MACROPHAGE-CD4$^+$ T CELL INTERACTIONS IN HIV-1 SPREAD

Thesis submitted for the degree of Doctor of Philosophy at the University of Oxford,

Trinity Term 2013

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Abstract

Macrophages and $CD4^+$ T cells are the main targets for productive human immunodeficiency virus-1 (HIV-1) infection *in vivo*. HIV-1 transmission at immune cell synapses, such as the multi-molecular HIV-1-induced virological synapse (VS) formed between T cells, is a more efficient method of viral dissemination than infection by cell-free virus, with important implications for HIV-1 prophylaxis and eradication. Infected macrophages can transfer HIV-1 to $CD4^+$ T cells in a contact-dependent manner. However, the mechanism(s) of intercellular HIV-1 transmission between primary macrophages and $CD4^+$ T cells are poorly defined.

Here I investigate the organisation of the macrophage-T cell VS, which like the VS formed between T cells is dependent on envelope glycoprotein (Env) binding to CD4, and is stabilised by ICAM-1/LFA-1 interactions. However, unlike the T cell VS, where polarised budding of virions occurs at the donor cell membrane, VS-transmission from macrophages involves actin-dependent relocation of HIV-1 contained within the macrophage virus-containing compartment. Macrophage VS-mediated transmission results in highly efficient productive T cell infection at multiplicities capable of largely overcoming antiretroviral inhibition. Compared with cell-free infection of T cells, VS-transmission by macrophages is equally susceptible to broadly neutralising antibodies (bNAbs) against gp120, but relatively resistant to bNAbs targeting the membrane-proximal external region of gp41, probably via steric hindrance.

In a related project, I investigated how HIV-1-infected $CD4^+$ T cells might infect macrophages. Interaction of macrophages with HIV-1-infected $CD4^+$ T cells resulted in rapid phagocytic uptake of the infected T cells, which correlated with efficient productive macrophage infection. This route of transmission also permitted macrophage infection with transmitted/founder HIV-1 clones that inefficiently replicate in macrophages following cell-free inoculation, potentially implicating macrophages in the mucosal amplification of transmitted HIV-1.

Overall, these data indicate an important role for cell-to-cell transmission between macrophages and $CD4^+$ T cells in various aspects of HIV-1 pathogenesis.

"I have learned from my mistakes, and I am sure I can repeat them exactly"

Peter Cook

Acknowledgements

Particular thanks go to my supervisors, Prof Quentin Sattentau, Dr Rebecca Moore and Prof Sir Andrew McMichael, for support, guidance and inspiration.

For funding I am grateful to the Wellcome Trust and Balliol College, and to Prof Rajesh Thakker and Prof Paul Klenerman, past and present directors of the Wellcome Trust Clinical Fellows Programme. I am also grateful to my graduate studies advisor, Prof David Greaves, and my college advisor Dr Piers Nye, for guidance.

Several people contributed directly to this thesis. James Williams in Dr John Frater's laboratory did the integration PCR assays (Figure 4.2). In the Sattentau laboratory, Amy Baxter provided expertise in ImageStream^{TM} and we collaborated on the L/D uptake experiments in Figures 5.4 and 5.5; Torben Schiffner prepared VRC01, 10E8 mAbs and Fabs, and provided the Env images for Figures 1.1, 1.5 and 4.7; and Gemma Needham helped me produce and titre many of the HIV-1 infectious molecular clones (IMCs).

I thank our collaborators Dr John Kappes and Dr Christina Oschsenbauer for kindly contributing IMCs, and others including Dr Ben Chen, Dr Una O'Doherty and Dr John Mascola for providing additional reagents and/or expertise.

I am also grateful to past and present Sattentau lab members for daily help/advice, and am indebted to the many volunteers who provided blood donations.

Most importantly, all my thanks to Sarah: for everything, always. This thesis is dedicated to our new baby James - you beat me to it!

Abbreviations

A, C, T, G - Adenine, Cytosine, Thymidine, Guanidine
AICD - Activation-Induced Cell Death
AIDS - Acquired Immuno-Deficiency Syndrome
APOBEC3 - Apolipoprotein B messenger RNA (mRNA)-editing Enzyme Catalytic polypeptide-like 3
ART - Antiretroviral Therapy
AZT - Azidothymidine
bNAb - Broadly Neutralising Monoclonal Antibody
CA - Capsid (Gag)
CCR5 - C-C Chemokine Receptor 5
CD4bs - CD4-Binding Site
CD4i - CD4-Inducible
CFAR - Centre For Aids Reagents
CKR - Chemokine Coreceptor
CNS - Central Nervous System
CPE - Cytopathic Effect
CSF - Cerebrospinal Fluid
CTL - Cytotoxic Lymphocyte
CXCR4 - CXC Chemokine Receptor 4
DC - Dendritic Cell
DC-SIGN - Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DMEM - Dulbecco's Modified Eagle's Medium
DMSO - Dimethyl Sulfoxide
DNA - Deoxyribonucleic Acid
dNTP - Deoxyribonucleotide Triphosphate
DRV - Darunavir
EC - Elite Controller
EC_{50} - 50% Effective Concentration
EDTA - Ethylene diamine tetra acetic acid
EFZ - Efavirenz
ELISA - Enzyme-Linked Immunosorbent Assay
EM - Electron Microscopy
Env - Envelope glycoprotein
ER - Endoplasmic Reticulum
ESCRT - Endosomal Sorting Complexes Required for Transport
Fab - Antigen Binding Fragment (Antibody)
FBS - Foetal Bovine Serum
Fc - Crystallisable Fragment (Antibody)
FC - Flow Cytometry
FcR - Fc Receptor
Gag - Group antigens (retroviral structural protein)
GALT - Gut-Associated Lymphoid Tissue
Gp120 - 120 kDa Envelope Glycoprotein Surface Subunit
Gp41 - 41 kDa Envelope Glycoprotein Transmembrane Subunit

Group M, N, O, P - M = Major; N = Non-M non-O; O = Outlier; P = *pending the identification of further human cases*
HAD - HIV-Associated Dementia
HIV-1 - Human Immunodeficiency Virus-1
HIV-2 - Human Immunodeficiency Virus-2
HTLV-1 - Human T cell Leukaemia Virus-1
IA-SEM - Ion Abrasion Scanning Electron Microscopy
IAP 1/2 - Inhibitor of Apoptosis 1/2
IC_{50} - 50% Inhibitory Concentration
IFN - Interferon
IL-2 - Interleukin-2
IN - Integrase
InSTI - Integrase Strand-Transfer Inhibitor
IRF3 - Interferon-Regulated Factor-3
IS - Immunological Synapse
ISG - Interfern-Stimulated Genes
IU - Infectious Units (virus)
IU - International Units (cytokine)
kDa - kiloDalton
KIR - Killer Inhibitory Receptor
LE/MVB - Late Endosome/Multivesicular Body
LEDGF/p75 - Lens Epithelium-Derived Growth Factor
LPS - Lipopolysaccharide
LSCM - Laser Scanning Confocal Microscopy
LTNP - Long-Term Non Progressor
LTR - Long Terminal Repeat
LucR - *Renilla* Luciferase
M-CSF - Macrophage-Colony Stimulating Factor
MA - Matrix (Gag)
mAb - monoclonal Antibody
MALT - Mucosa-Associated Lymphoid Tissue
mDC - Myeloid DC
MDDC - Monocyte-Derived DC
MDM - Monocyte-Derived Macrophage
MIP-1α/β - Macrophage Inflammatory Protein-1α/β
MOI - Multiplicity Of Infection
MPER - Membrane Proximal External Region
MRC - Macrophage Replication Capacity
MSM - Men who have Sex with Men
MVC - Maraviroc
NAb - Neutralising Antibody
NADPH - β-Nicotinamide adenine dinucleotide phosphate (reduced form)
NC - Nucleocapsid
Nef - Negative Factor
NF-κB - Nuclear Factor κB
NFAT - Nuclear Factor of Activated T cells
NIBSC - National Institute for Biological Standards and Control
NK Cell - Natural Killer Cell

NLR - NOD-Like Receptor
NNRTI - Non-Nucleoside Reverse Transcriptase Inhibitor
NOD - Nucleotide-binding Oligomerisation Domain-containing protein
NRTI - Nucleoside Reverse Transcriptase Inhibitor
NVP - Nevirapine
PAMP - Pathogen Associated Molecular Pattern
PBMC - Peripheral Blood Mononuclear Cell
PBS - Phosphate Buffered Saline
PCR - Polymerase Chain Reaction
pDC - Plasmacytoid DC
PE - Phycoerythrin (Fluorochrome)
PHA - Phytohaemagglutinin
PI - Protease Inhibitor
PI3K/Akt - Phosphatidylinositide 3-kinase/Ak thymoma
Pol - Polymerase (HIV)
PR - Protease (HIV)
PRR - Pattern Recognition Receptor
PS - Phosphatidyl Serine
PTEF-b - Positive Transcription Elongation Factor-b
PWID - People Who Inject Drugs
RAL - Raltegravir
Rev - Regulator of Expression of Virion proteins
RIG-I - Retinoic acid-Inducible Gene 1
RLR - RIG-I Like Receptor
RLU - Relative Light Units
RNA - Ribonucleic Acid
RPMI - Roswell Park Memorial Institute Medium
RRE - Rev-Response Element
RT - Reverse Transcriptase
RTC - Reverse Transcription Complex
SAMHD1 - Sterile Alpha Motif and HD domain protein 1
SCID - Severe Combined Immunodeficiency Syndrome
SGA - Single-Genome Amplification
SHIV - Simian-Human Immunodeficiency Virus
SIV - Simian Immunodeficiency Virus
SQV - Saquinavir
T-20 - Enfurvitide
T/F - Transmitted/Founder (Virus)
Taq - Thermus acquaticus
TAR - Tat-Responsive element
Tat - Trans-Activator of Transcription
Tetherin/BST-2 - Tetherin/Bovine Stromal cell antigen-2
TGF-β – Transforming Growth Factor-β
TLR - Toll-Like Receptor
TNF-α - Tumor Necrosis Factor-α
TRAIL - TNF-Related Apoptosis-Inducing Ligand
Treg - Regulatory T cell
TREX1 - Three-prime Repair Exonuclease 1

TRIM5α - TRIpartite Motif containing family protein 5α
UI - Uptake Index
VCC - Virus-Containing Compartment
vDNA - Viral DNA
Vif - Viral Inhibitory Factor
Vpu - Viral Protein Unique
Vpx - Viral Protein X
vRNA - Viral RNA
VS - Virological Synapse

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1 INTRODUCTION

1.1 Preface

The following sections provide general background to this thesis.

1.2 Human immunodeficiency virus-1

1.2.1 HIV/AIDS epidemiology

34 million people are currently infected with human immunodeficiency virus 1 (HIV-1) (De Cock et al., 2012). The clinical consequences of advanced infection - known as the acquired immunodeficiency syndrome (AIDS) - were first recognised in a cluster of men who have sex with men (MSM) in California in 1981 (Anon, 1981, Gottlieb et al., 1981). Even before HIV-1 was identified as the causative pathogen in 1983 (Barre-Sinoussi et al., 1983, Popovic et al., 1984) the principal transmission routes (sexual, parenteral and mother to child transmission) had been mapped out in careful epidemiological studies (Jaffe et al., 1983a, Jaffe et al., 1983b).

At this time, AIDS cases in Europe occurred in similar at-risk populations to the US (mostly MSM and people who inject drugs [PWID]), however a greater burden of heterosexual infection was evident in central Africa (Van de Perre et al., 1984, Piot et al., 1984). Various factors have been implicated in the rapid subsequent spread of HIV-1 throughout sub-Saharan Africa including genital ulcer disease, population mobility, urbanisation, low rates of condom use and infrequent male circumcision (reviewed in (De Cock et al., 2012)). The legacy of this unchecked dissemination is apparent today: nine southern African countries with 2% of the world's population contribute ~30% of the HIV cases (reviewed in (De Cock et al., 2012)). Global HIV-1 incidence peaked in 1997 then began to fall, seemingly impacted by a combination of

increased public awareness and modification of risk behaviours, together with control measures such as screening of blood products, provision of clean injecting equipment, promotion of condom use and male circumcision. The development and rollout of effective combination antiretroviral therapy (ART) has helped to stabilise the HIV-1 epidemic (reviewed in (El-Sadr et al., 2012)), yet uncontrolled transmission continues at unacceptable levels in certain risk groups such as MSM (Beyrer et al., 2012). Evidence from the HIV prevention trials network 052 study (Cohen et al., 2011a) suggested that 'treatment as prevention' - the provision of ART to infected people with a view to preventing sexual transmission to serodiscordant partners - could have an important impact on HIV-1 control in the next decade (Eaton et al., 2012).

1.2.2 HIV-1 origins and biology

HIV-1 is a lentivirus of the *retroviridae* family. Molecular epidemiology studies have identified the origin of pandemic HIV-1 in a cross-species transmission event of a related lentiviral species - chimpanzee simian immunodeficiency virus (SIVcpz) - in Cameroon in the early twentieth century, one of several similar zoonotic transmission events (reviewed in (Tebit and Arts, 2011, Sharp and Hahn, 2011)) each of which contributed a separate lineage of HIV-1 (groups M, N, O and P). The minor groups N, O and P constitute less than 1% of global HIV-1 cases and have remained geographically restricted to Cameroon, however pandemic group M (major) HIV-1 had spread to central Africa and the Americas by the 1960s, subsequently reaching Europe and Asia. Over this relatively short timespan multiple subtypes (clades) of M-group HIV-1 have been identified (A-D, F-H, J, K) with over 40 circulating recombinant forms.

The genetic diversity of HIV-1 reflects its rapid (duration 1-2 days) and highly mutation-prone life cycle. Aspects of the life cycle unique to retroviruses are reverse transcription and integration of the proviral DNA into the host genome, which depends initially on the production of double-stranded linear DNA from single-stranded viral RNA (vRNA) by reverse transcriptase (RT) (Baltimore, 1970, Temin and Mizutani, 1970). The highly error-prone process of HIV-1 reverse transcription generates mutations at a rate of approximately $2x10^{-5}$ per nucleotide per replication cycle (Pathak and Temin, 1990b, Pathak and Temin, 1990a); this, combined with the propensity for recombination due to RT template switching if the cell is simultaneously infected with more than one virus (Hu and Temin, 1990), underpins the genetic diversity of HIV-1.

1.2.3 HIV-1 structure, life cycle and antiretroviral therapy

Mature enveloped HIV-1 virions are 100 - 200 nm in diameter. The lipid envelope is unstable and susceptible to rapid extracellular degeneration, conferring a half-life of approximately 5 hours (Perelson et al., 1996). The HIV-1 genome is ~10,000 nucleotides long and encodes three proteins essential to the organisation and generation of new virions - Gag (matrix, MA; capsid, CA; nucleocapsid, NC), Pol (RT, integrase, IN and protease, PR) and Env - as well as six accessory proteins: Tat, Rev, Vpr, Vpu, Nef, and Vif (Figure 1.1). The principal functions of the accessory proteins are to promote viral transcription, counteract host retroviral restriction factors and evade host immunity (discussed below). Detailed understanding of the molecular mechanisms governing viral replication has enabled the development of antiretroviral drugs capable of blocking various stages of the HIV-1 life cycle (Figure 1.1) that will be discussed in the relevant sections below.

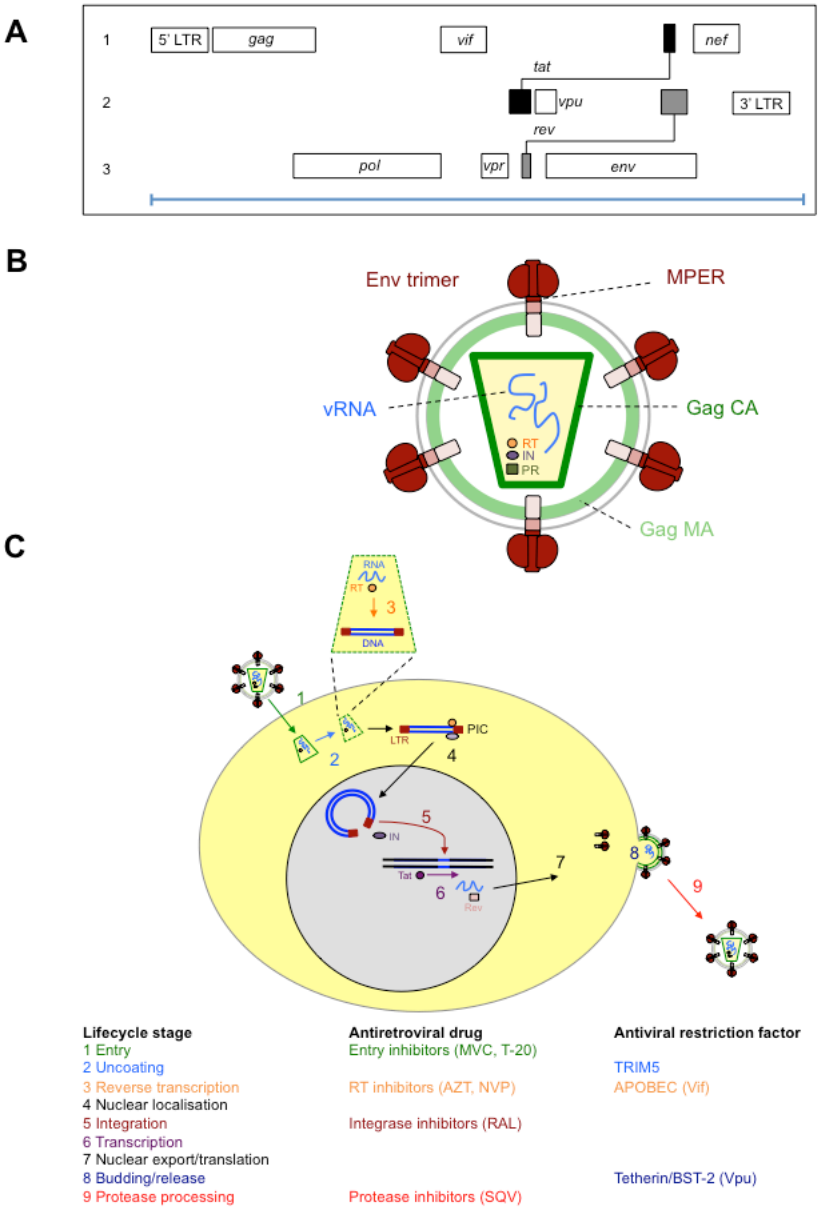

Figure 1.1 HIV-1 structure and lifecycle

A) The HIV-1 RNA genome encodes 9 genes. Adapted from the HIV Sequence Database (http:www.hiv.lanl.gov/sequence/HIV/map). The scale bar represents 10 kilobases.

B) The HIV-1 virion is encapsulated by a host-derived lipid bilayer envelope containing 7-14 160 kDa envelope glycoproteins (Envs) comprising a trimer of gp120 molecules atop the membrane proximal external region (MPER) of gp41, as well as the transmembrane and cytoplasmic domains. The circular virion structure is maintained by a scaffold of Gag matrix protein (p17, MA). Two copies of the genomic ssRNA as well as the viral enzmyes reverse transcriptase (RT), integrase (IN) and protease (PR) are contained within the Gag capsid lattice (p24, CA).

C) The process of HIV-1 replication from entry to budding and maturation. Also displayed are licensed antiretroviral agents (these are class examples) and host restriction factors that act upon these stages. Adapted from (Arts and Hazuda, 2012).

1.2.3.1 Binding and entry

HIV-1 entry is mediated by heavily glycosylated envelope glycoprotein (Env) gp160 trimers (also known as spikes) present on the virion surface (~7-14 per virion) (Chertova et al., 2002). The Env spike is a mushroom-like arrangement with the membrane proximal external region (MPER) subunit gp41 forming a compact stalk upon which three surface gp120 subunits assemble to create a cage-like structure with an internal cavity between the subunits (Figure 1.2A) (Zanetti et al., 2006, Liu et al., 2008, Hu et al., 2010, White et al., 2010).

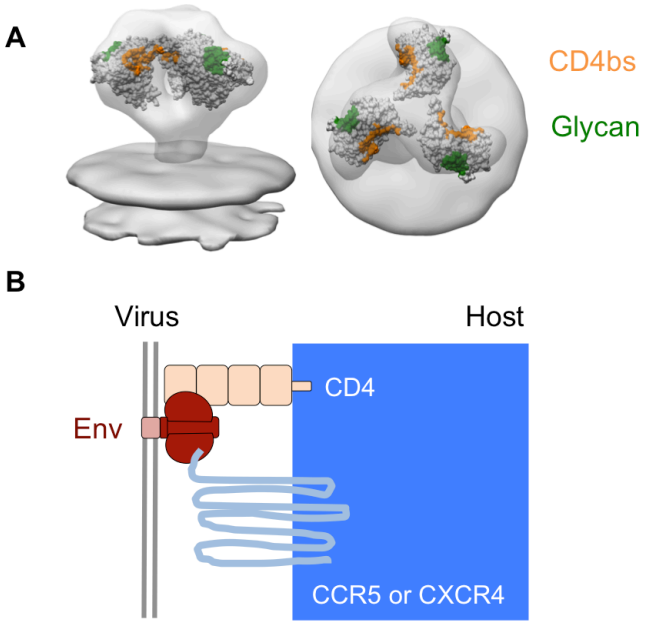

Figure 1.2 Env-receptor interaction
A) HIV-1 Env trimer structure showing the CD4bs and the N332 region targeted by glycan-specific anti-HIV neutralising monoclonal antibodies e.g. 2G12. Images were generated by superimposing the crystal structure of gp120 (PDB ID: 1GC1) into the electron tomography data of gp160 (EMD ID 5019+5022) using UCSF Chimera 1.6.2. Images courtesy of Torben Schiffner.
B) Process of CD4-coreceptor binding. HIV-1 Env binds CD4 inducing a conformational change in Env which exposes the chemokine co-receptor (usually CCR5 or CXCR4) binding site on the V3 loop. Co-receptor binding destabilises the trimer, permitting gp41-mediated membrane fusion through formation of the six-helix bundle.

HIV-1 attaches to cells through non-specific interactions with host cell molecules such as heparan sulfate proteoglycans (Saphire et al., 2001, Mondor et al., 1998), C-type lectins (Ugolini et al., 1999, Geijtenbeek et al., 2000) and integrins (Ballana et al., 2009, Arthos et al., 2008). Although not necessary for infection, viral adsorption enhances the probability of subsequent engagement of the specific HIV-1 entry receptors CD4 (Dalgleish et al., 1984) and a chemokine co-receptor (usually CXCR4 (Feng et al., 1996) or CCR5 (Dragic et al., 1996, Deng et al., 1996)) by the Env surface subunit gp120 (Figure 1.2B). CD4 binding is initially a relatively low affinity interaction (Ugolini et al., 1999) inducing an allosteric change in gp120 that reduces the gp120-CD4 K_{off} and exposes the co-receptor binding site. The coreceptor-gp120 interaction in turn destabilises the Env spike and triggers gp41-mediated membrane fusion over ~1 hour (reviewed in (Wilen et al., 2012)). The importance of co-receptor binding is emphasised in individuals with a heritable deficiency of CCR5 expression (due to a homozygous 32-base pair deletion in *CCR5*) who are resistant to infection by CCR5-using HIV-1 (Samson et al., 1996). The fusion event is pH-independent but it can occur at either the cell membrane (reviewed in (Wilen et al., 2012)) or from within macropinocytic (reviewed in (Mercer and Helenius, 2009)) or endosomal (Miyauchi et al., 2009) vesicles. The relative dominance and implication of these differential entry routes is still unclear.

Two licensed antiretroviral agents target these entry processes (Figure 1.1). The allosteric inhibitor maraviroc (MVC) - a uniquely host-directed antiretroviral - binds to hydrophobic pockets within the second transmembrane helix of CCR5 (Dragic et al., 2000), preventing both gp120 and chemokine binding. The gp41 fusion complex, a six helix bundle comprising two homologous domains (three N helices in an interior trimeric coiled coil onto which three antiparallel C helices pack) can be blocked by the interposition of a heterologous mimic such as the alpha helical peptide enfurvitide (T-20) (Kilby et al., 1998).

1.2.3.2 Reverse transcription

Viral fusion results in the release of the viral core into the cytoplasm and the initiation of reverse transcription, a process known as uncoating. Although the kinetics of reverse transcription are partly cell-type dependent, dictated by the availability of nucleotide precursors (reviewed in (Amie et al., 2013)) that are in turn regulated by the phosphohydrolase activity of sterile alpha motif domain and HD domain-containing protein 1 (SAMHD1) (Goldstone et al., 2011), reverse transcription begins within hours of viral entry (reviewed in (Hu and Hughes, 2012)). Reverse transcription occurs in the reverse transcription complex (RTC) - a multimolecular assembly of MA, CA, NC, IN and Vpr of undefined structure. Premature uncoating is thought to be a host innate defence mechanism against cross-species retroviral infection induced by the TRIpartite Motif containing family of proteins such as 5α (TRIM5α), which blocks reverse transcription through interaction with CA (Stremlau et al., 2004) possibly destabilising the RTC (Stremlau et al., 2006).

Viral DNA (vDNA) synthesis proceeds with the following steps: i) cellular $tRNA^{Lys3}$ primer binding to the primer binding site at the 5' end of the vRNA; ii) long terminal repeat (LTR) cDNA minus strand (strong stop) synthesis and removal of the 5' vRNA end by RT (RNase H domain); iii) repositioning of RT and complete minus strand synthesis from the 3' end; iv) RNase H degradation of RNA (sparing the polypurine tract); v) plus (second) strand cDNA synthesis originating at the polypurine tract with temporary circularization of the cDNA sequence; and finally vi) breakage of the cDNA circles and completion of plus strand synthesis to generate linear dsDNA with a LTR positioned at each end (reviewed in (Hu and Hughes, 2012)).

The apolipoprotein B messenger RNA (mRNA)-editing enzyme catalytic polypeptide-like 3 (APOBEC3) proteins (in particular, APOBEC3G (Sheehy et al., 2002)) are a family of cytidine deaminases packaged into newly assembled virions that induce G-to-A hypermutation in plus-strand cDNA (Sheehy et al., 2002) resulting in impaired cDNA synthesis and deleterious (or occasionally advantageous) sequence mutations. The HIV-1 accessory protein Vif effectively counteracts this process (Sheehy et al., 2002) by polyubiquitination-dependent proteosomal degradation of APOBEC (Sheehy et al., 2003, Marin et al., 2003).

RT was the earliest target for antiretroviral drugs: the first antiretroviral agent azidothymidine (AZT) (Mitsuya et al., 1985) was in clinical use by 1987 (Yarchoan et al., 1986, Fischl et al., 1987). There are two main classes of RT inhibitor: the nucleoside RT inhibitors (NRTIs, e.g. AZT), which are nucleoside analogues incorporated into the cDNA leading to chain termination (Mitsuya et al., 1985); and the non-nucleoside RT inhibitors (NNRTIs, e.g. nevirapine, NVP) which bind a noncatalytic allosteric pocket on RT preventing chain elongation (Merluzzi et al., 1990) (Figure 1.1).

1.2.3.3 Integration

Integration of viral cDNA underpins HIV-1 latency by linking replication of the viral genome to cellular proliferation, and also represents the precursor for new virion production. Integration is catalysed by integrase (IN) (Engelman and Craigie, 1992, Dyda et al., 1994) and occurs within 15 h of viral entry. Upon completion of reverse transcription the RTC transitions to the pre-integration complex (PIC), a large nucleoprotein complex structurally related to the RTC but now containing viral (v)DNA (Bowerman et al., 1989). The PIC must localise to the nucleus for replication in non-dividing cells, a process involving viral proteins MA, CA and IN and host

nuclear pore proteins, however the mechanisms underpinning nuclear localisation have not been satisfactorily resolved (reviewed in (Craigie and Bushman, 2012)).

The steps of vDNA integration are: i) removal of two nucleotides from the 3' ends (3' processing) which allows the 3' ends to attack a pair of phosphodiester bonds in the target DNA separated by five nucleotides; ii) covalent joining of the 3' ends to the 5' ends of the target DNA at the integration site; and iii) removal of the two unpaired bases at the 5' ends of the vDNA closing the single-strand gap between viral and target DNA and ligating the 5′ ends (reviewed in (Craigie and Bushman, 2012)) (processes ii-iii are known as DNA-strand transfer). Integration occurs preferentially at active integration units (Schroder et al., 2002), and data implicate host factors such as lens epithelium-derived growth factor (LEDGF/p75) in facilitating this targeted integration (Ciuffi et al., 2005).

Integrase strand transfer inhibitors (InSTIs) such as raltegravir (RAL) (Hazuda et al., 2000) are relatively recent additions to the antiretroviral arsenal. They block DNA-strand transfer by binding to the specific complex between integrase and the vDNA and interacting with two essential magnesium cofactors in both the integrase active site and the DNA (Hazuda et al., 2000). Labile non-integrated DNA forms including 2-LTR circles (a consequence of non-homologous end joining) accumulate when integration is blocked *in vivo* (Buzon et al., 2010). It remains controversial whether viral transcription can occur from unintegrated linear DNA (reviewed in (Sloan and Wainberg, 2011)).

1.2.3.4 Transcription

Host regulation of transcription balances the contrasting fates of productive HIV-1 infection or viral latency. Provirus transcription initiates at the LTR promoter (Rittner

et al., 1995) under the control of host transcription factors such as Nuclear Factor κB (NF-κB) (Liu et al., 1992) and Nuclear Factor of Activated T cells (NFAT) (Kinoshita et al., 1998) and is therefore inextricably linked to cellular activation status. Transcription is also dependent on additional host factors, such as the Positive Transcription Elongation Factor-b (PTEF-b) complex (Zhu et al., 1997, Wei et al., 1998, Bieniasz et al., 1998) and epigenetic modifications including histone deacetylation and methylation, which are also tied to global cellular activation; as a result resting infected $CD4^+$ T cells exhibit very low levels of viral production (Stevenson et al., 1990).

However, while host RNA polymerase II is responsible for transcribing the provirus, the efficiency of the process is critically dependent on the viral accessory protein Tat (Sodroski et al., 1985b, Sodroski et al., 1985a). Initial LTR-driven provirus transcription is stuttering and inefficient but produces sufficient levels of Tat to trigger a powerful positive feedback loop (Weinberger et al., 2005, Burnett et al., 2009): Tat binds to the LTR at the *tat*-responsive element [TAR] (Dingwall et al., 1989)) enhancing the efficiency of transcription elongation in complex with PTEF-b (Zhu et al., 1997, Wei et al., 1998, Bieniasz et al., 1998). Thus host regulation of Tat expression acts as a molecular switch to drive cells towards latency or virus production (reviewed in (Karn and Stoltzfus, 2012)). Tat remains of considerable interest as a drug target: unfortunately most compounds have failed to show sufficient potency (reviewed in (Arts and Hazuda, 2012)) although there have been recent indications of progress (Lalonde et al., 2011).

HIV RNA transcripts subsequently undergo varying degrees of processing which dictates their route of nuclear export (reviewed in (Karn and Stoltzfus, 2012)). Unspliced (genomic RNA and the *gag* and Gag-Pro-Pol polyprotein message) and incompletely spliced (*env* and *vpu*) transcripts undergo host-mediated degradation,

and therefore depend on the accessory protein Rev (Sodroski et al., 1986), which binds to transcript Rev-Responsive Elements (RRE) (Malim et al., 1989) for nuclear export via host nuclear pore complexes. By contrast, *tat*, *rev* and *nef* mRNA is completely spliced and so is independently transported by host mechanisms, elegantly ensuring that translation of Rev and Tat occurs first - to facilitate both the nuclear export of other transcripts and the positive feedback transcription switch (Weinberger et al., 2005, Burnett et al., 2009).

1.2.3.5 Assembly and budding

Virion assembly involves packaging of all the necessary components for a subsequent round of infection: the structural proteins Env and Gag, the enzymes RT, IN and PR, two ssRNA molecules and host $tRNA^{lys3}$ primer, as well as some accessory proteins (reviewed in (Sundquist and Krausslich, 2012)) (Figure 1.1). Host APOBECs are also incorporated into virions in the absence of Vif (reviewed in (Malim and Bieniasz, 2012)). Cytosolic polysomes translate unspliced vRNA to the polyproteins Gag and Gag-Pro-Pol (containing the Gag polyprotein and the enzymes as domains of Pol) at a 20:1 ratio (Jacks et al., 1988). Env, Vpu and the other accessory proteins are translated in the rough ER from singly and multiply spliced mRNA transcripts respectively (see Section 1.2.3.4). Spherical virion assembly depends on protein-protein and protein-RNA interactions mediated by small oligomers of Gag (containing the MA, CA and NC domains) at lipid rich 'raft' domains in the plasma membrane (Sundquist and Krausslich, 2012, Ono and Freed, 2001, Jouvenet et al., 2009). Incorporation of genomic RNA within the assembling virion is directed by the interaction of Gag with the RNA packaging sequence (Ψ) (reviewed in (D'Souza and Summers, 2005)). Gag targeting to the plasma membrane is achieved by myristoylation of MA (Ono et al., 2004). The membrane proteins Env and Vpu separately traffic to the plasma membrane, although the precise

mechanisms for trafficking all viral proteins to the plasma membrane remain incompletely defined (reviewed in (Sundquist and Krausslich, 2012)).

Budding by viral membrane fission is mediated by the host Endosomal Sorting Complexes Required for Transport (ESCRT) machinery (reviewed in (Sundquist and Krausslich, 2012)). Detailed analysis of the viral membrane revealed enrichment of lipid raft components such as sphingomyelin, phosphatidyl serine (PS), phosphatidyl ethanol and cholesterol (Brugger et al., 2006), implicating raft domains as the virion budding site. Multiple cell-type dependent (Lawn et al., 2000) host molecules (Ott, 2008) are also incorporated into virion membranes, some of which may indirectly influence infectivity (Fortin et al., 1997), however protease (PR)-dependent virion maturation, which occurs during and after budding, is essential for assembly of the conical capsid and virion infectivity (Pettit et al., 1994, Murakami et al., 2004, Moore et al., 2009a). The host restriction factor tetherin/BST-2 (Van Damme et al., 2008, Neil et al., 2008) prevents viral release following budding by tethering virions to the plasma membrane. Tetherin is an unusual transmembrane type II protein with a transmembrane anchor at the N-terminus and a GPI anchor at the C-terminus, and assembles in a coiled-coil arrangement (reviewed in (Malim and Bieniasz, 2012)). It is not a retrovirus specific factor because it acts on a diverse array of enveloped viruses, but the HIV-1 accessory protein Vpu (and in some SIV strains also Nef or Env) counteracts it by reducing its cell surface expression, either through endosomal or proteosomal degradation (reviewed in (Malim and Bieniasz, 2012)). Upon viral budding, tetherin coiled-coils attach the virion to the plasma membrane, and also virions to each other, through an as yet undefined mechanism.

1.2.4 HIV-1 pathogenesis

The natural history of untreated HIV-1 infection is well established and conceptually can be divided into several stages (Table 1.1).

Stage	Description
Transmission	Infection is initiated by a single or a small number of viral sequences (the 'transmitted/founder' virus), followed by widespread dissemination and rapid genetic diversification mediated by the combined effects of error prone replication and selection pressure exerted by an ultimately ineffective host immune response.
Acute	Profound depletion of $CD4^+$ T cells in blood and secondary lymphoid tissues at this stage is associated with excessive immune activation and $CD4^+$ T cell immunodeficiency, while CNS dissemination initiates neuropathology.
Chronic	A period of apparent homeostasis characterised by stable viral production - the viral load set point - and partial recovery of circulating $CD4^+$ T cell numbers.
AIDS	Opportunistic infections, malignancies or dementia herald the onset of AIDS around 8-10 years following initial infection

Table 1.1. Stages of HIV-1 infection

1.2.4.1 Acute infection

80% of HIV-1 transmission events occur by the mucosal route, (Cohen et al., 2011b) which is generally an inefficient process (reviewed in (Hladik and McElrath, 2008)) but is enhanced by cofactors such as genital ulcer disease, lack of circumcision and either acute or advanced HIV-1 infection in the transmitter (Powers et al., 2008). Single genome amplification and sequencing (SGA) of vRNA or DNA (which avoids *taq* polymerase errors, resampling or template switching, reviewed in (Keele, 2010)) combined with a stochastic model of initial genetic diversification (Lee et al., 2009) revealed that a single or small number of transmitted/founder (T/F) viruses are responsible for the initial transmission event (Keele et al., 2008, Salazar-Gonzalez et al., 2009, Bar et al., 2010, Keele, 2010, Li et al., 2010), findings supported by deep

sequencing (Fischer et al., 2010). Higher multiplicity transmission follows rectal mucosal (Li et al., 2010) or parenteral exposure (Bar et al., 2010), but these routes still only involve a handful of viral sequences (up to 16 in the case of parenteral exposure (Bar et al., 2010)). Interestingly T/F viruses invariably use the CCR5 co-receptor for entry (reviewed in (Keele, 2010, Shaw and Hunter, 2012)). Factors governing the tight mucosal bottleneck and preferential transmission of R5-using viruses remain unresolved (reviewed in (Shaw and Hunter, 2012)) but several factors including target cell range and innate defence mechanisms have been implicated (reviewed in (Haase, 2010, Hladik and McElrath, 2008, Borrow et al., 2010)). A recent comparative study showed T/F clones exhibited a higher density of Env spikes, higher infectivity for $CD4^+$ T cells and more efficient replication in the presence of type I interferon (IFN) than chronic Envs, phenotypes that would favour preferential mucosal transmission (Parrish et al., 2013). These findings contradict earlier studies that failed to attribute a phenotypic transmission signature to T/F molecular clones (Ochsenbauer et al., 2012, Parrish et al., 2012).

Mucosal $CCR5^+$ $CD4^+$ T cells are the earliest target cells in SIV transmission studies (Haase, 2010, Zhang et al., 1999, Zhang et al., 2004), and these data are supported by studies of HIV-1 target cells in vaginal explants (Hladik et al., 2007) and by the T cell tropism of HIV-1 T/F molecular clones *in vitro* (Baalwa et al., 2013, Li et al., 2010, Ochsenbauer et al., 2012, Parrish et al., 2012, Salazar-Gonzalez et al., 2009). Initial suggestions that transmission is favoured by preferential binding to the α4β7 integrin expressed by mucosal $CD4^+$ T cells (Cicala et al., 2009, Arthos et al., 2008) have not been confirmed for cloned T/F viruses (Parrish et al., 2012).

Subsequent to the transmission event there is a period of local viral amplification in the mucosa and local lymphoid tissues, during which time virus is undetectable in the blood but a subclinical acute phase response can be identified (Kramer et al., 2010).

This is known as the eclipse period (reviewed in (Shaw and Hunter, 2012)). Within 7-21 days of transmission viraemia becomes detectable through explosive $CD4^+$ T cell infection in secondary lymphoid tissues, particularly the mucosa-associated lymphoid tissues (MALT) (Brenchley et al., 2004, Mehandru et al., 2004) and is associated with the systemic production of a cascade of cytokines (Stacey et al., 2009) and a clinically apparent non-specific viral illness (Ho et al., 1985). Viral dissemination to the CNS occurs at this stage (Schnell et al., 2010) and it has also been proposed that a small proportion of infected $CD4^+$ T cells transitioning to a resting memory phenotype in acute infection establish the long-lived reservoir of latently infected cells (reviewed in (Siliciano and Greene, 2011)).

1.2.4.2 T cell response

$CD8^+$ T cells are central to the host defence against intracellular pathogens. In HIV-1, while antiviral $CD8^+$ T cell responses have been associated with establishing the viral load set point after the peak of viraemia (reviewed in (Walker and McMichael, 2012)), they fail to more effectively control established HIV-1 infection in the majority of patients. This is despite the fact that anti-HIV-1 $CD8^+$ T cells: are readily identified and target a broad range of epitopes (Addo et al., 2003); suppress viral replication *in vitro* (Walker et al., 1986); contribute to early control of peak viral replication (Goonetilleke et al., 2009); induce viral escape mutations with a fitness cost (Schneidewind et al., 2007); and are necessary for set point control in SIV models (Jin et al., 1999, Schmitz et al., 1999). Moreover, polymorphisms in the HLA class I locus (Fellay et al., 2007, Pereyra et al., 2010) have been linked to the rare capacity of some individuals to control viral replication to undetectable levels in the plasma without ART (elite controllers, EC). The viral protein Nef has an important role in down-regulating major histocompatibility (MHC) class I expression in infected cells that contributes to less efficient $CD8^+$ T cell recognition (Schwartz et al., 1996), and

Nef-deleted viruses are associated with slower disease progression (Learmont et al., 1992). However Nef cannot fully account for failure of $CD8^+$ T cell control since Nef-deleted viruses retain the ability to cause AIDS in some cases (Gorry et al., 2007) and sequence analysis in long term non-progressors (LTNPs) has showed no clear association with specific Nef signatures (Pushker et al., 2010). Exhaustion of $CD8^+$ T cells clearly contributes to the loss of control during transition to AIDS (Ribeiro-dos-Santos et al., 2012). Precise details of the key protective functions of antigen-specific $CD8^+$ T cells in ECs need further definition, but the failure of $CD8^+$ T cell control in the majority of patients remains largely unexplained (reviewed in (Walker and McMichael, 2012)).

There is little evidence that naïve $CD8^+$ T cell responses can mediate protection against HIV-1 acquisition and attempts to generate a protective $CD8^+$ T cell response with non-replicating viral vectored vaccines in human trials have failed (Buchbinder et al., 2008), although recent promise was shown in SIV models with a replicating rhesus cytomegalovirus (CMV) vector (Hansen et al., 2011) which appears to induce an extremely broad and novel non-canonical MHC-II restricted $CD8^+$ T cell response (Hansen et al., 2013).

1.2.4.3 Antibody response

A predictable antibody response develops in acute HIV-1 infection which can be used in combination with the sequential appearance of viral (v)RNA and Gag protein in peripheral blood to stage acute infection (Fiebig et al., 2003). Antibodies are generally ineffective in controlling established HIV-1 infection, although recent case reports have suggested a possible partial contribution to viral load set point control (Moore et al., 2009b, Huang et al., 2010). The only target for functional anti-viral antibodies is the Env spike - the most polymorphic of all HIV-1 proteins - and several

Env characteristics contribute to the failure of functional antibody recognition, including rapid escape mutations (Bar et al., 2012, Wu et al., 2012), conformational flexibility and extensive masking of key epitopes by glycans (reviewed in (Kwong and Mascola, 2012)).

Of major interest is the potential for HIV-1 antibodies to confer protection against HIV-1 acquisition through either neutralisation or Fc receptor-mediated effector mechanisms (reviewed in (Overbaugh and Morris, 2012)), although in the only HIV-1 vaccine trial to show partial efficacy (Rerks-Ngarm et al., 2009) the protection was associated with IgG against the V1/V2 region of gp120 rather than neutralising antibodies (Haynes et al., 2012). Monoclonal antibodies with the capacity to neutralise a broad range of HIV-1 Envs (broadly-neutralising monoclonal antibodies - bNAbs, see section 1.7.2) have been isolated with increasing frequency over the past few years from infected individuals (reviewed in (Kwong and Mascola, 2012, Overbaugh and Morris, 2012)) and these bNAbs exhibit robust and repeatable protection in chimeric simian-human immunodeficiency virus (SHIV) challenge models (Hessell et al., 2009, Burton et al., 2011). Intravenous infusion of less potent bNAbs to patients with established HIV-1 slightly delays viral rebound but escape mutations rapidly develop (Trkola et al., 2005), however combinations of more potent bNAbs are highly effective in animal models (Klein et al., 2012). A great deal of effort is currently focussed on understanding how bNAbs develop in infected individuals (Liao et al., 2013) since this may inform strategies to induce these responses by vaccination.

1.2.4.4 Innate response

As discussed above, the innate immune system is involved in early recognition and response to HIV-1 infection and therefore its role in pathogenesis is of increasing

interest (reviewed in (Borrow et al., 2010)). The innate immune system is responsible for sensing pathogens and initiating and orchestrating inflammatory responses. Pathogen sensing involves the recognition of conserved Pathogen Associated Molecular Patterns (PAMPs) by cognate Pattern Recognition Receptors (PRRs). The main classes of PRRs are the Retinoic Acid-Inducible Gene I (RIG-I)-like receptors (RLRs), the Toll-Like Receptors (TLRs), and the Nucleotide Oligomerisation Domain (NOD)-Like Receptors (NLRs) (reviewed in (Takeuchi and Akira, 2010)). Specialised antiviral plasmacytoid dendritic cells (pDCs) sense HIV-1 through TLR 7/8, producing large quantities of type I IFN in response to HIV-1 (Beignon et al., 2005) or HIV-1 infected cells (Lepelley et al., 2011), and type I IFN dominates the initial cytokine storm induced by acute HIV-1 infection (Stacey et al., 2009). Although type I IFN potently inhibits HIV-1 replication *in vitro* it can also induce $CD4^+$ T cell activation that might contribute to immunopathogenesis in acute infection (reviewed in (Borrow et al., 2010)). Additional components of the innate immune system such as natural killer (NK) cells (Alter et al., 2009) have important effector roles against HIV-1. NK cells have also been implicated in set point control (reviewed in (Carrington and Alter, 2012)), although HIV-1 has evolved the capacity to evade NK responses by selective Nef-mediated downregulation of MHC class I proteins while sparing inhibitory NK receptors (killer-inhibitory receptors, KIRs) (Cohen et al., 1999). However, several fundamental aspects of the innate immune response to HIV-1 remain undefined, particularly whether its predominant effect is protective or detrimental (reviewed in (Borrow et al., 2010)).

1.2.4.5 Impact of ART and viral persistence mechanisms

Antiretroviral monotherapy selects for the rapid evolution of drug resistant HIV-1. Resistance mechanisms are variable, but tend to involve either altered drug binding sites (e.g. NNRTIs) or specific mechanisms to exclude the drug (e.g. NRTI thymidine

analogue mutations) (reviewed in (Arts and Hazuda, 2012)). This propensity for resistance limited the clinical utility of the early antiretroviral agents. However, by 1996 several distinct classes of antiretroviral drugs were available (NRTI, NNRTI and PI) and clinical trials of combination ART demonstrated that potent suppression of vRNA to levels below the limit of assay detection could be achieved in the majority of patients (Collier et al., 1996, D'Aquila et al., 1996, Staszewski et al., 1996). With viral replication suppressed, circulating $CD4^+$ T cell numbers (but not mucosal T cells) recover (Autran et al., 1997, Lederman et al., 1998) and the clinical progression of immunodeficiency is halted. It is not an exaggeration to claim ART as the single most important breakthrough in 30 years of clinical HIV-1 research - overnight HIV-1 essentially became a treatable chronic infection.

However, it quickly became apparent that ART fails to eradicate HIV-1, even with several years of therapy, because viral rebound occurs not long after treatment discontinuation in the majority of patients (reviewed in (Sigal and Baltimore, 2012)). The costs and long-term toxicity of lifelong ART are important concerns, and differences in life expectancy compared to uninfected individuals persist depending upon the timing of ART initiation (May et al., 2011, Rodger et al., 2013), although in well-controlled patients with CD4 counts sustained at > 500 cells/mm^3 there is minimal evidence of excess mortality (Rodger et al., 2013). Antiretroviral resistance is also a constant threat requiring high levels of treatment adherence. Finally, recent studies using highly sensitive assays reveal that vRNA remains detectable in the plasma of most patients on ART (reviewed in (Doyle and Geretti, 2012)) meaning viral production is never completely suppressed.

The source of viraemia during ART remains controversial (reviewed in (Doyle and Geretti, 2012)): proposed mechanisms are: i) virus production by long-lived cells such as resting memory T cells reactivated from latent infection (Chun et al., 1997,

Finzi et al., 1997, Finzi et al., 1999) or ii) ongoing viral replication, defined as cycles of infection in which new infected cell populations are founded (reviewed in (Eisele and Siliciano, 2012)), either due to replication in tissues where drug penetrance is impaired (reviewed in (Sigal and Baltimore, 2012)) or possibly through high multiplicity cell-to-cell transmission (Sigal et al., 2011). The relative contribution of latency and ongoing replication is a matter of continuing debate (reviewed in (Sigal and Baltimore, 2012, Eisele and Siliciano, 2012, Cory et al., 2013)) but it is not inconceivable that both are involved. Nevertheless this debate serves to highlight the fact that despite the clinical success of ART, our knowledge of the fundamental mechanisms governing HIV persistence needs to improve (Deeks et al., 2012)). This is particularly relevant when considering strategies for HIV-1 eradication (reviewed in (Frater, 2011)).

1.3 $CD4^+$ T cells

$CD4^+$ T cells (also known as T helper cells) are a heterogeneous population of lymphocytes expressing the αβ T cell receptor that co-ordinate cellular and humoral immune responses and are involved in immune regulation (reviewed in (Sant and McMichael, 2012)). They recognise their cognate peptide antigen bound to MHC class II molecules presented by antigen presenting cells (e.g. DC). If the DC provides suitable co-stimulation, T cell activation and clonal proliferation result. $CD4^+$ T cell effector functions are achieved through the production of cytokines. $CD4^+$ T cells are thus defined variously by their status in relation to antigen exposure (naïve - have not yet engaged the cognate antigen, memory - antigen experienced), activation/proliferation (resting or activated) and their pattern of cytokine production (reviewed in (Sant and McMichael, 2012)). T cell activation is tightly regulated, and most activated $CD4^+$ T cells rapidly undergo activation-induced cell death (AICD), although some activated memory cells transition to a long-lived resting phenotype

until their next antigen exposure (Sant and McMichael, 2012). In addition, a subset of $CD4^+$ T cells has a predominantly immune regulatory, rather than effector, function (regulatory T cells, Treg) (reviewed in (Chevalier and Weiss, 2013)).

$CD4^+$ T cells present in the lamina propria of mucosal tissues such as the gut are predominantly effector memory cells expressing the chemokine co-receptor CCR5 (reviewed in (Lackner et al., 2012)). These memory T cells are highly permissive to HIV-1 replication (reviewed in (Swanstrom and Coffin, 2012)) and are the earliest targets of transmitted HIV-1 (see Section 1.2.4.1). HIV-1-infected T cells are susceptible to rapid cell death due to viral cytopathic effects (CPE) (Costin, 2007) or $CD8^+$ T cell lysis (reviewed in (Walker and McMichael, 2012)), and by additional mechanisms including abortive infection (Doitsh et al., 2010) (Cooper et al., 2013), bystander apoptosis (reviewed in (Garg et al., 2012)) or AICD (Alimonti et al., 2003). As a result, widespread $CD4^+$ T cell infection and massive depletion occurs in the GALT during acute HIV-1 infection, requiring a high level of $CD4^+$ T cell turnover to maintain a sufficient threshold level for mucosal immune defence (thought to be approximately 5-10 % of the total) (reviewed in (Lackner et al., 2012)). This profound loss of memory $CD4^+$ T cells is associated with immune activation (through failure of mucosal barrier function, loss of regulatory control and/or additional effects of uninhibited replication of other pathogens such as herpes viruses), destruction of lymphoid follicular architecture, and eventual immune exhaustion leading to immunodeficiency (reviewed in (Lackner et al., 2012)). However, there is no satisfactory explanation for the observation that SIV infections in natural hosts (e.g. SIVcpz or SIVsm), which are also characterised by high levels of viral replication and acute depletion of mucosal T cells, do not also result in immune activation or immunodeficiency (reviewed in (Lackner et al., 2012)). In the case of HIV-1, R5-using viruses do not infect naïve $CD4^+$ T cells - which do not express CCR5 - although X4-using viruses evolve late in the course of HIV-1 infection in

approximately 50% of individuals that replicate efficiently in all subsets due to broad T cell expression of CXCR4 (Schuitemaker et al., 1992, Brumme et al., 2005). HIV-1 replication in non-cycling (resting) T cells is inefficient (Stevenson et al., 1990) thus a small fraction of infected memory T cells that transition back to a resting state following infection form the latent HIV-1 reservoir (reviewed in (Siliciano and Greene, 2011)).

1.4 Macrophages

Macrophages are tissue-resident, terminally differentiated myeloid-lineage cells (reviewed in (Wynn et al., 2013)). They are widely dispersed throughout most organs, including the CNS (perivascular macrophages and related myeloid-lineage microglia), lymphoid tissue, gut and genital tract mucosa, and lung (alveolar macrophages). The lifespan of macrophages *in vivo* varies from weeks to years and in the steady state they need only infrequent replenishment by monocytes; indeed, many resident macrophages such as microglia exhibit self-renewal properties and can persist lifelong (Ginhoux et al., 2010). Under inflammatory conditions, monocytes are recruited to tissues where they differentiate into macrophages - these inflammatory monocyte-derived macrophages (MDM) are generally short-lived (reviewed in (Geissmann et al., 2010)).

As sentinels of the innate immune system, macrophages initiate inflammatory responses by producing cytokines and chemokines upon recognition of pathogens or molecular markers of host-cell damage by PRRs (see 1.2.4.4). They are also responsible for clearance of pathogens, cells and cellular debris by phagocytosis (reviewed in (Sarantis and Grinstein, 2012)). While macrophages in different tissues share some common characteristics, such as phagocytic ability, they also display considerable functional heterogeneity (reviewed in (Gordon and Martinez, 2010)).

This tissue-specific heterogeneity in turn influences the permissiveness of tissue macrophages to HIV-1 infection (reviewed in (Collini et al., 2010)). For example, gut macrophages appear relatively restrictive to productive infection whereas genital tract macrophages are highly susceptible (Shen et al., 2009, Shen et al., 2011). The gut macrophage phenotype (reduced CD4/CCR5 expression, reduced NF-κB translocation) can be recapitulated *in vitro* by the application of intestinal stromal conditioned media to MDM and is dependent on TGF-β (Shen et al., 2011), highlighting the important role of the tissue cytokine microenvironment in regulating macrophage permissiveness (reviewed in (Bergamaschi and Pancino, 2010)).

The capacity to infect macrophages is a conserved feature of mammalian lentiviruses and most prototypic lentiviruses are exclusively macrophage tropic (Miller et al., 2000). Indeed, some pathogeneic lentiviruses (e.g. the Maedi-Visna virus of sheep) cause clinical features reminiscent of AIDS despite not infecting T cells. Thus while the expanded tropism of HIV-1 for $CD4^+$ T cells (particularly mucosal effector memory T cells) is clearly associated with immune dysfunction, the contribution of macrophage infection to viral pathogenesis, particularly within the CNS, is increasingly recognised (reviewed in (Cobos-Jimenez et al., 2011, Koppensteiner et al., 2012b)). The functional consequences of HIV-1 infection for macrophages, in terms of dysfunction of innate immunity, are rather more controversial (reviewed in (Collini et al., 2010)). However, HIV-1-infected macrophages appear to play an important role in HIV-1 persistence, which I will discuss briefly below.

Infected macrophages are present in patients with advanced HIV-1 infection (Embretson et al., 1993, Gartner et al., 1986a, Gartner et al., 1986b, Koenig et al., 1986, Meltzer et al., 1990). The preponderance of studies from the pre-ART era relates to the availability of autopsy specimens in a period when progression to AIDS and death was commonplace, but infected macrophages have also been identified at

additional stages of HIV-1 infection. For example, 7-12% of vRNA producing cells in lymph node samples in patients in acute, early and late infection were $CD3^-$ (i.e. myeloid origin) (Zhang et al., 1999). In the first year of infection macrophages ($CD68^+$) comprised a median of 7% (range, 2%-19%) of the target cells in evaluated HIV-1^+ lymphoid tissue samples (Schacker et al., 2001). Following the initiation of ART macrophages contribute to the slower second phase decline of viraemia that originates in long-lived infected cells (Perelson et al., 1997) and rebounding viral sequences during structured treatment interruption are distinct from proviral DNA in circulating $CD4^+$ T cells, implicating an alternative cellular source (Chun et al., 2000, Davey et al., 1999, Zhang et al., 2000). Until recently little attempt has been made to accurately quantify the macrophage reservoir in patients on ART because of the invasive methods needed to sample tissues but concerted efforts are now underway (Cory et al., 2013). Recent studies of patients on ART have identified evidence of infected macrophages in the genital tract (Deleage et al., 2011) and the duodenal mucosa (Zalar et al., 2010). While the relative percentage of HIV-1-infected duodenal macrophages is low, the abundance of macrophages in the gut suggests the absolute number during ART could be relatively high (reviewed in (Cory et al., 2013)). Although preliminary, these data indicate that macrophages might contribute to HIV-1 persistence during ART.

1.5 Viral determinants of macrophage tropism

Cell-free HIV-1 infection of macrophages, which occurs *in vitro* through a dynamin-dependent macropinocytic-like uptake mechanism prior to fusion, is a relatively inefficient process (Carter et al., 2011). Compared with $CD4^+$ T cells, macrophages express limiting levels of CD4 (Collman et al., 1990, Raposo et al., 2011) and following entry, the virus faces inhospitable intracellular conditions where limiting concentrations of nucleotide precursors such as deoxynucleotide triphosphosphates

(dNTPs) are mediated by the dNTP hydrolase activity of the sterile alpha motif and HD domain protein 1 (SAMHD1) (reviewed in (Amie et al., 2013)). SAMHD1 potently inhibits HIV-1 replication in DCs (Laguette et al., 2011, Hrecka et al., 2011), and accounts for the dependence upon the SIV/HIV-2 accessory protein Vpx (which induces ubiquitin-proteasome-dependent degradation of SAMHD1) for DC replication (Laguette et al., 2011, Hrecka et al., 2011). Although Vpx enhances macrophage HIV-1 infection (Sunseri et al., 2011, Kaushik et al., 2009), SAMHD1 fails to entirely prevent macrophage infection because of the unique ability of HIV-1 to scavenge the low dNTP concentrations present in macrophages, through the evolution of a high affinity reverse transcriptase (Diamond et al., 2004). Therefore Vpx is not conditional for HIV-1 replication in macrophages. In addition to the tissue-specific variations in permissiveness to HIV-1 infection described above, mediated in large part by distinct tissue microenvironments, important but poorly defined donor-dependent influences on the efficient of HIV-1 replication by macrophages have also been reported (Bol et al., 2009).

In addition to these strong host effects there is also considerable variation in the ability of viral isolates to replicate in primary macrophages. The replication capacity of CCR5-using (R5) HIV-1 in macrophages can vary by up to 1000-fold *in vitro*, and many primary R5 isolates are unable to replicate in macrophages (Gray et al., 2005, Peters et al., 2006). There is considerable interest in exploring the determinants of HIV-1 macrophage tropism from the perspective of the virus, which I summarise in the following sections. These are adapted from a previously published review (Duncan and Sattentau, 2011).

1.5.1 Defining macrophage tropism

The concept of viral tropism for macrophages is both categorical (replication or not), and continuous (a spectrum of replication capacity). Here I use macrophage tropic (mac-tropic) to describe viruses capable of infecting macrophages *in vitro* in single-cycle or spreading infection assays using MDM, and consider viruses that do not replicate in single-cycle or spreading infection assays to be non-macrophage tropic. To conceptualise infection efficiency of mac-tropic isolates, I also use the term 'macrophage replication capacity' (MRC), an arbitrary measure of *in vitro* virus replication depending on the assay used. It has been recently recommended that the use of the term "replication" be restricted to describing a new round of productive infection (reviewed in (Eisele and Siliciano, 2012)), however many studies of mac-tropism have been done in single cycle assays, thus I also use it to refer to a single cycle of replication. Reference laboratory-adapted R5 HIV-1 isolates (including YU2, BaL, and SF162) demonstrate higher MRC than most primary isolates or molecular clones obtained without amplification *in vitro*, and can provide a positive control for qualitative and relative quantitative assessment of tropism phenotypes of primary isolates. These terms allow a clearer distinction between the complementary but distinct qualitative and quantitative aspects of tropism phenotype. Methodological considerations for obtaining primary viruses also warrant discussion in the context of tropism assessment.

Isolation of primary virus from cultured tissue samples or donor peripheral blood mononuclear cells (PBMCs) has the potential to drive adaptation to a specific cell-type that could confound assessment of the tropism phenotype, and may also select for viruses with more rapid *in vitro* replication kinetics that do not represent the dominant species *in vivo*. Bulk or limiting dilution PCR amplification of full-length molecular clones or *env* sequences avoids these issues, but is subject to *taq* polymerase errors (either mutations or recombination) as well as template

resampling or bias in amplification due to primer selection as discussed above (1.2.4.1).

1.5.1.1 Beyond co-receptor usage

HIV-1 tropism classification systems have gone through several iterations over the years (reviewed in (Goodenow and Collman, 2006)). When the principal co-receptors for HIV-1 were initially identified, it appeared that viral tropism for macrophages and R5 co-receptor preference were closely correlated. Although this is generally the case, there are exceptions (reviewed in (Goodenow and Collman, 2006)); for example, it was noted that some X4-using viruses could productively infect macrophages (Jayakumar et al., 2005, Verani et al., 1998, Simmons et al., 1998), whilst many R5 isolates could not (Peters et al., 2006), indicating that the tropism phenotype is related to, but distinct from, co-receptor preference. A reasonable proposal to modify the co-receptor classification to incorporate macrophage tropism (Goodenow and Collman, 2006) has not been widely adopted; for clarity here I qualify cellular tropism alongside viral co-receptor preference.

1.5.1.2 Association with disease stage

Up to 50% of patients who progress to AIDS do so with a viral quasispecies that has switched co-receptor preference from R5 to X4 {Gray, 2005 #512}. The remainder of individuals progress to AIDS without co-receptor switching, retaining the CCR5 preference of the initial transmitted virus(es) (Tuttle et al., 2002, Gray et al., 2005). In these individuals the mac-tropism of R5 isolates amplified by PBMC co-culture has been associated with disease stage in cross-sectional studies (Tuttle et al., 2002, Li et al., 1999). R5 isolates from individuals with advanced HIV-1 infection or AIDS can display enhanced *in vitro* MRC compared to early infection isolates (Tuttle et al., 2002, Li et al., 1999). It is unclear whether this apparent association with disease

progression may result from evolution of tropism with time to counter progressive loss of target $CD4^+$ T cells (Gorry et al., 2004), or whether macrophage replication is a viral virulence mechanism in its own right (reviewed in (Kuroda, 2010)). Although only one study (Richards et al., 2010) has been specifically designed with the principal aim of assessing the dynamics of tropism with time, other longitudinal studies have provided further support for the proposed association between disease stage and mac-tropism/MRC for R5 isolates observed in cross-sectional studies (Masciotra et al., 2002, Etemad et al., 2009, Richards et al., 2010). Richards and colleagues (Richards et al., 2010) examined changes in R5 mac-tropism and MRC over time of clade B Envs amplified by PCR from proviral DNA or vRNA in three subjects. Isolates were obtained periodically from the first 3 weeks to several years post-infection. The *env* sequences were cloned into an isogenic replication competent virus backbone. A highly divergent tropism phenotype was observed for early infection Envs, which may at least in part relate to the non-SGA method of sampling, which is prone to *taq* polymerase errors (see above). Clones from later stages of infection were more frequently mac-tropic, and in 2/3 subjects all late-stage clones were mac-tropic. In one subject who progressed to AIDS, MRC was highest at the time of AIDS diagnosis. Similarly Mascriota and colleagues (Masciotra et al., 2002) measured the *in vitro* tropism phenotype of primary isolates acquired longitudinally (ranging from 3–5 isolates) from 13 individuals by PBMC co-culture. Although the exact time of seroconversion was unknown, these patients were clinically classified as rapid, late or LTNP based on $CD4^+$ T cell count and time to AIDS. The authors hypothesised that mac-tropism of isolates would decline over time in progressors. This hypothesis was discounted, but insufficient data were presented to identify whether there was, by contrast, a temporal increase in mac-tropism or MRC. A representative data plot (Figure 3 in (Masciotra et al., 2002)) with two subjects per group was presented. Consistent with the alternative hypothesis that mac-tropism increases with disease progression, most isolates from late or rapid

progressors were mac-tropic. By contrast, 4/5 of the non-mac-tropic isolates were obtained from LTNP. There was also an increase in MRC over time in 1/2 rapid and late progressors, but no change in 2/2 LTNP. Finally, Etemad and colleagues (Etemad et al., 2009) amplified the V1-V5 region of clade A *envs* by PCR from PBMC in early and chronic infection (from 8 subjects) and cloned these into an isogenic clade A *nef*-deleted replication competent backbone, and tested MRC. No consistent patterns were observed, which may possibly relate to the earlier disease stage of the chronic isolates (median 41 months post-seroconversion) tested here in comparison to other studies, or to the absence of *nef*. In 3/8 subjects, chronic isolates replicated more efficiently than early isolates, whilst in 4/8, no clear increase was seen. In 1/8 a decrease in MRC was observed. However the data from this individual may have been biased by the relatively brief sampling interval between isolates (early isolate - 6 months, chronic isolate - 24 months) and the availability of only a single chronic isolate for comparative testing.

Overall, it appears that more isolates from individuals with advanced HIV-1 or AIDS are mac-tropic, and demonstrate higher MRC than earlier isolates (Li et al., 1999, Tuttle et al., 2002, Richards et al., 2010), in agreement with the hypothesis that tropism for macrophages evolves over time. This is supported by data from macaque SHIV models (Igarashi et al., 2001). However, the prospects for future longitudinal studies in humans, which are required in order to better understand the dynamics of tropism, are limited by ethical constraints and moves towards earlier initiation of anti-retroviral therapy (ART) in chronic infection.

The selection pressures on viral populations *in vivo* that may influence this evolution in tropism are incompletely understood. $CD4^+$ T cell attrition and associated B cell dysfunction, which occur as the disease progresses (Brenchley et al., 2006), may influence the neutralising antibody (NAb) response in advanced HIV-1. However,

Richards and colleagues (Richards et al., 2010) demonstrated that the divergent mac-tropism identified in early PCR amplified Envs occurred prior to the development of NAbs. Intriguingly, when examining samples from the latest time point a few years post seroconversion, NAbs from the individual with the fewest mac-tropic Envs had the greatest degree of homologous neutralisation activity. One could speculate that this might suggest an inverse relationship between mac-tropism of viral isolates and the exposure to, and activity of, NAbs later in disease. However, this is far from established.

Tropism for macrophages has been proposed as a direct marker of pathogenesis in HIV-1 (reviewed in (Kuroda, 2010)), although there are few *in vivo* data in humans to support this hypothesis. In macaque SIV models increased monocyte turnover was observed and appeared to better predict disease progression than viral load or CD4 T cell count (Hasegawa et al., 2009, Burdo et al., 2010). Whether increased turnover of bone marrow and peripheral blood monocytes occurs in response to direct monocyte cell death in the blood or bone marrow, monocyte repopulation of dying tissue macrophages, or as a consequence of increased monocyte recruitment in response to inflammation following microbial translocation across immune-depleted gastrointestinal mucosa, is unclear. It is also unclear whether increased monocyte turnover also occurs in HIV-1 infection, although markers of monocyte and macrophage activation have been identified in HIV-1 patients in several studies (reviewed in (Herbein and Varin, 2010)). In addition, these markers appear to persist despite peripheral blood $CD4^+$ T cell recovery after ART (Wallet et al., 2010). An interesting report on HIV-1 elite controllers previously identified an as yet undefined partial restriction to HIV-1 replication in macrophages, which occurred at the post-entry level, suggesting that host cell range may also be involved, to some degree, in HIV-1 control (Saez-Cirion et al., 2011).

1.5.1.3 Transmitted/Founder viruses

Early reports implicated macrophages as the principal target for transmitted virus based on the mac-tropic phenotype of these isolates (Zhu et al., 1993, van't Wout et al., 1994). Mac-tropism was proposed to be a component of the genetic bottleneck observed for mucosal transmission of R5 HIV-1 (reviewed in (Margolis and Shattock, 2006)). However, recent data challenge this paradigm. Most studies report evidence of low MRC of T/F viruses despite equivalent replication kinetics in $CD4^+$ T cells (Ochsenbauer et al., 2012, Salazar-Gonzalez et al., 2009). Restriction at the level of entry appears to determine this phenotype (Isaacman-Beck et al., 2009). These data suggest that cell-free T/F viruses are unlikely to efficiently infect macrophages to high levels early in infection, perhaps indicating that evolution of MRC occurs downstream of transmission of the founder sequence. However these data do not account for observations of infected macrophages *in vivo* in acute/early infection discussed above, suggesting that an alternative mechanism of more efficient macrophage infection might operate *in vivo*.

1.5.1.4 Compartmentalisation

Virus isolates from different tissue compartments demonstrate both intra- and inter-host diversity in tropism phenotype, with isolates from brain consistently demonstrating enhanced mac-tropism/MRC by comparison with isolates from other tissues such as blood and lymphoid tissues (Koenig et al., 1986, Ghorpade et al., 1998, Gorry et al., 2001, Smit et al., 2001, Peters et al., 2004, Dunfee et al., 2006, Peters et al., 2006, Dunfee et al., 2007, Peters et al., 2007, Thomas et al., 2007, Rossi et al., 2008, Dunfee et al., 2009, Gray et al., 2009). Genetic compartmentalisation between tissues has been widely studied (van Marle et al., 2007, Tovanabutra et al., 2007, Ritola et al., 2005, Burkala et al., 2005, Abbate et al., 2005, Kemal et al., 2003, Becquart et al., 2002, Marras et al., 2002, Haddad et al.,

2000, Coombs et al., 1998), and has also been noted between macrophages and $CD4^+$ T cells (Fulcher et al., 2004, Potter et al., 2004, Llewellyn et al., 2006, Xu et al., 2008), although recent studies adopting the SGA approach suggest that clonal proliferation or amplification within tissues may have over-estimated previous measures of genetic diversity identified using bulk or limiting dilution PCR methods (Bull et al., 2009a, Bull et al., 2009b, Anderson et al., 2010, Gantt et al., 2010, Salazar-Gonzalez et al., 2011). Earlier studies also suggested $CD4^+$ T cell-associated virus was capable of replicating in macrophages *in vitro* (Massari et al., 1990). In a study in which compartmentalised variants in blood monocytes were cloned and phenotyped, R5 mac-tropic viruses predominated, although R5X4 dual-tropic viruses that infected both MDM and $CD4^+$ T cells were also isolated (Xu et al., 2008). Within the CNS, a cross-sectional analysis associated compartmentalisation with disease stage (Harrington et al., 2009). An interesting SGA-sequencing study of longitudinally acquired cerebrospinal fluid (CSF) and blood samples also provided evidence of independent and dynamic sequence variation in the CNS (Schnell et al., 2010).

Compartmentalised viruses may evolve by stochastic variation within a spatially confined location, or as a result of adaptation to the available target cells, microenvironment and immune pressures (Kosakovsky Pond et al., 2008). The majority of mac-tropic/high MRC isolates are brain-derived (Fischer et al., 2010). In the brain the most abundant target cells are perivascular macrophages and macrophage-lineage microglial cells, which unlike gut macrophages are highly permissive despite expressing low levels of CD4 and co-receptors CCR5 and CXCR4 (Peters et al., 2007). The alternative co-receptor CCR3 can also be used by some brain-derived Envs (He et al., 1997, Aasa-Chapman et al., 2006). Moreover, host immune pressure from NAbs is contained by the blood-brain barrier (Richards et al., 2010). Taken together with data suggestive of differential HIV-1 permissiveness in

distinct tissues (see 1.4), these data suggest that adaptation to tissue conditions, rather than stochastic variation, is the dominant influence in determining the viral tropism of compartmentalised variants (Borderia et al., 2007).

1.5.1.5 Influence of recombination

Recombination occurs frequently during HIV-1 replication by switching of reverse transcriptase between heterologous RNA strands, which are present within individual cells as a result of multiple infection (Wodarz and Levy, 2010) (see 1.2.2). It has been suggested, using single fluorescently-tagged viruses, that recombination occurs more frequently in macrophages than $CD4^+$ T cells (Levy et al., 2004), although this finding has not been reproduced in other studies (Chen et al., 2005).

In a recent study employing SGA-sequencing of chronic isolates *in vivo*, inter-compartmental recombination events were shown to result in transfer of R5 Env determinants that dramatically altered mac-tropism/MRC, conferring brain-like MRC to recombinant lymph node isolates (Brown et al., 2011). As well as implicating Env as a key determinant of mac-tropism, these data raise the possibility that in later infection, recombination events between R5 brain-derived viruses and other non-mac-tropic tissue-derived R5 viruses could expand intra-host macrophage infection capability. Estimates of the frequency of recombination were approximately 10–15% (Brown et al., 2011). The mechanism(s) underpinning the necessary precursor events to intra-host recombination (such as inter-compartmental migration of cell-free or cell-associated virus, and subsequent multiple-virus infection of cells within the new compartment) need to be explored. As an efficient mode of HIV-1 dissemination (Sattentau, 2008), cell-to-cell transmission has been implicated in multiple infections per cell (Sigal et al., 2011, Del Portillo et al., 2011, Russell et al., 2013), and may therefore also be implicated in increasing the likelihood of recombination events *in vivo*. Recent data provide evidence of transit of sequences between brain to

periphery, occurring at the meninges (Lamers et al., 2011). Therefore, the brain could represent a reservoir of mac-tropic virus with high MRC, which can emerge in later stages of infection.

1.5.2 Molecular determinants of macrophage tropism: entry

Comparative studies of amino acid signatures in Env gp120 in high MRC *versus* non-mac-tropic isolates have identified several putative molecular determinants of R5 and X4 tropism for macrophages. These studies have predominantly utilised isolates obtained by co-culture with PBMC or amplified by PCR (with the inherent caveats described above), although the SGA-sequencing approach has been recently employed (Brown et al., 2011). In most studies, the *env* sequence was cloned into pseudoviruses, and tropism defined as entry and reporter gene expression in a single-cycle assay. Multiple single amino acid differences in the regions of R5 Env involved in CD4 binding principally distinguish between mac-tropic and non-mac-tropic isolates, as do incompletely characterised variations in the length and number of potential N-linked glycosylation sites in V1/2 regions of Env. In addition, there are some indications of an association between sensitivity to a limited number of CD4 binding site (CD4bs)-directed NAbs and R5 mac-tropism/MRC (Peters et al., 2008, Dunfee et al., 2009).

1.5.2.1 CD4 binding

Adaptation to efficiently enter cells expressing low surface CD4 has been observed in several studies using R5 mac-tropic brain isolates (reviewed in (Peters et al., 2007)), and R5 mac-tropism and/or MRC was shown to correlate with sensitivity to inhibitors of CD4-gp120 interactions (Peters et al., 2008, Peters et al., 2004, Gray et al., 2005, Peters et al., 2007, Duenas-Decamp et al., 2009, Brown et al., 2011), indicating that the macrophage tropic phenotype is strongly dependent on Env-CD4

binding affinity and/or exposure of the CD4 binding site (CD4bs). Molecular determinants include the N283 substitution in the C2 region, which enhances the binding affinity of gp120 to CD4 (Dunfee et al., 2006), and loss of the N-linked glycosylation site N386 in V4 (Dunfee et al., 2007). N386D mutagenesis of non-mac-tropic R5 Envs conferred increased MRC (Dunfee et al., 2007). N386 alone, or in combination with proximal residue R373 (Duenas-Decamp et al., 2008) was also suggested to mediate resistance to the CD4bs bNAb antibody b12. These molecular determinants accounted for around 50% of the enhanced mac-tropism/MRC of the pseudotyped Envs tested. Additional determinants on the N-terminal flank of the CD4 binding loop within the CD4bs, that influence CD4bs exposure, have also been associated with enhanced macrophage tropism and altered sensitivity to the tetrameric soluble (s)CD4-based inhibitor PRO 542 (Duenas-Decamp et al., 2009). A single E153G substitution within the gp120 V1 loop, distant from the CD4bs, was associated with enhanced R5 MRC, and was abrogated by the reciprocal mutation G153E (Musich et al., 2010). The exact mechanism remains uncertain, but it probably involves increased exposure of the CD4bs due to a V1-induced conformational change in the V3 loop, which incidentally also conferred increased sensitivity to the CCR5 binding antibody 477-52D (Musich et al., 2010). Moreover, variations in the V1/V2 region were recently identified as conferring mac-tropism to recombinant viruses identified by SGA sequencing (Brown et al., 2011).

Although mac-tropic Envs display increased sensitivity to inhibitors of CD4 binding, implicating the CD4bs (Peters et al., 2004, Gray et al., 2005, Peters et al., 2007, Peters et al., 2008, Duenas-Decamp et al., 2009, Brown et al., 2011), the relationship between the R5 Env properties of mac-tropism or MRC, and neutralisation sensitivity to NAbs targeting the CD4bs, is less clear. Originally, Gray and colleagues demonstrated an association between R5 mac-tropism/MRC and sensitivity to b12 (Gray et al., 2005), confirmed separately by Dunfee and colleagues (Dunfee et al., 2009). However, by contrast with previous studies, Dunfee and colleagues did not

identify an association between R5 MRC and sensitivity to sCD4 (Dunfee et al., 2009). N386D mutagenesis of the R5 reference strains YU2 and JRFL enhanced MRC by 1–2 orders of magnitude, and increased sensitivity to b12 by approximately 2-fold (Dunfee et al., 2009). However, not all studies confirm the association with b12 sensitivity. For reasons that are unclear, Brown and colleagues identified an inverse association with either b12 or 2G12 sensitivity (Brown et al., 2011) despite demonstrating a clear association between sensitivity to sCD4 and MRC. Variation in mac-tropism also emerged prior to autologous NAb responses in the study by Richards and colleagues (Richards et al., 2010), suggesting that the properties of NAb sensitivity and mac-tropism may be distinct, although there appeared to be a temporal association between reduced MRC and higher NAb activity in later isolates. Taken as a whole, the data are inconclusive with regard to an association between NAb sensitivity and mac-tropism or MRC, and more studies are required.

1.5.2.2 Co-receptor binding

Similar to the differential interaction with CD4, variable sensitivity to CCR5 inhibitors has also been observed for mac-tropic R5 Envs (reviewed in (Peters et al., 2007)). Reduced sensitivity to fusion inhibitors of chronic, compared to early R5 Env isolates spanning V1-5, was not associated with enhanced MRC despite increasing the efficiency of productive infection in cell lines and PBMCs, for reasons which are unclear (Etemad et al., 2009). However, a recent study employing a luciferase reporter system and a panel of R5 *env* sequences identified a correlation between entry efficiency in $CCR5^{low}$ cell lines with stable CD4 expression and MRC suggesting the mac-tropic Envs could more efficiently use low levels of co-receptor (Sterjovski et al., 2010). Inhibition by maraviroc was also reduced in the mac-tropic R5 Envs, implying that conformational changes that enhanced R5 binding also impaired small molecule inhibition. Duenas-Decamp and colleagues (Duenas-

Decamp et al., 2009) observed V3 loop determinants of R5 mac-tropism. They suggested that a conformational change in the V3 loop increased exposure of the CD4bs.

Whether enhanced co-receptor binding is linked to the macrophage tropism phenotype for X4 Envs has been less studied. Although the majority of mac-tropic brain-derived Envs are R5, some brain isolates can differentially use either CCR5 or CXCR4 for entry into macrophages. Non-brain R5X4 viruses, on the other hand, use X4 preferentially for macrophage entry (Gray et al., 2009). In an elegant mutagenesis study of an unusual brain-derived R5X4 virus it was shown that S306 in V3 conferred enhanced R5-entry via decreased dependence on the N terminus of CCR5, whereas in contrast R306 conferred enhanced X4-entry via decreased dependence on the N-terminus of CXCR4. This study provides a putative molecular determinant of co-receptor preference for a mac-tropic R5X4 envelope (Gray et al., 2009).

Recently, additional determinants were identified that conferred mac-tropism to more unusual X4-using mac-tropic Envs (Cashin et al., 2011). These included variants at position 326 in V3, and at positions 261 and 263 within the gp41 interactive region of gp120. The presence of isoleucine at position 326 was critical for mac-tropism across divergent X4 Envs. These X4 Envs were sensitive to large deletions in the N-terminus of the chemokine coreceptor CXCR4, suggesting that co-receptor binding and mac-tropism for X4-using viruses are linked.

1.5.3 Molecular determinants of macrophage tropism: post-entry

Humans have co-evolved with retroviruses, and some 8% of the human genome (more numerous than the protein coding regions) is thought to comprise redundant retroviral elements (Bannert and Kurth, 2004). There are a number of host restriction factors that partially regulate cellular permissiveness to HIV-1 infection post-entry, some of which are type I IFN-inducible (e.g., Tetherin/BST-2, SAMHD1 and the APOBECs). Those relevant to macrophage tropism are reviewed in (Bergamaschi and Pancino, 2010). The fact that restriction factors are unable to prevent ongoing HIV-1 replication in the host indicates that the virus has evolved successful strategies to overcome such restrictions, which primarily involve the non-structural accessory proteins of HIV-1, such as Vif (which targets APOBEC3G) and Vpu (which targets tetherin) (Kirchhoff, 2010). Viral infectivity is also associated with other regulatory proteins such as Nef and Tat. HIV-1 accessory proteins are the focus of a great deal of research (reviewed in (Kirchhoff, 2010) and (Malim and Emerman, 2008)). Here I discuss specifically the role of the HIV-1 accessory proteins in determining MRC.

1.5.3.1 Tat

Rather unsurprisingly, reduced *tat* mRNA expression in MDM has been shown to correlate with reduction in steady-state R5 virus production (Sonza et al., 2002), which can be recovered by the addition of exogenous Tat protein (Sonza et al., 2002). Alterations of *tat* expression have been linked to changes in the balance of host cellular RNA processing factors, rather than a reduction in *tat* mRNA stability (Dowling et al., 2008). Truncation mutants of *tat* (wherein the second coding exon is deleted) have highlighted the importance of the second coding exon for MRC in MDM (Neuveut et al., 2003) despite preserved single-cycle replication of these *tat* mutants in reporter cell lines, which implies the second coding exon may have additional activity in MDM separate from transcriptional activation (Neuveut et al.,

2003). In a study of LTR sequences, amplified from patients during progression to AIDS by nested PCR and cloned into luciferase-expressing pseudoviruses, there was an increase in the *in vitro* single-cycle transcription activity of late stage HIV-1 LTR in various target cells (including MDM) in a subset of patients, suggesting the enhanced replication capacity of isolates observed later in disease (see section 1.5.1.2) may be partially mediated by LTR transactivation activity (Hiebenthal-Millow et al., 2003). However, compartmentalisation of PCR-amplified *tat* sequences in neural *versus* lymphoid tissues was not convincingly demonstrated in a recent study, and no enhancement of LTR transactivation activity by brain-derived *tat* sequences in patients with HIV-associated dementia (HAD) was observed (Cowley et al., 2011), again suggesting that if Tat is important in determining MRC this function may be distinct from LTR transactivation. In keeping with this hypothesis, exogenous Tat protein has been shown to enhance MRC through upregulation of the IL-7R, leading to enhanced IL-7R signalling in response to IL-7 (Zhang et al., 2006). Whether this or a similar mechanism, rather than LTR transactivation, accounts for the enhancement of MRC by *tat* remains to be conclusively determined.

1.5.3.2 Vif

The viral infectivity factor (Vif), as the name suggests, enhances infection. *vif*-deleted viruses are either unable to establish spreading infections in MDM (Gabuzda et al., 1994, Kawamura et al., 1994), or can only sustain very low levels of MRC (Chowdhury et al., 1996). The activity of Vif is dominated by its targeting of APOBEC3G (Sheehy et al., 2002). Paradoxically, in long-term MDM culture, reduced virion infectivity over time was correlated with a reduction in APOBEC3G expression (independent of cytotoxicity), suggesting that Vif may also target an additional host factor(s) that may impact on virion infectivity, possibly through impaired processing/assembly of Gag capsid (Miyagi et al., 2008). However, few studies have

examined specific molecular determinants of Vif in relation to macrophage replication.

1.5.3.3 Nef

The negative factor (Nef) is a 25-27kDa myristoylated protein with multiple functions in mediating immune evasion and pathogenicity (Kirchhoff, 2010, Malim and Emerman, 2008) that impact upon *in vivo* disease progression (Deacon et al., 1995, Kestler et al., 1991, Kirchhoff et al., 1995). Nef-deleted viruses display impaired MRC *in vitro* (Malykh et al., 1995, Miller et al., 1994), and *in vivo* have been linked to long-term non-progression (LTNP), for example in recipients of blood-products contaminated with *nef*-deleted HIV-1 (Learmont et al., 1992). However, evidence is lacking to relate the reduced pathogenicity of the *nef*-deleted virus with alterations in macrophage tropism phenotype: a longitudinal analysis of R5 blood isolates (PBMC-isolated) in one long-term survivor who eventually progressed to AIDS did not demonstrate an increase in the low level of MRC during disease progression (Gorry et al., 2007).

To examine the role of *nef* in MRC, a mutagenesis study was conducted using the R5 macrophage-tropic reference strain SF162 (Brown et al., 2004). Deletion of *nef* resulted in delayed replication kinetics and reduced MRC, in keeping with previous data. Not surprisingly, significant inter-donor variation in MRC was observed, but there was a donor-dependent association between reduced MRC and amino acid mutations in the PXXP motif of Nef (Brown et al., 2004). Interestingly, mutations in *nef* associated with loss of CD4 down-regulatory function were associated with reduced MRC (Brown et al., 2004), in contrast to an earlier study in cell-lines (Goldsmith et al., 1995) but in keeping with studies in primary $CD4^+$ T cells (Lundquist et al., 2002) and *ex vivo* lymphoid tissue (Glushakova et al., 2001). CD4

down-regulation of infected cells has been proposed as a mechanism involved both in the efficient release of mature budding virions from the host cell membrane (Cortes et al., 2002, Ross et al., 1999) and in enhancing incorporation of gp160 into the viral envelope (Lama et al., 1999). Recent data indicate that HIV-1 virions in infected macrophages may bud from a plasma membrane-bound, surface-connected "quasi-intracellular" compartment (Bennett et al., 2009, Welsch et al., 2011, Welsch et al., 2007), suggesting that these mechanisms of CD4 down-regulation could be involved in efficient Nef-mediated virus particle release from macrophages. But whilst compartmentalisation of *nef* sequences amplified by PCR from neural and blood/lymphoid tissue was observed in a recent study in patients with HAD (Gray et al., 2011), the CD4 down-regulation activity of these *nef* sequences was conserved in both CNS and blood/lymphoid sequences, despite clearly distinct mac-tropism (Gray et al., 2011). Molecular sequence determinants of MRC in *nef* were not assessed. This study and others described above suggest that the determinants of CD4 down-regulation may not be causally associated *in vivo* with the macrophage tropism phenotype. Multiple additional functions of *nef* unrelated to MRC may impact pathogenesis (reviewed in (Kirchhoff, 2010)). However, in a recent systematic analysis of *nef*-mutations in LTNPs, no consistent molecular signatures were associated with LTNP status, and variation in *nef* was suggestive of rapid sequence evolution rather than deterministic mutations favouring attenuated pathogenicity (Pushker et al., 2010). Further work is required to establish whether the well-established molecular determinants of *nef* functional activity are linked to the macrophage tropism phenotype, and to identify specific molecular determinants in *nef* mediating MRC.

1.5.3.4 Vpu

HIV-1 viral protein U (Vpu) is a 16 kDa protein produced late in the viral replication cycle. The two main functions of Vpu are to counteract the host type-1 IFN-induced restriction factor tetherin/BST-2 to allow viral particle release (Neil et al., 2008, Van Damme et al., 2008) and to degrade intracellular CD4 (reviewed in (Kirchhoff, 2010)). Viruses deleted for *vpu* demonstrate low MRC in macrophages (Schubert et al., 1995), which has been associated with increased tetherin/BST-2 expression and reduced particle release (Schindler et al., 2010). Counteraction of tetherin is more important for *in vitro* replication in MDM than in lymphocytes or in *ex vivo* lymphoid tissues (Schindler et al., 2010). Interestingly, some R5 isolates with high MRC (e.g., AD8 and YU-2) have start codon mutations in *vpu*, which were previously believed to be redundant and could perhaps be compensated by Env (Schubert et al., 1999), however a study of chimeric *vpu*-deleted viruses expressing YU2 and AD8 Envs, which had low MRC *in vitro*, suggested Env cannot overcome the loss of Vpu (Richards and Clapham, 2007). In addition, a role for *vpu* in reducing virus particle release independent of tetherin down-regulation in some cell-lines (but not macrophages) has been identified (Miyagi et al., 2009). There is no current satisfactory explanation for the curious paradox of high MRC isolates with start codon mutations in *vpu*, but it is tempting to speculate on a more complex role for tetherin in macrophage infection.

1.5.3.5 Vpr/Vpx

The lentiviral accessory viral proteins R and X (Vpr and Vpx, which have a common genetic origin) are also involved in determining tropism for macrophages. Vpr is unique to HIV-1, and functions by triggering G2 cell cycle arrest and possibly regulating the nuclear transport of the pre-integration complex (reviewed in (Ayinde et al., 2010)). Deletion of Vpr reduces MRC *in vitro*, although additional roles are also

proposed for Vpr, and its *in vivo* relevance is unclear (reviewed in (Ayinde et al., 2010)). Vpx is present in HIV-2 and some SIV strains, but not HIV-1, and is indispensable for HIV-2/SIV replication in myeloid cells such as macrophages and dendritic cells (reviewed in (Ayinde et al., 2010)). As discussed above, when packaged with HIV-1, Vpx enhances HIV-1 mac-tropism (Sunseri et al., 2011, Kaushik et al., 2009) by proteosomal degradation of SAMHD1, preventing a block to efficient reverse transcription (Hrecka et al., 2011, Laguette et al., 2011), linked to the increased availability of dNTP precursors which are normally kept low in non-cycling cells like macrophages by the phosphohydrolase activity of SAMHD1 (Goldstone et al., 2011) (section 1.2.3.2). However, proteosomal degradation of SAMHD1 is not conditional for HIV-1 replication in macrophages.

1.5.4 Uncertainties

We continue to have an incomplete understanding of the viral determinants of tropism for this important target cell, such as which host and virus factors drive the apparent compartmentalisation of isolates between brain and other tissues, and what molecular mechanisms confer efficient HIV-1 replication post-entry. It should also be acknowledged that to date, most data on tropism phenotype comes from *in vitro* assays using MDM, which may not be representative of certain tissue macrophages in terms of permissiveness to viral infection. However, significant progress has been made, particularly regarding putative amino-acid changes determining the CD4 binding efficiency of R5 Envs. Improved sequencing techniques should allow questions to be addressed regarding the mediators of tropism evolution and its role in disease pathogenesis. An important unresolved question relates to the presence of HIV-1 infected macrophages in acute infection despite apparent inefficient macrophage replication of cloned T/F viruses.

1.6 Macrophage infection: a viral evasion strategy

Given the multiple barriers to efficient macrophage infection, the existence of HIV-1 macrophage tropism can be interpreted as either an evolutionary remnant of the prototypic lentiviruses or a conserved viral advantage. In the following sections I outline the accumulated evidence that suggests that HIV-1 infection of macrophages may in fact be a strategic tactic for viral persistence, through manipulation of macrophage longevity and simultaneous evasion of host immune and therapeutic suppression (Figure 1.3). This evasion activity happens both inside and outside the cell: HIV-1 can enter and infect macrophages without triggering innate immune sensing (reviewed in (Manel and Littman, 2011)), while in parallel newly-produced virions shelter in extracellular virus containing compartments (VCCs) which are relatively inaccessible to immune attack (reviewed in (Tan and Sattentau, 2013)). This paradigm also applies to escape from antiretroviral drug pressure: macrophages provide inhospitable intracellular conditions for the efficient activity of some antiretrovirals and are exposed to lower concentrations of drug by inhabiting antiretroviral sanctuaries such as the brain (reviewed in (Gavegnano and Schinazi, 2009)), whilst simultaneously the reserves of fully-assembled mature virions in VCCs are not amenable to ART inhibition. In the following sections I summarise these elegant mechanisms governing HIV-1 persistence in primary macrophages.

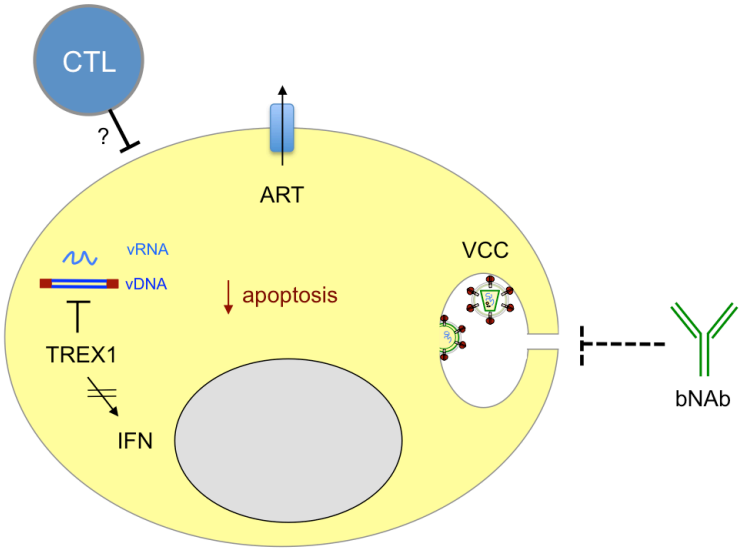

Figure 1.3 HIV-1 evasion mechanisms in macrophages
Macrophages are infected without triggering a type I IFN response, in part due to degradation of viral cDNA reverse transcription intermediates by host endonucleases such as TREX1. HIV-1 infection results in upregulation of survival pathways in macrophages (e.g. telomerases, the PI3K/Akt pathway, inhibitors of apoptosis 1/2, and HIV-1 Env-dependent production of the pro-survival cytokine M-CSF). Macrophages have active transport mechanisms (e.g P-glycoprotein efflux pumps) to remove antiretroviral drugs. Virions bud into sheltered virus-containing compartments (VCCs) in macrophages that are surface-connected through narrow tubular conduits, but may be impermeable to anti-HIV-1 neutralising antibodies. Data are presently conflicting on whether infected macrophages are resistant to cytotoxic T cell killing.

1.6.1 Intrinsic evasion

1.6.1.1 HIV-1 infects macrophages without triggering innate immune recognition or type I interferon production

As discussed above, a principal macrophage function is the initial recognition and response to pathogens. The innate response to virus infection predominantly involves sensors of nucleic acid, including endosomal PRRs (e.g. TLRs 3, 7/8 and 9) and various cytosolic sensors (e.g. RIG-I-like receptors, RLRs) (reviewed in (Borrow et al., 2010)). The canonical innate antiviral response is mediated by the production

of type I IFN, which triggers an antiviral state in host cells by inducing the transcription of multiple IFN-stimulated genes (ISGs) (Sadler and Williams, 2008) including several host HIV-1 restriction factors, such as tetherin, APOBEC, SAMHD1 and TRIM-5α (reviewed in (Hughes et al., 2012)). Type I IFN potently inhibits HIV-1 replication in macrophages (Meylan et al., 1993, Baca-Regen et al., 1994). It is worth noting that this restriction is independent of SAMHD1 dNTP hydrolase activity, since Vpx or saturating dNTP concentrations cannot overcome the IFN-induced RT block (Goujon et al., 2013, Dragin et al., 2013), and several other ISG mediators of HIV-1 restriction have been identified in macrophages, such as viperin (Nasr et al., 2012). Thus avoiding type I IFN production is a highly desirable viral strategy, particularly in macrophages, and it is not surprising that HIV-1 has evolved mechanisms to achieve this.

The absent IFN response in HIV-1 infected MDM (Gendelman et al., 1990) has been linked with a remarkable lack of transcriptional signal of HIV-1 infection, particularly within IFN-stimulated genes under conditions of uniform MDM infection with purified cytokine-free HIV-1 stocks (Tsang et al., 2009). This hyporesponsive phenotype was observed with exposure to several strains of HIV-1, but was associated with preserved type I IFN induction to control IFN stimuli such as poly I:C, implicating viral evasion of innate sensing rather than infection-induced dysfunction of type I IFN production as the principal mechanism (Tsang et al., 2009). However, these data have not been widely confirmed by other groups (Woelk et al., 2004, Coberley et al., 2004), which might reflect the difficulty in producing cytokine-free virus stocks (Brown et al., 2008) or in achieving uniform infection of macrophages *in vitro* (Bol et al., 2009), since incomplete infection of the population could result in indirect activation of uninfected cells through toxicity of by viral products such as gp120 released by infected cells (Tsang et al., 2009). A recent study reporting evidence of ISG induction (in the absence of measurable IFN production) observed that entry inhibitors such as

T-20 blocked the induction of ISGs, suggesting that contaminating cytokines were not responsible (Nasr et al., 2012). However, in this study approx. 13-60 % of the total MDM population were infected depending on the day of use, thus bystander activation cannot be excluded. Other recent studies found very minimal (Diget et al., 2013) or no evidence (Solis et al., 2011) of ISG expression in MDM compared to MDM exposed to controls such as Sendai virus, consistent with the findings of Tsang *et al*.

The mechanism of attenuated type I IFN production appears to be multifaceted. Reduced type I IFN production has been associated with failure of interferon-regulated factor-3 (IRF3) nuclear translocation in macrophages (Nasr et al., 2012). This may be linked to HIV-1 PR-dependent sequestration of RIG-I (Solis et al., 2011), since RIG-I has been implicated in vRNA sensing in the cytosol of infected macrophages (Berg et al., 2012). However it is probable that other, as yet unindentified, sensors of viral nucleic acid are involved (Manel and Littman, 2011), and evasion of these sensors in MDM should result in downstream failure of IRF3 nuclear translocation (Nasr et al., 2012). Interestingly, uninfected MDM respond to purified vRNA (Solis et al., 2011); unfortunately the ability of infected MDM to respond to control treatment with vRNA (which would presumably be attenuated due to the PR-dependent sequestration of RIG-I) was not tested (Solis et al., 2011).

Recent data suggest that while myeloid cells have a preserved capacity to sense viral nucleic acids such as vDNA, host mechanisms involved in restraining intracellular nucleic acid accumulation might limit this activity. Several host proteins including SAMHD1 and the exonuclease TREX1 degrade cytosolic nucleic acid or nucleic acid precursors (reviewed in (Manel and Littman, 2011, Yan and Lieberman, 2011)). This host regulatory mechanism also degrades viral nucleic acid intermediates, such as retroviral DNA, potentially limiting the cytosolic sensing of

products of viral replication (reviewed in (Manel and Littman, 2011, Yan and Lieberman, 2011)). Knockdown of *TREX1* in macrophages resulted in vDNA accumulation and substantial type I IFN production in response to HIV-1, which was IRF3 and viral reverse transcription-dependent, and associated with an enhanced capacity for non-HIV-1 ssDNA sensing (Yan et al., 2010). Similarly, in experiments using the HIV-2/SIV accessory protein Vpx to overcome the SAMHD1 block to productive infection in DCs, enhanced type I IFN expression in response to HIV-1 infection was observed (Manel et al., 2010). In this study, *de novo* viral capsid protein was proposed as the viral PAMP, but this was only under conditions of Vpx-induced SAMHD1 degradation (Manel et al., 2010), therefore a more complex relationship might exist between viral nucleic acid and capsid in the presence of SAMHD1 (Manel and Littman, 2011).

1.6.1.2 HIV-1 prolongs the lifespan of infected macrophages

Evasion of type I IFN signalling is a good example of viral opportunism, but does not imply that the virus lacks the means to manipulate homeostatic cellular processes of macrophages. Several viruses have evolved the ability to influence basic cellular processes such as cell survival by down-regulating pro-apoptotic pathways (reviewed in (Barber, 2001)). For example, adenoviruses express receptor internalisation and degradation (RID) proteins that induce the internalisation of cell-surface expressed death receptors such as Fas and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) (Gooding et al., 1988). HIV-1 exerts a similar pro-survival effect on macrophages (Swingler et al., 2007a, Chugh et al., 2008, Fernandez Larrosa et al., 2008, Tachado et al., 2008, Busca et al., 2012, Reynoso et al., 2012). In contrast to $CD4^+$ T cells, macrophages display a profound resistance to viral CPE underpinned by reduced susceptibility to HIV-1 induced apoptosis (Aquaro et al., 2002). Myriad manipulations of pro-survival or anti-apoptotic pathways in macrophages have been

reported, such as increased telomerase activity protecting against oxidative stress (Reynoso et al., 2012), increased activity of the PI3K/Akt pathway (Chugh et al., 2008), resistance to Vpr-induced apoptosis through inhibitors of apoptosis 1/2 (IAP 1/2) (Busca et al., 2012), and HIV-1 Env-dependent production of the pro-survival cytokine macrophage colony stimulating factor (M-CSF), which down regulates TRAIL receptor expression and up regulates the anti-apoptosis genes Bfl-1 and Mcl-1 in infected macrophages (Swingler et al., 2007a). While most of these data come from MDM, alveolar macrophages from HIV-1 infected patients also exhibit increased activity of the PI3K/Akt pathway (Tachado et al., 2008). This multifaceted promotion of macrophage survival is advantageous to HIV-1: infected macrophages efficiently transmit HIV-1 to $CD4^+$ T cells in a contact-dependent manner (Carr et al., 1999, Groot et al., 2008, Mann et al., 1990). Thus a single infected macrophage could conceivably transmit HIV-1 to several thousand $CD4^+$ T cells over its lifespan (reviewed in (Aggarwal et al., 2013)), a highly efficient method of intra-host viral dissemination. However, there are no data regarding whether prolonged survival of HIV-1-infected macrophages is true *in vivo*.

1.6.1.3 Macrophages are less susceptible to antiretroviral inhibition

Macrophages present barriers to the optimal intracellular activity of ART. RT inhibitors require phosphorylation before strand incorporation during cDNA synthesis (Mitsuya et al., 1985), however kinases required for cellular phosphorylation are less abundant in macrophages (Perno et al., 2006, Sigal and Baltimore, 2012). Intracellular drug concentrations correlate with antiviral activity (reviewed in (Eisele and Siliciano, 2012)), however macrophages and microglia express multiple efflux transporters, including P-glycoprotein and multidrug resistance transporters, to pump drugs out of the cell (Dallas et al., 2003, Dallas et al., 2004, Jorajuria et al., 2004, Dallas et al., 2013). Antiretroviral drug concentrations in macrophages are

consequently lower (Gavegnano et al., 2013). In addition, the EC_{50} of ART in established infected macrophages is significantly higher than the EC_{50} for prevention of initial infection (reviewed in (Gavegnano and Schinazi, 2009)). Although the mechanism is unknown, it may partly reflect changes in the availability of nucleotide precursors or cellular kinases following established macrophage infection similar to that described for co-infection with the parasite *Leishmania* (Mock et al., 2012). This failure to inhibit replication in infected cultures could also reflect the capacity of macrophages for prolonged maintenance (over a period of months) of replication competent HIV-1 in the absence of ongoing replication (Sharova et al., 2005b). This capacity for prolonged virion storage derives from the unique extracellular location of viral assembly in macrophages.

1.6.2 Extrinsic evasion

1.6.2.1 The macrophage virus-containing compartment is cell extrinsic but inaccessible to antibodies

The site of viral particle assembly and budding in macrophages has been a source of controversy (reviewed in (Benaroch et al., 2010, Tan and Sattentau, 2013)). Initial ultrastructural studies identified viral particle budding into apparently intracellular compartments in macrophages, (Joshi et al., 2009, Nydegger et al., 2003, Pelchen-Matthews et al., 2003, Raposo et al., 2002) enriched in markers of late endosomes/multivesicular bodies (LE/MVB) (Chertova et al., 2006). However, later EM studies using differential staining of the cell plasma and endosomal membranes revealed viral budding at the plasma membrane and from within cell membrane limited compartments (Deneka et al., 2007, Welsch et al., 2007). Subsequent studies showed these VCC had a neutral pH (Jouve et al., 2007) and were connected to the

surface by narrow channels (Bennett et al., 2009). Thus the VCC in macrophages appears to be a predominantly extracellular structure bound by the plasma membrane, but positioned deep within the cell as a result of the extensive involution of macrophage cell membranes - i.e. are “pseudo-internal”. EM tomography has been used to further characterise the VCC, which exhibits considerable morphological heterogeneity, varying in appearance from lattice-like webs to more defined spherical compartments, which could actually represent contiguous components of a more complex labyrinthine structure (Welsch et al., 2011). Whilst the variable topology might imply differential function or regulation, it is also possible that these appearances simply derive from taking snapshots of a dynamic VCC during fixed-cell imaging studies, and this is an area that needs further definition (reviewed in (Benaroch et al., 2010)).

The potential importance of the VCC to HIV-1 persistence lies in two principal observations: first, the VCC represents a reservoir for prolonged maintenance of extracellular infectious virions (Sharova et al., 2005b), avoiding HIV-1 sensing or degradation by host nucleases but preventing the rapid degeneration of the viral envelope in the extracellular fluid phase; second, the narrow surface connections to the VCC appear to prevent passive diffusion of extracellular applied antibodies (Chu et al., 2012b, Deneka et al., 2007, Koppensteiner et al., 2012a). Studies performed at 4^{o}C (to eliminate active transport) indicate that most externally applied antibodies fail to access the VCC over 1 hour (Chu et al., 2012b, Koppensteiner et al., 2012a). At 37^{o}C there is a delay of approximately 3 hours before antibodies reach the VCC (Deneka et al., 2007). Thus a 1-hour incubation at 37^{o}C followed by washout was insufficient to neutralise HIV-1 within the VCC with the potent broadly neutralising monoclonal antibody (bNAb) VRC01 (Koppensteiner et al., 2012a). Whether antibody-mediated neutralisation is possible during longer incubations and/or with different antibodies remains untested. Yet these preliminary data suggest that storage of replication competent HIV-1 in VCCs may aid viral evasion of host

defences. Curiously, the host restriction factor tetherin/BST-2, which acts to prevent virion release at the cell membrane, has also recently been implicated in maintenance of the VCC (Chu et al., 2012a). This implies a delicate balance between viral particle release at the cell membrane and storage within the VCC, with as yet unknown consequences for viral persistence (reviewed in (Tan and Sattentau, 2013)), and could account for the potentially linked observation that highly macrophage tropic primary viruses isolated from the brain (e.g. YU2 and AD8) have deletions in *vpu* (reviewed in (Duncan and Sattentau, 2011)).

1.6.2.2 Inefficient cytotoxic T cell surveillance of infected macrophages?

Infection of macrophages has been proposed as a lentiviral escape measure from CTL inhibition. Recently Vojinov *et al* (Vojnov et al., 2012) sorted antigen-specific $CD8^+$ T cells from SIV-infected macaques, and showed that most CTLs had reduced capacity to inhibit viral replication in infected MDM, although they were capable of efficient MHC class I dependent inhibition of replication in $CD4^+$ T cells from the same animals. In parallel assays using bulk $CD8^+$ T cells, the same phenotype was observed. The functionality of the $CD8^+$ T cell population was not explored, either in terms of interferon-gamma production or markers of cytotoxic potential (e.g. granzme or perforin). However, these data suggest that the efficiency of viral suppression is target cell dependent, and imply that infected macrophages, by virtue of their prolonged lifespan, might represent a CTL-resistant reservoir of HIV-1 (Vojnov et al., 2012). Indeed, in the murine SCID model of HIV-1, infection with macrophage-tropic viral isolates, rather than exclusively T cell tropic isolates, favours viral evasion of CTL pressure (Schutten et al., 2001). Possible mechanisms include the prolonged period of productive infection following viral down-regulation of MHC class I in macrophages (Vojnov et al., 2012), or the presence of infected macrophages in immune sanctuary sites, such as the brain (discussed below).

However, these findings contrast with data on HIV-1- (Fujiwara and Takiguchi, 2007, Mwimanzi et al., 2011) or SIV- (Sacha et al., 2009) specific CTL clones, which are reported to efficiently inhibit viral replication in infected MDM *in vitro*. These inconsistencies might be explained by differences in the biology of T cell clones expanded *in vitro*, compared to native CTLs isolated *ex vivo* (Vojnov et al., 2012). It is possible, for example, that the process of prolonged culture and repeated re-stimulation selects for clones with enhanced suppressive capacity (Chung et al., 2007, Vojnov et al., 2010). Unfortunately, similar studies using freshly isolated CTLs from HIV-1 patients are unavailable, although a report some years ago indicated that $CD8^+$ T cells isolated from patients with lymphocytic alveolitis, an inflammatory HIV-associated disease characterised by dense lymphocytic lung infiltrates, are capable of lysing autologous alveolar macrophages (Plata et al., 1987). The impact on viral replication, and the relative efficiency of macrophage lysis compared to $CD4^+$ T cell lysis was not assessed. Moreover, it is unclear how representative these data are, considering they derive from patients with a rare inflammatory pathology in the context of advanced HIV-1 infection. Taken together, there are presently insufficient data to conclude whether macrophage infection represents a true evasion mechanism from CTL control of viral replication.

1.6.2.3 Macrophages occupy anatomical sanctuaries

The anatomical location of infected macrophages also favours viral persistence. Immune privilege in tissues such as the CNS is a safeguard of peripheral immune tolerance (reviewed in (Carson et al., 2006)). However, immune privilege also provides the ideal opportunity for pathogens to evade protective immune responses. Lymphocyte and antibody access to the CNS is limited (Wilson et al., 2010) and perivascular macrophages/microglia are the principal HIV-1 targets in the CNS

(Persidsky and Poluektova, 2006, Yadav and Collman, 2009). Neurotropism is also a relatively conserved feature of lentiviruses, (Patrick et al., 2002) and HIV-1 CNS infection has significant pathogenic implications (Yao et al., 2010). Many highly macrophage tropic primary viruses were isolated from the brains of infected individuals (reviewed in (Duncan and Sattentau, 2011)), where there is convincing evidence of HIV-1 genetic compartmentalisation (i.e. independent evolution within a restricted anatomical location) (reviewed in (Blackard, 2012), see section 1.5.1.4). An interesting feature of macrophage-tropic brain Envs summarised above is the capacity to exploit low CD4 levels on macrophages paired with a heightened sensitivity to CD4bs NAb (see Section 1.5.2.1). Low titre NAb develop relatively early in the course of HIV-1 infection, and the virus responds promptly by developing escape mutations (Bar et al., 2012). But the exquisite sensitivity of macrophage tropic isolates to NAb points to viral adaptation to macrophage replication in the absence of NAb pressure (see Section 1.5.1.4).

Similarly, anatomical sanctuaries provide an escape route from ART inhibition. Distribution of orally administered ART to different parts of the body is variable (reviewed in (Cory et al., 2013)). Penetration of ART into important tissue sites of viral replication, such as the brain, lymph node and gut, is impaired (reviewed in (Cory et al., 2013, Sigal and Baltimore, 2012)) and these sites are termed pharmacological sanctuaries (reviewed in (Eisele and Siliciano, 2012)). Data from animal models indicate that ongoing viral replication occurs in pharmacological sanctuaries despite suppression of plasma viraemia (Bourry et al., 2010, Horiike et al., 2012, North et al., 2010). Moreover, in patients on apparently suppressive ART, viral production has been measured in pharmacological sanctuaries (Solas et al., 2003, Cu-Uvin et al., 2010, Trono et al., 2010, Deleage et al., 2011, Sigal and Baltimore, 2012, Cory et al., 2013), although no consistent correlation with drug concentration has been observed (Solas et al., 2003), perhaps because the

concentration of active free drug better predicts ART activity than protein-bound or total drug concentration (reviewed in (Eisele and Siliciano, 2012)). Taken together with data on the intracellular activity of ART outlined above, a combination of mechanisms appears to account for the reduced exposure to ART in macrophages.

1.7 HIV-1 cell-to-cell transmission

1.7.1 Importance and mechanisms of cell-to-cell transmission

For more than two decades it has been known that HIV-1 spreads more efficiently by direct transfer from one cell to another than by cell-free diffusion (reviewed in (Sattentau, 2008)). Indeed, the related retrovirus human T cell leukaemia virus-1 (HTLV-1) only appears to disseminate cell-to-cell *in vivo* (reviewed in (Nejmeddine and Bangham, 2010)). HIV-1 infects immune cells ($CD4^+$ T cells and macrophages) that exist densely packed in lymphoid tissue and frequently form cell-cell contacts (reviewed in (Sattentau, 2010)). Dendritic cells are much less permissive to HIV-1 infection, but are nevertheless able to efficiently capture and transmit HIV-1 to uninfected $CD4^+$ T cells (reviewed in (McDonald, 2010)). Thus, it has long been assumed that cell-cell spread between immune cells is the dominant mode of dissemination *in vivo*, although confirming this poses significant technical challenges. Progress has been made with humanised mouse models of HIV-1, in which $CD4^+$ T cells have been recently identified as efficient vectors of HIV-1 dissemination *in vivo* (Murooka et al., 2012) and retroviral cell-to-cell transmission (Sewald et al., 2012) has been observed in real-time. More controversially, challenge of monkeys with cell-associated virus preparations results in inter-host viral transmission in the macaque model of SIV, suggesting a role for cell-to-cell spread in mucosal transmission (Salle et al., 2010).

1.7.1.1 Synaptic infection mechanisms

In early studies using cell lines and lab-adapted viruses cell-cell fusion events (syncytia formation) were identified as a probable mechanism of cell-to-cell transmission, although it subsequently became obvious that syncytia are much less frequent with primary cells and primary (mainly CCR5-using) viruses (reviewed in (Sattentau, 2010)). It is now established that HIV-1 cell-to-cell spread between donor and target cells occurs at structurally distinct intercellular contact zones termed *virological* (Jolly et al., 2004) and *infectious* synapses (McDonald et al., 2003). The main factor that determines which structure is used for transfer is the infection status of the donor cell: Virological synapses (VS) are formed by HIV-infected cells, whilst infectious synapses (IS) are formed by uninfected cells (e.g. DCs) that have captured free HIV-1 (Figure 1.4).

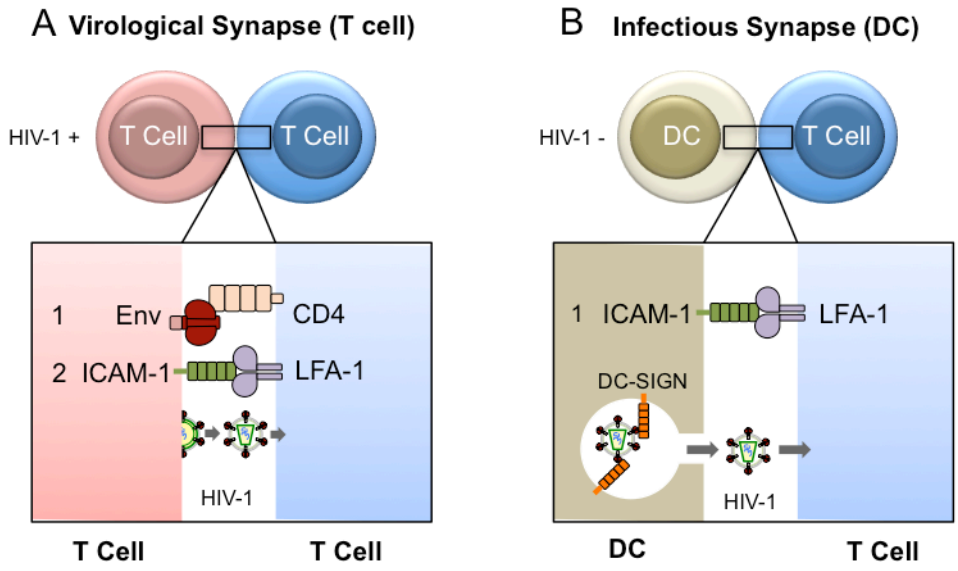

Figure 1.4 Synaptic structures
A) VS architecture (T cell). The primary signal for assembly is Env-CD4 binding (1). Subsequent stabilisation is achieved by interactions between ICAM-1 and LFA-1 (2). Virus buds at the interface and infects the target cell in a CD4-dependent manner.
B) IS architecture (dendritic cell, DC). The synapse forms as a result of cell-cell adhesion molecule interactions (1), and results in release of mature HIV-1 virions from the temporary surface-accessible viral storage compartment within into which mature HIV-1 particles were previously captured by various cell-surface molecules including DC-SIGN.

1.7.1.2 Virological synapses and actin-dependent membrane extensions

The canonical feature in the formation of the VS is the binding of Env expressed by the infected donor cell to CD4 on the target cell (Jolly et al., 2004) (Figure 1.4). The cell-cell interface is stabilised by adhesion molecules (Jolly et al., 2007), and a macromolecular structure assembles which is reminiscent of, but is distinct from, the immunological synapse (reviewed in (Vasiliver-Shamis et al., 2010)). The secretory apparatus of the infected donor T cell is hijacked by HIV-1 and polarises to the cell-cell contact zone (Jolly et al., 2011). It is possible for one infected T cell to assemble multiple VSs with several target cells simultaneously (polysynapses) (Rudnicka et al., 2009). Additional mechanisms for directed retroviral cell-to-cell transfer have been described, mainly comprising actin-dependent membrane projections, such as nanotubes and filopodia, which permit virion cell-membrane "surfing", but remain dependent on host receptors for fusion (reviewed in (Martin and Sattentau, 2009)). Although the relative importance of these various mechanisms is unclear, VS-mediated transfer of HIV-1 seems to dominate *in vitro* (Rudnicka et al., 2009) and has been observed *in vivo* (Sewald et al., 2012).

1.7.1.3 Infectious synapses and DC trans-infection

Myeloid-lineage dendritic cells (mDC) capture HIV-1 using a variety of receptors including C-type lectins (e.g. Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin, DC-SIGN (Geijtenbeek et al., 2000)), heparan sulphate proteoglycans (de Witte et al., 2007) Siglec-1 (Izquierdo-Useros et al., 2012) and others (Puryear et al., 2012), a process that does not directly result in infection (reviewed in (McDonald, 2010)). The captured virions are maintained in an infectious state within complex surface-connected plasma membrane invaginations (Cavrois et al., 2007, Frank et al., 2008, Yu et al., 2008, Felts et al., 2010). When the DC subsequently encounters an uninfected target cell such as a $CD4^+$ T cell, the surface

bound virus becomes liberated from its invaginated holding compartment, binding CD4 on the target cell and infecting it in a process known as *trans*-infection (McDonald et al., 2003). The organisation of the IS is dependent on adhesion molecule interactions but is Env/CD4-independent (Rodriguez-Plata et al., 2013).

1.7.1.4 HIV-1 entry in cell-to-cell transmission

As summarised previously, the conventional entry pathway for HIV-1 virions is receptor-activated fusion at the target cell plasma membrane at neutral pH. Recent evidence suggests that cell-free viral fusion can occur at either the plasma membrane or from within endosomal compartments. It is unclear whether one mode of entry dominates the other and whether the mode of entry is cell-type dependent, but regardless of this the overall receptor dependence and tropism of the virus appear equivalent.

As is the case with cell-free infection, discrepant data have been reported regarding the possibility of endocytic uptake of intact virions by the target cell following cell-to-cell transmission (reviewed in (Martin and Sattentau, 2009)). This discrepancy might be reconciled by recent descriptions of a process of endocytic uptake and maturation-dependent fusion of immature virions (Dale et al., 2011) (Figure 1.5). In these experiments, donor cells were transduced 48 hours previously with HIV-1 molecular clones therefore transfer was restricted to newly budding virus (Chen et al., 2007, Dale et al., 2011, Durham et al., 2012). Immature virions did not undergo immediate fusion with the plasma membrane, but appeared to be temporarily endocytosed until maturation was complete, resulting in delayed fusion kinetics (Dale et al., 2011). This intracellular maturation process was viral protease-dependent (Dale et al., 2011). By contrast, chronically infected donor cells used by other groups transfer viral particles that appear capable of immediate membrane fusion without a

maturation step (Monel et al., 2012, Jolly et al., 2004, Martin et al., 2010) (Figure 1.5). Thus the probability of endocytic uptake might be determined by the chronicity of donor cell infection and the rate of virion maturation (reviewed in (Sattentau, 2010)). However, it is worth noting that the delayed maturation step described by the Chen laboratory has yet to be confirmed for other recombinant HIV-1 clones in synaptic transmission models. Moreover, the mechanism of viral entry (i.e. plasma membrane or endosomal fusion) does not appear to influence subsequent productive infection, since infection has been reported to be highly efficient in both acute and chronic transmission models (reviewed in (Sattentau, 2010)).

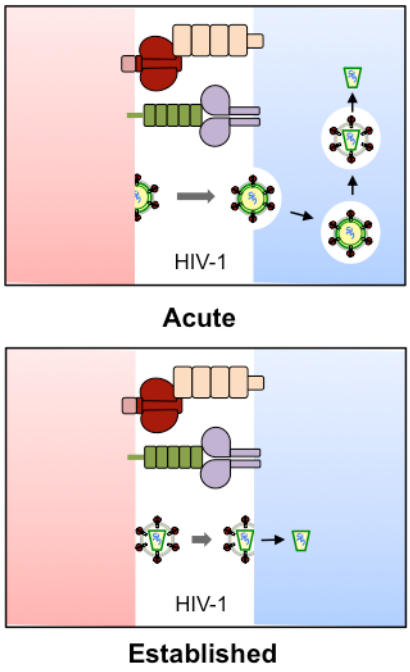

Figure 1.5 Mechanisms of VS transmission
Endocytic uptake in synaptic transfer is dictated by virion maturation. Top panel: Immature virions from acutely transduced donor cells are temporarily taken up into endosomal compartments until maturation-induced conformational change in Env permits CD4-dependent fusion. Bottom panel: donor cells in which productive infection is established transfer mature virions that immediately fuse at the cell membrane.

1.7.2 Inhibition of cell-to-cell transmission by NAb

Cell-to-cell spread *in vivo* has been implicated in the initial mucosal amplification of HIV-1 following transmission (reviewed in (Haase, 2010)), viral broadcasting through motile $CD4^+$ T cells (Murooka et al., 2012), and possibly even the initial transmission event itself (Salle et al., 2010); each of these steps is of primary importance to a prophylactic vaccine that seeks to prevent establishment of infection. Thus it is critical to resolve whether cell-to-cell spread represents an antibody evasion mechanism. This was suggested by early studies, which found that patient sera capable of neutralising cell-free virus infection were unable to neutralise infection in co-cultures of patient and donor PBMC over multiple rounds of infection (Gupta et al., 1989). Today, with the accumulation of a larger panel of bNAbs there are better tools with which to approach this question. However significant uncertainty remains.

BNAbs target a variety of Env epitopes (Figure 1.6). Most are on the gp120 subunit, including the CD4 binding site (targeted by b12 and VRC01 (Wu et al., 2010, Zhou et al., 2010, Burton et al., 1994, Zhou et al., 2007)), a cluster of high-mannose glycans (recognised by 2G12 and the PGT mAbs (Buchacher et al., 1994, Sanders et al., 2002)) and the co-receptor binding site which is only exposed upon CD4 engagement - the CD4-inducible epitope (CD4i, targeted by the non-neutralising mAb 17b (Kwong et al., 2000, Kwong et al., 1998, Thali et al., 1993)). Another conformational epitope on gp120 is at the apex of the spike where the V1/V2 loops contact to stabilise the trimer (Walker et al., 2009). This epitope, which is recognised by bNAbs PG9 and PG16, is formed in correctly assembled Env trimers (Mao et al., 2013), but not monomeric gp120 (Walker et al., 2009). The only bNAb epitope on gp41 known to date is the MPER, targeted by mAbs 4E10 and 2F5) (Buchacher et al., 1994). Until very recently (Huang et al., 2012), all MPER NAbs showed membrane-binding activity and it was hypothesised that this was required due to the membrane-embedded nature of the MPER peptide (Sun et al., 2008) as well as the

narrow steric restrictions imposed by the gp120 subunit (Mao et al., 2013). However a potent non-membrane binding MPER antibody 10E8 has been recently identified, implying that autoreactivity is not conditional for MPER-targeted neutralisation (Huang et al., 2012).

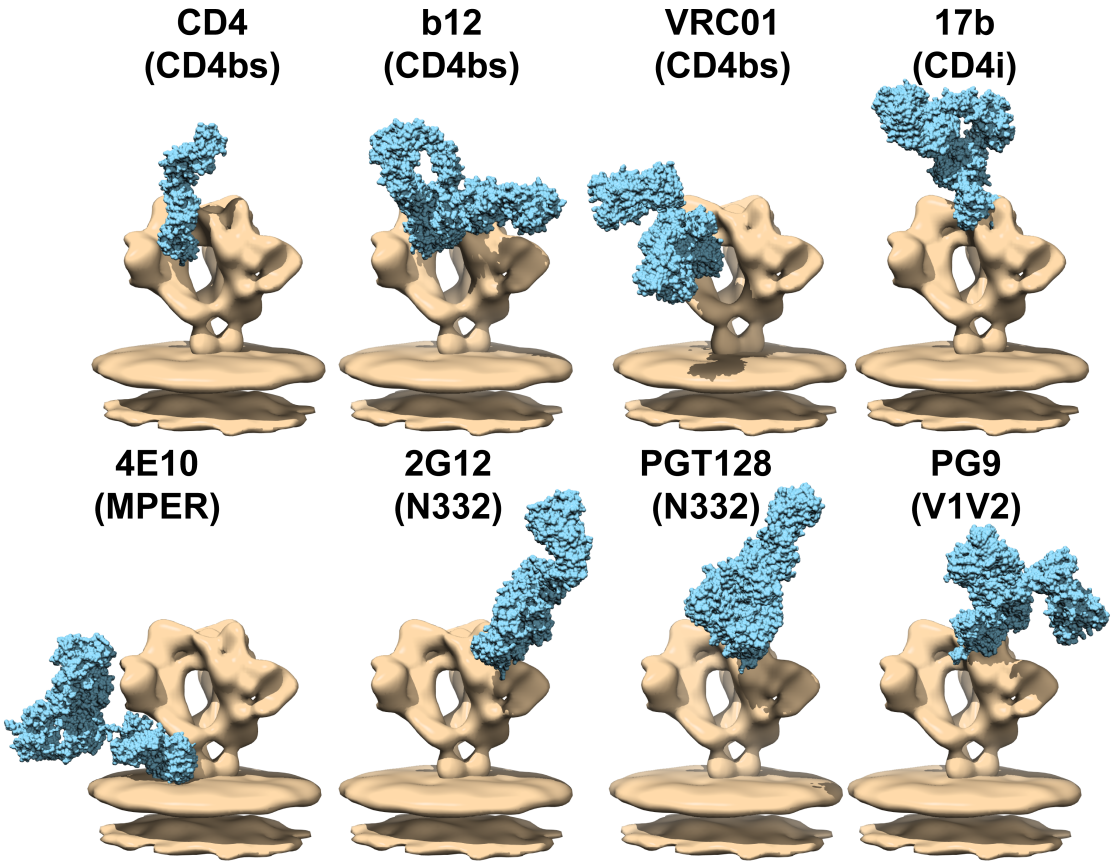

Figure 1.6 Targets of bNAbs
Env trimers (fawn) bound by antibodies (blue) commonly used in cell-to-cell transmission studies. Images courtesy of Torben Schiffner, prepared in UCSF Chimera 1.6.2.

Data using blocking antibodies suggest that target cell infection via the VS or IS remains dependent on CD4 binding (Groot et al., 2008, Massanella et al., 2009, Sanchez-Palomino et al., 2011, Abela et al., 2012, Durham et al., 2012, Felts et al., 2010) (Table 1.2). Fewer studies have examined inhibitors of co-receptor binding (Hubner et al., 2009, Felts et al., 2010, Dale et al., 2011) and viral fusion (Ketas et al., 2003, Frank et al., 2008, Martin et al., 2010, Durham et al., 2012), but these clearly indicate that synaptic infection is also co-receptor and fusion-dependent. In this respect, synaptic infection is qualitatively indistinguishable from cell-free infection and therefore the prospect of neutralising synaptic infection by targeting Env with antibodies seems viable.

Donor	Target	VS/IS	Transfer	Infection	Inhibitor	Ref
T Cell (JT)	T Cell (MT4)	VS	yes	n.d.	Leu3a	(Chen et al., 2007)
MDM	CD4	VS	yes	n.d.	Q4120	(Groot et al., 2008)
T Cell (MOLT)	CD4	VS	yes	yes	Leu3a	(Massanella et al., 2009)
DC	CD4	IS	n.d.	yes	M-T477	(Felts et al., 2010)
T Cell (JT)	T Cell (MT4)	VS	n.d	yes	Q4120	(Martin et al., 2010)
T Cell (MOLT)	CD4	VS	yes	n.d.	Leu3a	(Sanchez-Palomino et al., 2011)
PBMC	TZM-bl/PBMC	VS	n.d.	yes	DARPin 57.2	(Abela et al., 2012)
T Cell (JT/CD4)	T Cell	VS	n.d.	yes	Leu3a	(Durham et al., 2012)

Table 1.2 Inhibition of cell-to-cell transfer/infection by anti-CD4 antibodies
JT = Jurkat T cell line; MT4 = MT4 T cell line; VS = virological synapse; IS = infectious synapse; CD4 = Primary $CD4^+$ T cell; n.d. = not done. Transfer means that viral antigen was measured in the target cell; Infection means that viral replication was measured in the target cell.

Several recent studies have sought to address the function of NAbs in models of HIV-1 synaptic infection, either at the VS or the IS. At first glance the conclusions of these studies appear contradictory, however much of this is a direct result of the

heterogeneity of the experimental methods. The principal questions I seek to answer in this section concern:

- Do bNAbs inhibit synaptic infection?
- Is the efficiency of bNAb inhibition reduced for synaptic infection relative to cell-free infection?

Studies analysing synaptic transmission of HIV-1 have been conducted with experimental systems involving both cell lines and primary cells, and both acute and chronic models of virus transfer; these studies also vary in the accuracy with which they distinguish the consequences for the target cell: namely viral uptake and infection.

1.7.2.1 Transfer versus infection

It is important to distinguish viral transfer and target cell infection in studies of inhibition of synaptic infection; many of the conflicting data in the literature can be resolved by taking account of experimental approaches that fail to separately analyse these related, but independent, events. For example, in VS-mediated models the anti-gp41 antibodies 2F5 and 4E10 do not block gp120-CD4 engagement, therefore do not inhibit VS-mediated transfer (Chen et al., 2007, Massanella et al., 2009, Sanchez-Palomino et al., 2011). They do however inhibit infection (Massanella et al., 2009, Martin et al., 2010, Abela et al., 2012, Durham et al., 2012). In the case of IS-mediated transmission, DC *trans*-infection was assessed as 2F5-resistant by measuring intracellular Gag in T cells at 30 minutes after co-culture (when only transfer would be measured, and for the reasons outlined above, this should not be inhibited by 2F5). Interestingly, by 48 hours, a time-point when the assay might measure a component of *de novo* Gag production in target cells corresponding to

infection, synaptic neutralisation activity was only marginally lower (19%) than cell-free neutralisation (Ganesh et al., 2004). Another study showed that although Gag transfer was not inhibited by 2F5, it was potently inhibited by the CD4 binding-site NAb b12 (van Montfort et al., 2007). Thus, erroneous conclusions about bNAb resistance might otherwise be reached if a transfer endpoint such as Gag staining by flow cytometry, in the absence of controls showing this represents *de novo* production, is used as a surrogate for infection (Chen et al., 2007). Productive infection is best differentiated from transfer by endpoints such as vDNA production or reporter virus expression of fluorescent protein and/or luciferase (reviewed in (Sattentau, 2010)).

1.7.2.2 Cycles of infection

Like the distinction between transfer and productive infection, interpretation of antibody activity can be influenced by the number of cycles of infection studied. There is evidence that the activity of NAb might reduce over multiple cycles (Gupta et al., 1989, Koppensteiner et al., 2012a, Ganesh et al., 2004). Whether this is due to viral escape mutations or loss of antibody potency is unclear. However, as the main objective of prophylactic antibodies is to prevent initial viral entry, here I concentrate on studies of entry inhibition over a single cycle.

1.7.2.3 Synaptic structure

The molecular architecture of the synapse can influence how researchers interpret bNAb activity. For example, because the initial signal for VS assembly is Env engagement of CD4 (Figure 1.4), blocking CD4-Env interactions inhibits the formation of the VS (Jolly et al., 2004), which in turn inhibits both viral transfer and infection (Chen et al., 2007, Massanella et al., 2009, Martin et al., 2010, Durham et al., 2012). By contrast, the initial signals for IS assembly depend on other molecular

interactions, such as those between ICAM-1 and LFA-1 (McDonald, 2010, Rodriguez-Plata et al., 2013) (Figure 1.4). Therefore Env-directed inhibitors do not block IS formation (Felts et al., 2010) or viral transfer at the IS (Ganesh et al., 2004, van Montfort et al., 2007), but remain capable of inhibition of single cycle infection (Ketas et al., 2003, Cavrois et al., 2007, Yu et al., 2008, Sagar et al., 2012, Su et al., 2012).

1.7.2.4 DC subtype and maturation

The subtype of DC may also be an important factor in studies of IS-mediated transmission. Experiments have been done with lymph node follicular DC (Heath et al., 1995), purified blood myeloid DC (mDC) (Ganesh et al., 2004, Yu et al., 2008), skin Langerhans's cells (Cavrois et al., 2007), and monocyte-derived DC (MDDC) (Frankel et al., 1998, Ketas et al., 2003, Cavrois et al., 2007, van Montfort et al., 2007, Frank et al., 2008, Yu et al., 2008, Felts et al., 2010, Sagar et al., 2012, Su et al., 2012). Thus, a consistent picture of NAb activity has been difficult to obtain. Until more mechanistic studies have been conducted, we can only speculate about the influence of, for example, using purified blood mDC versus *in vitro* MDDC; however, NAb activity appears maintained when MDDC are used as donors (Frankel et al., 1998, Ketas et al., 2003, Cavrois et al., 2007, Frank et al., 2008, Yu et al., 2008, Felts et al., 2010, Sagar et al., 2012, Su et al., 2012), whilst relative resistance (with the caveats discussed above) has been reported for mDC (Ganesh et al., 2004).

Even within a single DC subtype there is additional heterogeneity due to cellular maturation status. DC maturation describes the phenotypic and functional changes that occur in the time between antigen uptake in the mucosal tissues and antigen presentation in lymph nodes. During maturation, the DC downregulates phagocytic ability and upregulates co-stimulatory molecule expression. DC maturation can be

triggered *in vitro* by a variety of signals, including lipopolysaccharide (LPS), cytokines and FcR binding (reviewed in (McDonald, 2010)). Previous studies have shown that mature DC are resistant to HIV-1 infection, whilst immature DC are capable of limited HIV-1 replication (reviewed in (McDonald, 2010)). On the other hand, mature DC are much more efficient at HIV-1 *trans*-infection (reviewed in (McDonald, 2010)). Therefore, DC maturation can dictate whether transfer occurs through the Env-dependent VS (infected immature DC, sometimes known as *cis*-infection) or the IS (uninfected mature DC - *trans*-infection). As a result, spread from immature DC may be VS or IS-mediated. In addition, in studies where *trans*-infection is measured using supernatant p24 production or reporter-virus luciferase activity from donor-target co-cultures, some neutralisation-resistant background signal may arise from infected DC. In a recent study, Su *et al* observed increasing immature DC infection over time, measured by intracellular Gag, during co-culture with $CD4^+$ T cells (Su et al., 2012). This intracellular Gag in DC could be inhibited by antiretroviral treatment (and NAbs), implying *de novo* production due to infection. Thus DC infection may confound assessment of NAb transmission-blocking activity. It is also worth noting that DC maturation can be induced by co-culture with T cells (Su et al., 2012), and conversely HIV-1-pulsed DC activate T cells (Frank et al., 2008), thus immunological cross-talk may exert a subtle but important influence on replication kinetics in co-culture models.

Bearing in mind these multiple methodological caveats, the evidence is surprisingly conclusive that bNAbs are capable of inhibiting synaptic infection at both the VS and the IS (Table 1.3). What mechanistic information can we glean from these studies? Firstly, these data support conclusions that synaptic infection occurs through external transfer of intact virions (Jolly et al., 2004, Monel et al., 2012). In addition, the CD4-dependency of synaptic infection we have already mentioned above (Table 1.2) is

strengthened by data suggesting that it can be inhibited by CD4bs bNAbs, such as b12 and VRC01, if they are present at sufficient concentrations (Table 1.3).

Target	Inhibitor	VS		IS	
		Transfer	Infection	Transfer	Infection
Gp120 CD4-bs	sCD4	yes (Chen et al., 2007)	yes (Durham et al., 2012)	n.d.	yes (Yu et al., 2008, Cavrois et al., 2007)
	CD4-IgG_2	n.d.	yes (Abela et al., 2012)	n.d.	yes (Yu et al., 2008, Ketas et al., 2003)
	b12	yes (Sanchez-Palomino et al., 2011, Massanella et al., 2009, Martin et al., 2010, Chen et al., 2007)	yes (Sanchez-Palomino et al., 2011, Massanella et al., 2009, Martin et al., 2010, Durham et al., 2012)	no (Ganesh et al., 2004)	yes (van Montfort et al., 2007, Su et al., 2012, Sagar et al., 2012)
	VRCO1	n.d.	n.d.	n.d.	yes (Su et al., 2012, Sagar et al., 2012)
Gp120 Glycan	2G12	yes (Sanchez-Palomino et al., 2011, Martin et al., 2010)	yes (Martin et al., 2010, Durham et al., 2012, Abela et al., 2012)	n.d.	yes (Su et al., 2012, Sagar et al., 2012)
Gp120 conf.	PG16	n.d.	n.d.	n.d.	yes [20]
Gp41 MPER	2F5	no (Sanchez-Palomino et al., 2011, Massanella et al., 2009, Chen et al., 2007)	yes (Massanella et al., 2009, Martin et al., 2010, Durham et al., 2012, Abela et al., 2012)	no (van Montfort et al., 2007)	yes (Su et al., 2012, Sagar et al., 2012)
	4E10	no (Sanchez-Palomino et al., 2011, Massanella et al., 2009)	yes (Massanella et al., 2009, Martin et al., 2010, Durham et al., 2012, Abela et al., 2012)	n.d.	yes (Su et al., 2012, Sagar et al., 2012)

Table 1.3 Anti-Env inhibitor activity in cell-to-cell transmission
VS = virological synapse; IS = infectious synapse; n.d. = not done; conf. = conformational epitope; MPER = membrane proximal external region. Transfer means that viral antigen was measured in the target cell; Infection means that viral replication was measured in the target cell.

1.7.2.5 Relative efficiency compared to cell-free infection

If bNAbs are able to inhibit synaptic infection, the next question, which may have a major bearing on prophylactic vaccine efficacy, is what is the relative efficiency of synaptic and cell-free inhibition by bNAbs? Unfortunately, fewer studies have been

performed to specifically address this question (Table 1.4). Again methodological heterogeneity limits definitive conclusions, but the emerging pattern seems to suggest a relative reduction in the efficiency of synaptic neutralisation for some bnAbs, particularly those targeting gp120.

1.7.2.6 Env conformation

Both acutely and chronically infected donor cells have been used in studies of the T cell VS. As discussed above, transmission from acutely transfected donors may involve endocytosis of immature virions, followed by delayed CD4-dependent fusion (Dale et al., 2011). By contrast, chronically infected cells produce mature viral particles expressing Envs that efficiently fuse at the target-cell surface membrane following CD4 engagement (Jolly et al., 2004).

Target	Epitope	bNAb	Virological Synapse			Infectious Synapse
			T cell Acute	T cell Chronic	Other	
Gp120	CD4-bs	b12	reduced (Durham et al., 2012)	equal (Martin et al., 2010)*	reduced (Abela et al., 2012)	reduced (Sagar et al., 2012)
		VRCO1	n.d.	n.d.	reduced (Abela et al., 2012)	reduced (Sagar et al., 2012)
	CD4-i	17b	reduced (Durham et al., 2012)	equal (Martin et al., 2010)	reduced (Abela et al., 2012)	n.d.
	Glycan	2G12	equal (Durham et al., 2012)*	equal (Martin et al., 2010)*	reduced (Abela et al., 2012)	reduced (Sagar et al., 2012)
	Conf.	PG16	n.d.	n.d.	n.d.	reduced (Sagar et al., 2012)
Gp41	MPER	2F5	reduced (Durham et al., 2012)	equal (Martin et al., 2010)	equal (Abela et al., 2012)	equal (Sagar et al., 2012)
		4E10	reduced (Durham et al., 2012)	equal (Martin et al., 2010)	equal (Abela et al., 2012)	equal (Sagar et al., 2012)

Table 1.4 Relative efficiency of cell-to-cell compared to cell-free neutralisation
Equal = no significant difference in IC_{50}; reduced = significantly higher IC_{50} for cell-to-cell transmission; n.d. = not done. T cell acute refers to transfected donor cells; Chronic refers to donor cells used after multiple cycles of replication. * non-significant reduction in cell-to-cell efficiency.

This difference probably accounts for some of the reduced efficiency of VS-mediated bNAb inhibition reported for acutely transfected donor cells (Durham et al., 2012) (Table 1.4**)**. Acutely transduced cells appear to transfer virions that express Env in conformations that are less easy to neutralise by gp120 or gp41-directed antibodies (Durham et al., 2012). Interestingly, the neutralisation-sensitive Env conformation observed in chronic models (Martin et al., 2010) can be recapitulated in acute models by deletion of the cytoplasmic tail of gp41 (Durham et al., 2012). It is curious that enhanced neutralisation sensitivity occurs despite increased Env cell surface expression levels (Durham et al., 2012). This paradox could be explained by increased exposure of neutralisation epitopes, or by restoring the conventional viral entry mechanism (Jolly et al., 2004) which appears to be more neutralisation

sensitive (Martin et al., 2010). The lack of a significant change in 2G12 activity (which binds glycan epitopes that may be less susceptible to conformational changes (Kwong et al., 2002)) supports the concept of changes in tertiary structure correlating with enhanced neutralisation. It is currently unclear why plasma membrane rather than endosomal fusion might be more neutralisation sensitive, but there might be a kinetic element, since the exposure of virions to externally applied antibodies at the synaptic junction could be reduced by endosomal uptake, unless the antibody was also taken up in the endosome. It is also unclear whether endosome acidification occurs within the period of maturation, and if so, whether this interferes with the binding activity of NAbs.

By contrast to these models of the T Cell VS, Albela *et al* (Abela et al., 2012) took a novel approach using co-culture systems that eliminated cell-free infection. These models (e.g. using reporter cell lines or PBMC transfected with a rhesus retroviral restriction factor that inhibits cell-free infection) do not entirely correspond to either the acute or chronic VS models described above. Unlike Martin *et al* (Martin et al., 2010), they observed a differential and significant reduction in synaptic compared to cell-free neutralisation for gp120-directed antibodies, particularly for those targeting the CD4bs, but not for host receptor inhibitors or gp41 bNAbs 4E10 and 2F5 (Abela et al., 2012), a finding that was independently observed in an entirely different system of mature MDDC *trans*-infection by Sagar *et al* (Sagar et al., 2012). Although it is difficult to be sure how representative the models described by Albela *et al* are to synaptic infection events *in vivo*, together these data suggest that the synaptic route of infection may have a qualitative impact on the efficiency of CD4bs bNAb inhibition (Abela et al., 2012, Sagar et al., 2012). Moreover, in the chronic VS model, whilst no statistically significant differences in IC_{50} were identified, there was also a trend to reduced IC_{50} for the CD4bs inhibitors b12 and 2G12 (Martin et al., 2010).

Despite a lack of consistency in experimental systems, there are indications that cell-to-cell spread may have a qualitative effect on the activity of NAb, particularly those targeting gp120 (Table 1.4). Some additional mechanistic explanations for this potentially important observation are discussed below.

1.7.2.7 Multiplicity

Cell-to-cell spread is a more efficient mechanism for HIV-1 transmission (reviewed in (Mothes et al., 2010)) and is associated with an increased multiplicity of infection (MOI) in target cells (Del Portillo et al., 2011, Sigal et al., 2011). If the probability of antibody inhibition is inversely proportional to the MOI, as has been proposed for antiretroviral drugs (Sigal et al., 2011), then higher multiplicity synaptic infections should increase the concentration of antibody needed for functional activity (i.e. the IC_{50}). However, Sagar *et al* equalised the infectivity of virus delivered by cell-free inoculation or DC trans-infection, finding that some gp120 directed NAbs still had reduced potency in the cell-to-cell context, (Sagar et al., 2012) suggesting that additional qualitative factors beyond MOI might be involved.

1.7.2.8 Steric and kinetic barriers

To act effectively, antibodies must be able to access the synapse (or the endosomal compartment into which virus is taken). Steric restriction may therefore dictate the partial loss of neutralisation efficiency observed. Ultrastructural studies have provided an important glimpse of the architecture of virological and infectious synapses, and have been reviewed elsewhere (Sattentau, 2010). A recent 3D ion abrasion scanning electon microscopy (IA-SEM) study (Felts et al., 2010) examining the DC-$CD4^+$ T Cell synapse, revealed thin sheets of DC membrane that envelop most of the T cells in contact, as well as interdigitation of T cell and DC membranes. The distance in the intercellular synaptic cleft was < 500 nm, and HIV-1 was

observed, as it has been in HIV-1 infected macrophages (Welsch et al., 2011), in a complex network of surface-connected compartments that have recently been shown to be relatively inaccessible to some NAbs (Koppensteiner et al., 2012a). These data provide a structural basis for steric restriction, at least at the IS, which may account for the improved efficiency of auto-reactive MPER antibodies which appear able to bind DC in the absence of HIV-1 (Sagar et al., 2012). It will be instructive to explore whether these findings are recapitulated by the newly identified non-auto-reactive gp41 bNAb 10E8. By contrast, 3D reconstruction of the T cell VS suggests a more open structure (Martin et al., 2010), which remains accessible to bNAbs such as b12 (Martin et al., 2010), and could account for data suggesting CD4bs NAb activity is relatively preserved in VS models of established T cell infection (Martin et al., 2010).

It may appear that the epitope-specific differences suggest that steric impedance is not the dominant mechanism, but one must consider distribution of epitopes on the Env trimer (Fig. 1.2). In cell-line based studies (Abela et al., 2012), and in one study of the DC IS (Sagar et al., 2012), NAbs with epitopes at the tip of the trimer (i.e. CD4-i, conformational, CD4bs) appear to suffer more from a relative restriction of synaptic activity than those at the base (i.e. 2F5 and 4E10). This form of epitope-specific steric inhibition is suggested by experiments with a Fab fragment of b12, which displayed equal inhibitory activity in synaptic and cell-free infections, in contrast to the intact antibody (Sagar et al., 2012). These data imply that this epitope is not entirely shielded in the IS, but that it maybe inaccessible to the full-length antibody. It remains unclear, and rather counter-intuitive, why the potentially more accessible epitopes are more affected by steric hindrance than the MPER region, which is buried close to the membrane and partially obscured by the gp120 trimer. Clearly further studies are needed to explore the impact of epitope distribution on synaptic bNAb activity involving a larger panel of antibodies.

As well as a steric inhibition, there are also kinetic limitations imposed by synaptic infection. Time-of-addition studies show a significant loss of transmission-blocking activity for CD4bs antibodies such as b12 if added 1 hour following co-culture (Martin et al., 2010), despite b12 remaining able to access the T cell VS (Martin et al., 2010). By contrast, MPER antibodies might act at a later time-point after gp120-receptor engagement, since in contrast to CD4bs antibodies they are able to inhibit infection post attachment in a cell-free infection model (Binley et al., 2003) and 2F5 has been shown to preferentially recognise Env after CD4 binding (Binley et al., 2003, Sattentau et al., 1995).

1.8 Perspectives

There are several gaps in our knowledge on the role of macrophages in viral persistence and the implications of cell-to-cell transmission for antibody activity that require specific attention.

Attempts should be made to define the lifespan of infected macrophages *in vivo* (for example in SIV models), and comprehensive assessments of the extent of macrophage infection in patients during ART are a priority. Comparative studies of primary CTL suppression of macrophage and $CD4^+$ T cell HIV-1 replication could better define the role of macrophage infection in escape from cell-mediated immune pressure. Finally, the mechanism and implications of macrophage cell-to-cell transmission in the face of ART, which has been proposed to be an ART-resistant method of viral dissemination between T cell lines, should be defined.

Whilst evidence seems to converge on the probability that NAbs can neutralise synaptic infection, one has to bear in mind that many of these broad and potent NAbs were isolated from patients showing exceptionally strong neutralising

responses (Walker et al., 2009, Wu et al., 2010, Li et al., 2007, Simek et al., 2009). In contrast, neutralising polyclonal sera, although much less broadly and potently neutralising (Vujcic and Quinnan, 1995, Chen et al., 2007), might better reflect the nature of the antibody response induced by natural HIV-1 infection.
In VS-mediated infection patient sera inhibit synaptic infection at a reduced efficiency compared to cell-free infection, although the neutralisation ability seems to vary significantly between different sera (Gupta et al., 1989, Chen et al., 2007, Hubner et al., 2009, Dale et al., 2011). Whether this is due to the pattern of epitope targeting, as has been observed in some studies for NAbs, is unclear. Furthermore, in studies using patient sera it remains unclear what influence other factors present in the serum (such as cytokines) might have on the assay. Thus, to avoid potential biasing influences of such factors, purified IgG rather than serum should be used in future studies. Neutralisation of IS-mediated infection by the IgG fraction of polyclonal sera has not yet been studied.

Moreover, few viral isolates have been tested in studies of synaptic transfer to date. Most studies have used lab-adapted HIV-1 strains that may be less biologically relevant. Since primary isolates and molecular clones may have distinct Env conformations, as far as practical, a broad panel of T/F viruses (Salazar-Gonzalez et al., 2009, Ochsenbauer et al., 2012) should be tested in synaptic transfer models. For example, a study involving a range of primary viruses including transmitter/founder viruses in the IS model demonstrated virus strain-specific restriction of synaptic transfer for the bNAb PG16 (Sagar et al., 2012). Thus, there may be viral determinants of synaptic neutralisation that are yet to be revealed.

Finally, the lack of studies of bNAb activity in primary cell models of HIV-1 cell-to-cell transmission is a major deficiency. It is unclear if findings in highly permissive cell lines or adherent 293 T cell engineered to express HIV-1 receptors can be

extrapolated to the activity of bNAbs under physiological conditions more relevant to the *in vivo* situation.

1.9 Statement of hypothesis

My central hypothesis was that cell-to-cell transmission of HIV-1 between the main productively infected target cells *in vivo* (macrophages and $CD4^+$ T cells) is a more efficient mode of viral dissemination than cell-free infection, and I sought to investigate the mechanism(s) involved. Once infected, macrophages can efficiently transfer HIV-1 virions to uninfected $CD4^+$ T cells through an apparent virological synapse, although the structure and organisation of this synapse remains poorly defined. Moreover, as I have summarised above, cell-free infection of macrophages is inefficient and many viruses are unable to robustly infect macrophages by this cell-free route. However macrophages are professional phagocytes, and an established hypothesis in the Sattentau laboratory on my arrival was that macrophages interact with and engulf infected $CD4^+$ T cells, possibly becoming infected in the process.

I therefore refined my broad hypothesis to address four specific questions regarding mechanisms of HIV-1 cell-to-cell transmission between macrophages and $CD4^+$ T cells of relevance to HIV-1 pathogenesis.

1. What is the architecture/organisation of the macrophage-to-T cell VS?

2. Do macrophages transmit a high multiplicity infection to $CD4^+$ T cells at the VS, and can this overcome antiretroviral inhibition?

3. Does VS-mediated transmission from macrophages to $CD4^+$ T cells represent a strategy for NAb evasion?

4. Do interactions between HIV-1 infected $CD4^+$ T cells and macrophages, particularly involving phagocytic uptake, result in macrophage infection? If so,

is cell-to-cell transmission more efficient than cell-free inoculation, particularly for transmitted/founder viruses?

2 MATERIALS AND METHODS

2.1 Viruses

Name	Clade	Type	Env	Insert	Source	Produced
BaL	B	Primary Ref	BaL	N/A	CFAR	PBMC
BaLΔEnv	B	Pseudo Ref	-	N/A	CFAR	293T
NL-mCherry	B	IMC T/F	CH077	mCherry	CHAVI	293T
NL-eGFP	B	IMC Ref	BaL	GFP	CHAVI	293T
NL-iGFP	B	IMC Ref	JRFL	GFP	B. Chen	293T
NL-LucR-T2A.ecto	B	IMC Ref	BaL	LucR	CHAVI	293T
NL-LucR-T2A.ecto	B	IMC Ref	YU2	LucR	CHAVI	293T
NL-LucR-T2A.ecto	B	IMC T/F	CH040	LucR	CHAVI	293T
NL-LucR-T2A.ecto	B	IMC T/F	THRO	LucR	CHAVI	293T
NL-LucR-T2A.ecto	B	IMC T/F	RHPA	LucR	CHAVI	293T
NL-LucR-T2A.ecto	B	IMC T/F	WITO	LucR	CHAVI	293T
NL-LucR-T2A.ecto	B	IMC T/F	REJO	LucR	CHAVI	293T
NL-LucR-T2A.ecto	B	IMC T/F	SUMA	LucR	CHAVI	293T
NL-LucR-T2A.ecto	B	IMC T/F	WEAU	LucR	CHAVI	293T
NL-LucR-T2A.ecto	B	IMC X4 Ref	NL4.3	LucR	CHAVI	293T
NL-LucR-T2A.ecto	C	IMC Ref	MW965	LucR	CHAVI	293T
NL-LucR-T2A.ecto	C	IMC T/F	Du422	LucR	CHAVI	293T
NL-LucR-T2A.ecto	C	IMC T/F	Du151.2	LucR	CHAVI	293T
NL-LucR-T2A.ecto	C	IMC T/F	3426	LucR	CHAVI	293T
NL-LucR-T2A.ecto	C	IMC T/F	CAP45	LucR	CHAVI	293T
NL-LucR-T2A.ecto	C	IMC T/F	1086.B2	LucR	CHAVI	293T
NL-LucR-T2A.ecto	C	IMC T/F	2010.F5	LucR	CHAVI	293T
NL-LucR-T2A.ecto	C	IMC T/F	0393.C3	LucR	CHAVI	293T
NL-LucR-T2A.ecto	C	IMC T/F	ZM249M	LucR	CHAVI	293T
NL-LucR-T2A.ecto	C	IMC T/F	1176.A3	LucR	CHAVI	293T

The Centre for HIV/AIDS Immunology (CHAVI) renilla luciferase (LucR)-expressing infectious molecular clones (IMCs) in this table were generated and kindly provided by C. Ochsenbauer and J. Kappes, University of Alabama, Birmingham. All viruses were R5-using except NL-LucR-T2A.NL4.3 (NL4.3) which was X4-using.

2.2 Antibodies

2.2.1 Cellular immunophenotyping

2.2.1.1 Primary antibodies

Clone	Target	Isotype	Species	[μg/mL]	Source
UCHT1	CD3	IgG_1	Mouse	6.25	P. Beverley
UCHT1	CD3	IgG_{2a}	Mouse	6.25	P. Beverley
L120	CD4	IgG_1	Mouse	10	CFAR
2D7	CCR5 (CD195)	IgG_{2a}	Mouse	10	BD
L130	LFA-1β (CD18)	IgG_1	Mouse	10	BD
LB-2	ICAM-1 (CD54)	IgG_{2b}	Mouse	10	BD
H4A3	LAMP-1 (CD107a)	IgG_1	Mouse	10	BD
454720	CD81	IgG_{2b}	Mouse	10	R&D

BD = BD Biosciences.

2.2.1.2 Secondary antibodies

Target	Fluorochrome	Species	[μg/mL]	Source
Human IgG_1 Fab	Alexa 647	Goat	5-10	Jackson
Biotin	TRITC	-	5-10	Jackson
Rabbit IgG	Alexa 488/555	Goat	5	Invitrogen
Mouse IgG_1	Alexa 488/546/647	Goat	5	Invitrogen
Mouse IgG_{2a}	Alexa 488/546/647	Goat	5	Invitrogen
Mouse IgG_{2b}	Alexa 488	Goat	5	Invitrogen

2.2.1.3 Directly conjugated antibodies/reagents

Clone/ Compound	Target	Isotype	Conj.	Species	[µg/mL]/ Dilution	Source
UCHT1	CD3	IgG_1	FITC	Mouse	0.0625	BD
UCHT1	CD3	IgG_1	APC	Mouse	0.0625	BD
HCD14	CD14	IgG_1	APC	Mouse	0.25	BL
L161	CD1c	IgG_1	FITC	Mouse	1:5	BL
HIT2	CD38	IgG_1	APC	Mouse	1:5	BD
L243	HLA-DR	IgG_{2a}	PerCP-Cy 5.5	Mouse	1:20	BL
FR10-11G9	CD26	IgG_{2a}	Biotin	Mouse	3	Miltenyi
	-	IgG_{2a}	Biotin	Mouse	3	Miltenyi
2G12	gp120	IgG_1	Biotin	Human	10	T. Schiffner
b12	gp120	IgG_1	Biotin	Human	10	T. Schiffner
Phalloidin	F-Actin	-	TRITC	-	0.2	Sigma
Live/Dead	Intracellular amines	-	Red	-	1:1000	Invitrogen
Annexin-V	PS	-	FITC	-	1:20	BD

BD = BD Biosciences; BL = BioLegend.

2.2.2 Anti-HIV-1 antibodies/inhibitors

Reference	Target	Isotype	Species	[μg/mL]/ Dilution	Source
b12	gp120	IgG_1	Human	10	NAC
2G12	gp120	IgG_1	Human	10	NAC
PGT121	gp120	IgG_1	Human	10	NAC
VRC01	gp120	IgG_1	Human	10	J. Mascola
10E8	gp41	IgG_1	Human	50	J. Mascola
2F5	gp41	IgG_1	Human	50	NAC
4E10	gp41	IgG_1	Human	50	NAC
HIVIG	Env	Polyclonal	Human	10	NAC
“B pool”	Env	Sera	Human	1:1000	M. Connors
sCD4		-	-	50	Progenics
D1D2-Igαtp	Env	IgG-fusion	Human	50	J. Arthos
CD4-IgG	Env	IgG-fusion	Human	50	Progenics
ARP342/4C9	p18 Gag	IgG_{2a} sup	Mouse	1:500	CFAR
ARP0431	p17 Gag	Sera	Rabbit	1:1000	CFAR
ARP0432	p24 Gag	Sera	Rabbit	1:1000	CFAR
KC57*	p24/33/39/55	IgG_1	Mouse	10	BC
D7320	p24	Polyclonal	Sheep	10	Aalto

NAC = Neutralising Antibody Consortium; CFAR = Centre for AIDS Reagents; BC = Beckman-Coulter. Sup = supernatant * Directly-conjugated to FITC or RD-1 (PE).

2.2.3 Functional antibodies

Clone	Target	Isotype	Species	[μg/mL]	Source
13B8.2	CD4	IgG_1	Mouse	10	BC
2D7	CCR5 (CD195)	IgG_{2a}	Mouse	10	BD
L130	LFA-1β (CD18)	IgG_1	Mouse	10	BD
MEM-25	LFA-1α (CD11a)	IgG_1	Mouse	10	Abcam
MAB24	LFA-1α (CD11a)	IgG_1	Mouse	10	Hycult
LB-2	ICAM-1 (CD54)	IgG_{2b}	Mouse	10	BD/SC
HA58	ICAM-1 (CD54)	IgG_1	Mouse	10	Abcam
B-T1	ICAM-2 (CD102)	IgG_1	Mouse	10	AbD Serotec
101-1D2	ICAM-3 (CD50)	IgG_{2a}	Mouse	10	SC
MA4	ICAM-3 (CD50)	Sup	Mouse	Neat	A. Devitt
6163	CD14	Sup	Mouse	Neat	A. Devitt
HSn 7D2	Sialoadhesin	IgG_1	Mouse	10	Abcam
Polyclonal	TIM-4	IgG	Goat	5	R&D
326203	Calreticulin	IgG_{2b}	Mouse	5	R&D
Polyclonal	MF-GE8	IgG	Goat	5	R&D
CBRM1/5	Mac-1 (CD11b)	IgG_1	Mouse	10-25	BL
M1/70	Mac-1 (CD11b)	IgG_{2b}	Rat	10	BL
ICRF44	Mac-1 (CD11b)	IgG_1	Mouse	10-25	BL

2.3 Antiretroviral compounds

Drug	Code	Class	Target	Solvent	Storage	Source
Azidothymidine	AZT	NRTI	RT	H_20	18-21 ^oC	CFAR
Nevirapine	NVP	NNRTI	RT	DMSO	18-21 ^oC	CFAR
Raltegravir	RAL	INSTI	IN	H_20	-20^oC	CFAR
Enfurvitide*	T-20	Fusion inhibitor	gp41	H_20	-20^oC	CFAR
TAK779	-	Entry inhibitor	CCR5	H_20	-20^oC	CFAR

*T-20 was obtained from Roche through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.

2.4 Peptides/Chemical inhibitors

Compound	Code	Target	Solvent	Storage	[μM]
Jasplakinolide	JAS	F-Actin	DMSO	-20°C	1-5
Latrunculin A	LAT	F-Actin	DMSO	-20°C	1-2
Cytochalasin D	CytD	F-Actin	DMSO	-20°C	10
Dynasore	DYN	Dynamin GTPase	DMSO	-20°C	80
Dimethyl amiloride	DMA	Macropinocytosis	DMSO	-20°C	50
5-(N-ethyl-N-isopropyl) amiloride	EIPA	Macropinocytosis	DMSO	-20°C	50
Blebbistatin	BLEB	Myosin II motor	DMSO	-20°C	100
Paclitaxel	PAX	Microtubules	H_20	-20°C	10
Colchicine	COL	Tubulin	DMSO	-20°C	10
Nocodazole	NOC	Microtubules	DMSO	-20°C	10
Chlorpromazine	CPZ	Clathrin-mediated endocytosis	H_20	-20°C	10
O-Phospho-L-serine	PLS	PS binding	H_20	-20°C	1
O-Phospho-L-tyrosine	PLT	Control	H_20	-20°C	1
Dextran sulphate	$DexS0_4$	SR	H_20	-20°C	10*
Mannan	Mannan	MR	H_20	-20°C	20*
peptide RGDS	RGDS	Integrin αvβ3	H_20	-20°C	50
peptide RGES control	RGES	Control	H_20	-20°C	50
cyclised RGD	cRGD	Integrin αvβ3	CH_3CO_2H	-20°C	50
cyclised RAD control	cRAD	Control	CH_3CO_2H	-20°C	50
Annexin-V	rAV	PS	H_20	-80°C	6*

* Dose in μg/mL. All Sigma except rAV (BD) and integrin peptides (BACHEM). PS = phosphatidylserine. SR = scavenger receptor; MR = mannose receptor.

2.5 Cell culture media

- Adherent cell lines: DMEM plus 10% foetal bovine serum (FBS, Sigma), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma).
- Primary $CD4^+$ T cells: RPMI plus 10% FBS (Sigma), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma) and 10 IU/mL IL-2 (CFAR).
- MDM: X-Vivo-10 plus 1% pooled human serum (multiple buffy coat donors or purchased from Sigma).

2.6 Suppliers

Reagents were purchased from the following suppliers:

- Aalto, Ireland
- Abcam, UK
- AbD Serotec, UK
- Appleton Woods, UK
- BACHEM, Switzerland
- Beckman-Coulter, USA
- BD Biosciences, UK
- BioLegend, UK
- Centre for AIDS Reagents, NIBSC, UK
- eBiosciences, UK
- Eurofins MWG Operon, Germany
- Invitrogen, UK
- Jackson ImmunoResearch, USA
- Lonza, UK
- Millipore, UK
- Miltenyi Biotech, UK

- NHS National Blood Service, UK
- Nalgene, Denmark
- OZ Biosciences, France
- PAA, UK
- Pharmacia, Sweden
- Progenics, USA
- Promega, UK
- Polysciences, Germany
- Qiagen, UK
- Sartorius, UK
- Santa-Cruz Biotechnology, USA
- Sigma-Aldrich, UK
- Takara Bio (Clontech), France

2.7 Cells

2.7.1 Primary Cell Isolation

2.7.1.1 PBMC Preparation

PBMCs were isolated from healthy HIV-uninfected donor blood obtained from leucocyte cones or buffy coats supplied by the Oxfordshire National Blood Service, John Radcliffe Hospital, Oxford or by venepuncture of healthy donors. Healthy donors were provided with a written information sheet and gave signed informed consent prior to venepuncture. University of Oxford policy is that formal Research Ethics Committee (REC) approval is waived for venepuncture conducted in accordance with University of Oxford guidance. Venepuncture was performed using a 21-gauge butterfly needle into 10 mL heparinised Vacutainers® (BD). PBMCs were isolated by density-gradient centrifugation as follows: 30 mL whole blood was layered onto 15 mL Histopaque® (Sigma) in a 50 mL conical tube and centrifuged with no brake at 1870 rpm for 25 min at room temperature. The mononuclear cell layer was carefully aspirated with a 2 mL Pasteur® pipette and washed three times in phosphate buffered saline (PBS, Sigma) by centrifugation at 1700 rpm for 10 min at room temperature. PBMCs were used immediately for isolation of monocytes, cryopreserved or resuspended at ~2.5 x 10^6 cells/mL in RPMI supplemented with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin (hereafter referred to as RPMI-10) supplemented with 10 IU/mL IL-2 (CFAR) +/- stimulation with 1 μg/mL phytohaemagglutinin (PHA, Sigma) for 3 d.

2.7.1.2 Monocyte Isolation by Adherence

Monocytes were isolated from PBMCs by adherence as previously described (Groot et al., 2008). Briefly, PBMCs were plated on 15 cm^2 tissue culture plates at ~2.5 x

10^6 cells/mL in RPMI and incubated at 37°C for 1 h. Supernatants containing the non-adherent PBMC fraction were removed, washed in fresh medium and stimulated as described in 2.7.1.1. Adherent cells were cultured overnight in X-Vivo® 10 (Lonza) supplemented with heat-inactivated 1% pooled human plasma (hereafter referred to as macrophage medium). The following day adherent cells were incubated at 4°C for 1h and reseeded at 0.5 x 10^6 cells/mL in appropriate plates, and differentiated into macrophages by culturing for 7 d in fresh macrophage medium. Half the total volume of macrophage medium was exchanged at 3-4 d. MDM generated by this method were approximately 90% positive for the monocyte/macrophage markers CD14 and HLA-DR and negative for myeloid dendritic cell markers CD1c (BDCA-1) and the T cell marker CD3 (see Chapter 3).

2.7.1.3 Magnetic Bead Enrichment

Untouched $CD4^+$ T cells were isolated to > 95% purity from PHA-stimulated PBMCs using MACS® magnetic selection technology according to manufacturers' instructions ($CD4^+$ T Cell Isolation Kit, Miltenyi).

Untouched monocytes were enriched to 94% purity from unstimulated PBMCs using MACS® magnetic selection technology according to manufacturers' instructions (Monocyte Isolation Kit II, Miltenyi). Monocytes were seeded at 0.5 x 10^6 cells/mL in macrophage medium and allowed to differentiate into macrophages as described above.

2.7.1.4 Percoll Density Gradient Centrifugation

Monocyte isolation using isotonic (285 mOsm) 1.046 g/mL Percoll (Pharmacia) density gradient centrifugation was initially tested using an established protocol (Colotta et al., 1984). Percoll-isolated MDM were > 90% pure by day of infection (7 d) as described in 2.7.1.2 (data not shown).

2.7.1.5 Cryopreservation

Where necessary cells were cryopreserved in 1.8mL cryovials (Nalgene) at 5-10 x 10^6 cells/mL in 90% FBS and 10% dimethyl sulfoxide (DMSO, Sigma) and stored at -80°C until use. Vials of cells thawed in a water bath at 37°C were rapidly transferred to pre-warmed medium containing 10% FBS and washed twice prior to counting and further use.

2.7.1.6 MDM Lifting

If required, MDM were lifted by incubation with 12 mM lidocaine (pH 7.4, Sigma) and 5mM EDTA in PBS (pH 8.0, Sigma) at 37°C for 10 min or 4°C for 1 h, followed by gentle lifting with a rubber policeman.

2.7.2 Cell Lines

Adherent human endothelial kidney (HEK) 293T (Yang et al., 1999) and TZM-bl (JC53) cells (Derdeyn et al., 2000, Wei et al., 2002) were passaged in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin (hereafter referred to as DMEM-10). Adherent cell lines were lifted with 0.25 mg/mL trypsin and 1 mM EDTA.

2.8 Virus preparation

2.8.1 Primary Isolates

HIV-1 BaL obtained from CFAR was amplified in PHA/IL-2 activated PBMCs pooled from 2-3 independent donors. Approximately 2 x 10^9 cells were infected at MOI 10^{-2} for 4h at 37°C then diluted to a total volume of 100 mL RPMI-10 supplemented with

10 IU/mL IL-2 (CFAR). Every 3 days post-infection (d.p.i.) 50-90 mL supernatant was removed, clarified by centrifugation at 1500 rpm for 10 min and frozen in single use 0.5-1 mL aliquots at -120°C or concentrated (see 2.8.4). An equal volume of fresh IL-2-containing RPMI-10 medium was replaced. At ~ 9 d.p.i. 10^8 cryopreserved PBMC were thawed, activated for 3 d with PHA/IL-2 and added as feeder cells.

2.8.2 Infectious Molecular Clones (IMCs)

Plasmid DNA was used to transform competent *E.coli* (OneShot Stbl3®, Invitrogen) and DNA was purified by MaxiPrep® (Qiagen) according to manufacturers instructions and quantified by UV spectrophotometry (Nanodrop). 293 T cells were seeded at a density of 0.5 x 10^6 cells/mL (8 mL per 10 cm^2 tissue culture plate) in DMEM-10 24 h prior to transfection by PEI precipitation to ensure 70-80% confluence on the day of transfection. 2.5 mL PEI at a final concentration of 0.4 μM (prepared fresh on the day of use from a 10 mM stock solution) was added drop-wise to 25 μg plasmid DNA diluted in 2.5mL DMEM whilst vortexing. The PEI-DNA transfection mixture was incubated for 20 min at room temperature before 5 mL was gently added to the cells which were subsequently incubated at 37°C. After 4 h the transfection mixture was removed and 5 mL warm DMEM-10 was added. Supernatants were harvested at 48-72 h, filtered through a disposable 0.45 μm pore filter (Sartorius) and frozen in 0.5 - 1 mL single use aliquots at -120°C or concentrated (2.8.4).

2.8.3 Pseudoviruses

The IMC transfection protocol was modified for pseudovirus preparation as follows: 18μg backbone vDNA and 6μg gp160 Envelope vDNA were co-transfected per 5 mL final transfection mix as outlined in 2.8.2, and supernatants were harvested at 24h and 48h post-transfection.

2.8.4 Virus Concentration

Filtered or clarified virus-containing supernatants were concentrated 25-100 fold using LentiX^{TM} concentrator according to manufacturers' instructions (Clontech), resuspended in fresh RPMI-10 or PBS and frozen in 25-100 μL single use aliquots at -120°C.

2.8.5 Viral Purification

Virus-containing supernatants were purified by centrifugation through 1 MDa Amicon® centrifugal filter units according to manufacturers' instructions (Millipore).

2.8.6 Primary Cell HIV-1 Infection

2.8.6.1 CD4^{+}T Cell

Activated CD4^{+} T Cells were incubated in 1-2 mL of virus inoculum (MOI ranging from 10^{1} to 10^{-3} depending on experimental design) at 37°C for 4-6 h and diluted to 2 x 10^{6} cells/mL in RPMI-10 with 10 IU/mL IL-2. Every 4-5 d additional IL-2 was added to maintain the concentration in the medium at 10 IU/mL. Cells were used at 2-7 d.p.i. depending on the experimental set-up.

2.8.6.2 MDM

MDM were incubated with low volume of virus inoculum (50 - 500 μL) at MOI 10^{1} - 10^{-3} at 37°C for 4-6 h, and medium topped up to a final volume of 0.2 - 2 mL (depending on plate volume). Cells were typically used at 7 d.p.i. when approximately 30% of MDM were HIV-1 Gag^{+} (MOI 10^{-1}) by flow cytometry.

2.8.7 Synchronous Magnetic Bead Infection (Magnetofection)

Synchronous high-level infection of PHA/IL-2 activated $CD4^+$ T cells was achieved by magnetofection using magnetic particles as previously described (Sacha and Watkins, 2010) according to manufacturers' instructions (OZ Biosciences).

2.8.8 Viral Infectivity Titration

Infectivity of viral stocks was assessed on TZM-bl cells according to a standard protocol (Seaman et al., 2010). Briefly, viral stocks at a starting dilution of 1:6-1:50 were titrated in a 3-fold dilution series in 100μL DMEM-10 and incubated with 10^4 TZM-bl cells/100μL/well in DEA-dextran (25 μg/mL) for 24h. Supernatant was removed and cells were washed once in PBS and lysed with 100 μL Reporter Lysis Buffer (Promega) and stored overnight at -80^{o}C, or lysed with Glo-Lysis Buffer (Promega) and immediately analysed. For luciferase quantification cell lysates were mixed 1:1 with Bright-Glo luciferase assay solution (Promega) at room temperature and luminescence measured at 1000 ms^{-1} integration with a SpectroMax M3 or M5 (Molecular Devices) plate reader. Background values from uninfected wells were multiplied by two and subtracted from test wells. Infectious unit calculations were done using ID-50 version 5.0 (John L. Spouge) using a Poisson model. For infections, MOI was expressed as the ratio of Tzm-bl infectious units (IU) to cells. Thus MOI 0.1 represented a ratio of 1 IU to 10 primary cells. Given the extensive donor-dependent variability in infectibility of primary cells, particularly MDM (summarised in Chapter 1), there is not a consistent, predictable relationship between MOI and per-cell infection levels [REF Bol], as has been conventionally associated with permissive cell lines [REF Field's Virology]. Thus, the term MOI is used in this thesis simply as a standardised measure of infectious virus exposure to permit comparisons between virus isolates and experimental conditions.

2.9 Flow Cytometry

2.9.1 Cell Staining Protocols

Live cell staining was performed in wash buffer (0.5% BSA, 2 mM EDTA in PBS) at 4^{o}C for 30 min for primary unconjugated or directly conjugated antibodies followed by three washes in wash buffer +/- 30 min incubation with the relevant fluorochrome-conjugated secondary antibody followed by three washes. Live cells were then either analysed immediately, or permeabilised in permeabilisation buffer (PB, 0.1% saponin [Sigma] 3% FBS [Sigma]) for 30 min, and intracellular staining performed for 1 h at 4^{o}C in PB, followed by three washes. Alternatively, cells were fixed with 4% formaldehyde for 15 min at room temperature, and antibody incubations were performed for 1 h at room temperature with the same washes and permeabilisation steps. For live cell staining of HIV-1 infected cells, primary antibody incubation was done as above followed by washes and fixation with 4% formaldehyde for 15 min at room temperature. Secondary staining was done as for fixed cells. Antibodies used are summarised in 2.2.1.

2.9.2 Cellular Identification

As well as size/granularity discrimination, the following cell surface markers were used to identify the principal cell types under investigation: $CD4^{+}$ T cells: $CD3^{+}$ $CD4^{+}$ ($CD14^{-}$). Monocytes: $CD4^{+}$ $CD14^{++}$ $CD16^{+/-}$ ($CD3^{-}$ $CD56^{-}$). MDM: $CD14^{+}$ ($CD3^{-}$ $CD4^{+/-}$ $CD16^{+}$). MDM were differentiated from mature MDDC based on surface marker expression ($CD14^{+}$ $CD1c^{-}$ $HLA\text{-}DR^{+}$), phagocytic activity (latex beads), and HIV-1 permissiveness.

2.9.3 VS-mediated HIV-1 Transfer Assay

$CD4^+$ T cells were co-cultured in duplicate/triplicate with HIV-1-infected MDM for 3 h in RPMI-10, non-adherent cells were gently aspirated in warm RPMI-10, washed and fixed, stained for CD3, permeabilised and stained for intracellular Gag, and analysed on a FACSCalibur (BD Biosciences). At least 5 x 10^3 gated events were collected for each replicate for each condition. Data were analysed using FlowJo 887. Non-viable cells and debris were gated out, and T cells were gated by FSC/SSC characteristics and $CD3^+$ staining. Gag positive cells were gated according to uninfected control $CD4^+$ T cells and/or isotype stained infected $CD4^+$ T cells (approximately 0.5% $CD3^+$ Gag^+). Transfer was expressed relative to the % $CD3^+$ Gag^+ cells in isotype or vehicle (DMSO or RPMI) control conditions: Gag transfer (%) = 100 x [(Gag $transfer_{inhibitor}$)/(Gag $transfer_{control}$)].

2.9.4 Infected T Cell Uptake Assay

For uptake experiments, samples were prepared as for multispectral flow cytometry (ImageStream^{TM,} see 2.15) in 6-well or 24-well plates. Antibodies used were: CD14-APC (BioLegend), CD14-FITC (BD Biosciences), CD3-FITC, CD3-APC, KC57-FITC/PE, together with appropriate fluorochrome-conjugated isotype controls at equal concentration. Cells were gated according to scatter characteristics and staining for CD14 (MDM) or CD3 (T cell). Samples were collected on a FACSCalibur and analysed with FlowJo 887. Uptake was expressed as % Gag^+ of parent ($CD14^+$).

2.9.5 Latex Microbead Phagocytosis Assay

The phagocytic activity of MDM and the blocking activity of inhibitors was assessed in a flow-cytometry based assay of the phagocytic uptake of 6.0 μm FITC-labelled latex microspheres (FluoresBrite, Polysciences). Beads were washed three times in PBS before adjusting to target concentration. MDM were pre-treated with inhibitors

(or vehicle control or untreated) for 1 h and then co-cultured with MOI 10^{-1} beads with or without washout of inhibitor for a further 2-3 h. MDM were therefore exposed to inhibitors for a total of 1 h or 3-4 h. MDM were then washed three times in 5 mM EDTA to remove unbound/unengulfed beads, before lifting in 5 mM EDTA/ 12 mM lidocaine, and analysis by flow cytometry (5000 gated events). Uptake was expressed as % $Bead^+$ MDM in treated conditions relative to DMSO vehicle control or untreated conditions as indicated.

2.10 p24 Enzyme-linked Immunosorbent Assay (ELISA)

Supernatants were removed, centrifuged/filtered (0.45 μm filter) to remove cells/debris and heat inactivated in a final concentration of 0.5% empigen (Sigma) at 56°C for 1 h. High-binding 96-well ELISA plates (Greiner) were coated overnight with 100 µL of 10 µg/mL anti-p24 (clone D7320, Aalto) in coating buffer (pH 8.5, 100 mM $NaHCO_3$). Buffers were prepared fresh from 10X tris-buffered saline (TBS) stock (1.44 M NaCl with 250 mM trizma-HCl in dH_20 and adjusted to pH 7.5). Plates were washed with 1x TBS plus 0.05% Tween-20 (wash buffer). Coating antibody was removed by three washes with 200 μL wash buffer using a 96-well plate washer (Dynex Technologies), and wells were blocked with 2% BSA/1X TBS for 30 minutes at room temperature to prevent non-specific binding. Following this, the plate was again washed three times.

Recombinant p24 protein (AG6054 Aalto) standards were titrated in a 2- fold dilution series from 200 ng/mL (or 50 ng/mL for T cell virion capture assay) in 10% FBS, 1X TBS, 0.5% empigen. 100 µL standards and samples in duplicate or triplicate were transferred to the ELISA plate and incubated at 37°C for 2 h with gentle shaking (75 rpm) followed by six washes with 200 μL wash buffer. 100 μL biotinylated anti-p24 antibody (BC1071-BIOT, Aalto 1:1000 dilution in 2% BSA with 20% FBS) was incubated for 2 h at room temperature. Plates were washed (6 x 200 μL wash buffer)

and incubated with 100 µL of Streptavidin-HRP (AbD Serotec; 1:10,000 in 1x TBS; 2% BSA with 20% FCS) for 1 h at room temperature. After washing (6 x 200 µL wash buffer) and 100 µL TMB-Turbo One-Step substrate (Pierce) was added to the plate for approximately 10-15 min before the reaction was stopped with 100 µL 0.5 M H_2SO_4, and absorbance read at 450 nm and 570 nm using a SpectroMax M3 or M5 plate reader. To correct for pipetting error values at 570 nm were subtracted from values at 450 nm, background values from negative control wells were subtracted and sample data were interpolated from a standard curve fit to a sigmoidal dose-response curve (variable slope) with baseline constrained to 0 in Prism 5.0 (GraphPad).

2.10.1 T Cell Viral Capture Assay

A magnetic bead virion capture assay, based on published data showing that CD26 is expressed on HIV-1 budding from $CD4^+$ T cell membranes (Lawn et al., 2000) was performed on clarified cell culture supernatants using biotinylated mAbs: mouse anti-human CD26-IgG_{2a}, and mouse anti-human IgG_{2a}-isotype control (Miltenyi Biotech, 3 µg/mL), according to manufacturers' protocol (Isolation of infectious HIV, MIltenyi Biotech). Captured HIV-1 was inactivated with 0.5% Empigen and analysed by p24 ELISA in duplicate as described above (2.10). Capture efficiency was expressed as % of starting p24 concentration.

2.11 Luciferase Assays

2.11.1 VS-Transmission Assay

Target $CD4^+$ T cells were co-cultured for 24 and 48 h at a 2:1 ratio with donor HIV-1^+ MDM 7 d.p.i. with *Renilla reniliformis* luciferase-expressing pNL4.3-LucR-T2A-BaL.ecto (Edmonds et al., 2010). In parallel conditions, co-cultures were also gently

shaken at 75 rpm at 37°C in 5% CO_2 (Martin et al., 2010, Sourisseau et al., 2007), or $CD4^+$ T cells were separated from HIV-1^+ MDM by a 3.0 μm transwell membrane (Sigma) (Groot et al., 2008, Martin et al., 2010), or $CD4^+$ T cells were incubated with virus-containing cell-free supernatants from HIV-1^+ MDM (collected at 7 d.p.i.) (Martin et al., 2010). At the relevant time-point $CD4^+$ T cells were gently removed (with <0.1% macrophage contamination in co-culture conditions determined by flow cytometry staining for CD3 and CD14, see Figure 3.8A), washed in PBS and lysed with Glo-Lysis buffer (Promega). For luciferase quantification cell lysates were mixed 1:1 with Ren-Glo assay solution (Promega) at room temperature and luminescence (relative light units, RLU) measured at 1000 ms^{-1} integration with a SpectroMax M3 or M5 plate reader. Background values from uninfected wells were subtracted.

2.11.2 Binding/Uptake Assay

Following 30 min to 2 h co-culture with HIV-$1_{NL\text{-}LucR\text{-}BaL}$ -infected $CD4^+$ T cells, MDM in duplicate-triplicate wells per condition were extensively washed with 5 mM EDTA to remove unbound/unengulfed T cells and cell lysates were prepared with 60 μL Glo-Lysis buffer (Promega) according to manufacturer's instructions. 50 μL cell lysate was mixed with 50 μL *Ren-Glo* Luciferase Assay solution (Promega) at RT in white 96 well plates. Luciferase activity was measured at 1000 ms^{-1} integration with a SpectraMax M3 or M5 plate reader. Background values in uninfected wells were subtracted, and uptake/association index in test wells was expressed relative to relevant untreated/vehicle/isotype control as follows: binding/uptake index (BUI) = $[(RLU_{inhibitor})/(RLU_{control})]$.

2.11.3 Productive Infection

At the relevant timepoint(s) post-infection, cell lysates were prepared as described above and either temporarily stored at -80^{o}C prior to analysis, or analysed immediately as described above.

2.11.4 Antiretoviral Therapy Susceptibility Assay

Activated CD4^{+} T cells were pre-incubated for 1 h with non-toxic concentrations of the nucleoside reverse transcriptase (RT) inhibitor azidothymidine (AZT), the non-nucleoside RT inhibitor nevirapine (NVP), the integrase inhibitor raltegravir (RAL, all CFAR, NIBSC) prior to co-culture with infected MDM at a donor to target cell ratio of 1:2, or cell-free infection at varying MOI as described above. Infections were allowed to proceed for 48 h (approximating a single cycle of replication) during which time antiretroviral drugs were maintained in the medium. At 48 h CD4^{+} T cells were gently removed (<0.1% macrophage contamination determined by flow cytometry staining for CD3 and CD14, Figure 3.8A), washed in PBS and lysed as described in 2.11.2. After subtracting background values, data were normalised to untreated controls to derive the transmission index, *Tx*: (infection$_{drug}$) / (infection$_{control}$) (Sigal et al., 2011). Antiretroviral titration data were fitted to a sigmoidal-dose response curve in GraphPad Prism 5.0 to obtain the 50% inhibitory concentration (IC_{50}) and the significance of differences in the IC_{50} were compared by extra sum-of-squares F-test.

2.11.5 Primary Cell Neutralisation Assay

Renilla luciferase-expressing HIV-1-infected MDM were pre-incubated with pooled purified human IgG (10 μg/mL) to block Fc receptors for 1 h at 37^{o}C before removal and pre-incubation with bNAbs or isotype for 1 h at 37^{o}C. In parallel cell-free virus stocks were pre-incubated with bNAbs or isotype. 5-fold dilutions of bNAbs from the starting concentration of 10-50 μg/mL were tested. Autologous CD4^{+} T cells were

cultured with HIV-1-infected MDM or cell-free viral stocks (MOI titrated to achieve equivalent luciferase expression in both conditions) for 48 h in RPMI-10 with 10 IU/mL IL-2, followed by gentle aspiration, lysis and luciferase analysis as described above. After subtracting background values, data were normalised to the isotype control to derive the % inhibition, expressed as 100 x [1-($RLU_{inhibitor}$/$RLU_{control}$)]. Inhibitor titration data were fitted to a sigmoidal-dose response curve in GraphPad Prism 5.0 and the significance of differences in the IC_{50} were compared by extra sum-of-squares F-test.

2.12 HIV-1 DNA qPCR

2.12.1 Sample Preparation

Cells (MDM or $CD4^+$ T cells) were washed in PBS, pelleted ($CD4^+$ T cells) and frozen at -80°C for later extraction with the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturers protocol.

2.12.2 HIV-1 *pol* DNA

Primers and probes for the detection of HIV-1 BaL *pol* and the cell reference human *β-globin* gene have been previously described (Martin et al., 2010). Singleplex PCR reactions were performed in triplicate in 20 μL total volume, with 2X Brilliant III Ultrafast master mix (Agilent), 375 nM forward and reverse primers, 125 nM probe, 0.3 μL ROX and 2 μL of sample DNA. A standard curve for *pol* and *β-globin* was generated for each qPCR reaction by 5-fold titration of single vDNA copy/cell standards prepared from ACH2 cells (Martin et al., 2010). Reactions were performed on a StepOne Plus (ABI) qPCR machine as follows: 1 cycle of 95°C for 3 min, followed by at least 40 cycles of 95°C for 5 s then 60°C for 10 s. The results of

triplicate technical replicates per sample for *pol* and *β-globin* were averaged and the ratio of *pol* to *β-globin* calculated to determine the relative abundance (expressed as vDNA copies/cell).

2.12.3 Total HIV-1 *gag* DNA

Cell copy number was quantified in triplicate at two dilutions using an adapted albumin qPCR as described (Mexas et al., 2012). The master mix contained 2 x Roche LightCycler 480 Probes MM, 200 nM probe FAM-CCTGTCATG CCCACACAAATCTCTCC-BHQ-1, 250 nM Albumin_F GCTGTCATCTCTTGTGGGCTGT) and 250 nM Albumin_R (AAACTCATGGGAGCTGCTGGTT) (Eurofins MWG Operon) with 10μL DNA sample in a total volume of 25 μL. $2x10^4$ input cells in 10 μL were assayed in triplicate for total HIV using 500 nM Probe (FAM-AGTRGTGTGTGC CCGTCTGTTG-BHQ-1), 500 nM LTR_OS (GRAACCCACTGCTTAASSCTCAA) and 500 nM LTR_AS (TGTTCGGGCGCCACTGCTAGAGA) (MWG) and 2x Roche LightCycler 480 Probes MM, in a total volume of 25 μL. Both qPCR amplifications were performed with one cycle of 95°C for 10 minutes; 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Data were analysed using Roche LightCycler software. $8x10^5$ reference cells containing one integrated copy of HIV per cell were used in duplicate as standards with cell and HIV copy numbers ranging in serial 10-fold dilutions from $1x10^5$ to $1x10^0$ DNA copies/reaction.

2.12.4 *Alu-gag* integrated HIV-1 DNA

Integrated HIV was measured using an adapted *alu*-PCR assay (Agosto et al., 2007). Briefly, 5 replicates of 7500 cells underwent a first round PCR amplification (95°C for 2 minutes; 20 cycles of 95°C for 15 seconds, 50°C for 15 seconds and 72°C for 2

minutes 30 seconds) using 100 nM *alu* GCCTCCCAAAGTGCTGGGATTACA and 600 nM *gag* reverse GTTCCTGCTATGTCACTTCC primers (Eurofins MWG Operon), 1.5U platinum taq (Invitrogen), 1.5mM $MgCl_2$ (Invitrogen), 300 μM dNTP (Appleton woods), 10X master mix (Invitrogen) in a total volume of 50 μL. 10 μL of the first round product was amplified in a nested protocol, using the 'total' *gag* assay described above. A first round PCR with 3 replicates using only the *gag* reverse primer (*gag* only) acted as a background 'unintegrated' control. Serially 3x diluted integration site standards (Liszewski et al., 2009) which take into account the integration site (ranging from 3750 to 15 copies per 7500 cells in duplicate) were used to construct a standard curve per plate. Integration levels per cell were calculated, only if the *alu-gag* and *gag*-only signals were statistically significantly different by t test ($P < 0.05$).

2.13 Live Cell Microscopy

Live-cell time-lapse imaging of MDM and $CD4^+$ T cell co-cultures was performed in 35 mm glass-bottomed imaging dishes (Maktec) with a 40X oil-immersion objective Axiovert 200 microscope with Axiovision MRm CCD camera and Colibri illumination. Image analysis was performed using AxioVision v 4.8.2. For short-term analysis of MDM to $CD4^+$ T cell VS transfer events, images were acquired every 2 min for up to 6 h. For longer-term conjugate analysis comparing HIV-$1_{NL\text{-}eGFP\text{-}BaL}$ +/- MDM, images were acquired every 5 min for up to 13 h. Image analysis of conjugate duration (min) and frequency (n. of conjugates formed per MDM) was performed for n = 10 MDM for each condition (GFP+ vs GFP-). Conjugates were scored if they persisted > 5 min (i.e. were present in two sequential images). Imaging of MDM interactions with $CD4^+$ T cells synchronously infected HIV-$1_{NL\text{-}mCherry\text{-}CHO77}$ was done every 2-5 min for up to 8 h.

2.14 Laser-Scanning Confocal Microscopy

Cell preparations on glass coverslips were fixed in 4% paraformaldehyde for 1 h at RT, washed in PBS containing 0.5% bovine serum albumin (BSA), +/- nuclear staining with 1 μg/mL Höechst (Sigma) in PBS (for 8 min). Cells were washed in PBS and surface stained for 1h at RT, followed by 3 washes in PBS/BSA then permeabilisation using 0.1% saponin/0.5% bovine serum albumin with 10% pooled human/goat serum (to block Fc-receptor binding) for 30 min, then intracellular staining was performed for 1 h at RT. Cells were washed three times with PBS/BSA, then once with dH_20, before mounting in Mowiol 4-88 (Calbiochem) and analysed using an Olympus FV1000 microscope. Images were acquired with either a 20X objective, or 40X, 63X and 100X oil-immersion objectives and processed (linear threshold adjustment and scale bars applied uniformly to each image) in Olympus Fluoview v2.0b. 3D reconstructions of z-stack images were processed in ImageJ. Images were transferred to Prism 5.0 for graphical presentation.

2.15 Multispectral Flow Cytometry (ImageStream™)

Samples were collected on an ImageStream^X (Amnis) instrument with a 40X or 60X objective (with extended depth of field, EDF collection filter for 60X objective). Cell area thresholds were set at 50 for T cells and 200 for MDM. Laser intensities were adjusted to single colour controls from the positive samples. Single colour compensation controls (10^3 events) were collected for each donor to permit construction of a compensation matrix, followed by sample collection ($2x10^4$ -$1x10^5$ events depending on experiment). A compensation matrix was generated using single colour controls for each donor with Ideas 5.0 software (Amnis), and applied to raw image files for the relevant donor. Gating strategies and data analysis are summarised in the relevant experimental chapters. Image processing was performed in Ideas 5.0, and images exported to Prism 5.0 for graphical presentation.

2.16 Spectrophotometry

2.16.1 MTS Metabolic Activity (Viability) Assay

This assay of metabolic activity was used as a highly relevant surrogate for cell viability. The assay measures reducing compounds which are produced during cellular metabolism capable of reducing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) into a soluble formazan product that can be measured by spectrophotometry.
The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) was used according to the manufacturer's instructions. Briefly, 20 µl of reagent solution was added to cells in 100 µL media in 96-well plates and incubated at 37°C with 5% CO_2 for 1-4 h. Absorbance was read at 490 nm using a SpectraMax M3 or M5 plate reader and background absorbance in reagent plus media only wells subtracted. Viability was expressed as a percentage of treated cells compared to relevant vehicle controls.

2.17 Data and Statistical Analysis

Statistical analysis was not performed for experiments involving technical replicates in a single donor. Due to difficulty ascertaining the normality of distribution, data were analysed using non-parametric statistical tests or log-transformed prior to analysis with parametric tests. Measures of central tendency were mean or median. Distribution of values around the central tendency was expressed as standard deviation (SD) for technical or experimental replicates in a single donor, while standard error of the mean (SEM) was used for replicates in multiple donors. For pairwise significance testing of continuous data t test/Mann-Whitney test were used as appropriate. For analysis of continuous data in three or more groups ANOVA (on

log-normalised data) or Kruskal-Wallis (K-W) ANOVA was used together with the appropriate post-test adjustment for multiple comparisons (e.g. Dunnett's [normalised data, parametric ANOVA] or Dunn's [unadjusted data, non-parametic test]) respectively. For correlation analysis Spearman's correlation coefficient was calculated. Significance of differences in IC_{50} for titration data was assessed using the extra sum-of-squares F-test. All statistical tests were two-tailed with an alpha value of < 0.05 considered statistically significant.

3 THE MACROPHAGE-T CELL VIROLOGICAL SYNAPSE

3.1 Introduction

It is well established that cell-to-cell spread of several enveloped viruses that enter cells at neutral pH, including retroviruses, enhances the efficiency of viral dissemination in several ways: by increasing the probability of target cell interaction; by reducing vulnerability to dispersal and envelope degeneration in the fluid phase; and potentially through restricting exposure to soluble host immune factors (Sattentau, 2008, Mothes et al., 2010).

In addition to these physical advantages, other factors may contribute to the enhanced efficiency of viral transmission between cells. For example, target cell receptor clustering at the intercellular junction enhances the efficiency of viral binding and entry (Sattentau, 2008, Mothes et al., 2010), whilst directed budding of virions between T cells delivers a higher multiplicity target cell infection (Sigal et al., 2011).

Whilst there have been considerable advances in our understanding of the molecular mechanisms governing HIV-1 transmission by T cells [reviewed in (Sattentau, 2010)] and DCs [reviewed in (McDonald, 2010)] very little is known about the structure, mechanisms and consequences of macrophage cell-to-cell transmission (Waki and Freed, 2010). This is despite recognition more than 20 years ago of the ability of HIV-1-infected MDM to induce HIV-1 replication in PBMCs more efficiently than cell-free supernatants (Mann et al., 1990). In this study, PBL infection by MDM was CD4-dependent, since it could be inhibited by the continued presence of CD4 blocking antibodies (Mann et al., 1990). A subsequent study showed that production of Gag p24 was cell-contact dependent, and that AZT could reduce the production of vDNA, suggesting *de novo* reverse transcription (Carr et al., 1999).

Several years passed before the structural basis of the HIV-1 IS formed between trans-infected DC and T cells was reported by McDonald *et al.* (McDonald et al., 2003), closely followed by the first description by Jolly *et al.* of the HIV-1 VS formed between X4 virus-infected T cell lines and primary $CD4^+$ T cells (Jolly et al., 2004). However it was another four years before descriptions of synapses between infected macrophages and primary $CD4^+$ T cells or T cell lines were published (Gousset et al., 2008, Groot et al., 2008). Groot *et al.* observed co-localisation of HIV-1 Env and Gag on MDM and CD4 on primary T cells suggestive of a VS (Groot et al., 2008). This study confirmed and extended previous findings by demonstrating that contact-dependent HIV-1 Gag transfer from macrophages to primary $CD4^+$ T cells measured by flow cytometry was rapid and could be inhibited by antibodies to both CD4 and Env (Groot et al., 2008). Electron microscopy (EM) revealed close apposition of macrophage and $CD4^+$ T cell membranes at the synapse (Groot et al., 2008), suggesting possible structural differences in the organisation and architecture of the macrophage-T cell VS compared to that formed by T cells (Martin et al., 2010), which is more open (Sattentau, 2010). Gousset *et al.* followed the assembly of tetracysteine-tagged Gag reporter virus pseudotyped with VSV-G in MDM, observing that Gag assembled in CD63- and CD81-enriched compartments, and that Gag rapidly localised to the synapse with uninfected immortalised Jurkat T cells (Gousset et al., 2008). They did not explore whether the virus-like particles (VLPs) lacking Env were transferred to T cells, but these data suggested that HIV-1 within the VCC relocates to the synapse (Gousset et al., 2008).

We can therefore construct a preliminary model of the VS formed by HIV-1-infected macrophages based on these data (Waki and Freed, 2010), but there remain significant gaps (Figure 3.1). There are no data on additional molecular components (for example the cytoskeleton and adhesion molecules) in the organisation of the

putative macrophage VS, and the role of the VCC in cell-to-cell transmission by macrophages remains poorly defined (Tan and Sattentau, 2013).

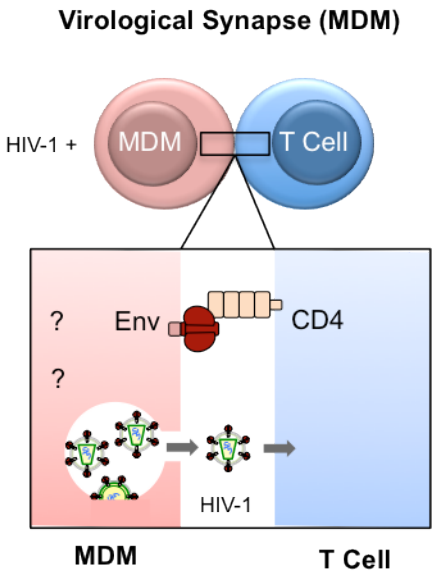

Figure 3.1 Macrophage VS model.
HIV-1 virions assemble and bud into complex surface-accessible intracellular compartments in MDM. These virions are efficiently transmitted to uninfected target cells such as PBL or $CD4^+$ T cells at the intercellular contact site. However, the molecular organisation of the VS is unknown.

3.1.1 Hypothesis

I hypothesised that MDM efficiently transmit HIV-1 from the VCC to $CD4^+$ T cells at an Env-dependent VS involving the actin cytoskeleton and adhesion molecule interactions.

3.1.2 Experimental objectives

I aimed to characterise the molecular architecture of the VS formed between HIV-1-infected MDM and autologous $CD4^+$ T cells and the implications for productive infection.

3.2 Model development

3.2.1 Primary cell isolation and differentiation

Although several insights into the mechanisms of cell-to-cell transmission have been achieved with immortalised cell lines, there is significant variability in cell-to-cell transmission between different immortalised cell lines (Zhong et al., 2013), and their relevance to the *in vivo* situation is questionable (Sattentau, 2010). I therefore optimised a primary cell model of MDM transmission to autologous $CD4^+$ T cells based on that described previously (Groot et al., 2008).

My initial priority was to enrich primary cells to high purity. Adherence selection of monocytes achieved suboptimal purity by day 7 post isolation (Figure 3.2A), required considerable numbers of PBMCs (Figure 3.2B) and requires a step of detachment and re-seeding that can adversely affect cell viability (Figure 3.2C). Moreover, inter donor differences in the starting percentage of monocytes by adherence could result in unpredictable final numbers of MDM, which was a disadvantage when scaling down experiments to a 96-well plate format. I therefore explored two alternative methods of monocyte isolation - Percoll density gradient centrifugation (Colotta et al., 1984) and two commercial kits for untouched magnetic bead enrichment of $CD14^+$ monocytes (from Miltenyi and StemCell Technologies). The Miltenyi kit consistently provided the highest purity of selection (Figure 3.3A). Similarly, $CD4^+$ T cell isolation using the Miltenyi untouched magnetic selection kit consistently yielded > 95% $CD3^+$ $CD4^+$ cells as previously described (Groot et al., 2008) (Figure 3.3B). Since MDDC also efficiently transmit HIV-1 to primary $CD4^+$ T cells it was important to ensure that the culture conditions used for macrophage differentiation (Groot et al., 2008) did not inadvertently generate MDDC. MDM cultured for 7 d in 1% pooled human serum expressed the macrophage-lineage marker CD14, but not the MDDC marker CD1c

(BDCA-1), indicating that the differentiation method reliably generated MDM (Figure 3.3C).

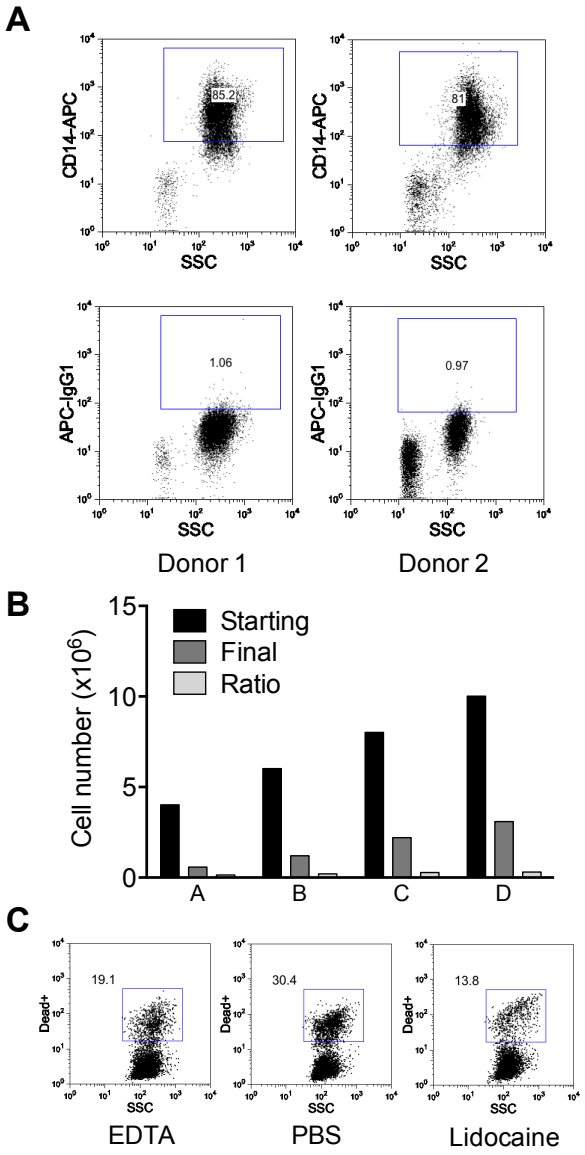

Figure 3.2 Adherence selection of MDM.

A) Representative FACS plots in two independent donors of purity of MDM at day 7 post-isolation by adherence selection assessed by flow cytometry staining for CD14-APC (top panels) and isotype (bottom panels).

B) Cell losses following adherence selection in 10 cm^2 TC-treated dishes are constant across varying seeding densities (A - 4, B - 6, C - 8, D - 10 x 10^6 cells/plate).

C) Viability of MDM lifted by gentle scraping following 10 min treatment with 5 mM EDTA, PBS or 5 mM EDTA/12 mM lidocaine at 37^oC followed by staining with Live/Dead Red viability stain (Invitrogen). Failure to exclude the marker identifies non-viable MDM ($Dead^+$).

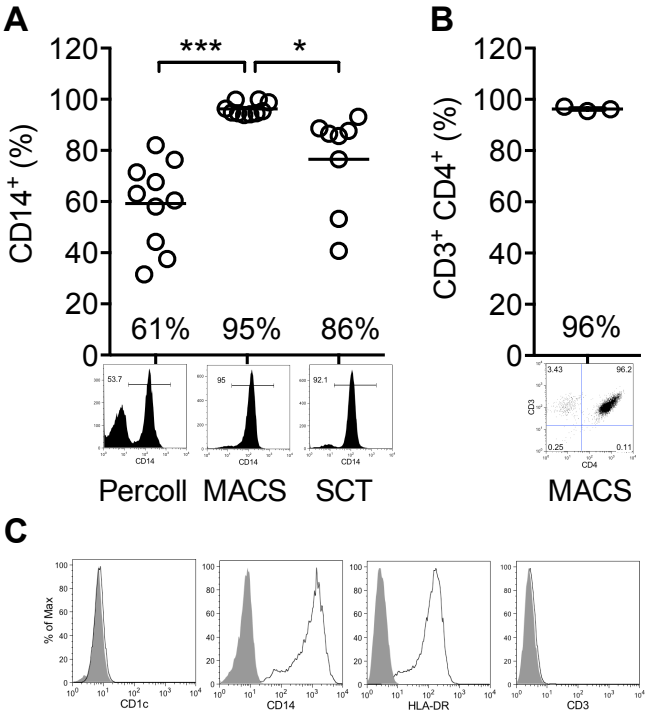

Figure 3.3 MACS selection of MDM and CD4$^+$ T cells and MDM identity.
A) Purity of monocyte isolation by Percoll density centrifugation, magnetic cell sorting (Monocyte Isolation Kit II, Miltenyi [MACS] or Monocyte Isolation Kit, StemCell Technologies [SCT]). Bars represent median % purity, *** P < 0.001, * P < 0.05, K-W ANOVA with Dunn's post test, n = 10 donors/condition represented by individual data points. Representative post-isolation FC plots are displayed below, showing cells surface stained with CD14-APC to identify monocytes.
B) Purity of CD4$^+$ T cell isolation (CD4$^+$ T cell isolation Kit, Miltenyi, MACS) in n = 3 donors. Representative post-isolation FC plot displayed below, showing surface staining with CD3-FITC and CD4-APC.
C) MDM cell surface marker expression at 7 days post MACS isolation. Cells were lifted with 5 mM EDTA/12 mM lidocaine and surface stained with CD1c-FITC, CD14-APC, HLA-DR-PerCP-Cy5.5 or CD3-FITC (unfilled line) together with appropriate fluorochrome-conjugated isotype controls (filled grey region).

3.2.2 Cell-free MDM infection

Because MDM permissiveness to HIV-1 infection shows considerable inter-donor variation (Bol et al., 2009), I next optimised conditions for cell-free MDM infection. Using various assays of productive infection I established that 6 hours incubation with virus in low volume followed by topping up of media (but not removal of the initial viral inoculum) provided the most efficient conditions for reliable cell-free infection with mac-tropic HIV-1_{BaL} in a 96 well plate format not used previously in the

laboratory (Figure 3.4A). I then confirmed that the sensitivity of my other assays for productive infection (i.e. p24 ELISA, luciferase reporter virus expression, and qPCR for HIV-1 *pol*) permitted experiments in 96 well plates (Figure 3.4B-D). Having established a reliable system for MDM infection, I could proceed to study cell-to-cell transmission of HIV-1 from infected MDM to non-adherent autologous $CD4^+$ T cells.

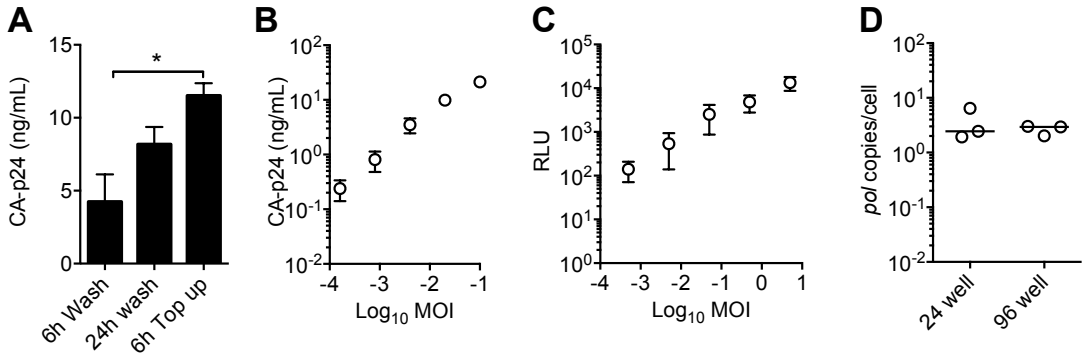

Figure 3.4 Optimising cell-free MDM infection for 96 well plate.
A) The efficiency of several conditions for MDM infection, including incubation with HIV-1_{BaL} (MOI 10^{-2}) for 6 h or 24 h in 50 μL followed by refreshing or topping up media to 200 μL, was tested by p24 ELISA at 7 d.p.i.. 6 h incubation followed by topping up was optimal. Mean ± SEM, n = 3 independent donors, * P = 0.05; K-W ANOVA with Dunn's post-test. The sensitivity of assays for productive HIV-1 infection in 96 well plates was confirmed by titrating MDM viral inocula across a range of MOI. MDM infection was measured by **B)** p24 ELISA, mean ± SD n = 2 donors and **C)** luciferase infectivity assay, mean ± SEM in n = 3 donors. **D)** Sensitivity of qPCR for HIV-1 *pol* was confirmed to be equivalent for MDM (HIV-1_{BaL} MOI 10^{-1}) in 96-well and 24-well plates (line = median of triplicate wells in n = 1 donor).

3.3 Rapid contact-mediated HIV-1 transfer to $CD4^+$ T cells

Using the experimental model described above (Figure 3.5A) I observed rapid HIV-1 transfer from the VCC within infected MDM to the interface with T cells by time-lapse live cell imaging of co-cultures of primary autologous $CD4^+$ T cells and MDM infected with an NL4.3-based GFP-Gag reporter virus bearing the R5-using Env JRFL (HIV-$1_{NL\text{-}Gag\text{-}iGFP}$, kindly provided by B. Chen). This rapid transfer resulted in a large quantity of GFP deposition on the T cell surface, which I interpreted as transferred virions (Figure 3.5B). Following transfer, T cells detached from MDM but retained the

GFP signal (Figure 3.5C), confirming a previous hypothesis regarding the transient nature of the macrophage VS (Groot et al., 2008).

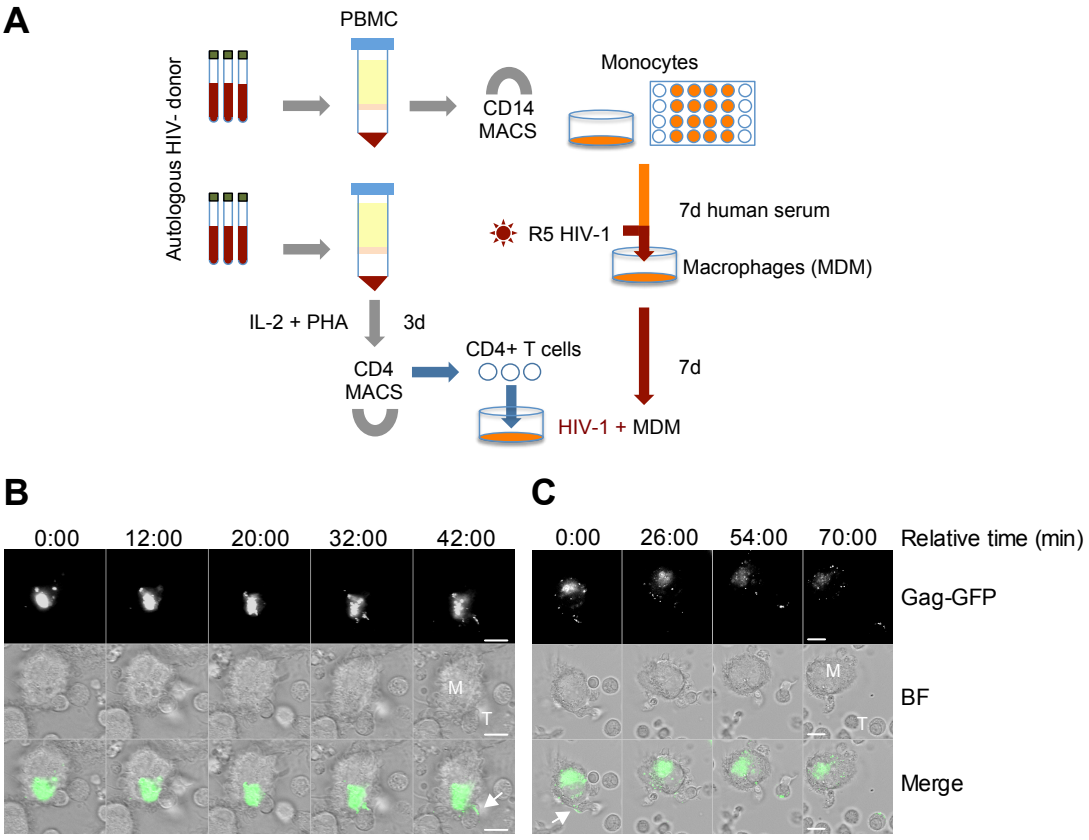

Figure 3.5 MDM transmit HIV-1 to autologous CD4$^+$ T cells.
A) Schematic of experimental model.
Live-cell time-lapse imaging of HIV-$1_{NL\text{-}Gag\text{-}iGFP\text{-}JRFL}$-infected MDM and autologous CD4$^+$ T cell co-cultures was performed in 35 mm glass-bottomed imaging dishes (MatTek) with 40X oil-immersion objective Axiovert 200 microscope with Axiovision MRm CCD camera and Colibri illumination. Image processing was performed using AxioVision 4.8.2. For analysis of MDM to CD4$^+$ T cell VS transfer events, images were acquired every 2 min for up to 6 h.
B) Still time-lapse images showing HIV-1 Gag transfer at the VS (arrowed) and **C)** detachment of a Gag$^+$ T cell following VS-mediated transfer. Scale bar = 10 μm. M = MDM, T = T cell.

To quantify the frequency and duration of cell-cell interactions I performed live-cell co-culture experiments with MDM infected with a mac-tropic diffusible GFP reporter infectious molecular clone (HIV-$1_{NL\text{-}eGFP\text{-}BaL}$, kindly provided by J. Kappes) under conditions that produced a mixed population of HIV-1 infected (GFP^+) and uninfected (GFP^-) MDM at 7 d.p.i. (Figure 3.6A). I observed frequent transient cell-to-cell interactions ('scanning' of MDM by T cells), however image analysis of all interactions with n = 10 MDM for each condition ($GFP^{+/-}$) revealed that the frequency of interactions with infected MDM was significantly higher than with uninfected MDM (median conjugates per cell GFP^+ = 5, GFP^- = 1.5, P = 0.001, Mann-Whitney test, Figure 3.6B), although the duration of interactions was the same (median duration = 35 mins, P = 0.550, Mann-Whitney test, Figure 3.6C).

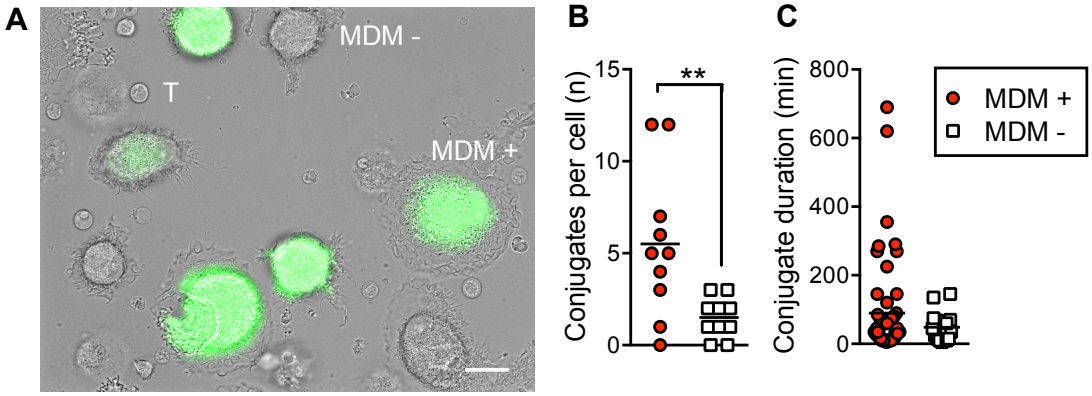

Figure 3.6 Frequent conjugate formation between HIV-1 infected MDM and uninfected $CD4^+$ T cells.

A) MDM infected with HIV-$1_{NL\text{-}eGFP\text{-}BaL}$ (green cells) were co-cultured with uninfected autologous $CD4^+$ T cells for 13 h on 35 mm glass-bottomed imaging dishes (MatTek) and imaged every 5 min with a 40X oil-immersion objective Axiovert 200 microscope with Axiovision MRm CCD camera and Colibri illumination. Image processing was performed using AxioVision v 4.8.2. Scale bar = 20 μm. T = $CD4^+$ T cell.

B) Image analysis of conjugate frequency (n. of conjugates formed per MDM) and C) duration (min) and was performed for n = 10 MDM for each condition (MDM+ v MDM-). Conjugates were scored if they persisted > 5 min (i.e. were present in two sequential images). Infected MDM engaged T cells significantly more frequently ** P = 0.001, Mann-Whitney test **(B)**, but there was no significant difference in average conjugate duration (P = 0.55), although a minority of infected MDM interactions were very long lasting **(C)**.

To examine the kinetics of HIV-1 transfer to T cells I used a flow cytometry-based transfer assay in which uninfected $CD4^+$ T cells were co-cultured with HIV-1_{BaL}-

infected MDM for varying times before gentle removal, fixation, permeabilisation and staining for CD3 and intracellular Gag (Figure 3.7A). Transfer was rapid (Figure 3.7B), and was not due to high multiplicity cell-free virion diffusion from infected macrophages, since co-cultures across a transwell membrane (which permits cell-free virion diffusion but not direct cell-to-cell contact (Martin et al., 2010)) resulted in a significantly lower percentage of T cells positive for intracellular Gag at 24 h ($P = 0.032$, Mann-Whitney test, Figure 3.7B). As previously described for the T cell VS (Sourisseau et al., 2007) higher ratios of target to donor cells enhanced the efficiency of transfer (Figure 3.7C), increasing the area under the curve by approximately 25 % (Figure 3.7D).

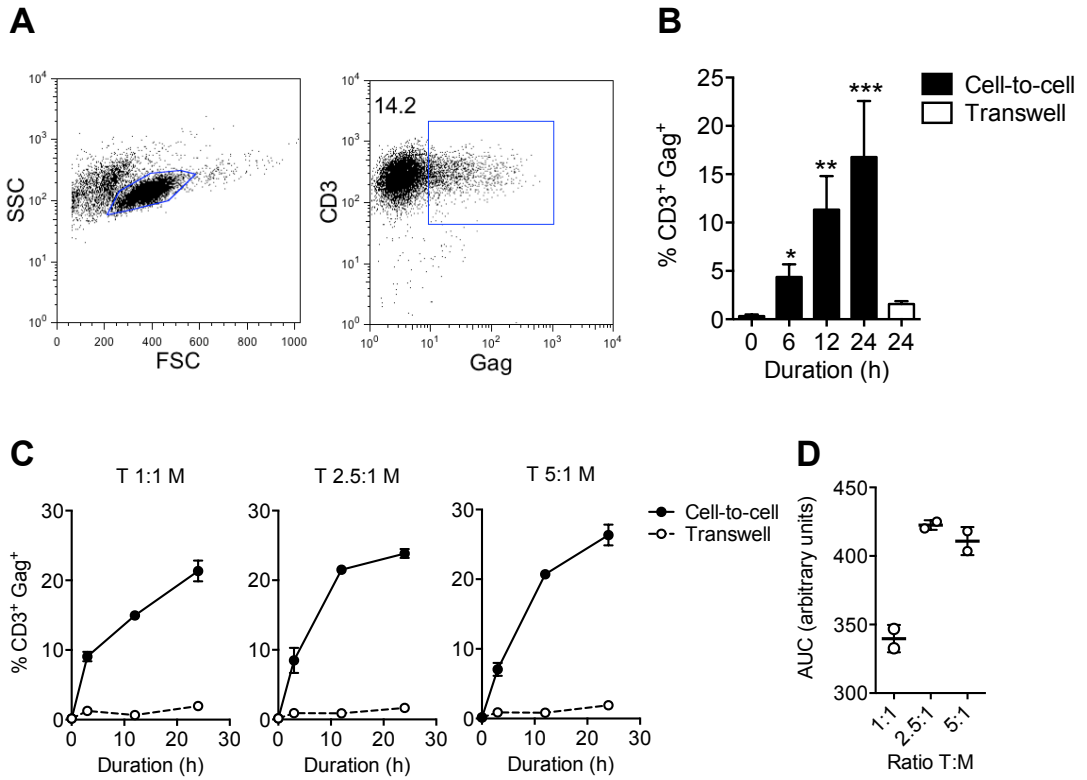

Figure 3.7 Contact-dependent HIV-1 transfer

A) Gag transfer measured by flow cytometry of permeabilised $CD4^+$ T cells stained for intracellular HIV-1 Gag. Displayed is the gating strategy based on scatter characteristics and expression of CD3 and Gag.

B) Kinetics of Gag transfer measured in the flow cytometry transfer assay. Bars = mean ± SEM in n = 10 independent donors (n = 4 for transwell), * P < 0.05; ** P < 0.01, *** P < 0.001, K-W ANOVA with Dunn's post-test compared to t = 0. Cell-free exposure through a virus permeable 3.0 μm transwell membrane resulted in no significant signal above background compared to t = 0.

C) Exposure to increasing ratios of target $CD4^+$ T cells results in more rapid and efficient contact-dependent transfer, as displayed by quantification of the area under curve **D)**. Mean ± SD of duplicate wells in n = 1 donor.

3.4 Transfer results in productive $CD4^+$ T cell infection

I reasoned that viral transfer measured by intracellular Gag staining might not necessarily imply productive transmission (since it could correspond to uptake of Gag protein or virions into a non-productive internal compartment). To assess whether efficient virion transfer led to productive T cell infection, I gently aspirated $CD4^+$ T cells (with <0.1% MDM contamination, Figure 3.8A) at various time-points post co-culture and measured the production of HIV-1_{BaL} *pol* or HIV-$1_{NL\text{-}LucR\text{-}BaL}$

luciferase expression (Figure 3.8B and Figure 3.8C-D). Both assays confirmed that the rapid kinetics of Gag transfer measured by flow cytometry corresponded to similar time-dependent increases in $CD4^+$ T cell infection.

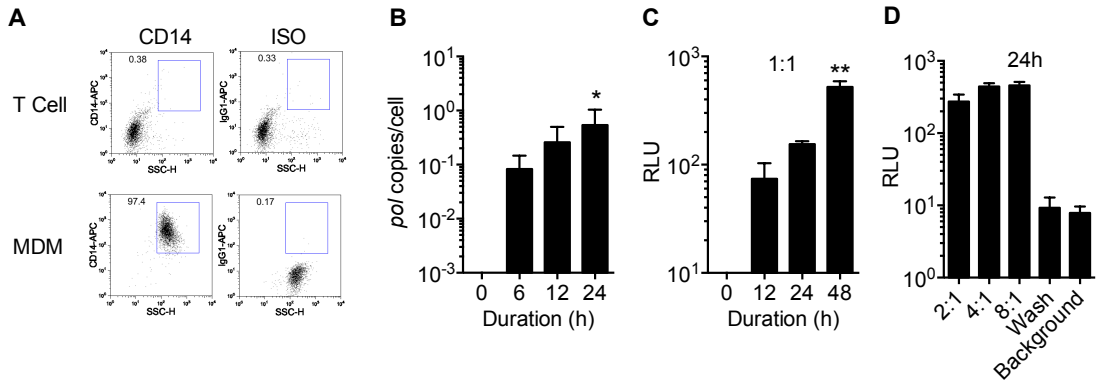

Figure 3.8 Transfer results in productive $CD4^+$ T cell infection.
A) Representative FC plots showing aspirated $CD4^+$ T cells following 48 h incubation with infected MDM (top panels) and lifted MDM (bottom panels). Cells were fixed and stained with CD14-APC or isotype and instrument voltages set for MDM. There was negligible MDM contamination of aspirated $CD4^+$ T cells (also seen in additional control experiments in n = 2 donors and by ImageStream^{TM} in n = 6 donors, data not shown). $CD4^+$ T cells were gently aspirated after co-culture with infected MDM at a 1:1 ratio and analysed for productive infection by **B)** qPCR for HIV-1 pol (HIV-1_{BaL}-infected MDM, mean ± SEM n = 4 independent donors), or **C)** luciferase expression (HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected MDM, mean ± SEM n = 3 independent donors). * $P < 0.05$; ** $P < 0.01$, K-W ANOVA with Dunn's post-test compared to t = 0. **D)** Increasing ratios of target $CD4^+$ T cells resulted in a saturable increase in luciferase signal in aspirated $CD4^+$ T cells after 24 h co-culture with HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected MDM. Aspiration of media alone from infected MDM wells (Wash) in parallel did not generate signal above non-infected T cells (Background), indicating negligible infected MDM contamination. Mean ± SD of quadruplicate wells in n = 1 donor.

3.5 HIV-1 transfer from the macrophage VCC is actin-dependent

HIV-1 in the VCC of infected MDM co-localises with tetraspanin molecules such as CD81 by LSCM (Tan and Sattentau, 2013) (Figure 3.9A-B). Interestingly during co-culture with primary T cells, CD81 and HIV-1 Gag were strongly polarised to the VS, suggesting relocation/reorganisation of components of the VCC to the intercellular interface (Figure 3.9C), a process I suspected to be cytoskeleton-dependent. Indeed, by LSCM I observed F-actin in association with the VCC in HIV-1_{BaL}-infected MDM (Figure 3.9D) and polarised towards the contact site with uninfected T cells (Figure 3.9E). To investigate a functional role for actin in this process, I pre-incubated MDM

with non-toxic (Figure 3.9F) concentrations of various cytoskeleton remodeling and uptake inhibitors including the endocytosis inhibitor dimethyl amiloride (DMA), the F-actin inhibitors jasplakinolide, latrunculin A and cytochalasin D, the microtubule inhibitors nocodazole, colchicine and paclitaxel. I also tested the myosin II motor inhibitor blebbistatin and the dynamin GTPase (associated with endocytosis and phagocytosis) inhibitor dynasore together with appropriate vehicle controls for 1 h, and then washed out the inhibitors to avoid confounding effects on the T cell cytoskeleton. I observed significant reductions in transfer to T cells over 3 h in actin inhibitor-treated MDM, strongly implicating actin cytoskeletal rearrangements in the reorganisation of the VCC (Figure 3.9G). However, inhibition of other cytoskeletal components, most notably microtubules, did not impact on viral transfer. This contrasts with reports that viral transfer at the T cell VS is dependent on polarised secretion mediated by the microtubular organising centre (MTOC) (Jolly et al., 2011).

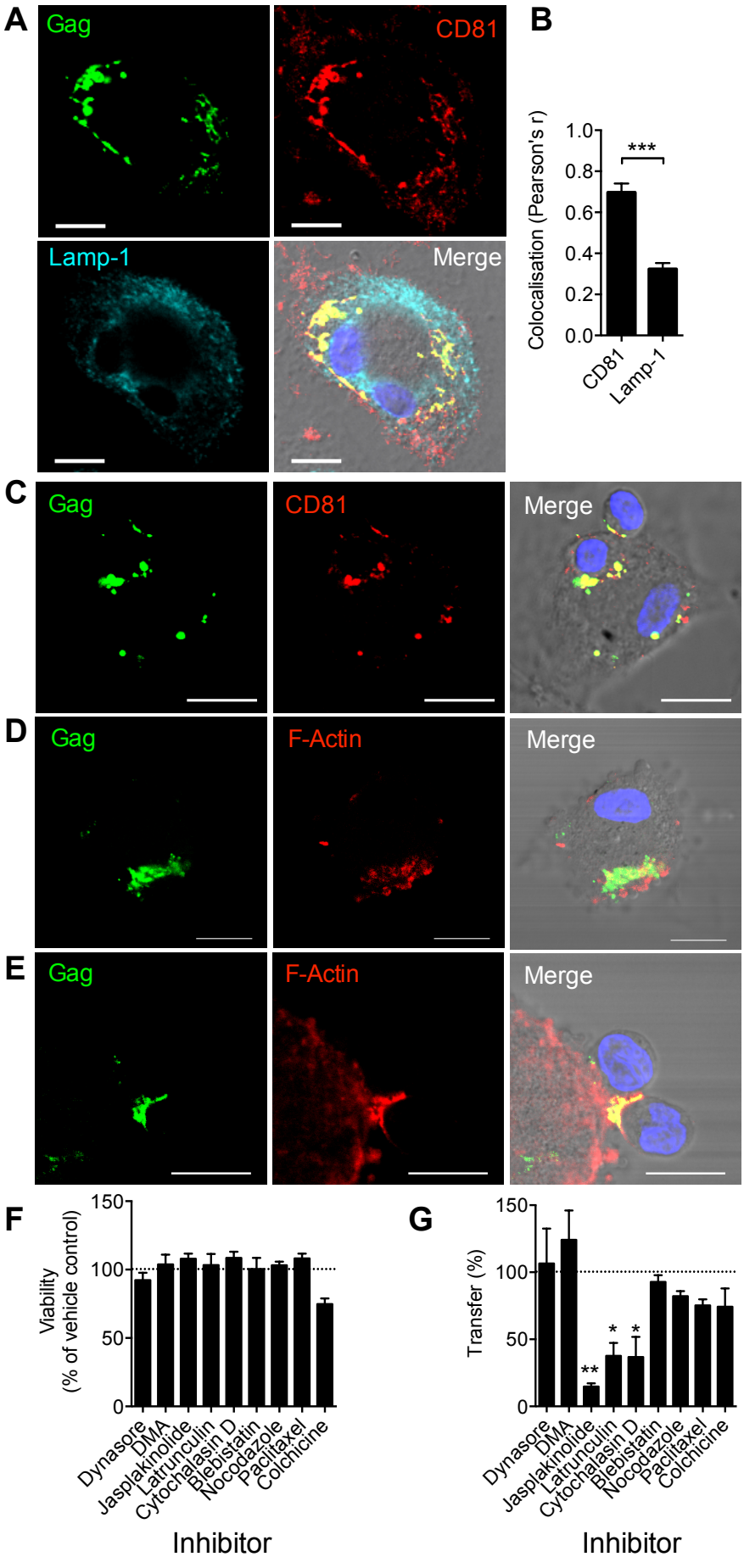
A
Gag
CD81
Lamp-1
Merge
B
Colocalisation (Pearson's r)
1.0
0.8
0.6
0.4
0.2
0.0
***
CD81
Lamp-1
C
Gag
CD81
Merge
D
Gag
F-Actin
Merge
E
Gag
F-Actin
Merge
F
Viability
(% of vehicle control)
150
100
50
0
Dynasore
DMA
Jasplakinolide
Latrunculin
Cytochalasin D
Blebistatin
Nocodazole
Paclitaxel
Colchicine
Inhibitor
G
Transfer (%)
150
100
50
0
**
*
*
Dynasore
DMA
Jasplakinolide
Latrunculin
Cytochalasin D
Blebistatin
Nocodazole
Paclitaxel
Colchicine
Inhibitor

Figure 3.9 HIV-1 transfer from the VCC is actin-dependent.
A) LSCM of permeabilized HIV-1_{BaL}-infected MDM showing co-localisation of HIV-1 p18 Gag (mouse IgG_{2a}) with CD81 (mouse IgG_{2b}) but not the lysosomal marker LAMP-1 (mouse IgG_1).
B) Co-localisation with p18 Gag was quantified in n = 10 positive MDM in Olympus Fluoview 2.0b.
C) Gag p18 and CD81 co-localised at the synaptic junction with autologous $CD4^+$ T cells 3 h post co-culture.
D) F-Actin (phalloidin-TRITC) associated with HIV-1 p18 Gag in the VCC of HIV-1_{BaL} infected MDM, and
E) co-polarised to the synaptic junction with autologous $CD4^+$ T cells 3 h post co-culture. Nuclei were stained with Hoechst and images acquired using an Olympus FV1000 microscope with x100 oil-immersion objective lens. Scale bars represent 10 μm.
F) Toxicity of inhibitors on MDM was assessed after 1 h treatment with the dynamin inhibitor dynasore (80 μM), the endocytosis inhibitor DMA (50 μM), the F-Actin inhibitors jasplakinolide (2 μM), latrunculin A (2 μM) and cytochalasin D (10 μM), the microtubule inhibitors nocodazole (10 μM), paclitaxel (10 μM) and colchicine (10 μM) and the myosin II inhibitor blebbistatin (100 μM) in serum-free medium by MTS assay. Viability was expressed as a percentage of the DMSO/media control. Mean ± SEM in n = 3 donors.
G) HIV-1_{BaL}-infected MDM were pre-treated with these inhibitors, washed three times in warm media, and incubated with autologous $CD4^+$ T cells for 3 h, when HIV-1 transfer was measured in T cells stained for CD3 and HIV-1 Gag by flow cytometry (FACSCalibur) and expressed as a percentage of the % $CD3^+$ Gag^+ cells in DMSO control wells. Bars = mean ± SEM in n = 2-5 independent donors, * $P < 0.05$, ** $P < 0.01$, K-W ANOVA with Dunn's post-test compared to DMSO control.

3.6 Transmission occurs at an Env-induced, adhesion molecule-dependent VS

To examine the molecular organization of the VS governing this highly efficient transfer process I combined fixed-cell imaging and functional approaches, imaging HIV-1_{BaL}-infected MDM in co-culture with autologous primary $CD4^+$ T cells by LSCM, and in parallel quantifying the impact of VS inhibitors using the flow cytometry-based functional assay of VS transfer described above. In LSCM experiments I pre-labeled T cells with the non-blocking CD4 antibody L120 (Healey et al., 1990, Jolly et al., 2004) to avoid labeling CD4 on MDM, and observed strong co-polarisation of HIV-1 Gag, Env and target cell CD4 at the intercellular junction (Figure 3.10A) characteristic of the VS that forms between infected and uninfected T cells (Jolly et al., 2004). Pre-incubation of primary T cells for 1 h with anti-CD4, and to a lesser degree anti-CCR5 blocking antibodies, significantly inhibited transfer (Figure 3.10B),

suggesting that transfer is dependent on Env binding to target cell receptors on $CD4^+$ T cells (Groot et al., 2008). This hypothesis was supported by pre-incubation of MDM with the anti-gp120 bNAb 2G12, which blocks CD4-Env engagement and thus also inhibits the conformational changes required for CCR5 binding (Figure 3.10B).

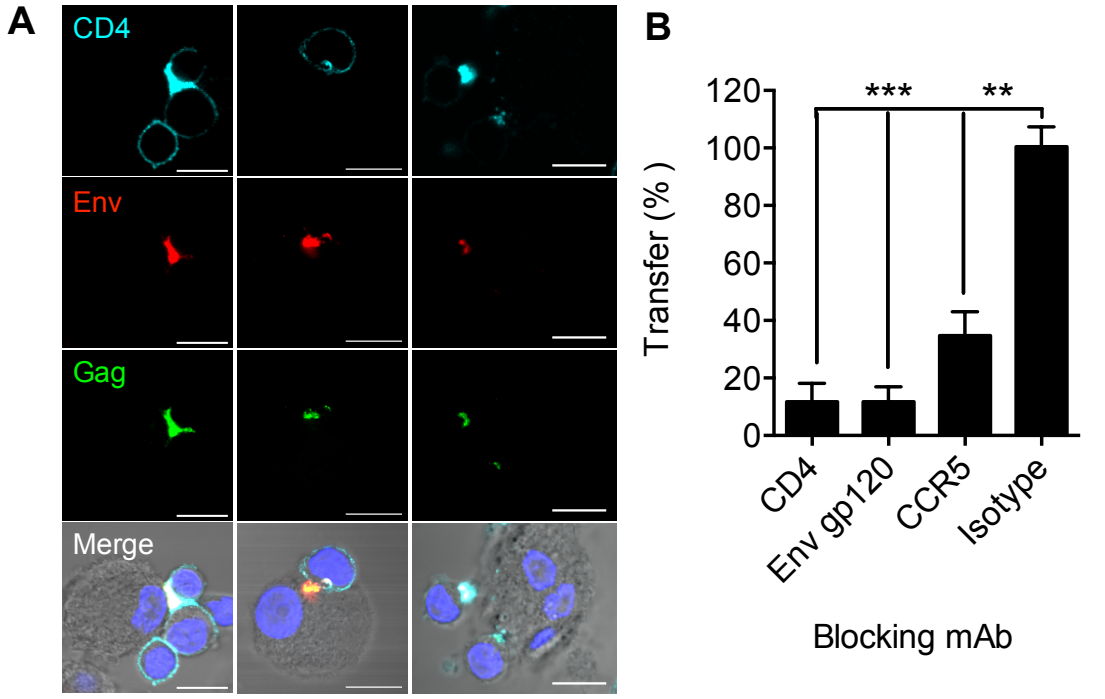

Figure 3.10 VS assembly is CD4-Env dependent.
A) LSCM of HIV-1_{BaL}-infected MDM co-cultured for 3 h with autologous $CD4^+$ T cells pre-incubated for 30 min with non-blocking anti-CD4 (L120, mouse IgG_1), permeabilised and stained for HIV-1 Gag (KC57-FITC) and Env (2G12-biotin) +/- appropriate secondary antibodies. Nuclei were stained with Hoechst and images acquired using an Olympus FV1000 microscope with 63-100X oil-immersion objective lens. Scale bars represent 10 μm.
B) Pre-incubation of $CD4^+$ T cells with blocking anti-CD4 (13B8.2) or anti-CCR5 (2D7) antibodies (10 μg/mL, mouse IgG_1) or pre-incubation of HIV-1_{BaL} infected MDM with anti-gp120 (2G12, 10 μg/mL, human IgG_1) after Fc block resulted in a significant reduction in HIV-1 transfer to $CD4^+$ T cells during 3 h co-culture. All mAbs were maintained during co-culture. Transfer was measured by flow cytometry (FACSCalibur) of aspirated $CD4^+$ T cells stained for CD3 and HIV-1 Gag, expressed as a percentage of the % $CD3^+$ Gag^+ cells in relevant isotype (mouse or human IgG_1) control wells. Bars = mean ± SEM in n = 3 independent donors, *** $P < 0.001$, ** $P < 0.01$, ANOVA with Dunnett's post-test compared to control.

Adhesion molecule interactions are also required for the stabilisation of immune cell synapses (Rodriguez-Plata et al., 2013). By LSCM I observed co-polarisation of ICAM-1, HIV-1 Env and CD4 at the synapse between HIV-1_{BaL}-infected MDM and uninfected $CD4^+$ T cells (Figure 3.11A). A functional role of ICAM-1-LFA-1 interactions was confirmed using two independent clones (LB-2 and HA58) of blocking ICAM-1 mAb that efficiently inhibited VS-mediated HIV-1 transfer (Fig. 3D). The degree of inhibition by the ICAM-1 mAbs was mirrored by the activity of a blocking antibody against LFA-1 (Figure 3.11B). By contrast, neither anti-ICAM-2 nor anti-ICAM-3 blocking mAbs showed inhibition. These results are consistent with previous studies showing that the ICAM-1-LFA-1 interaction dominates the VS-mediated spread of HIV-1 from infected to uninfected $CD4^+$ T cells (Jolly et al., 2007).

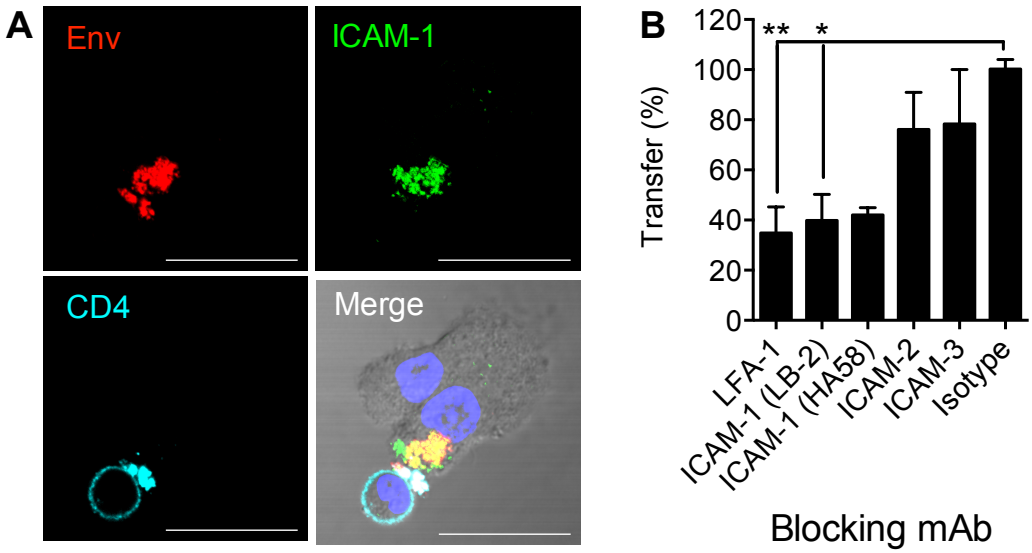

Figure 3.11 VS assembly is adhesion molecule dependent.
A) LSCM of HIV-1_{BaL}-infected MDM co-cultured for 3 h with autologous $CD4^+$ T cells pre-incubated for 30 min with non-blocking anti-CD4 (L120, mouse IgG_1), permeabilised and stained for HIV-1 Gag Env (2G12-biotin) and ICAM-1 (LB-2, mouse IgG_{2b}) followed by appropriate secondary antibodies. Nuclei were stained with Hoechst and images acquired using an Olympus FV1000 microscope with 100X oil-immersion objective lens. Scale bars represent 10 μm.
B) Pre-incubation of $CD4^+$ T cells with blocking anti-LFA1 (L130 n = 5 donors) or pre-incubation of HIV-1_{BaL} infected MDM with anti-ICAM-1 (LB-2, IgG_{2b} n = 5 donors, or HA58, IgG_1 10 μg/mL n = 2 donors), ICAM-2 (B-T1, IgG_1 10 μg/mL) or ICAM-3 (101-1D2, IgG_{2a} 10 μg/mL, both n = 2 donors) after Fc block followed by assessment of HIV-1 transfer to $CD4^+$ T cells during 3 h co-culture with HIV-1_{BaL}-infected MDM. All mAbs were maintained during co-culture. Transfer was measured by flow cytometry (FACSCalibur) of aspirated $CD4^+$ T cells stained for CD3 and HIV-1 Gag, expressed as a percentage of the % $CD3^+$ Gag^+ cells in isotype control wells. Bars = mean ± SEM * $P < 0.05$, ** $P < 0.01$, K-W ANOVA with Dunn's post-test compared to control.

3.7 Activated T cells participate in VS-mediated transfer

The experiments above were performed with PHA-activated $CD4^+$ T cells, but VS-mediated transmission also occurred, albeit at a lower level, to a subset of non-activated (unstimulated) $CD4^+$ T cells (Figure 3.12A). Moreover, transfer resulted in productive T cell infection by HIV-$1_{NL\text{-}LucR\text{-}BaL}$- and HIV-$1_{NL\text{-}LucR\text{-}YU2}$-infected MDM at 48 h measured by the luciferase assay (Figure 3.12B).

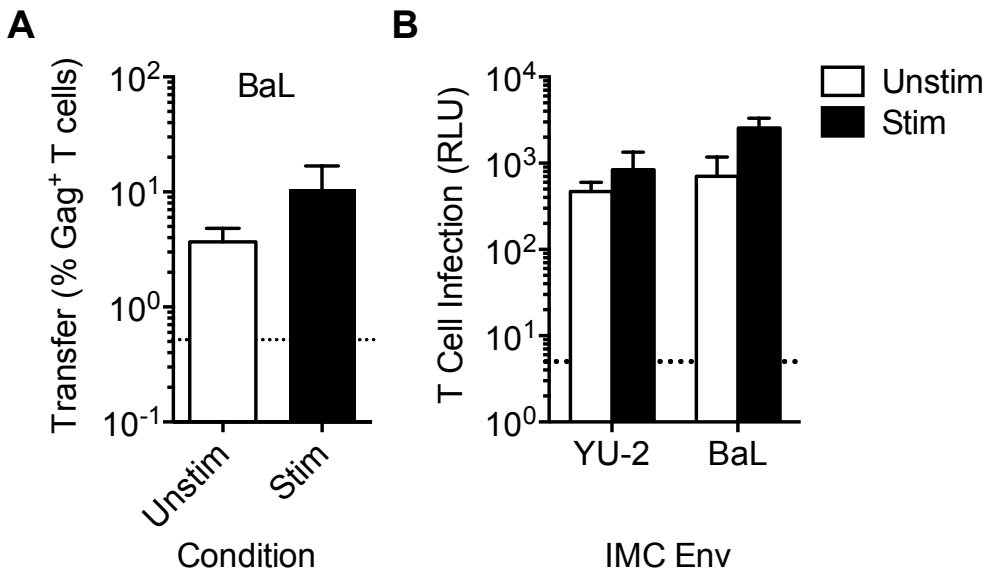

Figure 3.12 Macrophage VS-mediated transfer and infection of unstimulated CD4+ T cells

A) VS-mediated transfer from HIV-1_{BaL}-infected MDM to unstimulated (white bars) or phytohaemagglutinin (PHA)-stimulated (1 μg/mL for 3 d, black bars) autologous $CD4^+$ T cells was assessed using the flow-cytometry based transfer assay. Mean ± SEM in n = 3 donors, dotted line represents mock-infected control staining.

B) VS-mediated transmission from HIV-$1_{NL\text{-}LucR\text{-}BaL}$- or HIV-$1_{NL\text{-}LucR\text{-}YU2}$-infected MDM to unstimulated (white bars) or phytohaemagglutinin (PHA)-stimulated (1μg/mL for 3 d, black bars) autologous $CD4^+$ T cells was assessed by luciferase infectivity assay at 48 h post-co-culture. Mean ± SD in n = 2 donors, dotted line represents mock-infected control infection levels.

$CD4^+$ T cells expressing the activation markers CD38 and HLA-DR are more susceptible to cell-free HIV-1 infection *in vivo* (Meditz et al., 2011) and expression of CD38 and HLA-DR was induced upon stimulation with PHA or an anti-CD3/-CD28 mAb cocktail (Figure 3.13A-C). However, a subset of $CD4^+$ T cells within the freshly isolated T cell population also expressed HLA-DR and CD38 without PHA-stimulation (Figure 3.13D-E).

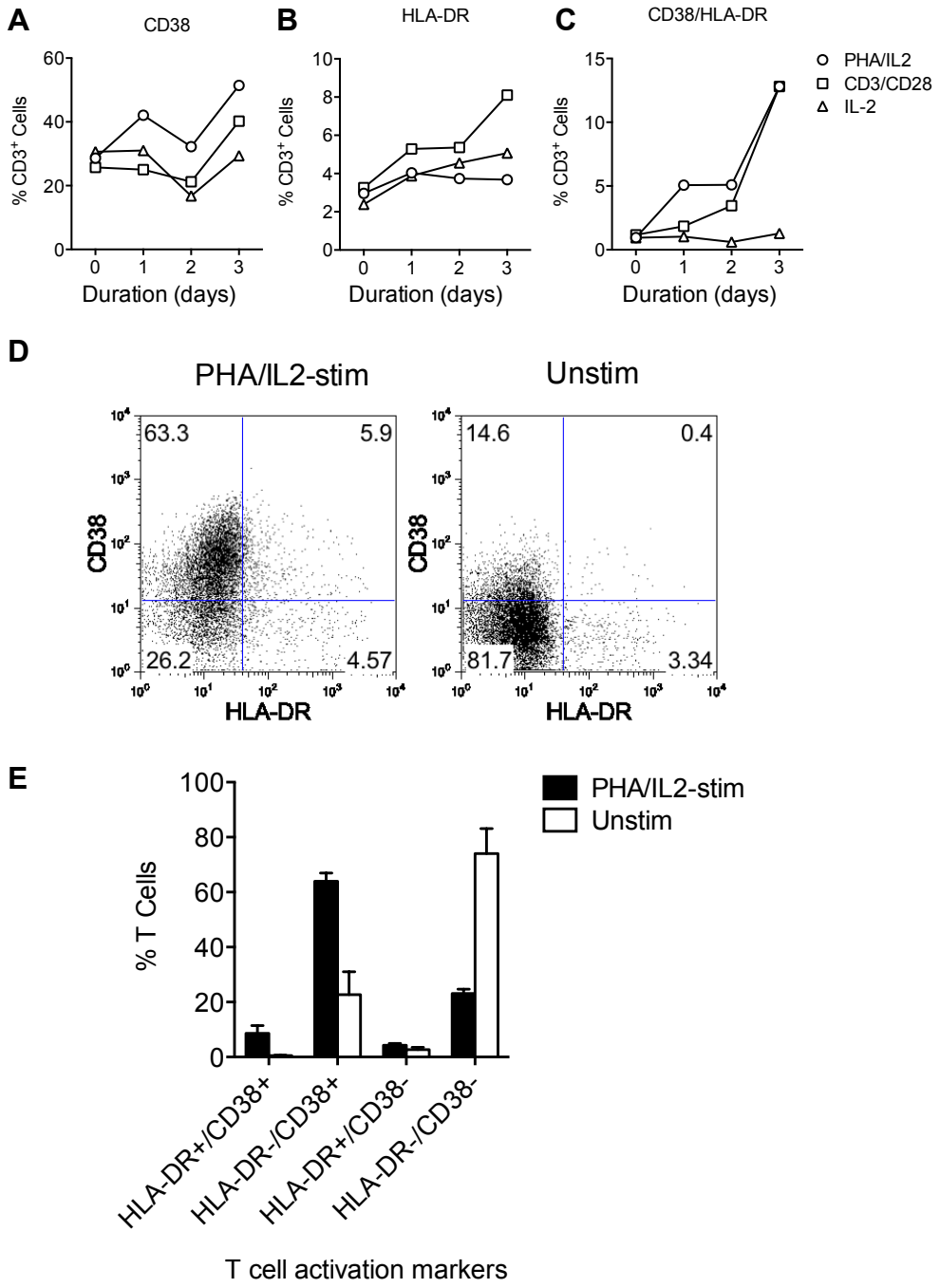

Figure 3.13 Expression of activation markers by unstimulated and stimulated primary CD4$^+$ T cells

Time-course of cell surface expression of activation markers CD38 (**A**, CD38-APC) HLA-DR (**B**, HLA-DR PerCP-Cy5.5) and both CD38/HLA-DR (**C**) by flow cytometric analysis of primary CD4$^+$ T cells cultured with PHA (1 μg/mL) plus IL-2 (10 IU/mL), or a cocktail of anti-CD3 and anti-CD28 mAbs (10 μg/mL), or IL-2 alone (10 IU/mL), n = 1 donor. **D)** Representative FC plot in a different donor after 3 d culture with PHA (1 μg/mL) plus IL-2 (10 IU/mL). **E)** Expression of activation markers CD38 and HLA-DR after 3 d culture with PHA (1 μg/mL) plus IL-2 (10 IU/mL) (PHA-stimulated, black bars) or IL-2 alone (unstimulated, white bars). Mean ± SEM in n = 3 donors.

Using the flow cytometry-based VS transfer assay, I examined whether VS-mediated transmission occurred preferentially to T cells expressing these activation markers under PHA-stimulated or unstimulated conditions. PHA-stimulated or unstimulated $CD4^+$ T cells were co-cultured with HIV-1_{BaL}-infected MDM for 6 h, aspirated, fixed and surface stained for CD38 and HLA-DR before permeabilisation and staining for HIV-1 Gag. In these experiments, Gag transfer was preferential to HLA-DR^+ and HLA-DR^+/$CD38^+$ cells in both the unstimulated and PHA-stimulated populations (Figure 3.14A). A similar phenotype was observed in experiments followed to 48 h (Figure 3.14B), suggestive of preferential infection.

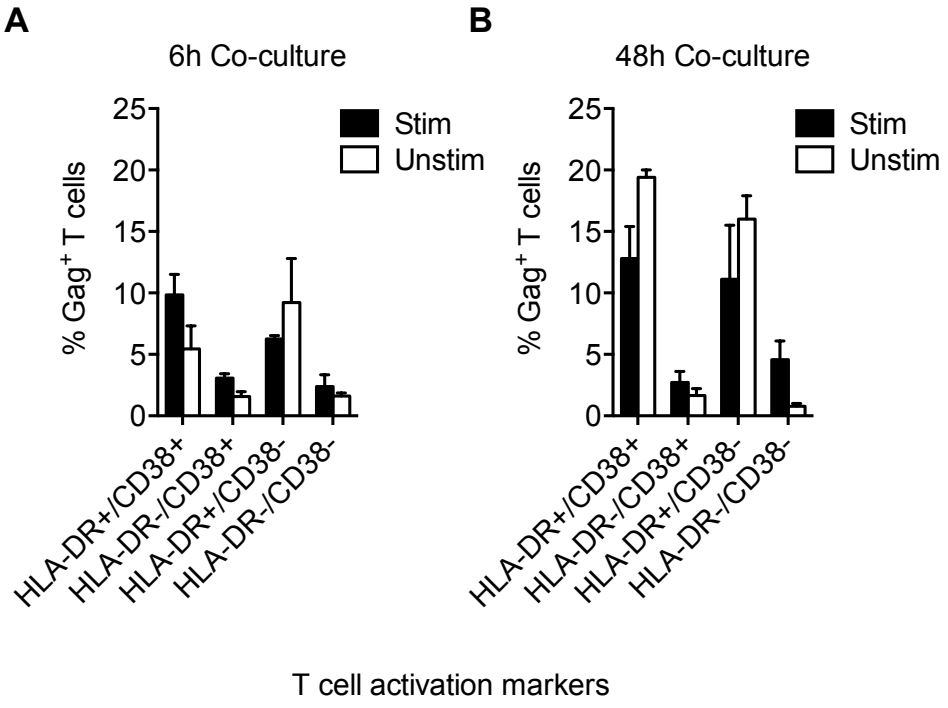

Figure 3.14 Preferential VS-mediated transfer of activated $CD4^+$ T cells by HIV-1_{BaL}-infected MDM

A) Preferential VS-mediated transfer from HIV-1_{BaL}-infected MDM over 6 h occurred to HLA-DR+/CD38+ and HLA-DR+/CD38- primary $CD4^+$ T cells previously cultured for 3 d in 1 μg/mL PHA plus 10 IU/mL IL-2 (PHA-stimulated, black bars) or IL-2 alone (unstimulated, white bars). Mean ± SEM in n = 2 donors.

B) Preferential VS-mediated transmission from HIV-1_{BaL}-infected MDM over 48 h occurred to HLA-DR+/CD38+ and HLA-DR+/CD38- primary $CD4^+$ T cells previously cultured for 3 d in 1 μg/mL PHA plus 10 IU/mL IL-2 (PHA-stimulated, black bars) or IL-2 alone (unstimulated, white bars). Mean ± SEM in n = 2 donors.

The increased efficiency of VS-mediated transfer to, and subsequent infection of, PHA-stimulated $CD4^+$ T cells (Figure 3.12) likely reflects the greater proportion of these subsets under stimulation conditions (see Figure 3.13E). These data showing differential susceptibility of T cells to VS-mediated transfer also imply active T cell participation in this process. To assess a functional role for F-actin in this process, I pre-treated $CD4^+$ T cells with non-toxic concentrations of jasplakinolide to depolymerise the actin cytoskeleton, or with the endocytosis inhibitor DMA or DMSO control, before washing out inhibitors and co-culturing $CD4^+$ T cells with HIV-1_{BaL}-infected MDM for 3 h. In these experiments, paralysis of the T cell actin cytoskeleton significantly reduced the efficiency of VS-mediated transmission compared to DMSO vehicle control (P = 0.008 Mann-Whitney) or the irrelevant inhibitor DMA (P = 0.0178 Mann-Whitney, Figure 3.15).

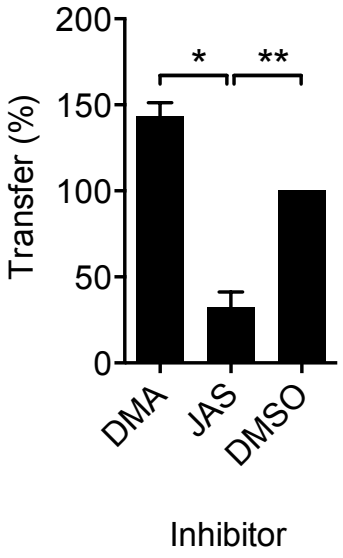

Figure 3.15 Macrophage VS-mediated HIV-1 transfer to PHA-stimulated $CD4^+$ T cells is dependent on target cell actin

PHA-stimulated $CD4^+$ T cells were pre-treated with non-toxic concentrations of DMA (100 μM), jasplakinolide (JAS, 5 μM) or DMSO for 1 h prior to washing in RPMI-10 and co-culture with HIV-1BaL-infected MDM at a 2:1 T:M ratio for 3 h. HIV-1 Gag transfer was assessed by flow cytometry staining of fixed, permeabilised T cells for intracellular Gag and expressed as a percentage of DMSO control treatment. There was a significant reduction in VS-mediated transfer to CD4+ T cells treated with JAS compared to DMSO (P = 0.008) or DMA treated cells (P = 0.016, Mann-Whitney test). Mean ± SEM in n = 4 (DMA) or 5 (JAS, DMSO) donors.

An additional possibility suggested by the T cell activation data is that immunological cross-talk between HIV-1-infected MDM and $CD4^+$ T cells could enhance $CD4^+$ T cell activation, and subsequently promote an increased efficiency of VS-transmission. To explore this hypothesis, I co-cultured unstimulated $CD4^+$ T cells with HIV-1_{BaL}-infected and uninfected MDM for 24 - 72 h, before aspirating T cells and analysing them for expression of CD38 and HLA-DR. This preliminary experiment in a single donor suggested that HIV-1_{BaL}-infected MDM did not activate unstimulated $CD4^+$ T cells compared to uninfected MDM, based on measurement of the percentage of cells expressing HLA-DR and CD38 over time (Figure 3.16).

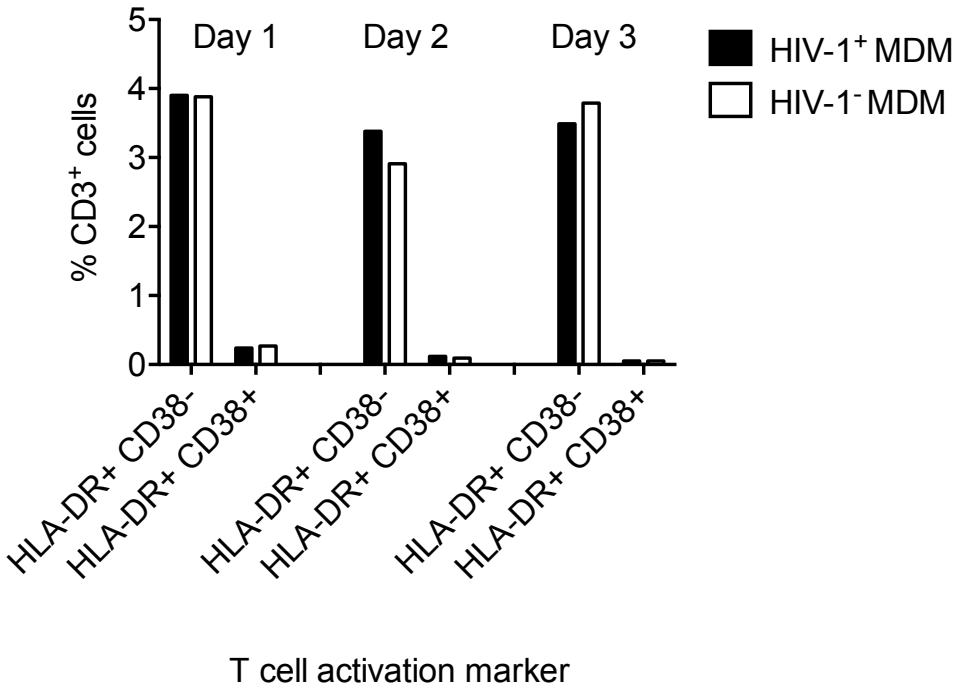

Figure 3.16 No evidence of HIV-1-infected MDM induced expression of $CD4^+$ T cell activation markers

Unstimulated $CD4^+$ T cells were co-cultured with HIV-1_{BaL}-infected or uninfected autologous MDM for 3 d in RPMI-10, and cell surface expression of activation markers CD38 (CD38-APC) and HLA-DR (HLA-DR PerCP-Cy5.5) assessed by flow cytometry. No difference in the proportion of cells expressing the activation phenotypes associated with preferential macrophage-T cell VS-mediated transmission was observed. n = 1 donor.

3.8 Discussion

I have defined for the first time the principal molecular components of the VS formed by HIV-1-infected primary MDM, an efficient and rapid mechanism of productive viral dissemination to primary $CD4^+$ T cells. The efficiency of this transmission route relates to the structure of the macrophage VS, which shares features of both T cell and DC synapses, and in this respect it is unique (Figure 3.17).

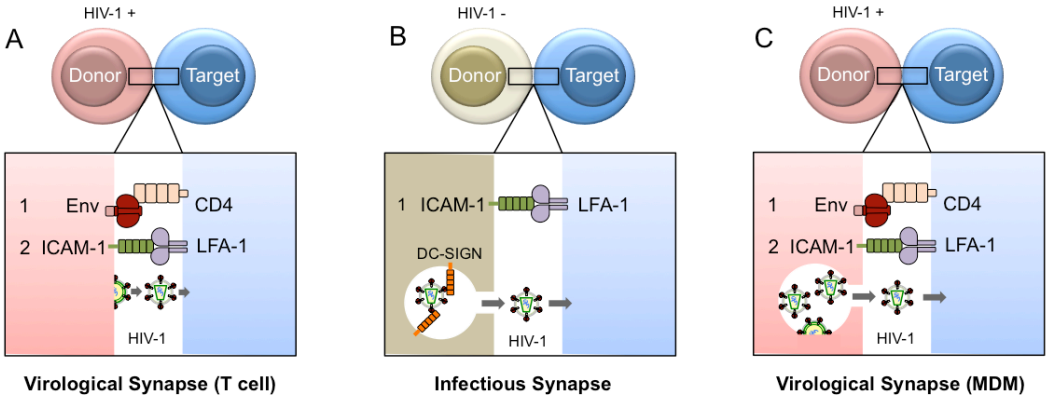

Figure 3.17 Macrophage VS model.
Displayed are the T cell VS and the DC IS alongside a model of the MDM VS based on the data presented in this Chapter. This model highlights the overlapping features of the MDM VS compared to other synaptic stuctures: the dependency on CD4-Env and the transmission of *de novo* produced virions observed for the T cell VS; and the relocation of virions from the VCC observed for the DC IS. Adhesion molecule interactions are important for efficient HIV-1 transmission at all immune cell synapses

In infected MDM virions are principally sequestered in the VCC and the lack of effect of the microtubule inhibitors including nocodazole, but dramatic action of actin paralysis with jasplakinolide, suggest that transmission does not depend on polarised secretion of virions involving the MTOC, as has been shown for T cells , but instead depends on actin-mediated rearrangement of the VCC. However, unlike the majority of DC IS (McDonald, 2010), the macrophage VS synapse is formed by productively infected cells and requires gp120-CD4 interaction for function. All immune cell

synapses implicated in efficient cell-to-cell transmission of HIV-1 appear to be stabilised by adhesion molecule interactions, with the LFA-1/ICAM-1 interaction most prominent (Rodriguez-Plata et al., 2013, Jolly et al., 2007, Groot et al., 2006a).

Intriguingly, I observed that a dynamic target cell cytoskeleton is also required for efficient transmission at the macrophage-T cell VS. Actin polymerisation is induced by the gp120-CD4 interaction (Barrero-Villar et al., 2009), and CD4 and CKR recruitment and polarisation are dependent on actin polymerisation (Jimenez-Baranda et al., 2007), accounting for the significant reduction in efficiency of VS-mediated transfer induced by jasplakinolide. Together with data showing preferential transfer to $CD4^+$ T cells expressing activation markers, this introduces the concept of active target cell participation in VS-transfer. This is consistent with data on DC-mediated transfer, in which virions in the DC surface-accessible compartment are engaged by $CD4^+$ T cell membrane protrusions (Felts et al., 2010).

My data showing an increased frequency of cell-cell contacts is consistent with previous observations of T cell chemotaxis by HIV-1-infected MDM, mediated by macrophage inflammatory protein (MIP)-1α and MIP-1β (Swingler et al., 1999). Although there was no significant difference in duration of T cell conjugates between infected and uninfected MDM, VS duration is relatively brief (around 30 - 60 mins) (Martin et al., 2010) compared to immunological synapse duration (several hours) (Vasiliver-Shamis et al., 2010) and the time-lapse studies of VS-mediated transfer were consistent with this timescale. I did not find evidence of T cell immune activation by infected MDM in a preliminary experiment in a single donor, but this does not exclude the possibility of T cell activation induced by HIV-1-infected macrophages, reported by other groups using more sensitive methods (Swingler et al., 1999).

There are several limitations to these data. The function of blocking antibodies and other inhibitors was predominantly assessed in VS-mediated transfer assays. Although the kinetics of transfer correlate with measures of productive infection - implying that functional impacts on VS-mediated transfer would correspond to a proportional effect on productive infection - this has not been directly demonstrated by these experiments. Moreover, additional aspects of the $CD4^+$ T cell phenotype that may be relevant to either transfer or productive infection (i.e. the relative expression levels of CCR5 or LFA-1), as well as the potential influence of actin inhibition on polarisation of target cell receptors have not been assessed.

However, efficient cell-to-cell transmission is likely to be the dominant mode of transmission in densely packed primary and secondary lymphoid tissues (Sattentau, 2010) where intense HIV-1 replication occurs (Cory et al., 2013). Recent progress with murine imaging models shows that retrovirus infected cells frequently engage uninfected cells (Murooka et al., 2012, Sewald et al., 2012) and suggests they are important vectors for HIV-1 dissemination. High efficiency directed cell-to-cell transmission of HIV-1 has important potential implications for host-mediated and therapeutic antiviral strategies: it has been reported to overcome antiretroviral inhibition possibly contributing to viral persistence by facilitating intermittent HIV-1 replication during antiretroviral therapy (Sigal et al., 2011) (see Chapter 4). In addition, host restriction factors such as tetherin/BST-2 (Jolly et al., 2010) may be saturated by the intensity of viral transmission at immune cell synapses.

I have defined a role for several molecular components of the macrophage-T cell VS in mediating efficient cell-to-cell transfer. My next aim was to extend these studies to quantify the multiplicity of infection occurring across the macrophage-T cell VS, to measure the relative efficiency of this process compared to cell-free infection, and to

understand the implications of macrophage VS-mediated transmission for key therapeutic interventions such as antiretroviral drugs and neutralising antibodies.

4 THERAPEUTIC BLOCKADE OF VS-MEDIATED TRANSMISSION

4.1 Introduction

The relative efficiency of VS-mediated transmission and whether it is amenable to conventional therapeutic and immune (antibody) intervention is relevant for several reasons. While it is appears from previous studies that the process of macrophage driven cell-to-cell transmission is more efficient than cell-free infection and therefore should result in a higher multiplicity target cell infection (Carr et al., 1999, Groot et al., 2008, Mann et al., 1990), this has not been directly demonstrated. Multiple lines of evidence support a key role for macrophages in HIV-1 persistence: infected macrophages resist viral cytopathic effects *in vitro* (Swingler et al., 2007b), harbour replication-competent virus for weeks to months (Sharova et al., 2005a), and reside in tissues where ART penetration is reduced (Sigal and Baltimore, 2012). Due to the potential importance of the macrophage reservoir (Eisele and Siliciano, 2012), together with evidence that infected tissue macrophages persist in patients during ART (Zalar et al., 2010), direct quantification of the multiplicity of transmission across the macrophage-T cell VS and specific assessment of the activity of inhibitors against this process will inform the role of macrophages in HIV-1 persistence.

By virtue of the large number of virions transmitted across the VS, cell-to-cell spread of HIV-1 appears to increase the probability of escape from antiretroviral inhibition, and has been proposed to account for ongoing replication in the face of ART (Sigal et al., 2011), a potential explanation for viral persistence (Eisele and Siliciano, 2012). This proposal is controversial, not least because others have suggested that ART has equivalent efficacy against both cell-free and cell-to-cell spread between T cells (Permanyer et al., 2012). However, much of the data in both studies were generated with immortalised cell-lines, meaning their relevance to transmission events *in vivo* is

uncertain. An important related question is whether resistance to antiretroviral inhibition is quantitative, resulting from a greater number of viruses transferred per cell (Sigal et al., 2011), or whether qualitative differences inherent to the mechanism of cell-to-cell infection between different target cell types (e.g. T cell-T cell or macrophage-T cell) are involved (Sattentau, 2010). The ability of ART to control HIV-1 replication and spread is important in the design of ART regimens and has direct relevance to viral reservoir maintenance and potential HIV-1 eradication (Durand et al., 2012).

As I outlined in Chapter 1, data are also conflicting on the relative activity of anti-HIV-1 bNAbs against cell-to-cell transmission compared to cell-free infection, in part due to heterogeneous experimental approaches that frequently involve non-physiological cell-lines and/or fail to differentiate viral transfer from productive infection (Martin and Sattentau, 2009). Whilst bNAbs are under intensive investigation as a prophylactic vaccine strategy (Kwong and Mascola, 2012), recent data in humanised mice also suggest the potential for therapeutic application (Klein et al., 2012). Thus beyond the fundamental importance of defining whether VS-transmission represents a route of antibody insensitive viral dissemination, exploring the sensitivity of this mode of transmission to bNAbs might inform their therapeutic potential. Currently no data are published on the activity of bNAbs at the macrophage-T cell VS.

Therefore I sought to define the relative efficiency of cell-to-cell transmission from MDM to autologous primary $CD4^+$ T cells and to establish whether this is amenable to antiretroviral inhibition. Additionally, as a result of the present degree of uncertainty about HIV-1 NAb evasion during cell-to-cell transmission, I attempted to address this question in a biologically relevant model system.

4.1.1 Hypothesis

I hypothesised that VS-mediated transmission from MDM to $CD4^+$ T cells was more efficient than cell-free infection, and that this process would result in high multiplicity infection of T cells. Further, I expected that such high multiplicity HIV-1 transmission would reduce the probability of inhibition by ART. Finally, I suspected that although bNAbs would inhibit VS-mediated transmission, there would be qualitative epitope-specific effects on neutralisation by bNAbs. Based on the current literature, I hypothesised that this would most likely involve bNAbs to the CD4bs.

4.1.2 Experimental Objectives

I aimed to define the relative efficiency of VS-mediated transmission compared to cell-free infection and to characterise the susceptibility of macrophage VS-mediated transmission to ART and bNAb.

4.2 MDM transmit high multiplicity infection to T cells

Macrophage cell-to-cell transmission to T cells is more efficient than cell-free infection (Carr et al., 1999, Groot et al., 2008, Mann et al., 1990), however the relative efficiency has not been precisely defined. Various methods have been used to distinguish cell-free infection from cell-to-cell transmission in co-culture models to allow comparisons of the relative efficiency of these routes of T cell infection, such as the use of transwell membranes that permit viral diffusion without cell contact (Groot et al., 2008), incubation with infected donor cell-free supernatants (Martin et al., 2010) or gentle shaking of co-cultures to prevent sustained cell-cell contacts (Sourisseau et al., 2007). There is no consensus in the literature over which is the optimal strategy (Martin and Sattentau, 2009). I therefore compared all of these methods of cell-free infection to VS-mediated transmission by MDM infected 7 days previously with HIV-$1_{NL\text{-}LucR\text{-}BaL}$ and observed 20-250 fold higher $CD4^+$ T cell infection

by VS-mediated transmission at 24 and 48 h (Figure 4.1A), indicating that the multiplicity of infection by VS-mediated transmission is significantly higher than cell-free infection. The shaking culture conditions did not adversely affect the metabolic activity of either cell type (Figure 4.1B-C).

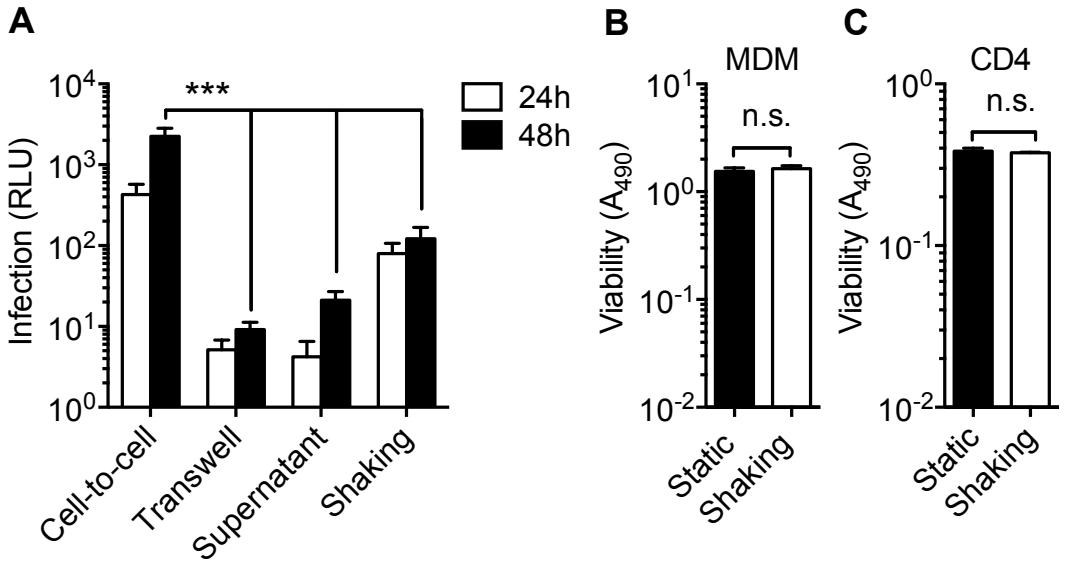

Figure 4.1 VS-transmission is more efficient than cell-free infection.
A) Luciferase expression levels in aspirated $CD4^+$ T cells after 24 h and 48 h direct co-culture with HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected MDM (cell-to-cell) or co-culture across a 3.0 μm transwell membrane (transwell), or exposure to cell-free supernatants collected from HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected MDM 7 d.p.i. HIV-1 (supernatant), or co-cultured with HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected MDM under gentle shaking conditions (75 rpm, shaking). Cell-to-cell infection levels were 250, 100 and 20-fold higher than transwell, supernatant and shaking controls respectively at 48 h. Mean ± SEM n = 5 independent donors. *** $P < 0.001$, K-W ANOVA with Dunn's post-test compared to cell-to-cell condition. **B)** MDM and **C)** $CD4^+$ T cell viability was assessed under static or gentle shaking conditions for 48 h by MTS metabolic activity assay. Mean ± SEM n = 5 (MDM) or n = 3 (CD4) independent donors.

To directly quantify the multiplicity of infection I collaborated with James Williams in Dr John Frater's laboratory, who used an adapted *alu*-PCR assay (Agosto et al., 2007) to measure integrated provirus DNA levels in T cells which I had infected by VS-mediated transmission from MDM compared to either transwell infection as above, or high-titre cell-free virus HIV-1_{BaL} (MOI 10^{-1}) inoculation at 48 h. James showed that VS-mediated transmission achieved a robust, high level of target cell integration (mean 2.0 (95% CI: 0.2, 3.8) integrated vDNA copies per cell Figure 4.2A), while transmission across a transwell produced T cell integration levels that were below the limit of detection of the assay (10^{-2} copies/cell). To confirm this high level of VS-mediated integration was not the result of contamination by infected MDM, I pre-treated T cells with the integrase strand-transfer inhibitor raltegravir (RAL, 2 μM), which significantly reduced the number of integrated vDNA copies per cell, indicative of *de novo* integration (Figure 4.2A). Intriguingly, when I compared the efficiency of integration for VS-mediated and cell-free infections, expressed as the ratio of integrated to total vDNA (Figure 4.2B), I found that this was equivalent for both transmission routes, indicating that post-entry effects on the efficiency of nuclear localisation are unlikely to be involved.

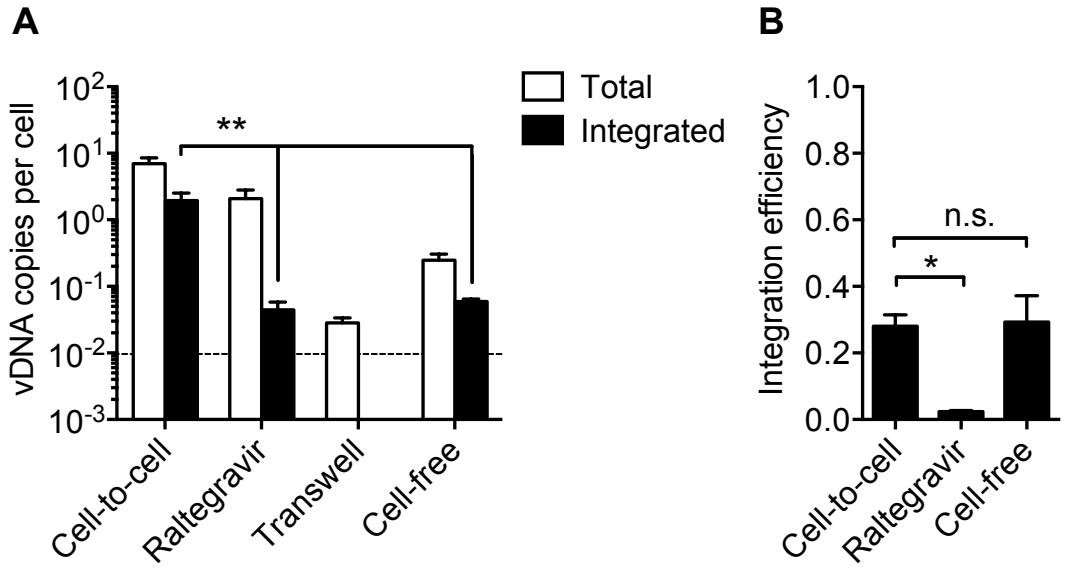

Figure 4.2 VS-transmission results in high multiplicity T cell infection.
A) Total (white bars) and integrated (black bars) HIV-1 vDNA in $CD4^+$ T cells was quantified by *gag* qPCR and *alu-gag* qPCR relative to human albumin respectively. Conditions analysed were: co-culture with HIV-1_{BaL}-infected MDM for 48 h alone; or in the presence of 2μM raltegravir (RAL); or in the presence of a 3.0 μm transwell membrane; or cell-free infection of $CD4^+$ T cells with HIV-1_{BaL} (MOI 10^{-1}). Transwell integrated vDNA levels were below the limit of detection (represented by dotted line). Mean ± SEM n = 4 independent donors. ** $P < 0.01$, K-W ANOVA with Dunn's post-test compared to cell-to-cell condition. **B)** Integration efficiency was expressed as the ratio of integrated to total HIV-1 Gag DNA as follows: $(vDNA_{integrated})/(vDNA_{total})$. * $P < 0.05$, n.s.= non-significant by K-W ANOVA with Dunn's post-test compared to cell-to-cell condition. Mean ± SEM n = 4 independent donors. qPCR assays performed by James Williams, used with permission.

4.3 High multiplicity cell-free infection overcomes ART inhibition

To ensure toxicity did not compromise my assessments of antiviral activity I first titrated the nucleoside RT inhibitor (NRTI) azidothymidine (AZT), the non-nucleoside RT inhibitor (NNRTI) nevirapine (NVP) and RAL on MDM and assessed toxicity after 48 h incubation in the MTS assay (Figure 4.3A). These experiments confirmed no evidence of dose-limiting toxicity. I also confirmed the drugs were functional by infecting the reporter cell-line TZM-bl with HIV-$1_{NL\text{-}LucR\text{-}BaL}$ in the presence or absence of 10-fold dilutions of AZT, NVP and RAL, which resulted in efficient dose-dependent inhibition (data not shown). To explore the hypothesis that the probability of antiretroviral inhibition is inversely proportional to MOI (Sigal et al., 2011), I incubated

primary $CD4^+$ T cells for 48 h with cell-free $HIV\text{-}1_{BaL}$ at high or low MOI in the presence or absence of 10 μM AZT (the *Cmax* in patients on ART (Wintergerst et al., 1995)), and analysed infection by flow cytometry for intracellular Gag (Figure 4.3B). Consistent with a recent report (Sigal et al., 2011), high multiplicity cell-free infection saturated NRTI inhibition activity despite efficient inhibition at low MOI. The transmission index (*Tx*) has been derived to assess the ability of ART to inhibit replication at different MOI (Sigal et al., 2011). *Tx* is essentially a ratio of inhibition in the presence of drug to inhibition in the absence of drug. Under very high multiplicity conditions $Tx \sim 1$, and at very low multiplicities $Tx << 1$ (Sigal et al., 2011). With increasing multiplicity cell-free infection of primary $CD4^+$ T cells, the activity of AZT was increasingly saturated, and *Tx* approached 1 (Figure 4.3C). Since VS-mediated transmission by MDM results in a high multiplicity $CD4^+$ T cell infection, this suggested that VS-mediated transmission by MDM could also be drug insensitive.

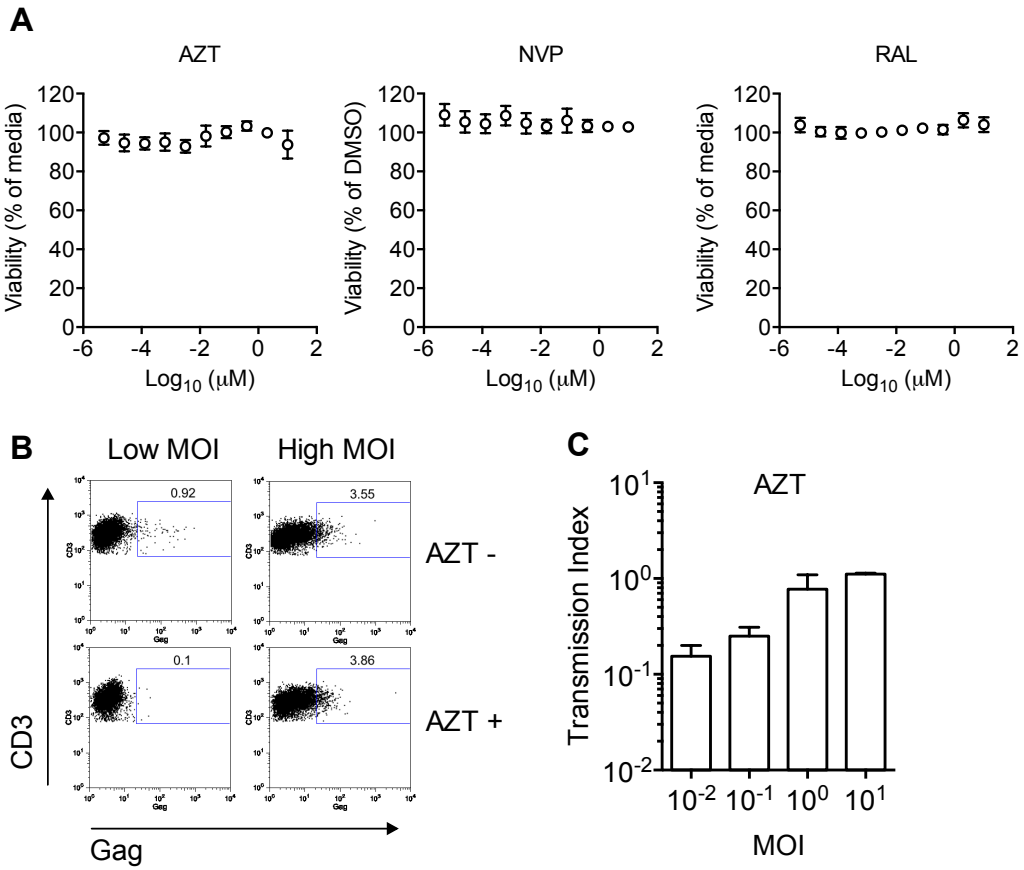

Figure 4.3 High multiplicity cell-free infection overcomes ART inhibition

A) Toxicity of azithothymidine (AZT), nevirapine (NVP) and raltegravir (RAL) was assessed on MDM at 48 h by the MTS metabolic activity assay and expressed as a percentage of the relevant vehicle control. No evidence of dose-limiting toxicity was observed. Mean ± SEM, n = 4 donors.

B) Representative FC plots of primary $CD4^+$ T cells pre-treated (AZT +) or not (AZT -) with 10 μM AZT and infected with HIV_{BaL} at low (10^{-2}) or high (10^1) MOI. Cells were fixed, permeabilised and stained for CD3 and Gag at 48 h post-infection.

C) AZT activity against cell-free HIV_{BaL} infection of primary $CD4^+$ T cells measured by flow cytometry and expressed as the transmission index, *Tx*, derived as: $(Infection_{AZT})/(infection_{no\ AZT})$. At low MOI *Tx* << 1, at high MOI, *Tx* ~1. Mean ± SD, n = 2 donors.

4.4 VS-transmission overcomes ART inhibition

To address this hypothesis I first co-cultured HIV-1_{BaL}-infected MDM with autologous $CD4^+$ T cells for 24 h in the presence or absence of AZT, NVP and RAL before analysis by flow cytometry for intracellular Gag (Figure 4.4A). While these data appeared to confirm my hypothesis, one must consider the confounding effects of continued and potentially non-infectious HIV-1 Gag transfer (see Chapter 3) which could not conceivably be inhibited by ART. Thus I turned to the luciferase infectivity assay (described earlier) to quantify expression of integrated virus in $CD4^+$ T cells. This readout is a direct measure of productive infection not confounded by measuring non-infectious transfer of Gag by flow cytometry or post-integration latency and/or the presence of non-integrated vDNA forms by PCR. Repeat cell-free inoculation experiments +/- AZT (as in Figure 4.3C) with luciferase as the readout for productive infection (Figure 4.4B) confirmed that the phenomenon of reducing inhibitory activity with increasing MOI can be recapitulated in the luciferase assay. I therefore co-cultured luciferase-expressing HIV-1-infected MDM pre-treated with approximate *in vivo Cmax* concentrations of AZT (Wintergerst et al., 1995), NVP (van Heeswijk et al., 2000) and RAL (Rizk et al., 2012) or vehicle control with uninfected autologous $CD4^+$ T cells, aspirated T cells and analysed T cell infection by luciferase activity of T cell lysates at 48 h (with < 0.1% MDM contamination, see Chapter 3). In repeat experiments in several donors, cell-to-cell infection of $CD4^+$ T cells was resistant to inhibition by all drugs tested, despite highly efficient inhibition of cell-free infection (Figure 4.4C), which corresponded to a considerably higher cell-to-cell *Tx* (Figure 4.4D).

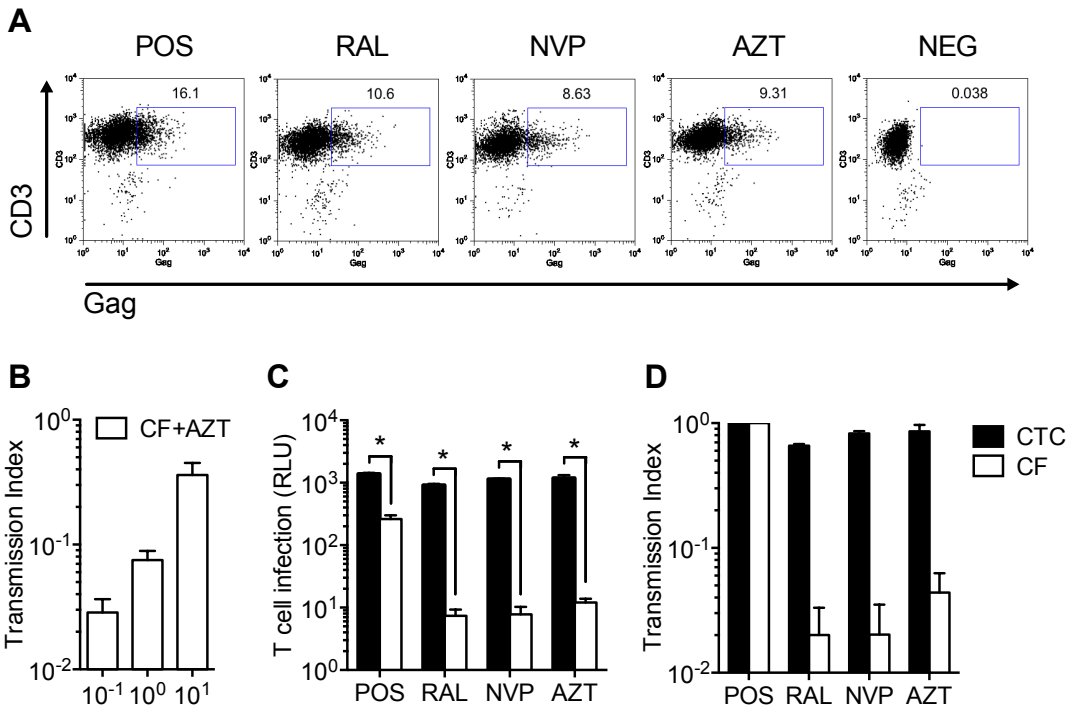

Figure 4.4 VS-transmission overcomes ART inhibition

A) CD4$^+$ T cells were pre-treated with approximate *Cmax* concentrations of RAL [2 μM], NVP [10 μM] or AZT [10 μM] or the relevant vehicle control (DMSO or media) prior to co-culture with autologous HIV-1_{BaL}-infected MDM at 1: 1 ratio for 24 h. T cells were gently aspirated, fixed, permeabilised and stained for CD3 and Gag.

B) Primary CD4$^+$ T cells pre-treated or not with 10 μM AZT and infected with $HIV_{NL\text{-}LucR^-YU2}$ at an MOI 10^{-1} to 10^{1}. Cells were lysed and analysed for luciferase expression at 48 h post-infection. AZT activity against cell-free $HIV_{NL\text{-}LucR^-YU2}$ infection was expressed as the transmission index, *Tx*, derived as: $(RLU_{drug})/(RLU_{no\ drug})$. Mean ± SD n = 1 donor.

C) CD4$^+$ T cells were pre-treated with approximate *Cmax* concentrations of RAL [2 μM], NVP [10 μM] or AZT [10 μM] or the relevant vehicle control (DMSO or media). T cells were then co-cultured with autologous $HIV_{NL\text{-}LucR^-YU2}$-infected MDM at 2:1 T:M ratio (CTC, black bars) or infected with cell-free $HIV_{NL\text{-}LucR^-YU2}$ (CF, white bars) for 48 h. T cells were gently aspirated lysed and analysed for luciferase expression at 48 h post-infection. Mean ± SEM n = 3-5 donors, * $P < 0.05$, K-W ANOVA with Dunn's post-test.

D) ART activity expressed as the transmission index, *Tx*, derived as: $(RLU_{drug})/(RLU_{no\ drug})$ from data in **C)** Mean ± SEM n = 3-5 donors.

4.5 VS-transmission is equally drug sensitive at low multiplicity

I reasoned that the impaired efficacy of ART against cell-to-cell transmission could result from qualitative differences in the mechanism of viral transfer at the macrophage-T cell interface as a result of VS formation (Sattentau, 2010), or might simply reflect a higher HIV-1 MOI by direct cell-to-cell spread compared to cell-free spread (Sigal et al., 2011). In support of the latter hypothesis, increasing cell-free MOI in the presence of AZT resulted in a stepwise increase in *Tx* in earlier experiments (Figures 4.3C and 4.4B). To directly address the effect of MOI under cell-to-cell transmission conditions, I titred initial MDM inocula to achieve different MDM infection levels (Figure 4.5A), with the aim of influencing the multiplicity of the resulting T cell infection level transmitted by MDM under cell-to-cell conditions (Figure 4.5B). Titration of MDM infection in this way, in parallel with increasing the cell-free inocula to which T cells were exposed, yielded equivalent T cell infection by both cell-free and cell-to-cell routes (see positive control (POS) in Figure 4.5C) allowing a comparison of the qualitative aspects of the different routes of infection. Interestingly, when MOI was controlled in this way, inhibition by antiretroviral drugs was equally effective by either route (Figure 4.5C-D). Moreover, under these conditions titration of ART concentration in both cell-to-cell and cell-free infections demonstrated that the effectiveness of inhibition was not influenced by the mode of viral transmission across a range of antiretroviral concentrations (Figure 4.6A-D). Thus the probability of inhibition by antiretrovirals is governed by the quantity of virus exposure, irrespective of transmission route.

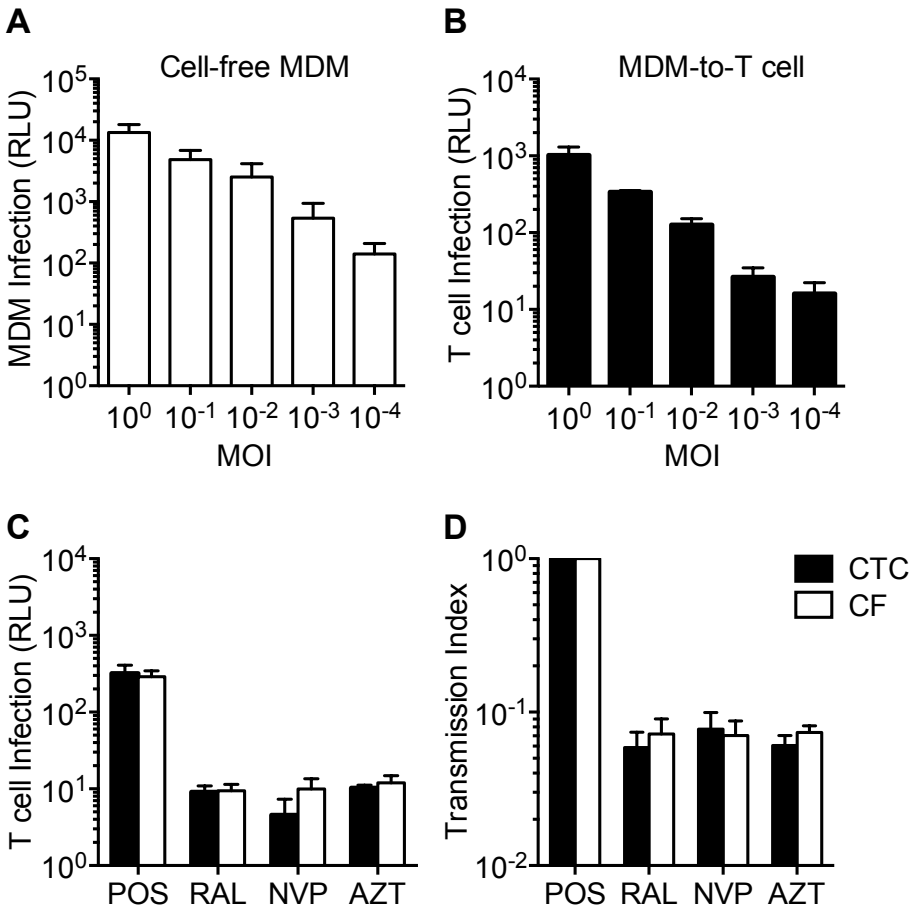

Figure 4.5 VS-transmission is equally drug sensitive at low multiplicity

A) MDM were infected with increasing MOI $HIV_{NL\text{-}LucR\text{-}YU2}$, lysed and analysed for luciferase expression at 7 d.p.i.. Mean ± SEM n = 3 donors.

B) Corresponding T cell infection levels measured by luciferase expression at 48 h post co-culture. MOI refers to the initial MDM inocula. Mean ± SEM n = 3 independent donors.

C) Titration of MDM HIV-1 inocula permitted control of $CD4^+$ T cell infection levels in the cell-to-cell (CTC, black bars) conditions to achieve equal multiplicity to cell-free (CF, white bars) conditions (see untreated positive control, POS), resulting in equivalent drug susceptibility **C)** and *Tx* **D)**. Bars represent mean ± SD of experiments in n = 3 donors.

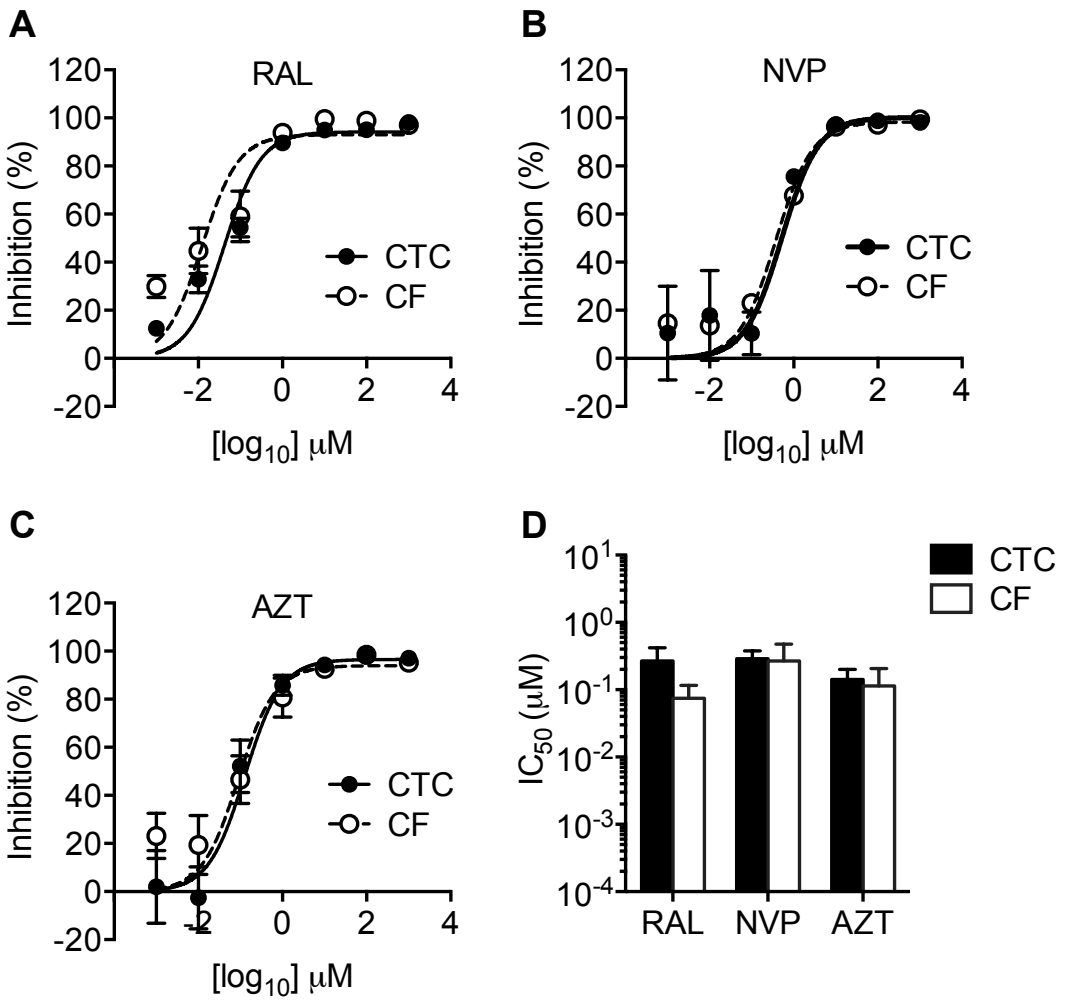

Figure 4.6 Equivalent inhibition across a range of ART concentrations
When multiplicity was equalised, the efficacy of **A)** RAL **B)** NVP and **C)** AZT was equivalent across a range of concentrations in a 10-fold dilution series, as reflected in **D)** equivalent 50% inhibitory concentration (IC_{50}) for cell-to-cell (CTC, black, solid line) and cell-free (CF, white, dotted line) infections. Mean ± SEM n = 3 donors.

4.6 VS-transmission is insensitive to MPER bNAbs

Having established a method to assess inhibition in cell-to-cell and cell-free infections at equivalent MOI, I next used this model to address the activity of bNAbs and other entry inhibitors targeting HIV-1 Env in the cell-to-cell context. Only one previous study has made any effort to equalise multiplicity when comparing the relative activity of entry inhibitors against different modes of infection (Sagar et al., 2012). Although some reports suggest that MOI may have only subtle effects on cell-free neutralisation activity (Seaman et al., 2010), I though it prudent to exclude any potential confounding effects by equalising multiplicity. I tested the relative activity of several bNAbs targeting three main regions of Env: the CD4bs (VRC01 and b12); a cluster of epitopes present on the glycan shield of gp120 (PGT121 and 2G12); and the overlapping epitopes on the MPER of gp41 (10E8, 2F5 and 4E10) (See Figure 1.5). These epitope regions on trimeric Env are displayed in Figure 4.7A. I used IMCs expressing two distinct Clade B macrophage-tropic Envs: BaL, which is relatively easy to neutralise (Tier 1) (Seaman et al., 2010) and YU2, which is more difficult (Tier 2) (Seaman et al., 2010). HIV-1-infected MDM were pre-incubated with 10 μg/mL pooled purified human IgG to block Fc receptors before pre-incubation with bNAbs or isotype for 1 h. In parallel, cell-free virus stocks were pre-incubated with bNAbs or isotype. I tested 5-fold dilutions of bNAbs from the starting concentration of 50 μg/mL (MPER, b12, 2G12) or 10μg/mL (VRC01, PGT121). Autologous $CD4^+$ T cells were cultured with HIV-1-infected MDM or cell-free viral stocks (MOI titrated to achieve equivalent luciferase expression in both conditions) for 48 h, followed by gentle aspiration, lysis and luciferase quantification. At 48 h post-infection, luciferase expression of the untreated T cell controls was equivalent in cell-to-cell and cell-free infections, confirming that MOI was equivalent (Figure 4.7B-C).

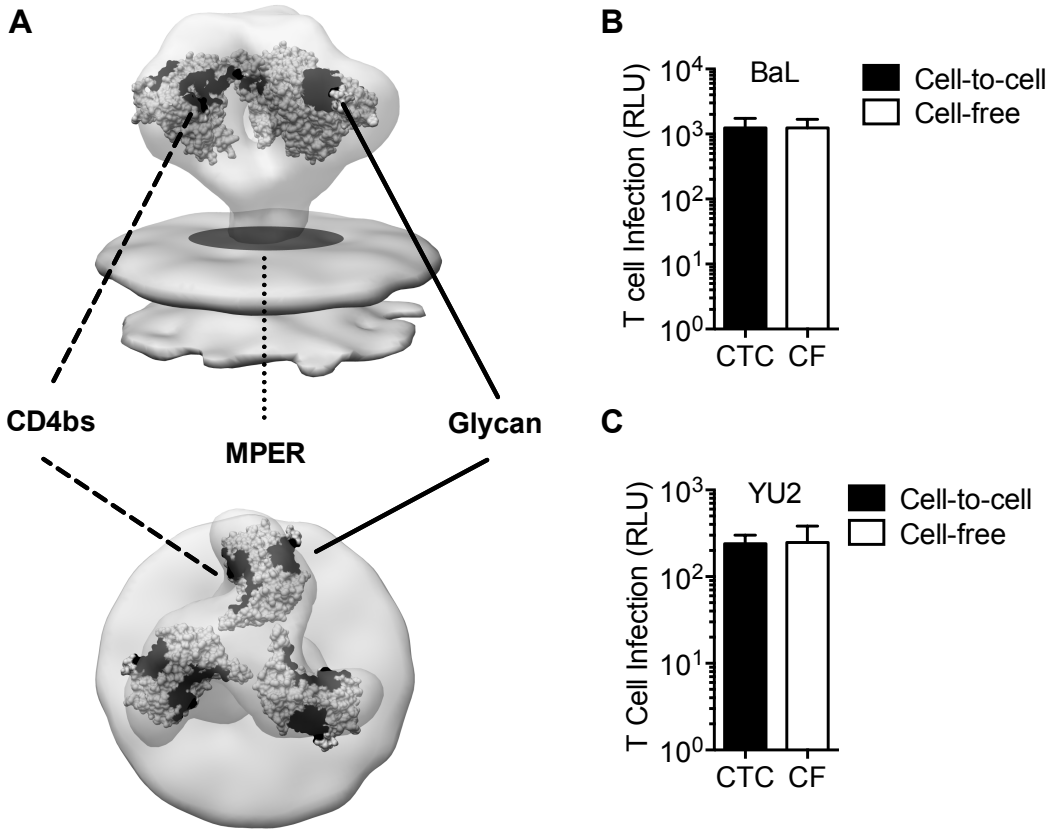

Figure 4.7 Assessing BNAb activity

A) Schematic HIV-1 Env trimer structure. Images were generated by superimposing the crystal structure of gp120 (PDB ID: 1GC1) into the electron tomography data of gp160 (EMD ID 5019+5022) using UCSF Chimera 1.6.2. Images courtesy of Torben Schiffner. Displayed are the CD4 binding site (CD4bs, interrupted line), the Glycan shield epitope (solid line) and the MPER epitope (dotted line).

MDM and T cell-free inocula were adjusted to produce equivalent infection levels at 48 h post-infection by the cell-to-cell (CTC, black) and cell-free (CF, white) routes for **B)** HIV-$1_{NL\text{-}LucR\text{-}BaL}$ and **C)** HIV-$1_{NL\text{-}LucR\text{-}YU2}$. Mean ± SEM n = 6 (BaL) and n = 4 (YU2) donors.

I observed a subtle but significant reduction in the activity of the CD4bs NAb b12 against BaL measured by the 50% inhibitory concentration (IC_{50}) (P = 0.002, F-test, Figure 4.8A), however b12 was not significantly impaired against YU2 (P = 0.612, F-test, Figure 4.8B) and the more potent CD4bs antibody VRC01 showed comparable activity against both BaL and YU2 in cell-to-cell and cell-free conditions (P = 0.634 and P = 0.347 respectively, Figure 4.8A-B). The activity of glycan-directed bNAbs PGT121 and 2G12 against BaL and PGT121 against YU2 was also unaffected by route of transmission (Figure 4.8A-B).

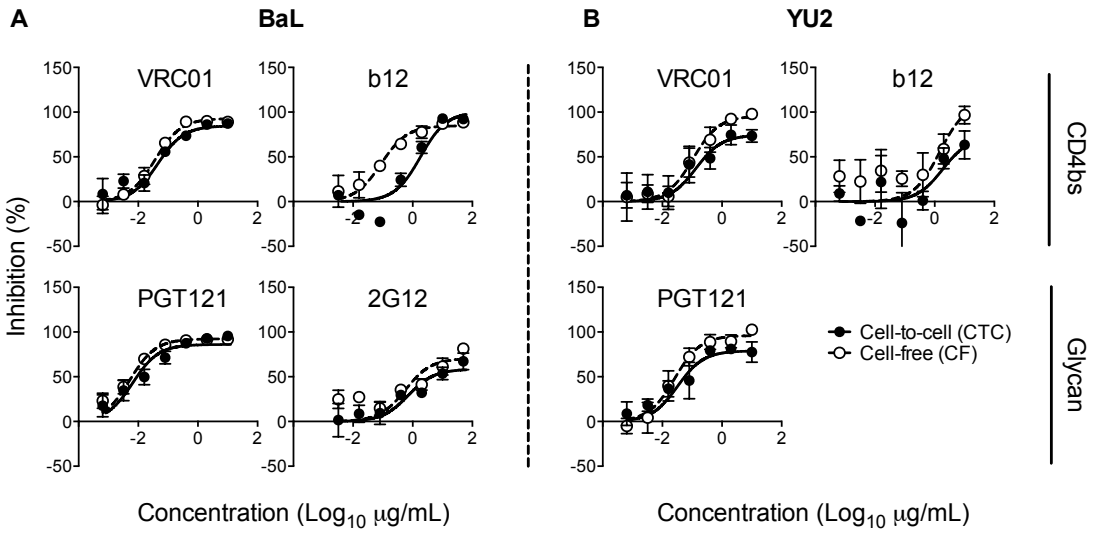

Figure 4.8 VS-transmission is sensitive to gp120 bNAbs
The activity of bNAbs targeting the CD4bs (VRC01 and b12) and the Glycan shield (PGT121 and 2G12) were tested against cell-to-cell (filled circles) and cell-free (open circles) infection of $CD4^+$ T cells with **A)** $HIV\text{-}1_{NL\text{-}LucR\text{-}BaL}$ and **B)** $HIV\text{-}1_{NL\text{-}LucR\text{-}YU2}$. Mean ± SEM n = 3-6 donors. The significance of differences in IC_{50} was assessed by F-test. A significant difference was observed for b12 (P = 0.002) against BaL. 2G12 was not tested against YU2.

However there was a striking reduction in activity of MPER-directed bNAbs 2F5, 4E10 and 10E8 against VS-mediated transmission of BaL (Figure 4.9A) and 2F5 and 10E8 against YU2 (Figure 4.9B, others not shown) despite preserved neutralisation of cell-free infection. Only data on 10E8 cell-to-cell inhibition of BaL could be fitted to a sigmoidal dose-response curve to derive the IC_{50}, which was significantly higher for cell-to-cell transmission (P < 0.0001, F-test, Figure 4.9A).

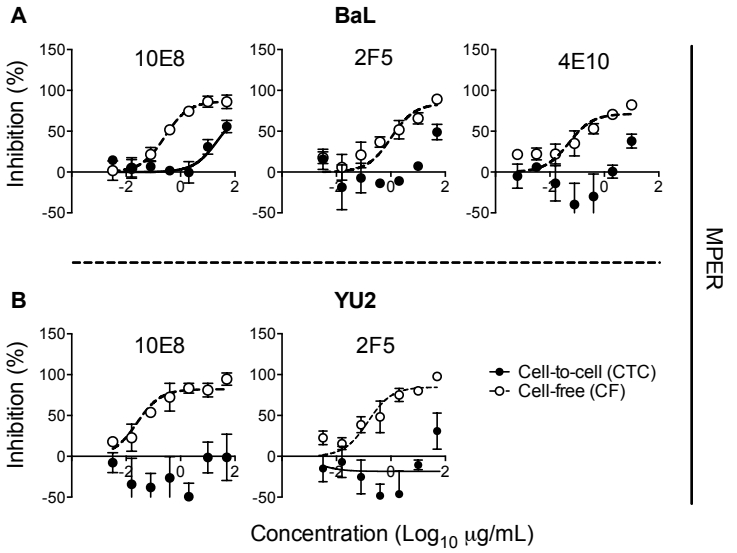

Figure 4.9 VS-transmission is resistant to MPER bNAbs
The activity of bNAbs targeting the MPER region (10E8, 2F5 and 4E10) were tested against cell-to-cell (filled circles) and cell-free (open circles) infection of $CD4^+$ T cells with **A)** HIV-$1_{NL\text{-}LucR\text{-}BaL}$ and **B)** HIV-$1_{NL\text{-}LucR\text{-}YU2}$. Mean ± SEM n = 3-6 donors. The significance of differences in IC_{50} was assessed by F-test. A significant difference was observed for 10E8 ($P < 0.001$) against BaL. IC_{50} values could not be fit for 2F5 and 4E10 (BaL), and 10E8 and 2F5 (YU2). 4E10 was not tested against YU2.

To exclude the possibility that these epitope-specific effects were due to differential bystander inhibition of macrophage replication by bNAbs during the co-culture I titrated a representative effective gp120 directed bNAb (PGT121) and a resistant gp41 MPER-directed bNAb on HIV-$1_{LucR\text{-}T2A\text{-}YU2}$-infected MDM at 7 d.p.i. However there was no evidence of a dose-response related inhibition of MDM replication over 48 h by either bNAb in a titration series in two donors or at saturating concentrations in four donors (Figure 4.10A-B). The activity of MPER bNAbs against VS-mediated transmission was also unchanged in repeat experiments done using a commercially available Fc blocking reagent (Fc-block, Miltenyi) or without blocking Fc receptors on MDM (data not shown), suggesting that the loss of function was not Fc-mediated (Sagar et al., 2012).

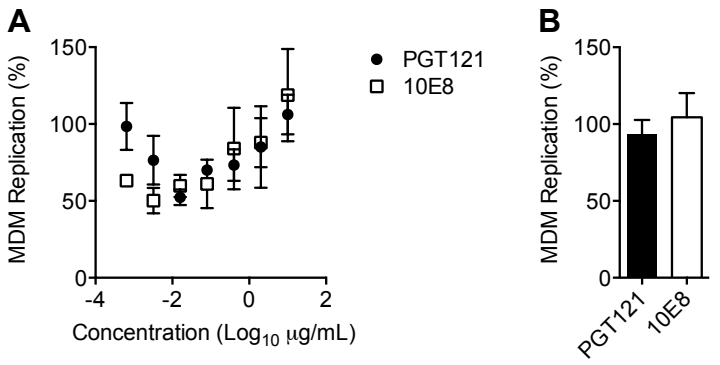

Figure 4.10 Differential activity of gp120 and gp41 bNAbs unrelated to inhibition of MDM replication
A) The inhibitory capacity against HIV-1 replication over 48 h was measured by luciferase expression in HIV-$1_{NL\text{-}LucR\text{-}YU2}$-infected MDM 7 d.p.i. for bNAbs with activity (PGT121) and resistance (10E8) to inhibition of cell-to-cell transmission across a range of concentrations. No evidence of dose-dependent inhibition was observed. Mean ± S.E.M n=2 donors.
B) The inhibitory capacity was also assessed at saturating concentrations for PGT121 (10 μg/mL) and 10E8 (50 μg/mL) in HIV-$1_{NL\text{-}LucR\text{-}YU2}$-infected MDM 7 d.p.i. over 48 h in n = 4 donors. No differential inhibition was noted.

However, the inhibitory activity of the 36 amino-acid peptide inhibitor of gp41-mediated fusion T-20 (Figure 4.11A-B), which has a molecular mass 33-fold lower than human IgG_1, was maintained. This suggested that there could be a steric component to the loss of activity of the gp41 mAbs.

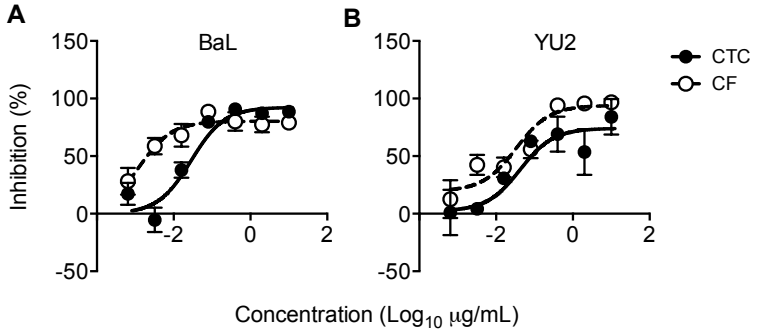

Figure 4.11 VS-transmission is sensitive to T-20
The activity of the 36-amino acid peptide inhibitor of gp41-mediated fusion T-20 was tested against cell-to-cell (CTC, filled circles) and cell-free (CF, open circles) infection of $CD4^+$ T cells with **A)** HIV-$1_{NL\text{-}LucR\text{-}BaL}$ and **B)** HIV-$1_{NL\text{-}LucR\text{-}YU2}$. Mean ± SEM n = 3 donors. The significance of differences in IC_{50} was assessed by F-test. A significant difference was observed for BaL (P = 0.001) but not YU2 (P = 0.154).

4.7 Insensitivity of VS-mediated transfer to MPER NAbs is partly steric

Reasoning that steric inhibition might be playing a role in mediating MPER restriction, I tested a 10E8 Fab' prepared by Torben Schiffner in the lab using a Fab preparation kit (Pierce). This had considerably improved activity against VS-mediated transmission of both HIV-$1_{LucR\text{-}T2A\text{-}BaL}$ (Figure 4.12A) and HIV-$1_{LucR\text{-}T2A\text{-}YU2}$ (Figure 4.12B), although like T-20, the IC_{50} for BaL remained significantly higher than cell-free infection (BaL P = 0.013; YU2 P = 0.400; F-test). There appeared to be no steric block to the activity of potent CD4bs mAbs such as VRC01 against cell-to-cell infection, which was also confirmed by testing the activity of a VRC01 Fab', which had equal activity to the full-length mAb (data not shown). Suspecting that a large molecular weight CD4bs directed inhibitor might be more susceptible to steric hindrance in the cell-to-cell context, I tested the dodecameric form of CD4 fused to IgG (Arthos et al., 2002) which has a molecular weight 5-fold greater than IgG. This showed a significantly higher IC_{50} for BaL (P = 0.027, F-test, Figure 4.12C) and a non-significant trend towards a higher IC_{50} for YU2 (P = 0.121, F-test, Figure 4.12D) in cell-to-cell compared to cell-free infections, suggesting that steric hindrance can also impact epitopes other than the MPER, but implying that the MPER is particularly susceptible. The reasons for this differential susceptibility to steric hindrance at the macrophage-T cell VS are unclear.

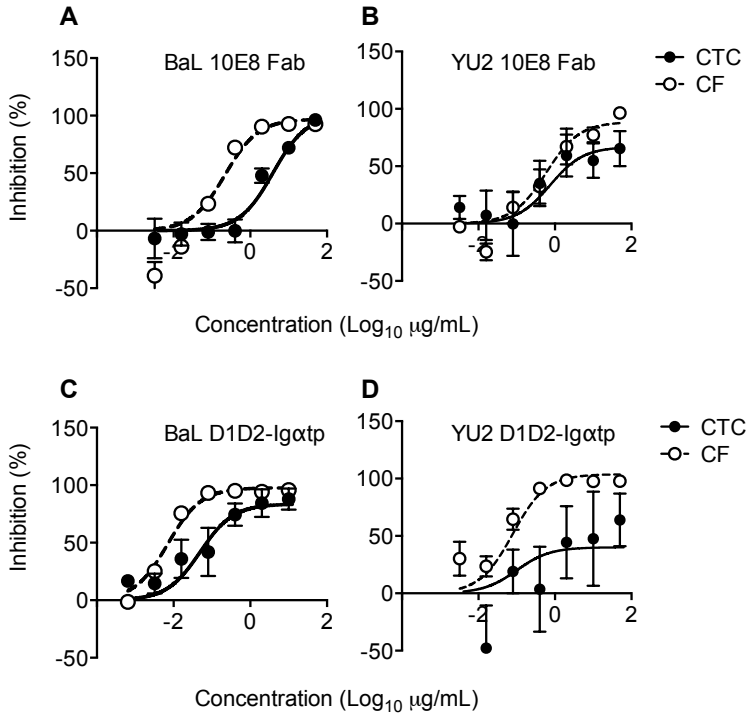

Figure 4.12 10E8 insensitivity is partly steric
Cell-to-cell (black circles) and cell-free (white circles) neutralisation experiments were repeated with 10E8 Fab' against **A)** HIV-$1_{NL\text{-}LucR\text{-}BaL}$ and **B)** HIV-$1_{NL\text{-}LucR\text{-}YU2}$ and dodecameric CD4 (D1D2-Igαtp) against **C)** HIV-$1_{NL\text{-}LucR\text{-}BaL}$ and **D)** HIV-$1_{NL\text{-}LucR\text{-}YU2.}$ There was a significant difference in 10E8 Fab' IC_{50} for HIV-$1_{NL\text{-}LucR\text{-}BaL}$ ($P = 0.013$, F-test n = 3 donors) but not HIV-$1_{NL\text{-}LucR\text{-}YU2}$ ($P = 0.400$, F-test n = 5 donors) and in D1D2-Igαtp IC_{50} for HIV-$1_{NL\text{-}LucR\text{-}BaL}$ ($P = 0.027$, F-test n = 3 donors) but not HIV-$1_{NL\text{-}LucR\text{-}YU2}$ ($P = 0.121$, F-test n = 5 donors).

4.8 Prolonged incubation does not rescue 10E8 activity

Previous studies of the MDM VCC have reported that antibodies against membrane molecules could access the VCC after prolonged incubation (e.g. 3 h) (Deneka et al., 2007) despite being restricted at 1 h (Chu et al., 2012b, Koppensteiner et al., 2012a, Tan and Sattentau, 2013), suggesting an active transport mechanism. I therefore explored whether prolonged incubation of HIV-$1_{LucR\text{-}T2A\text{-}BaL}$-infected MDM with 10E8 (50 μg/mL) or the 10E8 Fab' (16.67 μg/mL) could rescue VS-mediated neutralisation. However, no improvements were noted up to 6 h of pre-incubation (Figure 4.13A), suggesting that mAb access to the VCC could not be rescued by prolonged incubation.

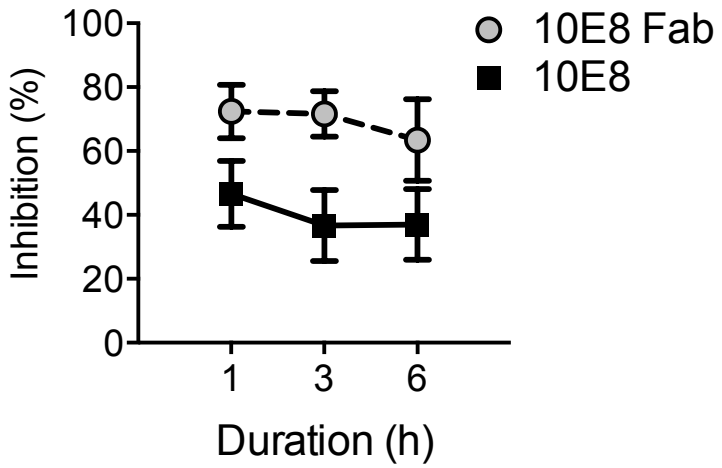

Figure 4.13 Prolonged incubation does not rescue 10E8 activity
HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected MDM were pre-incubated with 50 μg/mL 10E8 (black squares) or 16.7 μg/mL 10E8 Fab (grey circles) for 1, 3 or 6 h prior to co-culture with uninfected primary $CD4^+$ T cells. At 48 h, T cells were gently aspirated, lysed and analysed for luciferase expression and inhibition expressed as a percentage of the isotype control. Mean ± SEM n = 3-5 donors.

4.9 Discussion

Few studies have attempted to directly quantify the multiplicity of cell-to-cell transmission by measuring viral integration in target cells, and none have reported the multiplicity of macrophage-mediated VS transmission. Our finding of approx. 2 integrated copies of HIV-1 proviral DNA per target $CD4^+$ T cell is comparable to studies of VS-mediated transmission between T cells using fluorescence *in situ* hybridisation (FiSH)-based measurements in highly permissive T cell lines (3-3.8 per cell) (Del Portillo et al., 2011, Russell et al., 2013). Interestingly, by examining the ratio of total DNA to integrated forms, we observed no difference compared to cell-free infection of primary $CD4^+$ T cells, suggesting that the enhanced efficiency of synaptic transmission derives from the quantity of virions transferred rather than post-entry effects on the target cell favoring nuclear trafficking and/or integration. This is an important insight that supports efforts to target the entry pathway in cell-to-cell transmission.

In keeping with this observation, my data show that antiretroviral resistance in cell-to-cell transmission is proportional to viral exposure, and not due to an inherent feature of this viral dissemination route. This has important implications for HIV-1 treatment and eradication strategies. Data presented in Chapter 3 and previously published data suggest that the enhanced efficiency of cell-to-cell transmission derives from polarised budding of HIV-1 from the donor cell towards the target cell (Jolly et al., 2011), viral receptor clustering on the target cell (Groot et al., 2008, Jolly et al., 2004) and the close proximity of donor and target cells at the virological synapse (Groot et al., 2008, Martin et al., 2010, Sattentau, 2010). If cell-to-cell infection was a qualitatively drug-insensitive route of viral dissemination, the implication is that efforts to drive transcription from latently infected cells under ART would fail due to uninhibited expansion of the infected cell population (Sigal et al., 2011). However, I observed that cell-to-cell infection is equally susceptible to antiretroviral inhibition when the target cell viral challenge 'dose' is equivalent, suggesting that the increased efficiency is in fact not qualitative, but quantitative. This agrees with recent publications on the multiplicity of T cell-to-T cell spread between cell-lines (Del Portillo et al., 2011, Sigal et al., 2011, Permanyer et al., 2012, Russell et al., 2013), and supports the concept that aspects of HIV-1 cell-to-cell transmission relevant to antiretroviral activity (e.g. post-entry reverse transcription, integration etc.) are qualitatively similar to cell-free infection (Sattentau, 2010).

Therefore MOI appears to be the principal *in vitro* determinant of antiretroviral activity in the absence of resistance mutations (Permanyer et al., 2012, Sigal et al., 2011). The extremely high titre extracellular concentrations of virus needed to saturate antiretroviral activity are unlikely to be reached *in vivo* by cell-free spread, where virion infectivity is limited by fluid phase dilution and diffusion coupled with rapid Env degeneration (Sattentau, 2010). By contrast, sufficiently high multiplicities might be achieved in tissues in which target cells are densely packed, cell-to-cell transmission

occurs (Murooka et al., 2012, Sewald et al., 2012), and antiretroviral penetrance is reduced (Sigal and Baltimore, 2012). This may have important implications for efforts to “purge” latently infected cells.

In direct contrast to the situation for ART inhibition, where the quantitative effects of high multiplicity transmission dominate, my data suggest there are additional qualitative differences in the function of bNAbs in the cell-to-cell context. This is particularly evident for antibodies targeting various epitopes in the MPER region of gp41, such as 2F5, 4E10 and 10E8. The experiments using T-20 and a 10E8 Fab imply that much of this loss of activity is due to steric hindrance.

The wider literature is conflicting on whether synaptic transmission represents an antibody evasion route of dissemination (see Chapter 1), and the conclusions of several studies appear to depend considerably on methodological aspects of the cell-to-cell model system tested. A major limitation of the present literature is the lack of studies in primary cells. For example, no study has yet been performed to specifically assess bNAb function in HIV-1 transmission between primary $CD4^+$ T cells. Whether there are primary cell-type specific influences on antibody resistance in the VS-transmission context is unknown, and represents an important area for future study.

However, the possibility of cell-type specific differences in bNAb activity has been suggested by recent data. A comprehensive analysis of cell-to-cell transmission between various cell lines showed unexplained cell-line specific discrepancies in neutralisation activity of 2F5, 4E10 and 2G12 (Zhong et al., 2013). Adherent cell-line based model systems also report loss of CD4bs-directed neutralisation but preserved MPER antibody function (Abela et al., 2012), which agrees with one recent report of DC-mediated transfer (Sagar et al., 2012) but conflicts with another (Su et al., 2012). Other studies of acutely transfected T cell lines report more global resistance to

bNAb inhibition (Durham et al., 2012). Interestingly, these studies involve transmission of immature virions expressing Env in conformations that are unable to initiate immediate cell-membrane fusion (Dale et al., 2011). Deletion of the cytoplasmic tail of gp41 restored Env fusogenicity (Durham et al., 2012), and resulted in considerable improvements in neutralisation by gp120-directed bNAbs such as b12 (Durham et al., 2012). Intriguingly there was much less impact on the cell-to-cell resistance to the MPER antibody 4E10 (Durham et al., 2012).

My data on macrophage VS-mediated transfer show that the MPER-directed antibodies 10E8, 2F5 and 4E10 suffer most from loss of activity in a primary cell VS model, and the data using T-20 and 10E8 Fab suggest this is partially mediated by steric hindrance, consistent with EM imaging which shows tight apposition of MDM and T cell membranes at the synaptic junction (Groot et al., 2008). Indeed, other studies have shown the peptide inhibitor T-20 has relatively preserved activity in the cell-to-cell context (Abela et al., 2012, Durham et al., 2012). When I tested the large dodecameric CD4-IgG fusion protein, this also showed partial loss of activity against cell-to-cell transmission, suggesting that steric impedance can also play a role at other epitopes. However my data show, at least for the macrophage-T cell VS, that most gp120 antibodies are able to access and function at the VS. Why the MPER antibodies should be sterically restricted, when other antibodies of similar size appear to freely access the VS, is a critical unanswered question, but may relate to the buried nature of the MPER epitope region close to the virion membrane (Buchacher et al., 1994).

However, beyond the steric restraints observed for MPER antibodies, the activity of functional gp41-directed inhibitors (e.g. T-20 or 10E8 Fab) against cell-to-cell transmission remained modestly less efficient compared to cell-free activity, which might be explained by the inability of these gp41-directed inhibitors to block the

gp120-CD4 interaction necessary for VS (but not IS) formation (see Chapter 1). Alternatively (or in addition), MPER-directed antibodies have been shown to preferentially bind to Env post-CD4 engagement (Binley et al., 2003, Sattentau et al., 1995). Since plasma membrane fusion is a relatively rapid process (see Chapter 1), in the context of rapid virion transfer at the VS the rapid engagement of entry receptors polarised on the target cell might critically reduce the window of opportunity for post-attachment neutralisation.

In this and the preceding chapter I have outlined the organisation of the VS responsible for efficient transfer of HIV-1 from infected MDM to uninfected $CD4^+$ T cells, and have defined the potential importance of this mechanism of high multiplicity transmission with respect to therapeutic intervention. In the next set of experiments, I shifted my focus on cell-to-cell transmission to the opposite direction - i.e. from the infected $CD4^+$ T cell to the uninfected macrophage.

5 EFFICIENT CELL-TO-CELL INFECTION OF MACROPHAGES BY INTERACTION WITH HIV-1-INFECTED CD4$^+$ T CELLS

5.1 Introduction

Cell-free infection of macrophages is an inefficient process: several entry and post-entry restrictions are active in these terminally-differentiated, non-proliferating cells, which express 10-20 fold lower levels of CD4 (Collman et al., 1990, Raposo et al., 2011) and 22-330 fold lower levels of dNTP precursors (Amie et al., 2013). There is also significant donor-dependent variation in MDM permissiveness (Bol et al., 2009). As a result, many viruses capable of infecting CD4$^+$ T cells fail to efficiently replicate in MDM *in vitro*, as discussed in Chapter 1.

Various factors have been implicated in the tight genetic 'bottle neck' of HIV-1 mucosal transmission (Shaw and Hunter, 2012) and there is still no satisfactory explanation for the apparent selection for CCR5-using viruses in this scenario (see Chapter 1). However, it seems unlikely, based on *in vitro* MDM susceptibility of T/F molecular clones, that specific targeting of mucosal macrophages is responsible for this advantage (Ochsenbauer et al., 2012, Salazar-Gonzalez et al., 2009). Yet these *in vitro* data do not correspond to the *in vivo* situation: Infected macrophages can be identified early following mucosal transmission of macaques with SIV (Zhang et al., 1999) and in regional lymph nodes in humans with acute HIV-1 infection (Schacker et al., 2001, Zhang et al., 1999). This raises the question of how genital mucosal or lymphoid tissue macrophages become infected *in vivo* with viruses that are so inefficient at infecting MDM *in vitro*. The obvious explanation, that MDM are less permissive than genital macrophages, has been excluded by careful studies of *ex vivo* vaginal macrophage HIV-1 susceptibility - in fact, they are equally permissive to HIV-1 (Shen et al., 2009). In addition, there is a preponderance in humans of CCR5$^+$

macrophages in the rectal, compared to the colonic mucosa, indicating that permissive macrophages are present at mucosal transmission sites (McElrath et al., 2013), and suggesting an alternative, as yet unidentified, explanation for the reduced *in vitro* MDM susceptibility.

As described earlier, the main function of the macrophage is phagocytic clearance of dying and dead cells and pathogens. Data in SIV mucosal transmission studies consistently identify mucosal $CD4^+$ lymphocytes as the earliest targets of transmitted virus (Shaw and Hunter, 2012), and because T cells are susceptible to viral CPE (Lackner et al., 2012), it is possible that mucosal macrophages may ingest dead/dying or infected $CD4^+$ T cells. Such physiological cell-to-cell interactions between infected $CD4^+$ T cells and macrophages could be involved in initiating macrophage infection in the genital or rectal mucosa.

5.1.1 Hypothesis

It was an established lab hypothesis that cell-to-cell interactions between HIV-1 infected $CD4^+$ T cells and uninfected macrophages would lead to phagocytic uptake, and potentially macrophage infection. I hypothesised that this might enable infection with T/F viruses, which weakly infect macrophages *in vitro*.

5.1.2 Experimental objectives

I aimed to define whether MDM ingest infected $CD4^+$ T cells, understand the mechanism and mediators of uptake, and to explore the consequence for the MDM in terms of infection; and if infection resulted, to understand the relative efficiency compared to cell-free infection.

5.2 MDM phagocytose HIV-1-infected $CD4^+$ T cells

To explore whether MDM were capable of phagocytosing autologous HIV-1^+ $CD4^+$ T cells I combined live- and fixed-cell imaging approaches. I synchronously infected $CD4^+$ T cells with a reporter virus expressing the T/F Env CH077 (HIV-$1_{NL\text{-}mCherry\text{-}CH077}$) for 48 h, at which point approximately 10% of $CD4^+$ T cells were productively infected (as assessed by flow cytometry staining for HIV-Gag, data not shown). Synchronous infection was used to enhance infection efficiency (Sacha and Watkins, 2010). I imaged co-cultures of MDM with autologous HIV-$1_{NL\text{-}mCherry\text{-}CH077}$-infected $CD4^+$ T cells for up to 6 h, recording images every 2 min. MDM were seen to ingest mCherry positive $CD4^+$ T cells (Figure 5.1A). I also observed uptake of HIV-1_{BaL}-infected $CD4^+$ T cells by MDM after 6 h co-culture by fixed-cell LSCM studies, where the characteristic actin-rich phagocytic cup can be seen surrounding the engulfed infected T cell (Figure 5.1B).

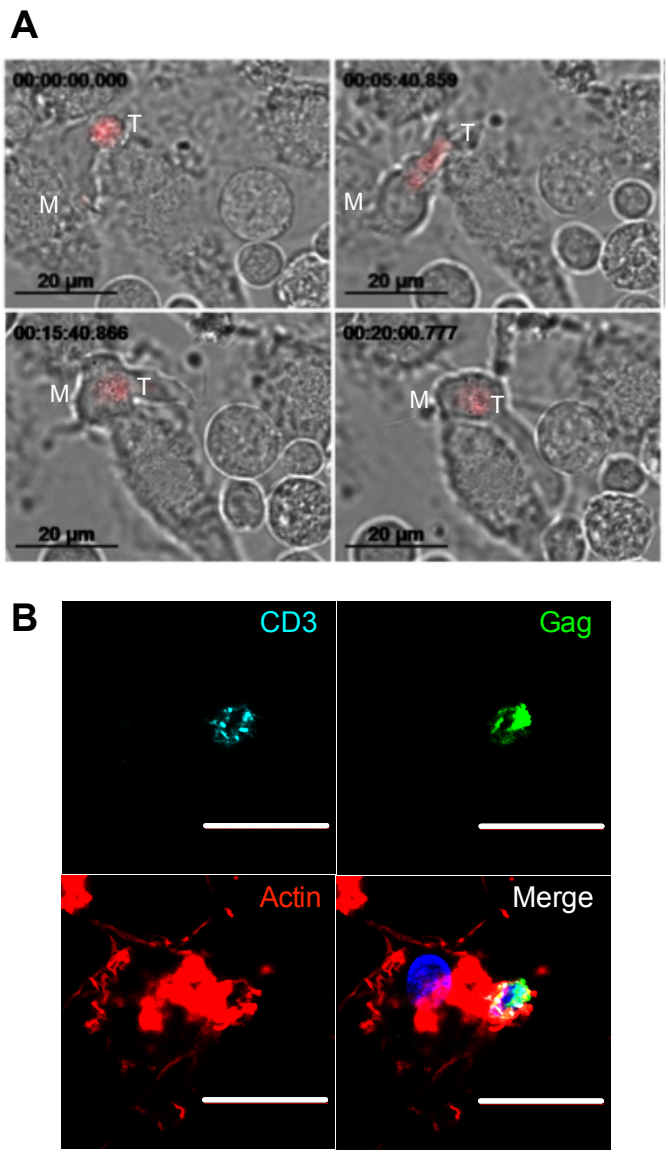

Figure 5.1. MDM phagocytose HIV-1 infected primary CD4$^+$ T cells

Live-cell time-lapse imaging of HIV-$1_{NL\text{-}mCherry\text{-}CH077}$-infected CD4$^+$ T cells (cherry, T) co-cultured with uninfected autologous MDM (M) was performed in 35 mm glass-bottomed imaging dishes (MatTek) with 40X oil-immersion objective Axiovert 200 microscope with Axiovision MRm CCD camera and Colibri illumination. Productive infection of CD4$^+$ T cells was confirmed by flow cytometry (data not shown). Images were acquired every 2 min and image processing was performed using AxioVision v 4.8.2. **A)** Representative time-lapse image series showing engulfment of an HIV-$1_{NL\text{-}mCherry\text{-}CH077}$-infected CD4$^+$ T cell by an uninfected MDM. Scale bar = 20 µm. **B)** LSCM of an HIV-1_{BaL}-infected CD4$^+$ T cell (CD3$^+$) being engulfed by an uninfected autologous MDM. The MDM actin cup surrounding the T cell can be visualised. MDM were co-cultured with HIV-1_{BaL}-infected CD4$^+$ T cells for 3 h prior to extensive washes in 5 mM EDTA to remove unbound/unengulfed T cells and fixed in 4% PFA. Fixed cell nuclei were stained with Hoechst and permeabilised cells were stained with anti-CD3 (mouse IgG_{2a}) and appropriate secondary, together with anti-Gag (KC57-FITC) and anti-F-actin (phalloidin-TRITC). Images were acquired using an Olympus FV1000 microscope with x100 oil-immersion objective lens. Scale bar = 20 µm.

5.3 Uptake is rapid and dose-dependent

To define the kinetics of uptake I developed a luciferase-reporter assay for quantifying binding/uptake of HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected $CD4^+$ T cells by autologous MDM. In these experiments, infected $CD4^+$ T cells were washed in RPMI-10 to remove cell-free virus and co-cultured for increasing durations (Figure 5.2A) or at increasing T cell:MDM ratios (Figure 5.2B). Unbound/unengulfed T cells were extensively washed away with 5 mM EDTA, MDM were immediately lysed, and luciferase (which is expressed following viral integration and therefore is only produced by infected $CD4^+$ T cells over this time period) was measured. This assay overcomes a potential complication of measuring vDNA production by qPCR - the potential for rapid vDNA production by MDM following VS-mediated transmission from T cells (Martin et al., 2010). Using the luciferase uptake assay I observed that binding/uptake was both time- and dose-dependent (Figure 5.2A and B). In separate control experiments I also observed a linear relationship between the number of HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected $CD4^+$ T cells lysed and the luciferase output (Figure 5.2C). Finally, luciferase signal in lysed HIV-$1_{NL\text{-}LucR}$-infected $CD4^+$ T cells was highly correlated with per cell infection assayed by flow cytometry (Figure 5.2D, spearman $r = 0.85$, $P < 0.001$.) Incubation of MDM with high-titre cell-free virus from infected $CD4^+$ T cells did not generate a signal, nor did analysis of luciferase activity in cell-free supernatants (data not shown), indicating the specificity of the assay for HIV-1-infected cells.

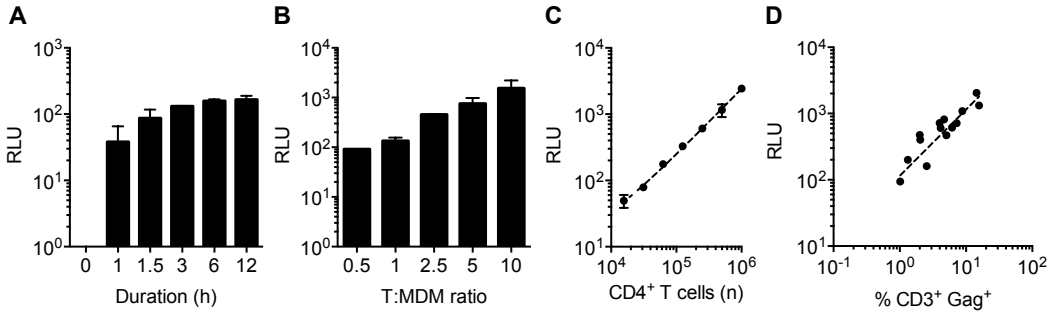

Figure 5.2 Rapid dose-dependent binding/uptake of HIV-1 infected CD4$^+$ T cells by MDM.

HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected CD4$^+$ T cells were co-cultured with uninfected autologous MDM for **A)** increasing durations at a 1:1 T cell:MDM ratio or **B)** at increasing T cell:MDM ratios for 6 h. Unattached/unengulfed T cells were extensively washed with 5 mM EDTA and MDM lysates analysed for luciferase expression (relative light units, RLU). Mean ± SD n = 2 independent donors. **C)** To explore the relationship between infected cell numbers and luciferase output, HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected CD4$^+$ T cells were titrated two-fold, lysed and analysed. Mean ± SD n = 2 independent donors. **D)** To explore the relationship between the percentage of infected cells and luciferase output, HIV-$1_{NL\text{-}LucR}$-infected CD4$^+$ T cells (n = 2 independent donors infected with n = 7 different IMCs) were fixed, permeabilised, stained for CD3 and HIV-1 Gag and analysed by flow cytometry (% CD3$^+$ Gag$^+$), or lysed and analysed for luciferase expression (RLU). Spearman r = 0.85, P < 0.001.

These data indicate that MDM do indeed interact with and engulf infected CD4$^+$ T cells, but raise the question of whether uptake of infected CD4$^+$ T cells is a specific response to HIV-1 infection, or occurs secondary to HIV-1-induced cytopathic effects (CPE) causing CD4$^+$ T cell death and subsequent clearance by MDM.

5.4 MDM phagocytose HIV-1-infected and dead/dying CD4$^+$ T cells

As discussed previously, the mechanism of HIV-1-induced viral CPE includes apoptosis of productively infected and bystander uninfected cells (Lackner et al., 2012), which can progress to secondary necrosis if apoptotic cells are not cleared (Garg et al., 2012). Massive T cell apoptosis occurs during acute HIV-1 infection particularly in the GALT (Lackner et al., 2012). I therefore examined apoptosis (by cell membrane expression of PS or intracellular expression of capsase-3) and necrosis (by the failure of cells to exclude a cell-impermeable viability dye) in infected and uninfected CD4$^+$ T cells by flow cytometry to understand the consequences of

HIV-1 infection. To prevent confusion arising due to multiple rounds of asynchronous infection, I synchronised infection by magnetofection and analysed cells at 48 h to approximate a single replication cycle. It should be noted that only viable cells can be reliably stained for apoptosis markers such as PS (stained with Annexin-V) since necrotic cells also high levels of exposed PS (Garg et al., 2012). Among the intermediate cell population containing viable and apoptotic cells (gated according to Figure 5.3A), Annexin-V or anti-caspase antibody staining were equally capable of identifying apoptosis (Figure 5.3B). There was also a higher than expected level of apoptosis in the uninfected CD4+ T cell culture (13.2%) compared to the infected CD4+ T cell cultures, potentially due to AICD (Figure 5.3B). Interestingly, approximately half of apoptotic cells in the infected $CD4^+$ T cell cultures did not express HIV-1 Gag, implicating either, or probably both bystander apoptosis and AICD (Figure 5.3C and D). Viability staining indicated a different pattern with median 21.9% (IQR: 20.8-25.1) viable infected cells, 3.67% necrotic/infected (IQR: 2.8-4.9), and 27.3% necrotic and uninfected (IQR: 25.1-31.6, data not shown), suggesting a higher level of bystander and/or AICD-induced necrosis and lower levels of direct infection-related necrosis.

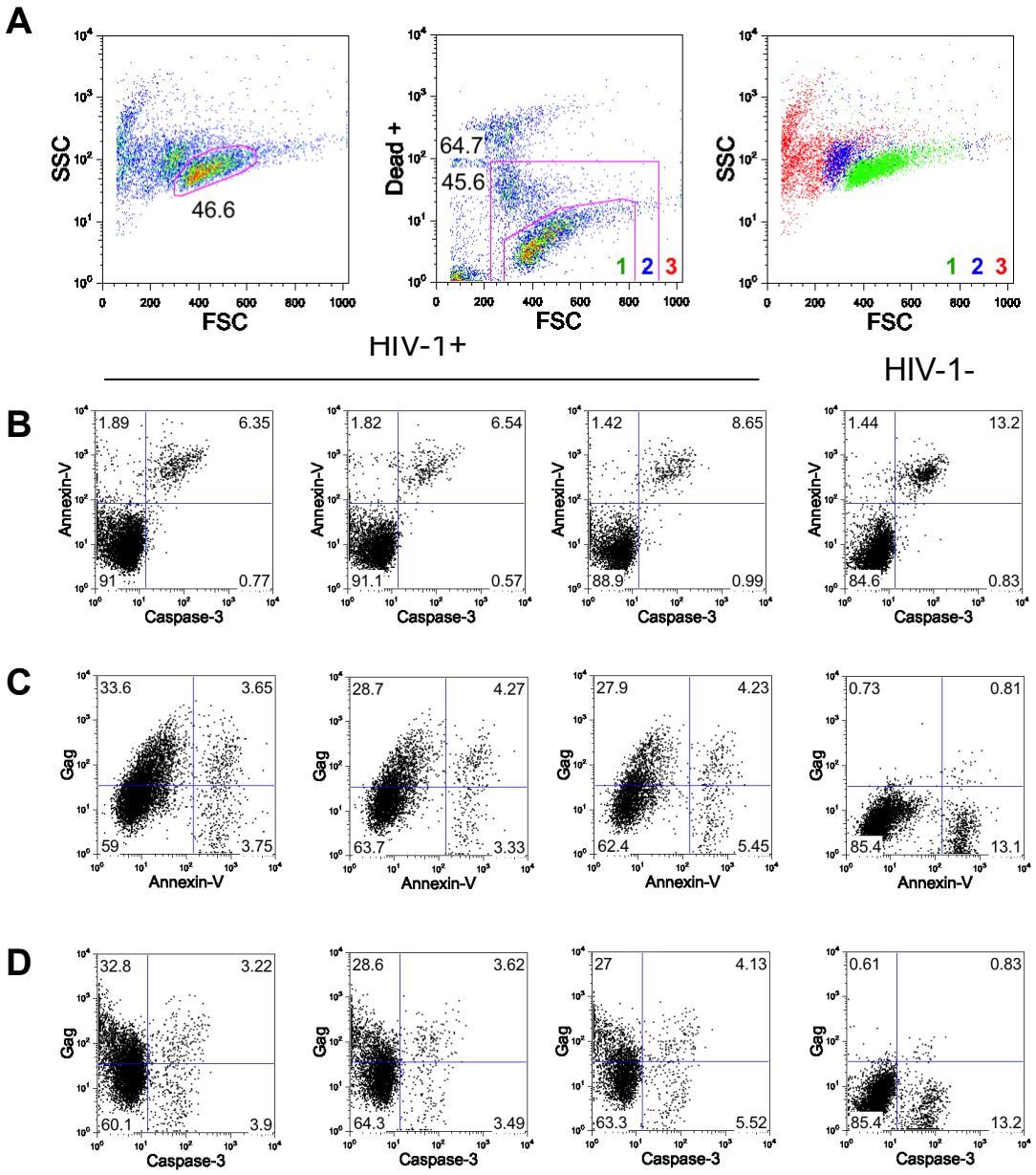

Figure 5.3. HIV-1 infected and apoptotic CD4$^+$ T cells

CD4$^+$ T cells from n = 3 donors were magnetofected with HIV-1$_{BaL}$ or mock (n = 1 displayed) and analysed by flow cytometry for markers of cell death including a viability stain (Live/Dead red), anti-caspase-3 PE, and Annexin-V FITC. Unfixed cells were stained with Live/Dead, followed Annexin-V FITC. Cells were then fixed, permeabilised and stained for caspase-3 and HIV-1 Gag. For apoptotic cell detection, debris and non-viable cells were gated out. Displayed in **A)** is the conventional viable T cell gate on FSC/SSC (left panel) and gating based on viability staining together with size (middle panel). Three cell populations could be identified based on intensity of the L/D stain: 1 = viable, 2 = intermediate and 3 = non-viable. Back-gating of these populations validated the viable FSC/SSC gate (right panel). Quantification of apoptosis was done on intermediate staining T cells (gate 2 in **A**). **B)** both Annexin-V and anti-caspase 3 reliably identified apoptotic cells, and equivalent proportions of infected and uninfected apoptotic cells were observed with Annexin-V **C)** and anti-caspase-3 **D)** respectively.

To understand the relative influence of these T cell phenotypes on MDM uptake, others in the laboratory explored apoptotic cell isolation techniques, which were unreliable for primary cells (personal communication from Amy Baxter). Without access to biological safety category 3 fluorescence activated cell sorting (FACS) facilities, I turned to multispectral flow cytometry (ImageStream^{TM}) experiments, performed with Amy Baxter, to explore the relative uptake of these cell subsets. In these experiments, we pre-labelled HIV-1_{BaL}-infected CD4$^+$ T cell populations (consisting of approximately 10% Gag$^+$ T cells) with the Live/Dead viability stain, co-cultured them with MDM for 3 h, extensively washed away unbound T cells with 5 mM EDTA and lifted the MDM in 12 mM lidocaine/5 mM EDTA. We then fixed and permeabilised MDM before staining with CD3, Gag and capsase-3 to identify T cells within the MDM. In parallel we stained T cells which had not been exposed to MDM. We characterised the T cell phenotypes using this combination of markers as illustrated in Figure 5.4A. Based on the expression of markers of HIV-1 infection (Gag, green), apoptosis (caspase-3, red) and necrosis (Live/Dead viability stain, orange) we could identify 8 unique populations of CD4$^+$ T cell (Figure 5.4A). These were:

1) viable, uninfected (CD3+/Gag-/caspase-3/LD-)

2) viable, infected (CD3+/Gag+/caspase-3-/LD-)

3) apoptotic, uninfected (CD3+/Gag-/caspase-3+/LD-)

4) necrotic, uninfected (CD3+/Gag-/caspase-3-/LD+)

5) apoptotic/secondary necrotic, uninfected (CD3+/Gag-/caspase-3+/LD+)

6) apoptotic, infected (CD3+/Gag+/caspase-3+/LD-)

7) necrotic, infected (CD3+/Gag+/caspase-3-/LD+)

8) apoptotic/secondary necrotic, infected (CD3+/Gag+/caspase-3+/LD+).

These cell populations were grouped for simplicity into 4 main phenotypes (uninfected, viable; infected, viable; uninfected, dead/dying; and infected, dead/dying). We then compared the proportion of these phenotypes present in the T

cell population to the proportion of these phenotypes present inside the MDM (Figure 5.4B). MDM were gated on the basis of positive CD3 staining, and 1000 CD3+ (uptake) events were scored. We used these data to calculate the uptake index (UI) as follows: UI = [(T cell phenotype$_{pre\text{-}co\text{-}culture}$)/(T cell phenotype$_{engulfed}$)], where UI = 1 was taken to indicate indiscriminate uptake, UI > 1 to indicate preferential uptake, and < 1 to indicate selective avoidance. By this analysis, MDM appeared to ignore uninfected viable T cells (UI 0.14, Figure 5.5), while preferentially taking up HIV-1 infected, apparently viable T cells (UI 2.1) to the same degree as uninfected dead/dying T cells (UI 2.1). HIV-1 infected dead/dying T cells showed highest UI (5.2) compared to uninfected viable cells, but this was not significantly higher than viable infected or dead/dying uninfected cells (Figure 5.5).

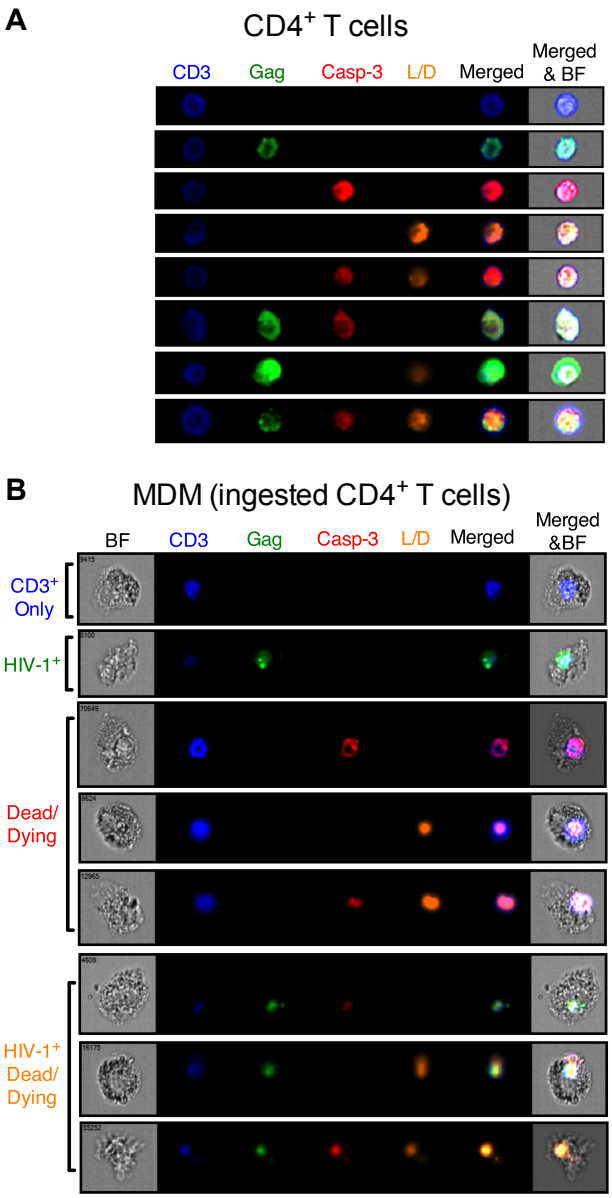

Figure 5.4. Differentiating the influence of T cell death and HIV-1 infection.
A) Representative ImageStream^{TM} images of T cells pre-co-culture selected for focus, size and aspect ratio, and labeled for the phenotypes analysed: CD3-PB (blue), Gag-KC57 (green), Caspase-3 PE (early apoptosis, red), live/dead (LD, necrosis, orange) and all channels merged with brightfield (BF). Eight distinct cell populations could be identified based on marker expression.
B) ImageStream^{TM} images of MDM plus T cells selected for focus, MDM based on size and aspect ratio, then CD3^{+} labelling from 50,000 total acquired events showing single engulfed T cells labeled for the different phenotypes analysed. Displayed are representative images showing the T cell populations in Figure 5.4A within MDM. Images courtesy of Amy Baxter.

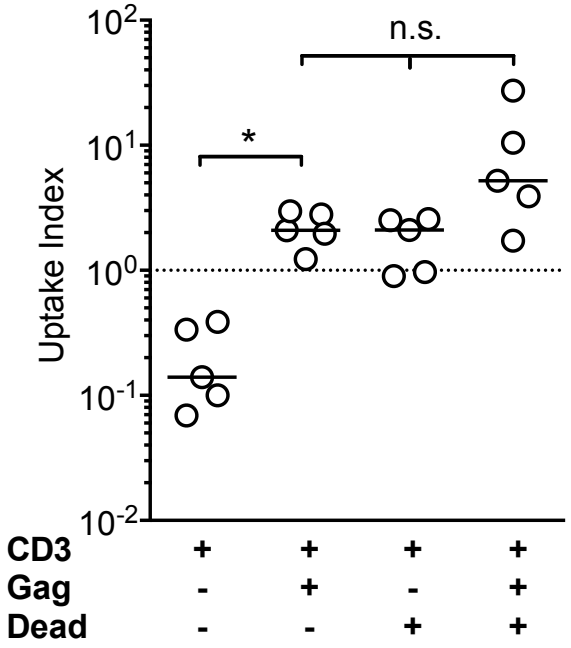

Figure 5.5 Relative uptake of CD4$^+$ T cell populations.
The uptake index was calculated as follows: UI = [(T cell phenotype$_{pre\text{-}co\text{-}culture}$)/(T cell phenotype$_{engulfed}$)], where UI = 1 is taken to indicate indiscriminate uptake, UI > 1 to indicate preferential uptake, and < 1 to indicate selective avoidance. MDM engulfed dead/dying (median UI = 2.1, IQR: 0.9-2.5), HIV-1 infected T cells (median UI = 2.1, IQR: 1.6-2.9) and infected and dead/dying T cells (median UI = 5.2, IQR: 2.8-18.9) but ignored viable T cells (UI 0.14, IQR: 0.08-0.36). UI for infected T cells was significantly above viable T cells (* = P < 0.05), but there was no significant difference between infected T cells and the other phenotypes. Bar = median, n = 5 independent donors, K-W ANOVA plus Dunn's post-test. Experiments done with Amy Baxter.

These studies suggested uptake of apparently viable as well as dead/dying HIV-1$^+$ T cells. I therefore sought to define the mechanisms involved in recognition/uptake of infected T cells by MDM.

5.5 T cell binding/uptake is actin-dependent

The actin cytoskeleton is central to the mechanics of phagocytosis (Sarantis and Grinstein, 2012), and the involvement of F-actin was implicated by earlier imaging studies (Figure 5.1B). Therefore I tested jasplakinolide, a functional (Figure 5.6A), non-toxic (Figure 5.6B) inhibitor of actin depolymerisation and remodelling. Binding/uptake of HIV-1$_{NL\text{-}LucR\text{-}BaL}$-infected CD4$^+$ T cells was strongly blocked by

jasplakinolide (Figure 5.6C). However, these data do not address the receptor/ligand interactions mediating infected $CD4^+$ T cell recognition.

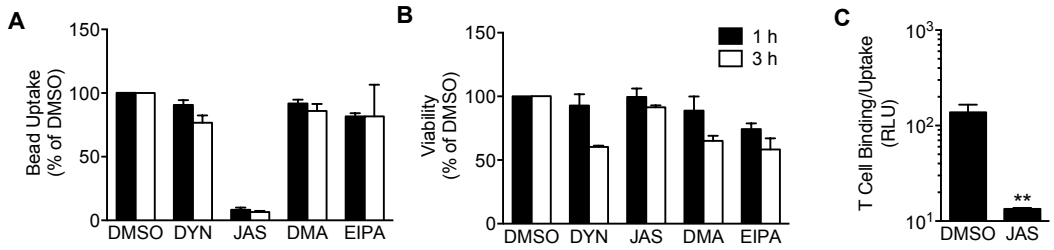

Figure 5.6 Phagocytic uptake is actin dependent
A) The phagocytic blocking activity of functional inhibitors of actin depolymerisation (jasplakonolide, 5 μM), endocytosis (DMA 50 μM and EIPA 50 μM) or dynamin GTPase activity (dynasore 80 μM), was assessed in a flow-cytometry based assay of the phagocytic uptake of 6.0 μm FITC-labelled latex microspheres. MDM were pre-treated with inhibitors for 1 h in duplicate and then co-cultured with MOI 10^{-1} beads (± prior washout of inhibitors) for a further 2 h. MDM were therefore exposed to inhibitors for a total of 1 h (washout, black bars) or 3 h (no washout, white bars). MDM were then washed in 5 mM EDTA, lifted with 5 mM EDTA/12 mM lidocaine and analysed by flow cytometry (5,000 gated events). Uptake was expressed as % $Bead^+$ MDM in treated conditions relative to DMSO vehicle control. Bars = mean ± SD n = 2 (3 h) or n = 4 (1 h) independent donors. **B)** The toxicity of inhibitors under these conditions was assessed using the MTS metabolic activity assay. Bars = mean ± SD n = 2 (3 h) or n = 3 (1 h) donors. **C)** The only functional non-toxic inhibitor testable in the 2 h binding/uptake assay was jasplakinolide, which showed a significant inhibition of HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected $CD4^+$ T cell uptake by MDM. Bars = mean ± SEM n = 4 donors. ** P = 0.004, Mann-Whitney test.

5.6 T cell binding/uptake is Env-independent

Since Env is the only cell surface expressed HIV-1 protein, I first sought to address whether conventional Env-receptor interactions were responsible for recognition and uptake of infected T cells. I took two approaches: first I analysed the relative uptake of T cells infected with VSV-G pseudotyped ΔEnv HIV-1; then, using the luciferase uptake assay described above, I tested a variety of inhibitors of Env-receptor interactions.

In the first set of experiments (Figure 5.7) I synchronously infected primary $CD4^+$ T cells with HIV-1_{BaL} or VSV-G pseudotyped HIV-$1_{BaL\Delta Env}$. At 48 h I co-cultured the T

cells (Figure 5.7A) at a 1:1 ratio with autologous MDM for 6 h before washing away unbound T cells, and lifting, fixing and permeabilising MDM before staining them for Gag (Figure 5.7B). Although the proportion of Gag^+ MDM was greater following exposure to HIV-1_{BaL}-infected T cells than HIV-$1_{BaL\Delta Env}$-infected T cells, this was due to a 3-fold higher infection level in the HIV-1_{BaL}-infected T cells (Figure 5.7C). When the proportion of Gag^+ MDM was expressed relative to the proportion of Gag^+ T cells, there was no difference (Figure 5.7D), suggesting that Env does not mediate binding/uptake.

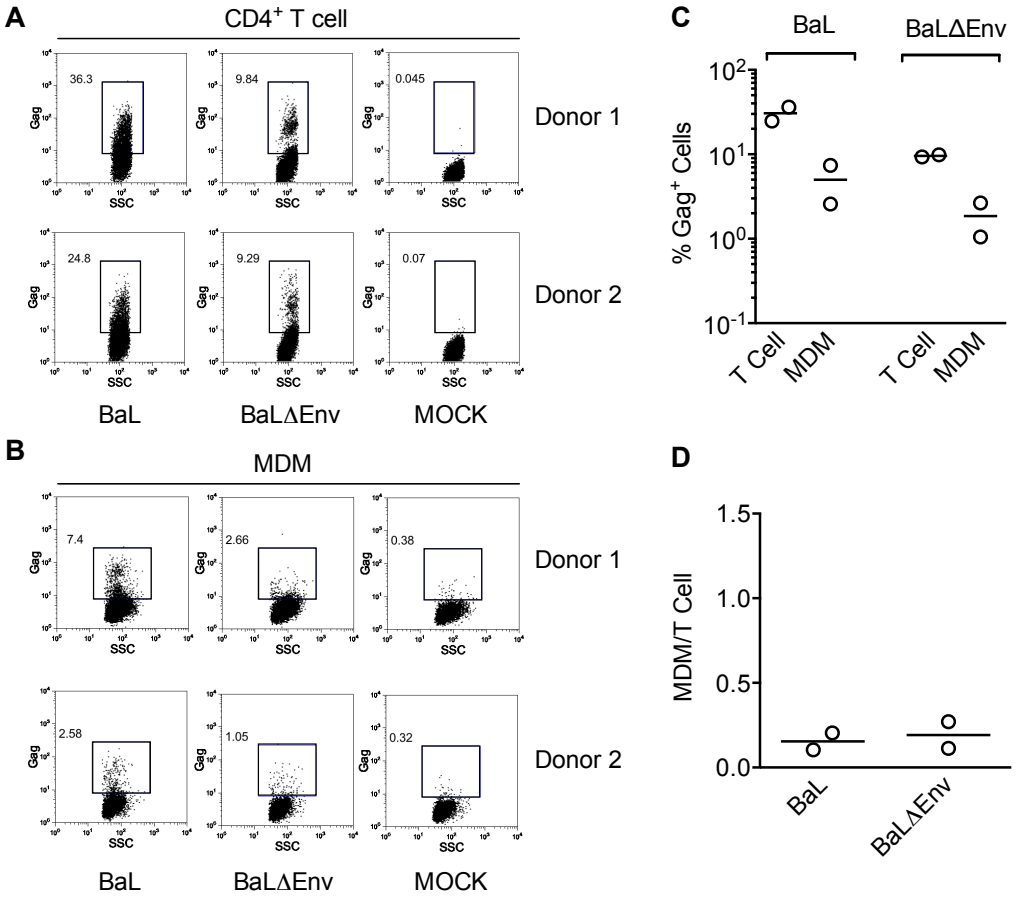

Figure 5.7 Infected T cell binding/uptake is Env-independent
A) Purified CD4$^+$ T cells from two donors were synchronously infected with HIV-1$_{BaL}$ or VSV-G pseudotyped HIV-1$_{BaLΔEnv}$ or mock-infected and used at 48 h post-infection. Displayed are FC plots showing % Gag$^+$ cells following fixation, permeabilisation, intracellular Gag staining (KC57-FITC) and analysis by flow cytometry (10,000 gated events). **B)** Infected CD4$^+$ T cells were washed and added to MDM at a 1:1 ratio and co-cultured for 3 h. MDM were extensively washed in 5 mM EDTA before lifting in 5 mM EDTA/12 mM lidocaine and fixation, permeabilisation, intracellular Gag staining and analysis by flow cytometry (5,000 gated events). **C)** % Gag$^+$ T cells and MDM are plotted (bars represent mean/median). A greater % Gag$^+$ T cells resulted in a greater proportion of Gag$^+$ MDM **D)** However, when relative T cell uptake was expressed by the uptake index as described in Figure 5.5 there was no difference.

To confirm this in the luciferase uptake assay I pre-incubated HIV-1$_{NL\text{-}LucR\text{-}BaL}$-infected CD4$^+$ T cells with various inhibitors of viral entry including NAbs for 1h, and in parallel pre-incubated MDM with known inhibitors of Env-binding for 1 h, prior to co-culture for 2 h in the continued presence of inhibitors. To avoid potential Fc-mediated enhancement of uptake I blocked MDM Fc receptors with 10 μg/mL purified human

IgG. The inhibitors tested were all functional at inhibiting cell-free infection of MDM at 48 h with MOI 10^{-1} HIV-$1_{NL\text{-}LucR\text{-}BaL}$ (Figure 5.8A). However, over 2 h co-culture none of the inhibitors showed significant inhibition (Figure 5.8B), suggesting that HIV-1 Env on T cells did not mediate MDM binding/uptake. I next explored whether apoptotic cell recognition was involved in infected T cell uptake.

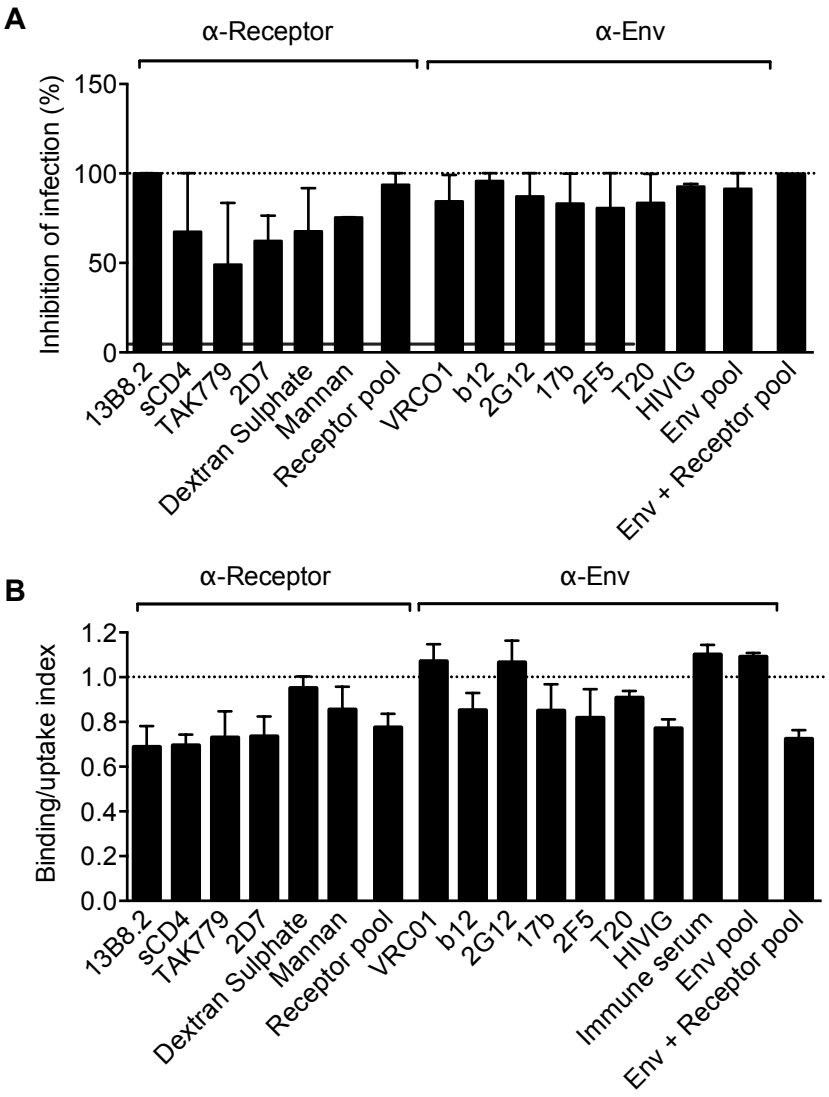

Figure 5.8 Blocking Env-receptor interactions does not prevent binding/uptake. **A)** Several inhibitors of Env binding to target cell receptors were tested for their ability to block cell-free MDM infection with HIV-$1_{NL\text{-}LucR\text{-}BaL}$ (MOI 10^{-1}), measured by luciferase expression in cell lysates at 48 h. MDM were pre-treated with pooled IgG to block Fc receptors. Receptor directed inhibitors were pre-incubated with MDM for 1 h, HIV-1 directed inhibitors were pre-incubated with virus for 1 h prior to infection in the continued presence of inhibitors. Inhibition was expressed as a percentage relative to the appropriate control (media/isotype, represented by dotted line). Bars = mean ± SD, n = 2 donors. **B)** The inhibitors were tested for their ability to block binding/uptake of HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected $CD4^+$ T cells by MDM following extensive washing in 5 mM EDTA to remove unbound/unengulfed T cells. MDM were pre-treated with pooled IgG to block Fc receptors. Receptor directed inhibitors were pre-incubated with MDM for 1 h, HIV-1 directed inhibitors were pre-incubated with infected $CD4^+$ T cells for 1 h prior to infection in the continued presence of inhibitors. None showed a significant reduction in binding/uptake relative to the control conditions (media/isotype, dotted line). Bars = mean ± SEM, n = 2-6 donors. K-W ANOVA plus Dunn's post-test.

5.7 Binding/uptake is independent of apoptotic cell recognition

Inhibitors of cell surface expressed PS and αvβ3 integrin binding have been implicated in blocking MDM uptake of apoptotic cells (Santiago et al., 2007, Kobayashi et al., 2007, Neher et al., 2011, Kurosaka et al., 2003). I therefore tested several inhibitors of PS and αvβ3 integrin binding (see Chapter 2) alone or in combination, but none were able to interfere with infected T cell binding/uptake in repeat experiments in multiple donors over 2 h, although the potent inhibition by EDTA potentially implicated a Ca^{++}-dependent interaction (Figure 5.9A). Reasoning that over a 2 h co-culture the blocking effects might be saturable, I repeated experiments reducing the co-culture duration to 30 min (Figure 5.9B). At 30 min, untreated uptake remained significantly above background (P = 0.006 t test, data not shown). However, no enhancement of blocking effects was seen for PS or αvβ3 integrin inhibitors, although the function of jasplakinolide and EDTA were maintained under these conditions (Figure 5.9C). Because of the central importance of phagocytosis to host immune defence and homeostasis (Sarantis and Grinstein, 2012), there is considerable redundancy inherent to this process. I therefore investigated other potential interactions that might drive MDM uptake of HIV-1-infected T cells.

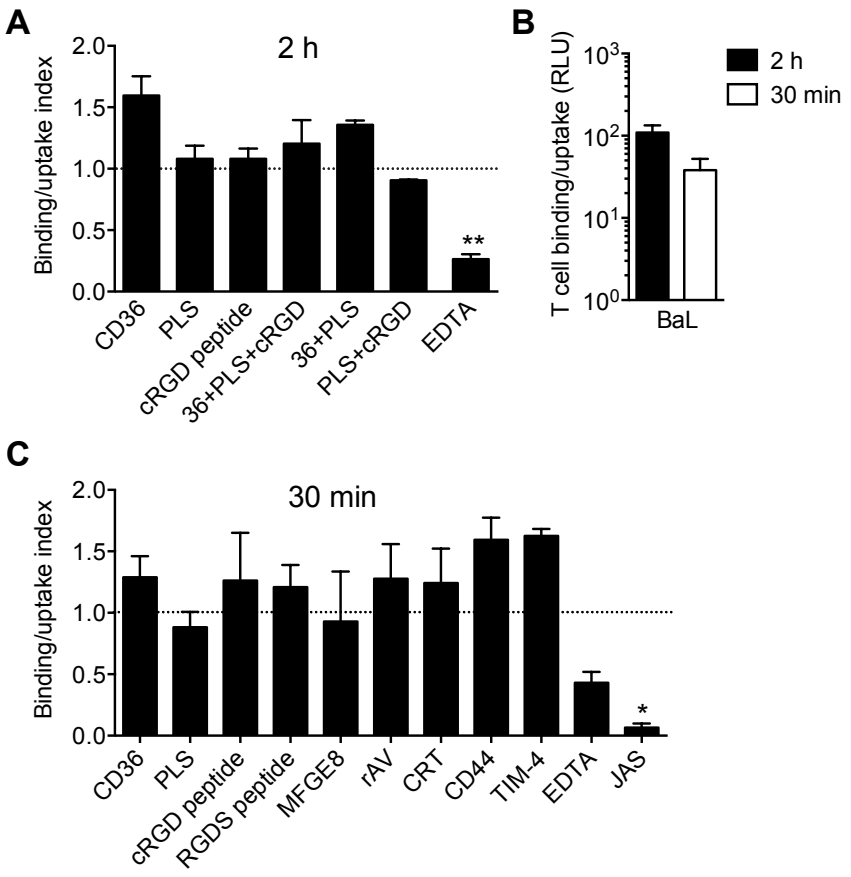

Figure 5.9 Binding/uptake is independent of apoptotic cell recognition
A) Several inhibitors reported to block apoptotic cell recognition or uptake by MDM were tested for their ability to inhibit binding/uptake of HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected $CD4^+$ T cells by MDM. MDM were pre-treated with pooled IgG to block Fc receptors. Receptor-directed inhibitors (e.g. anti-CD36) were pre-incubated with MDM for 1 h, PS-directed inhibitors (e.g. PLS) were pre-incubated with infected $CD4^+$ T cells for 1 h prior to co-culture at 1:1 ratio for 2 h in the continued presence of inhibitors, followed by extensive washing in 5 mM EDTA to remove unbound/unengulfed T cells and luciferase quantification on cell lysates. Inhibitor activity (binding/uptake index) was expressed as a ratio to the appropriate control (i.e. peptide, vehicle or isotype). Inhibitors tested included: anti-CD36 (scavenger receptor B), O-phospho-L-serine (PLS, blocking PS) and O-phospho-L-tyrosine (PLT) control, cRGD peptide (targeting αvβ3 integrin) and cRAD peptide control, or mixtures of these as illustrated. In addition, 2 mM EDTA was also tested to interfere with Ca^{++}-dependent interactions. Bars = mean ± SEM, n = 4-6 donors (n = 2 for mixtures). ** $P < 0.01$, K-W ANOVA plus Dunn's post-test. **B)** The binding/uptake of HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected $CD4^+$ T cells by MDM was explored at 2 h and 30 min co-culture to confirm sensitivity at the lower incubation time in duplicate wells in n = 1 independent donors. **C)** Inhibitor blocking studies were then repeated as described in **A)** with a 30 min co-culture period. Additional inhibitors and their controls included the anti-αvβ3 integrin RGDS peptide and RGES peptide control, and several additional inhibitors of PS recognition including anti-TIM4, anti-MFG-E8, anti-calreticulin (CRT) antibodies and recombinant annexin-V protein. In addition, anti-CD44 (which binds CD44 expressed on apoptotic microparticles) was tested. Jasplakinolide and EDTA were included in these experiments as positive controls. Bars = mean ± SEM, n = 3 donors. * $P < 0.05$, K-W ANOVA plus Dunn's post-test.

The activity of EDTA prompted me to explore various additional Ca^{++}-dependent integrin ligands. Anti-ICAM-1 (clone LB-2) and to a lesser degree anti-ICAM-2 but not ICAM-3 blocking antibodies pre-incubated on MDM and maintained during co-culture resulted in a significant reduction in HIV-1-infected $CD4^+$ T cell binding/uptake after 30 min co-culture (Figure 5.10A). However, unlike previous data on VS-mediated transmission, pre-incubation of anti-LFA-1 blocking antibodies produced an enhancement of uptake (Figure 5.10B), suggesting that ICAM-1/LFA-1 interactions are not involved. As previously reported (Marodon et al., 1999), I found that ICAM-1 expression is also upregulated approximately 6-fold on HIV-1-infected $CD4^+$ T cells in a donor-dependent manner (data not shown), suggesting that ICAM-1 expressed by HIV-1-infected $CD4^+$ T cells might be involved through binding to an alternative ligand on MDM. Another binding partner of ICAM-1 is Mac-1 (CD11b/CD18, also known as complement receptor 3) (Solovjov et al., 2005), expressed on both macrophages (Newman et al., 1984) and activated $CD4^+$ T cells (Wagner et al., 2001), which has been implicated in phagocytic uptake of pathogens by macrophages (Hawley et al., 2012). Two independent clones of blocking antibody to Mac-1 (ICRF44 and CBRM1/5) had no significant blocking activity when pre-incubated with MDM and either maintained during co-culture or washed out (Figure 5.10E). To address whether ICAM-1 on MDM or T cells is playing the dominant role, I pre-incubated $CD4^+$ T cells with two clones of anti-ICAM-1 blocking antibody (LB-2 and HA58) which were maintained during 30 min co-culture, which resulted in a similar degree of uptake inhibition for LB-2 (Figure 5.10D) as was observed with MDM pre-incubation (Figure 5.10A). However, washing out either ICAM-1 antibody before T cell-MDM co-culture prevented their inhibitory activity (Figure 5.10D). This suggested that ICAM-1 was acting on the MDM. Indeed, washing out ICAM-1 antibodies pre-incubated with MDM before T cell-MDM co-culture showed a similar level of inhibitory activity (Figure 5.10D). However, when both cell types were

incubated with a cocktail of anti-ICAM-1/Mac-1, or when MDM were incubated with anti-ICAM-1 and $CD4^+$ T cells with anti-Mac-1, there was a significant and dramatic reduction in uptake (Figure 5.10E), which was not seen with the combination of anti-ICAM-1 and anti-LFA-1 (Rebecca Moore, personal communication). Taken together, these data implicate macrophage ICAM-1 in binding/uptake of HIV-1 infected $CD4^+$ T cells, and based on these data it appears that that the ligand for ICAM-1 expressed by the $CD4^+$ T cell might be CD11b/CD18 (Mac-1) rather than CD11a/CD18 (LFA-1), although this remains to be confirmed.

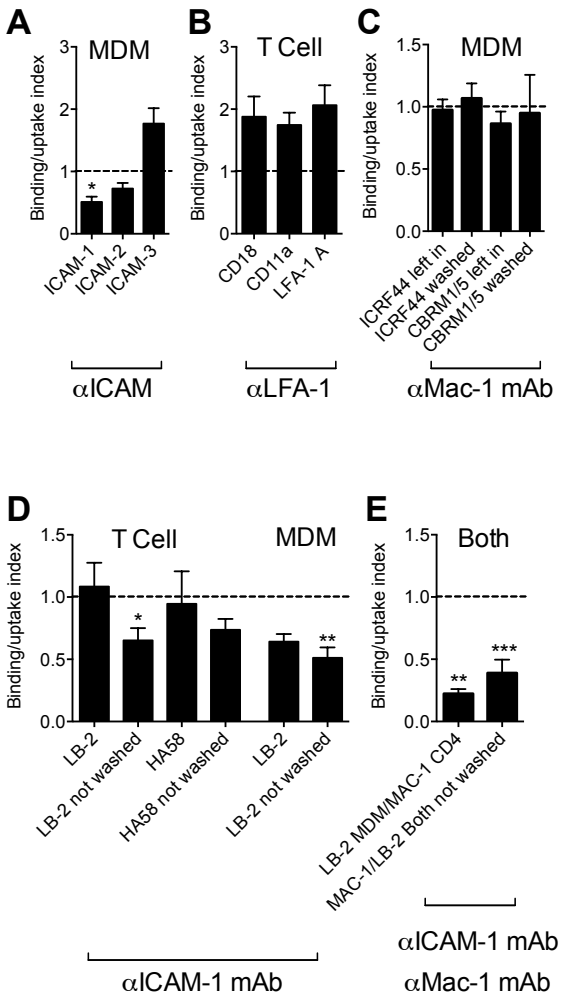

Figure 5.10 ICAM-1/Mac-1 interactions are involved in binding/uptake of HIV-1 infected CD4$^+$ T cells

A) MDM were incubated after Fc block with 20 μg/mL blocking mAbs against ICAM-1 (LB-2 IgG_{2b}), ICAM-2 (B-T1 IgG_1) or ICAM-3 (101-1D2 IgG_{2a}) or relevant isotype control for 1 h prior to co-culture with HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected CD4$^+$ T cells for 30 min at a 1:2 M:T ratio. Mean ± SEM, n = 2 - 5 donors. * P < 0.05, K-W ANOVA plus Dunn's post-test.

B) HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected CD4$^+$ T cells were incubated with 20 μg/mL blocking mAbs against CD18 (L130 IgG_1), anti-CD11a (MEM-25, IgG_1) or anti-activated LFA-1 (MAB24, IgG_1) for 1 h prior to co-culture with MDM for 30 min at a 1:2 M:T ratio. Mean ± SEM, n = 2 - 5 donors.

C) MDM were incubated after Fc block with 20-50 μg/mL blocking mAbs against Mac-1 (ICRF44 IgG_1 or CBRM1/5 IgG_1), or IgG_1 isotype control for 1 h +/- washing prior to co-culture with HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected CD4$^+$ T cells for 30 min at a 1:2 M:T ratio. Mean ± SEM, n = 2 - 5 donors. * P < 0.05, K-W ANOVA plus Dunn's post-test.

D) HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected CD4$^+$ T cells or MDM were incubated after Fc block with 20 μg/mL blocking mAbs against ICAM-1 (LB-2 IgG_{2b} or HA58 IgG_1), or relevant isotype control for 1 h +/- washing prior to co-culture with HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected CD4$^+$ T cells for 30 min at a 1:2 M:T ratio. Mean ± SEM, n = 2 - 5 donors. * P < 0.05, ** P < 0.01 K-W ANOVA plus Dunn's post-test.

E) HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected CD4$^+$ T cells were incubated with 20 μg/mL anti-Mac-1 mAb (CBRM1/5 IgG_1) and MDM with 20 μg/mL blocking mAb against ICAM-1 (LB-2 IgG_{2b}) + washing, or both cells were incubated with 10 μg/mL of each mAb for 1 h prior to co-culture for 30 min at a 1:2 M:T ratio. Uptake was compared to the relevant isotype control treatment. Mean ± SEM, n = 3 - 6 donors. ** P < 0.01, *** P < 0.001, K-W ANOVA plus Dunn's post-test.

Having identified that binding/uptake could be effectively blocked, even though I had failed to conclusively determine the ICAM-1-interacting partner, I turned to the consequences of this interaction for MDM, and specifically whether this resulted in productive MDM infection.

5.8 Uptake results in highly efficient MDM infection

To address whether productive MDM infection occurred, I used several complementary approaches. I observed time-dependent increases in luciferase expression in MDM lysates and p24 content of supernatants following 6 h exposure to HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected $CD4^+$ T cells (Figure 5.11A). There was also an increase in MDM infection signal at day 6 (luciferase and p24 content) with increasing duration of exposure to $CD4^+$ T cells (Figure 5.11B). Since there was also a corresponding increase in uptake (Figure 5.2A), this suggests a relationship between uptake and subsequent infection levels. To assess the relative efficiency of co-culture initiated infection I utilised the 3.0 μm transwell co-culture system to isolate the cell-free component of infection. In these experiments, HIV-1 production measured in separate experiments by various assays increased over time and was predominantly cell-contact dependent, since transwell conditions showed delayed replication kinetics by flow cytometry staining for intracellular Gag in MDM (Figure 5.11C), p24 ELISA (Figure 5.11D), or luciferase production (Figure 5.11E). Gag protein production measured by ELISA represented *de novo* productive infection, since pre-treating MDM with 5 μM AZT and maintaining it in the culture media completely abolished the production of p24 in supernatants (Figure 5.11F).

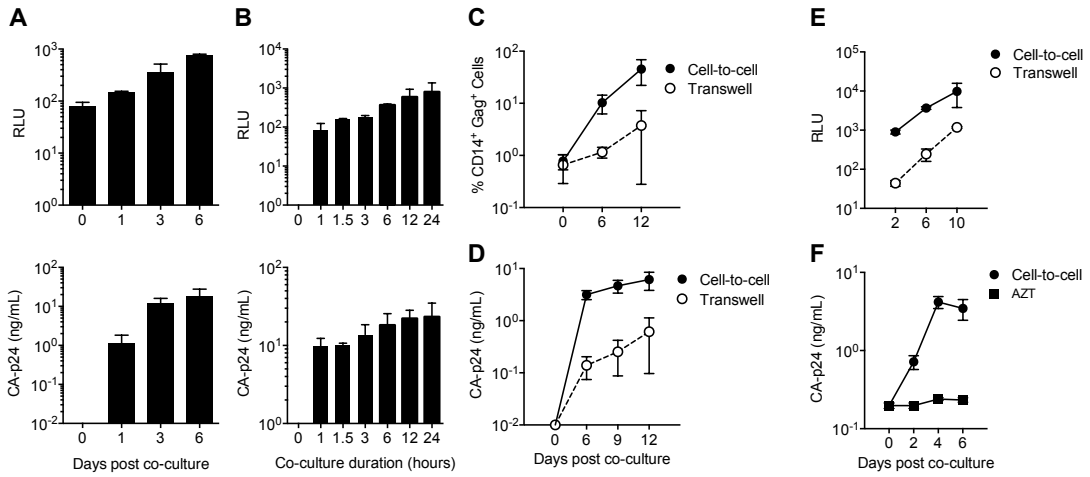

Figure 5.11 Binding/uptake results in productive MDM infection
HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected $CD4^+$ T cells were co-cultured with uninfected autologous MDM at a 1:1 ratio for **A)** 6 h or **B)** for increasing co-culture durations. Unattached/unengulfed T cells were extensively washed with 5 mM EDTA following the co-culture period, and MDM were subsequently cultured and productive infection analysed at the indicated time-points post co-culture by measuring luciferase expression in MDM lysates (top panels) or clarified supernatant Gag capsid p24 content by ELISA (bottom panels). Mean ± SD n = 2 donors. **C)** HIV-1_{BaL}-infected $CD4^+$ T cells were co-cultured with uninfected autologous MDM at a 1: 1 ratio for 6 h directly (Cell-to-cell, filled circles) or separated by a 3.0 μm transwell (transwell, open circles). Unattached/unengulfed T cells were extensively washed with 5 mM EDTA following the co-culture period, and MDM were subsequently cultured and productive infection analysed at the indicated time-points post co-culture by flow cytometry for CD14 and Gag or **D)** p24 ELISA. Mean ± SD n = 2 independent donors. **E)** Alternatively, these experiments were repeated with HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected $CD4^+$ T cells and infection analysed at the indicated time-points post co-culture by measuring luciferase expression in MDM lysates. **F)** Alternatively, MDM were pre-treated with, and maintained in 5 μM AZT (AZT, filled squares) or media (cell-to-cell, filled circles) and productive infection measured in clarified supernatants by p24 ELISA at the indicated time-points. Mean ± SD n = 2 independent donors.

If binding/uptake of HIV-1-infected T cells resulted in productive MDM infection, one would also predict that blocking it would prevent (or reduce) productive infection. Therefore I modified the binding/uptake assay to test for productive infection by removing T cells and culturing MDM for 6 d prior to lysis and measurement of luciferase expression. In parallel, I also quantified p24 Gag production into the MDM supernatant. These experiments proved that blocking uptake with jasplakinolide in a dose-dependent manner or blocking binding/uptake with EDTA (Figure 5.12A) reduced productive infection (Figure 5.12B). Indeed, the level of binding/uptake inhibition strongly correlated with inhibition of productive infection 6 d later

(Spearmans' r = 0.94, P < 0.001, Figure 5.12C). The level of p24 production into the MDM supernatants mirrored the luciferase infection data (Figure 5.12D). Parallel infections with cell-free virus supernatant from infected $CD4^+$ T cells also showed no significant signal above background (Figure 5.12B) supporting the concept that infection was not due to cell-free virus during this brief (30 min) exposure. Similarly, the blocking experiments performed for ICAM-1 and Mac-1 above (Figure 5.10D) resulted in a degree of infection inhibition at 6 days post-co-culture that correlated with the previous binding/uptake inhibition (Spearmans' r = 0.70, P < 0.001, Figure 5.12E). Interestingly, the apparent additive effect of anti-Mac-1 on infection inhibition (Figure 5.10E) was not as clear as that seen for inhibition of binding/uptake (Figure 5.12D). Thus, there is presently insufficient evidence to form a definitive conclusion on the role of Mac-1 in this system.

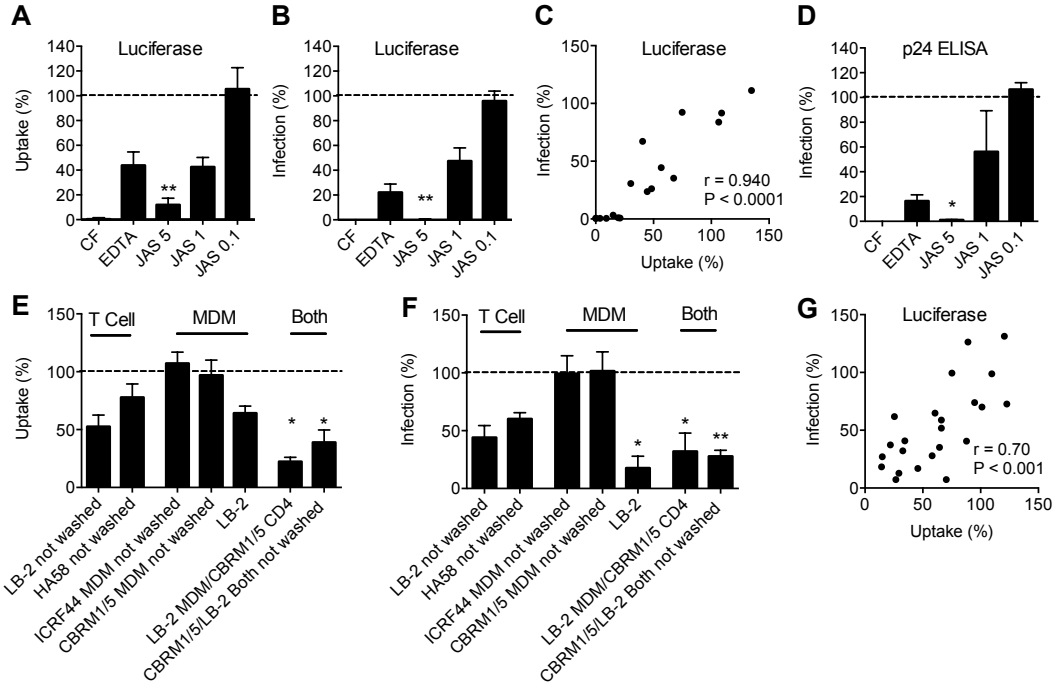

Figure 5.12 Blocking uptake/binding prevents productive infection

Top panels: MDM were pretreated with 2 mM EDTA or jasplakinolide (0.1, 1 and 5 μM) for 1 h prior to co-culture with HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected $CD4^+$ T cells for 30 min at a 1:2 M:T ratio. Unattached/unengulfed T cells were extensively washed with 5 mM EDTA following the co-culture period and MDM were either lysed immediately and analysed for luciferase expression **A)** or were subsequently cultured and productive infection analysed at day 6 post co-culture by measuring luciferase expression in MDM lysates **B).** Mean ± SEM, n = 5 donors. ** P < 0.01, K-W ANOVA plus Dunn's post-test. Data are expressed as a percentage of the relevant vehicle control.

C) Uptake and infection (data from **A** and **B**) were highly correlated by Spearman's correlation coefficient. **D)** Productive infection was also analysed at day 6 post co-culture in the experiment in **B)** by measuring p24 production in supernatants. Mean ± SEM, n = 5 donors. ** P < 0.01, K-W ANOVA plus Dunn's post-test. Data are expressed as a percentage of the relevant vehicle control.

Bottom panels: HIV-$1_{NL\text{-}LucR\text{-}BaL}$-infected $CD4^+$ T cells or MDM were incubated as indicated with anti-ICAM-1 20 μg/mL (LB-2 IgG_{2b} or HA58 IgG_1), anti-Mac-1 20 μg/mL (ICRF44 or CBRM1/5, IgG_1) or combinations as indicated (with washing unless otherwise indicated) for 1 h prior to co-culture with untreated cells at a 1:2 M:T ratio for 30 min. Unattached/unengulfed T cells were extensively washed with 5 mM EDTA following the co-culture period and either lysed immediately and analysed for luciferase expression **E)** or were subsequently cultured and productive infection analysed at day 6 post co-culture by measuring luciferase expression in MDM lysates **F).** Data displayed in **E)** were also presented in Figure 5.10D-E. Mean ± SEM, n = 2 - 6 donors. * P < 0.05, ** P < 0.01 K-W ANOVA plus Dunn's post-test. Data are expressed as a percentage of the relevant vehicle control. **G)** Uptake and infection (data from **E** and **F**) were highly correlated.

However, these data do not exclude the possibility that some of the signal of infection could originate from infected T cells producing *de novo* virus remaining attached to the MDM. To unambiguously show that MDM were productively infected I synchronously infected $CD4^+$ T cells with HIV-$1_{NL\text{-}eGFP\text{-}BaL}$, which produces a cell-intrinsic GFP signal upon productive infection. I co-cultured MDM with HIV-$1_{NL\text{-}eGFP\text{-}BaL}$-infected $CD4^+$ T cells for 6 h, with or without 5 μM AZT treatment in the presence or absence of a 3.0 μm transwell membrane, followed by extensive washing. I fixed and permeabilised MDM at 3 d post co-culture, a time-point when cell-free infection kinetics are delayed, before staining for CD3 to identify T cells and imaging by LSCM (Figure 5.13 A-C). I observed robust productive infection in MDM exposed to HIV-1-infected T cells that was inhibited by AZT and depended on cell-to-cell contacts. Quantitative image analysis of multiple randomly selected low power fields (n = 500 MDM per condition) revealed a significant 18-fold increase in infected MDM over the transwell and AZT conditions (Figure 5.13D).

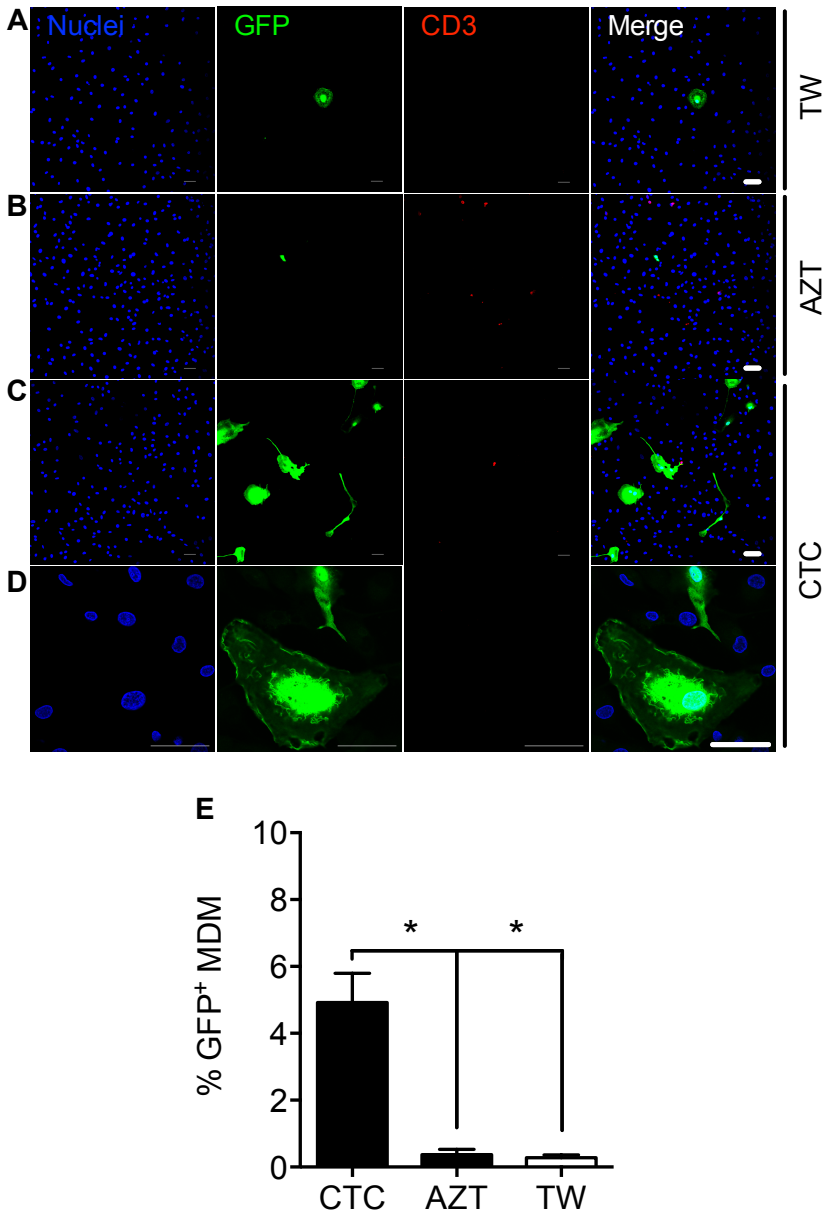

Figure 5.13 Visualising productive MDM infection

HIV-$1_{NL\text{-}eGFP\text{-}BaL}$-infected $CD4^+$ T cells were co-cultured for 6 h with uninfected autologous MDM at a 1:1 ratio (cell-to-cell, CTC), or uninfected MDM pre-treated with 5 μM AZT (which was maintained throughout the experiment) or separated by a 3.0 μm transwell (transwell, TW). Unattached/unengulfed T cells were extensively washed with 5 mM EDTA following the co-culture period, and MDM were subsequently cultured and productive infection analysed at day 3 post co-culture by fixing MDM and analysing GFP signal in MDM by LSCM. Fixed MDM preparations were also stained with Hoechst and anti-CD3. Multiple x20 magnification images were acquired for each condition and a miniumum of 500 MDM scored in image analysis to quantify infection levels. Displayed are representative x20 TW **(A)** AZT **(B)** and CTC **(C)** images. Higher magnification (x100 oil-immersion) images of GFP^+ MDM were also collected, displayed in **(D)**. Infection was expressed as a % of total MDM (minimum of 500 counted per condition per experiment in n = 4 independent donors). Mean ± SEM, * $P < 0.05$, K-W ANOVA plus Dunn's post-test. Scale bars = 20 μm.

5.9 Enhanced efficiency of T/F virus infection

Based on these data with the mac-tropic reference strain BaL it appears that the cell-to-cell interaction leading to T cell uptake results in considerably more efficient MDM infection that cell-free virus inoculation. I therefore hypothesised that this efficient infection route might overcome the reported low macrophage replication capacity (MRC) of T/F viruses. I first confirmed that cell-free inoculation of MDM (MOI 10^{-1}) with multiple pNL4.3-LucR-T2A IMCs bearing distinct T/F virus Envs leads to inefficient replication at d 6 compared to the mac-tropic reference Envs BaL (Clade B) and MW965 (Clade C) and in contrast to $CD4^+$ T cells (Figure 5.14A) as previously reported (Ochsenbauer et al., 2012, Salazar-Gonzalez et al., 2009). I then examined viral replication kinetics in MDM exposed to mac-tropic or T/F virus-infected $CD4^+$ T cells for 6 h +/- transwell followed by extensive washing, as before. The transwell condition was designed to permit direct comparison with cell-free infection efficiency (see Chapter 4). MDM infection values were normalised to the starting $CD4^+$ T cell infection levels (% $CD3^+$ Gag^+ by flow cytometry) for each virus to account for inter-virus variability (Figure 5.14B). In these experiments, co-culture resulted in considerably more efficient replication kinetics for T/F viruses than the equivalent duration of cell-free exposure (Figure 5.15C) as demonstrated by comparison of the area under the curve for each condition. This enhancement in replication efficiency was also evident for luciferase reporter expression (Figure 5.15D). In separate experiments in multiple donors, luciferase expression examined at 6 d.p.i. was significantly higher than the cell-free controls (data not shown). And as was also the case for the reference macrophage-tropic Env BaL, treatment with AZT prevented *de novo* p24 production, (Figure 5.14E). In addition, there was a close correlation between the level of infected T cell uptake immediately following co-culture and the level of intracellular Gag at 6 d.p.i. suggesting a link between uptake and subsequent infection efficiency (Figure 5.14F).

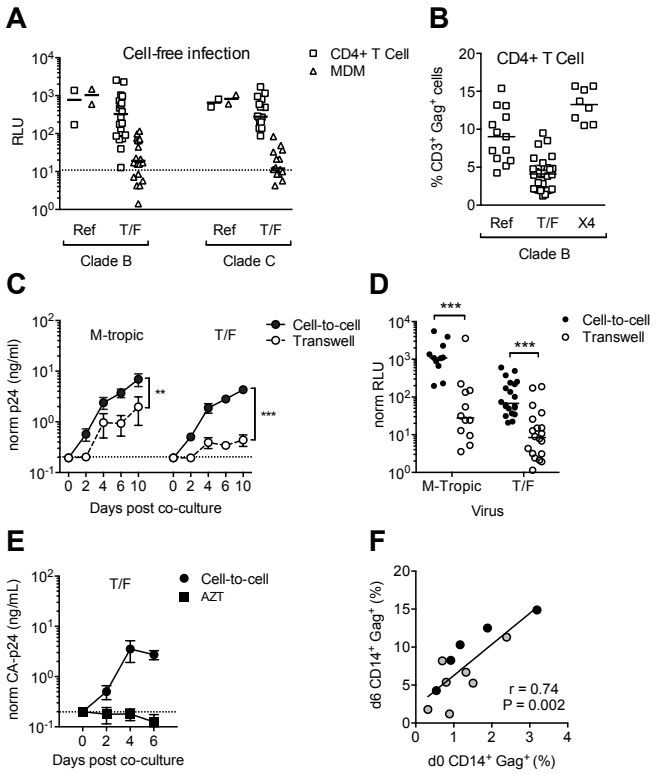

Figure 5.14 Uptake leads to efficient T/F infection

A) $CD4^+$ T cells (open squares) and MDM (open triangles) were infected in duplicate wells with cell-free luciferase expressing R5-using HIV-$1_{NL\text{-}LucR}$ (MOI 10^{-1}) IMCs expressing Clade B or Clade C macrophage-tropic (Ref) or T/F Envs, and analysed for luciferase expression at 6 d.p.i.. Each data point represents an individual donor infected with an individual IMC. Experiments repeated in n = 2 independent donors. Lines represent the median. Dotted line represents the background signal in mock-infected cells. T/F IMCs replicate efficiently in $CD4^+$ T cells but inefficiently in MDM. **B)** Infection levels at 6 d.p.i. by flow cytometry in $CD4^+$ T cells (open squares) infected as in **A)** with Clade B luciferase expressing R5-using HIV-$1_{NL\text{-}LucR}$ (MOI 10^{-1}) IMCs. Each data point represents an individual IMC in a single donor (total donor n = 13 in 6 different experiments). Lines represent the median. **C)** HIV-$1_{NL\text{-}LucR}$-infected $CD4^+$ T cells were co-cultured with uninfected autologous MDM at a 1:1 ratio for 6 h directly (Cell-to-cell, filled circles) or separated by a 3.0 μm transwell (transwell, open circles). Unattached/unengulfed T cells were extensively washed with 5 mM EDTA following the co-culture period, and MDM were subsequently cultured and productive infection analysed at the indicated time-points post co-culture by p24 ELISA. To account for the variability in starting T cell infection level in **B)** MDM data were normalised to T cell infection level (% $CD3^+$ Gag^+). IMCs expressed the Clade B macrophage-tropic Ref Envs BaL or YU2 (M-tropic), and the T/F Envs CH040, THRO, RHPA, WITO, REJO, or SUMA (T/F). Mean ± SEM n = 4 independent donors. Dotted line represents the limit of detection. **D)** In similar experiments MDM were lysed at 10 d.p.i. for luciferase quantification. Each data point represents an individual IMC infection in up to n = 6 independent donors. IMCs expressed the Clade B macrophage-tropic Ref Envs BaL or YU2, and the T/F Envs CH040, THRO, RHPA, WITO, REJO, or SUMA. Bars = median. **E)** Alternatively, MDM exposed to T/F-infected $CD4^+$ T cells were pre-treated and maintained in 5 μM AZT (AZT, filled squares) or media (cell-to-cell, filled circles) and productive infection measured in clarified supernatants by p24 ELISA at the indicated time-points. Mean ± SD n = 2 independent donors. Dotted line represents limit of detection prior to normalisation. ** $P < 0.01$, *** $P < 0.001$ Mann-Whitney test. **F)** Spearman's correlation of T cell uptake by MDM (% $CD14^+$ Gag^+ day 0) vs MDM infection (% $CD14^+$ Gag^+ day 6) measured by flow cytometry in n = 4 independent donors. IMCs expressed the Clade B macrophage-tropic Ref Env BaL (black circles) or T/F Envs CH040, THRO, RHPA (grey circles).

However, since T/F viruses replicate more efficiently in T cells, I was keen to exclude the possibility that these measurements were confounded by T cell contamination of MDM cultures. I set up separate experiments using the non-mac-tropic X4-using lab-derived NL4.3, which does not enter and replicate in MDM (Choe et al., 2001, Jayakumar et al., 2005) the mac-tropic HIV-$1_{NL\text{-}LucR\text{-}BaL}$, and the T/F HIV-$1_{NL\text{-}LucR\text{-}CH040}$. In these experiments, despite efficient $CD4^+$ T cell infection levels (Figure 5.15A), negligible viral replication at 6 d.p.i. was measured by various assays including flow cytometry for intracellular Gag, luciferase infectivity assay, p24 ELISA or an adapted virion capture assay which detects virions budding from T cells, through the use of a biotinylated mAb against CD26, expressed on the surface of virions budding from T cells but not MDM (Lawn et al., 2000), followed by capture with streptavidin magnetic beads (Figure 5.15B-E). These data were supported by direct visualisation of intracellular Gag in MDM by LSCM (Figure 5.15F).

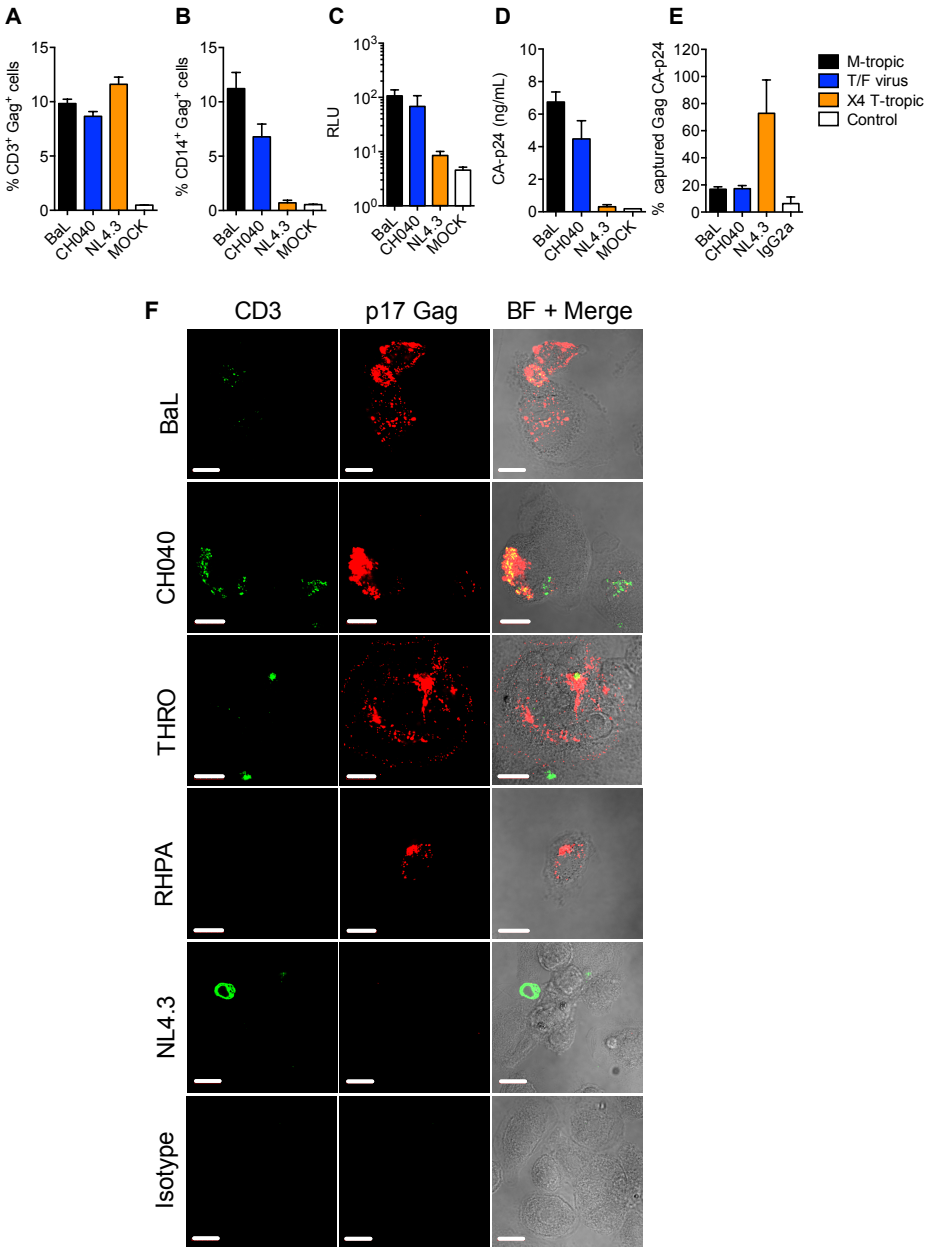

Figure 5.15 Infection is not due to T cell contamination

A) CD4$^+$ T cells were infected in parallel with the M-tropic IMC HIV-1$_{NL\text{-}LucR\text{-}BaL}$, the T/F IMC HIV-1$_{NL\text{-}LucR\text{-}CH040}$ or the X4 T-tropic IMC HIV-1$_{NL\text{-}LucR\text{-}NL4.3}$ and infection assessed at 6 d.p.i. by flow cytometry. Infected CD4$^+$ T cells were co-cultured with uninfected autologous MDM at a 1:1 ratio for 6 h. Unattached/unengulfed T cells were extensively washed with 5 mM EDTA following the co-culture period, and MDM were subsequently cultured and productive infection analysed at 6 d.p.i. by various assays, including **B)** flow cytometry, **C)** luciferase expression, and **D)** p24 ELISA. **E)** In addition, clarified supernatants were incubated with biotinylated anti-CD26 then virions captured using anti-biotin magnetic beads and analysed for p24 content by ELISA. This was expressed as a percentage of the starting p24 content determined in **D)**. Mean ± SEM n = 2-4 independent donors. **F)** MDM at 6 d.p.i. by 6 h co-culture with luciferase-expressing IMC-infected CD4$^+$ T cells were fixed, permeabilised, stained for CD3 and Gag and analysed by LSCM. Representative images acquired at x63 magnification with an oil-immersion objective from repeat experiments in n = 4 donors are displayed.

5.10 Discussion

The data presented here show that MDM rapidly phagocytose $CD4^+$ T cells resulting in more efficient MDM infection than cell-free virus inoculation, particularly for biologically relevant T/F viruses that inefficiently infect MDM by the cell-free route. Control experiments with NL4.3-LucR and the GFP-reporter virus show that the major cellular source of HIV-1 replication is MDM and not contaminating $CD4^+$ T cells.

Macrophage infection through the phagocytic uptake of pathogen-infected cells (Sarantis and Grinstein, 2012) has been described before for the pathogen *Leishmania major* (van Zandbergen et al., 2004) but has not previously been described for a pathogenic virus (Sarantis and Grinstein, 2012). The mechanism of macrophage infection via pathogen-infected cells in *Leishmania* remains unresolved, and is thought to occur either through parasite persistence within the phagosome following engulfment of intact apoptotic cells (in this case neutrophils), or potentially *via* parasite escape from dead/dying cells during the process of phagocytic uptake (Ritter et al., 2009).

Binding/uptake of infected $CD4^+$ T cells by MDM strongly correlated with subsequent productive infection of MDM, and inhibiting uptake resulted in a corresponding reduction in productive infection, which strongly supports the hypothesis that intimate cell-to-cell interactions underpin this efficient infection mechanism. Indeed, over the timescale used in these blocking experiments (30 min) the contribution of cell-free virus to productive infection of MDM was negligible. I made considerable efforts to identify the receptor-ligand interation(s) responsible for mediating this uptake. I was able to show that this was an actin cytoskeleton-dependent process that did not appear to involve HIV-1 Env or conventional ligands (such as PS) on apoptotic cells. The absence of a role for HIV-1 Env or CD4 also argue against a conventional VS,

and interestingly in imaging experiments we rarely observed VS formation as defined by co-capping of Gag/Env on T cells and CD4 on MDM at the site of intercellular contact. This is unusual, since HIV-1-infected $CD4^+$ T cells readily form a VS with uninfected $CD4^+$ T cells (Sattentau, 2010). However, it is possible that the interactions leading to phagocytic engulfment dominate over those leading to VS initiation, or occur more rapidly. The use of EDTA appeared to implicate a Ca^{++}-dependent interaction, and indeed an important role was confirmed for the adhesion molecule ICAM-1, as was also seen in the MDM-T cell VS (Chapter 3). It appears, based on experiments using several blocking antibodies against ICAM-1 and the integrin Mac-1 (CD11b/CD18), that ICAM-1/Mac-1 interactions may mediate this process. It is presently unclear whether this interaction simply facilitates cell-to-cell interaction between these cell types, or whether it is involved in the specific recognition and preferential uptake of infected and/or dying infected $CD4^+$ T cells. Regrettably, I made these observations at the end of my experimental work, when I had insufficient time to properly characterise the binding interaction, and this calls for further careful assessment of the $CD4^+$ T cell cell-surface expression of Mac-1/ICAM-1 in HIV-1 infection and apoptosis, and potentially also knockdown experiments to define the cell-type specificity of the interaction partners. Other members of the Sattentau laboratory are now continuing these experiments.

Regardless of this incompletely defined receptor-ligand interaction, my data implicate macrophages in the initial amplification of HIV-1 in the genital tract mucosa, and could account for the paradoxical situation described earlier, in which infected macrophages are identified in genital tract mucosa and draining lymph nodes *in vivo,* but MDM *in vitro* are resistant to efficient T/F virus replication; these data also indicate that the macrophage reservoir may be founded earlier in infection than previously assumed (Cobos-Jimenez et al., 2011). An additional implication of these data is that the route of macrophage infection could have an important influence on

in vitro assessments of HIV-1 mac-tropism; it may therefore be reasonable, although technically challenging, to incorporate cell-to-cell infection assays when assessing the cellular tropism of primary HIV-1 isolates. However, my data also show that mac-tropic strains such as BaL and YU2 replicate more efficiently than T/F viruses irrespective of route, therefore viral adaptation to replication in macrophages during HIV-1 disease progression, and/or within anatomical sanctuaries such as the CNS - where which $CD4^+$ T cells are scarce - probably remains highly relevant to the development of HIV-1 mac-tropism (see Chapter 1). This is supported by data with the non mac-tropic X4 virus NL4.3, which failed to infect MDM even during cell-to-cell interaction. Thus, cell-to-cell interaction does not breach conventional tropism barriers governed by Env, but rather drives an enhanced efficiency of infection with viruses that are already capable of entry and infection of MDM, albeit with low/very low MRC.

Acute HIV-1 infection is characterised by massive depletion of $CD4^+$ T cells in the GALT (Lackner et al., 2012). Much of this is bystander cell-death, although extensive productive infection of mucosal $CCR5^+$ $CD4^+$ T cells also occurs (Swanstrom and Coffin, 2012). Gut macrophages, unlike several other tissue macrophages, display considerably reduced permissiveness to HIV-1 replication (Shen et al., 2009), through down regulation of CD4/CCR5 and NF-κB, principally resulting from local exposure to TGF-beta (Shen et al., 2011). However they are also highly phagocytic, therefore exploring the consequences of this route of infection for gut macrophages will be of considerable interest.

$CD4^+$ T cells produce many virions in their short life span (relative to MDM) (Dimitrov et al., 1993) and one hypothesis to account for the enhanced efficiency of infection I observed is that this “Trojan horse” route exposes MDM to a high multiplicity infection. However, it is also possible that the interaction with HIV-1-infected,

dead/dying $CD4^+$ T cells (but not their soluble products) might result in macrophage activation, which could in turn influence MDM permissiveness or replication, as has been previously reported for several cytokines including M-CSF (Gavegnano et al., 2013). Indeed, interactions between macrophages and apoptotic lymphocytes drive enhanced macrophage replication of other human pathogens such as *Coxiella burnettii* (Benoit et al., 2008), and interactions with infected T cells have been implicated in pDC activation (Lepelley et al., 2011). My data using a transwell membrane show that soluble secreted factors (such as cytokines) are not responsible for enhanced MDM replication of T/F viruses. Further work is thus indicated to address whether MDM activation results from the cell-cell interaction.

This hypothesis is one of several areas of active investigation in the laboratory, which also include the nature of the macrophage compartment(s) that engulfed T cells are directed to, and the fate of the T cell post engulfment. Preliminary EM imaging (not shown) suggests that large numbers of virions might escape from the T cell during phagocytic uptake, which could position them close to limiting target cell receptors, and suggests that MDM infection may occur rapidly during interaction/uptake (as my data support), potentially implicating an alternative type of intercellular synapse (a 'phagocytic synapse') in the efficient infection of MDM. This requires further analysis. Certainly, my imaging and functional blocking data were not consistent with infection being mediated through a conventional Env-dependent VS, such as that outlined for MDM-T cell transmission in Chapter 3. However, the nature of the mechanism(s) leading to more efficient macrophage infection through interactions with, and phagocytic uptake of, HIV-1-infected $CD4^+$ T cells, clearly needs further definition.

6 CONCLUSION

6.1 Summary of findings

Macrophages and $CD4^+$ T cells are the principal cells capable of efficiently producing HIV-1 *in vivo*. Distinct aspects of HIV-1 biology in these cells complement viral replication. $CD4^+$ T cells are mobile (Murooka et al., 2012) and highly permissive (Swanstrom and Coffin, 2012) but susceptible to viral CPE (Lackner et al., 2012), while macrophages resist viral CPE (Swingler et al., 2007a) and shelter virions over prolonged periods (Sharova et al., 2005b) in distant tissue sites where immune surveillance/ART penetration may be reduced (Sigal and Baltimore, 2012). HIV-1 seeks refuge in the macrophage whilst replicating aggressively in the T cell. However, few studies have addressed interactions between these target cells in the context of HIV-1.

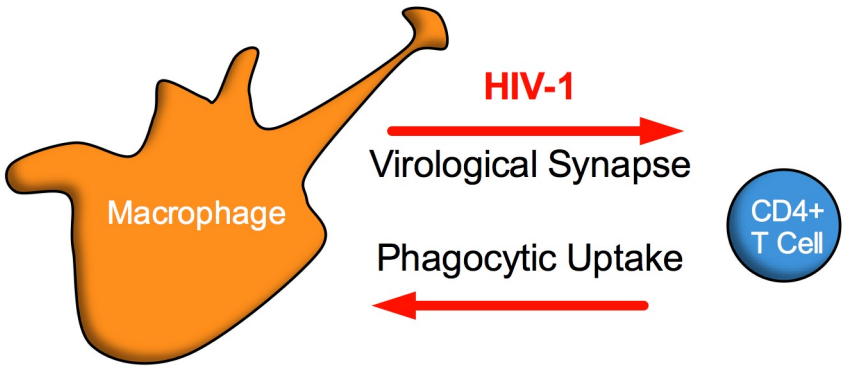

Figure 6 Cell-to-cell transmission between macrophages and $CD4^+$ T cells.

Cell-to-cell transmission is an effective and efficient mode of viral dissemination (Sattentau, 2008). In this thesis I have defined two distinct mechanisms used by HIV-1 for bidirectional spread between these viral producer cells (Figure 6). I have shown that HIV-1-infected MDM mediate frequent contacts with uninfected $CD4^+$ T cells and efficiently transmit a high multiplicity of infection through the VS, which shares

features of synapses formed by both T cells and DC (Chapter 3). Transmission at the VS also involves the active participation of the target T cell (Chapter 3), and the large quantity of viruses transmitted across the VS saturates antiretroviral activity (Chapter 4). Unlike some other cell-culture models of cell-to-cell transmission, transmission at the macrophage-T cell VS is susceptible to gp120-directed neutralising antibody inhibition (Chapter 4). However, there is a striking loss of activity for gp41-mediated neutralising antibodies compared to cell-free activity, apparently mediated by steric hindrance (Chapter 4).

In the opposite direction, I have shown that HIV-1 infected $CD4^+$ T cells act as vectors for efficient infection of macrophages in a novel cell-to-cell transmission route involving phagocytic uptake (Chapter 5 and Figure 6), which permits more efficient macrophage infection with T/F Env-bearing IMCs compared to cell-free infection of macrophages, albeit at a lower magnitude than highly mac-tropic reference strains (Chapter 5). Therefore, these data help to resolve the conflicting observation of infected macrophages *in vivo* with viruses that appear unable to replicate to high levels *in vitro*, and provide insight into the potential role of macrophages in the initial mucosal amplification of transmitted HIV-1 and how the macrophage reservoir may be established.

6.2 Discussion and future priorities

The most significant advance in HIV-1 over the last 30 years is combination antiretroviral therapy (ART), which has turned an invariably fatal disease on its head. Unless diagnosis is delayed or ART unavailable, contemporary HIV clinicians are more occupied with limiting drug toxicities, supporting treatment adherence and managing the consequences of aging in HIV-1 infected patients, than with treating opportunistic infections or terminal care. In lieu of a vaccine, ART has also shown

promise in impacting upon transmission and may prove an important tool in controlling the epidemic.

But the widespread use of ART has highlighted some stark gaps in our basic understanding of HIV-1 pathogenesis and persistence. Immune activation does not significantly abate with the onset of ART (Lackner et al., 2012). Nor does virus production, as highly sensitive PCR assays invariably find low-level production in "suppressed" patients (Doyle and Geretti, 2012). We do not know definitively whether this is the result of ongoing viral replication or continued reactivation of a small fraction of latently infected memory T cells (Eisele and Siliciano, 2012) - but the end result is that ART fails to eradicate established HIV-1 (Sigal and Baltimore, 2012).

It is increasingly suggested (Cobos-Jimenez et al., 2011, Koppensteiner et al., 2012b, Aggarwal et al., 2013, Cory et al., 2013), although not yet conclusively proven, that macrophages are relevant to HIV-1 persistence in the face of ART, not through reactivation of latent virus (Coleman and Wu, 2009) but by low-level productive infection in antiretroviral sanctuary sites (Cory et al., 2013). Defining new mechanisms of macrophage infection should therefore aid our understanding of HIV-1 persistence. There is also a view, supported by my data, that infected macrophages could be more important to pathogenesis than previously recognised, related to their prolonged duration of infection *in vitro* (although this has not been confirmed *in vivo*) and their capacity to efficiently disseminate HIV-1 to $CD4^+$ T cells (Kuroda, 2010, Aggarwal et al., 2013). Since cell-to-cell transmission is currently regarded as the dominant mode of transmission *in vivo* (Mothes et al., 2010), designing interventions to specifically target it seems sensible; a crucial first step is to define the mechanisms of HIV-1 cell-to-cell transmission.

To improve the physiological relevance of these cell-to-cell transmission experiments compared with previous studies, I did all experiments in autologous primary cells. However, there are several limitations to my data, beyond those specifically discussed in the relevant results chapters. Due to the macrophage tropism restrictions at entry (Duncan and Sattentau, 2011), I was only able to test three clade B Envs in macrophage-T cell VS transmission experiments - BaL, YU2 and JRFL. Testing a broader range of mac-tropic Envs would have been informative, particularly given the evidence of subtle differential activity of entry inhibitors against BaL and YU2 (e.g. the bNAb b12 and the peptide T-20, Chapter 4). In ART inhibition experiments I did not explore combinations of antiretroviral drugs, which may have led to more efficient inhibition - even under high viral exposures - than single drug treatment (Sigal et al., 2011), and would more accurately reflect the *in vivo* situation in treated patients. Furthermore, due to technical reasons and a lack of available tissues, I used MDM rather than tissue macrophages. Tissue-specific heterogeneity influences HIV-1 permissiveness and could have important impacts on the efficiency of infection following phagocytosis, or on the efficiency of VS-mediated transmission. Therefore *in vivo* or *ex vivo* studies using tissue macrophages are a critical next step (as outlined below). In line with most comparable studies in the literature, I used stimulated T cells, which may also reduce the *in vivo* relevance of some of my data, although I was able to confirm that macrophage VS-mediated transfer and subsequent productive infection occurred with unstimulated T cells. In several situations, I tested chemical inhibitors that may have unanticipated off-target effects (Carter et al., 2011). For this reason, genetic studies (knockdown or overexpression) are preferable. However, genetic manipulation of primary human macrophages is notoriously difficult (Carter et al., 2011), and may also induce unwanted off-target effects such as type I IFN induction. My use of chemical inhibitors and blocking antibodies should therefore be considered as a useful screening strategy (i.e. to rule out potential contributing factors). Human embryonic stem cell-derived macrophages,

which are more amenable to genetic manipulation (Karlsson et al., 2008), might obviate the need for chemical inhibitors and would be an appropriate future strategy - particularly to define and validate potential up-stream mediators of actin cytoskeletal rearrangement.

Most HIV-1 replication takes place in tissues (Deeks et al., 2012). *S*tudies of viral replication in humans have understandably been limited to analysis of conveniently accessible samples - e.g. blood, CSF and genital secretions. A renewed interest in HIV-1 eradication makes defining viral reservoirs and the cellular source of replication in tissues critically important (Deeks et al., 2012). My studies suggest that the level of macrophage infection governs the probability of drug resistant transmission: transmission to T cells by highly-infected MDM was largely drug resistant, whereas MDM infected at lower MOI transmitted a drug sensitive infection. Thus quantifying the *in vivo* burden of macrophage infection (i.e. by sorting Gag^{+} macrophages and quantifying integrated provirus numbers) might give some indication of whether replication could be initiated by macrophage-T cell transmission under ART. Modelling studies have indicated that if ongoing replication does occur during ART it is likely to be intermittent and self-terminating (Sigal et al., 2011), and therefore does not result in the accumulation of resistance mutations. One could speculate that T cell interactions with a small population of infected macrophages transmitting an antiretroviral-saturating infection would recapitulate this interrupted replication kinetic. Imaging studies of fixed human and SIV tissues might also provide a snapshot of macrophage-T cell interactions occurring *in vivo*, although the technical difficulties of defining the kinetics and dynamics of cell-to-cell transmission *in vivo* remain considerable (Sattentau, 2010). However, significant progress has been made with a humanised mouse model of HIV-1 (Murooka et al., 2012), which has defined a role for motile T cells in viral dissemination.

It would also be instructive to repeat the experiments summarised here in human *ex vivo* tissue macrophages or tissue explants. As mentioned before, heterogeneity exists in the phenotype and permissiveness of macrophages at various anatomical sites (Collini et al., 2010). Preliminary studies have shown MDM to be comparable to some tissue macrophages in terms of HIV-1 permissiveness (Shen et al., 2009), and MDM may represent quite well the phenotype of infiltrating monocyte-derived macrophages under inflammatory conditions (Wynn et al., 2013). It is unclear whether the permissiveness of MDM cultured in gut stromal conditioned media persists in the presence of gut inflammation, such as occurs during acute HIV-1 infection. Regardless, a comprehensive assessment of the ability of tissue-resident macrophages to participate in the cell-to-cell transmission events described here would provide important supportive evidence of their *in vivo* relevance.

The question of whether HIV-1 cell-to-cell transmission is an immune-evasion mechanism often recurs in the literature but remains unclear (see Chapter 4). For other viruses, such as paromyxoviruses and herpes viruses, cell-to-cell transmission can occur without virion exposure to the extracellular milleu, and thus evasion of soluble immune factors is robust (Sattentau, 2008). But in VS-transmission of HIV-1, the steps of target cell entry appear to be equivalent to cell-free infection (Sattentau, 2010). Thus the efficiency of cell-to-cell transmission derives from the close proximity of cells, directed release of virus, recruitment and availability of target cell receptors, and protection from the deleterious effects of dilution- and time-dependent envelope degeneration (Mothes et al., 2010). My data on entry inhibition by antibodies and other agents suggest that gp120 antibodies access the VS and prevent infection, whereas gp41 antibodies are sterically restricted. The reasons for this discrepancy are unclear. One testable hypothesis is that differential glycosylation profiles conferred by the producer cell may have a role in this epitope-specific steric hindrance. My data conflict with the conclusions of recent studies in cell-line based

systems (Abela et al., 2012, Durham et al., 2012). Cell-type dependent effects may be involved in this discrepancy, as has been recently shown for various T cell lines (Zhong et al., 2013), but insufficient data are yet available in primary cells to form a definitive conclusion.

Priorities for future work are summarised in the relevant results chapters (particularly Chapter 5). However, several related questions of interest could be addressed in future work based on the primary cell models developed/refined in this thesis. VS-mediated transmission to specific memory T cell subsets, and the consequences in terms of initiating productive or latent infection, might inform the role of macrophages in seeding latent T cell reservoirs. DC have been shown to activate T cells during IS transmission (Rodriguez-Plata et al., 2013) and preferentially transmit R5-using HIV-1 to R5-expressing effector memory T cells (Groot et al., 2006b). One could hypothesise that macrophages are capable of similar preferential transmission, but whether this results in productive infection or latency would likely depend upon the activation status of the T cell (Stevenson et al., 1990). The impact of antibody-independent immune pressure during intercellular transmission between macrophages and $CD4^+$ T cells, such as that mediated by $CD8^+$ T cells or type I IFN, remains of considerable interest, since data suggest macrophages may be less susceptible to $CD8^+$ T cell suppression of SIV replication (see Chapter 1) and cell-to-cell transmission between T cells can also overcome the antiviral activity of type I IFN (Vendrame et al., 2009). Finally, an intriguing role might exist for the IFN-inducible restriction factor tetherin/BST-2 in cell-to-cell transmission involving macrophages (Tan and Sattentau, 2013). Tetherin has been implicated in the organisation of the macrophage VCC (Chu et al., 2012a), and there are conflicting data on the impact of tetherin on cell-to-cell transmission between T cells (Casartelli et al., 2010, Jolly et al., 2010). Indeed, some highly mac-tropic viruses lack *vpu*, so

the potentially complex role of tetherin in macrophage-initiated VS transmission is worth exploring.

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