effect. If successful, further trials could target HSCs and possibly incorporate conditioning to enhance engraftment and reduction of latent reservoirs.

Gene therapy is still in its early stages, but more and more anti-HIV trials are being performed. Although progress is slow, gene therapy has great opportunity for the treatment of HIV infection, with continual improvements in areas such as vector design, transgene efficacy and transduction protocols. In the future, this method could potentially provide long term protection after only a single intervention, which would be a highly desirable alternative or complement to current drug regimens.

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