

Characterisation of the HIV-1

Reservoir and the Potential for Viral

Eradication

A thesis submitted in partial requirement for the degree of Doctor of Philosophy in
Clinical Medicine

By

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Trinity Term 2014

Abstract:

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The HIV-1 reservoir is the major barrier to eradication and cure of HIV-1 infection. The aim of this thesis was to characterise the HIV-1 reservoir in PHI and to investigate two potential reservoir clearance strategies.

Sensitive Q-PCR assays that quantify HIV-1 DNA reservoir level were developed and applied to characterise the HIV-1 reservoir in acutely infected patients enrolled in the SPARTAC trial. HIV-1 DNA levels were found to be a useful biomarker for predicting clinical progression and the time of viral remission in patients, after HAART interruption. Since low HIV-1 DNA reservoir levels were clinically beneficial, potential therapies aimed at decreasing the reservoir burden were investigated.

Thermotherapy was employed as a possible method for increasing CTL killing ability of HIV-1-infected cells. This may increase the likelihood of virological control and functional cure. SPION uptake impacted negatively on the natural killing ability of CTL that expressed the high affinity 868-TCR. However, localised thermotherapeutic heat generation was insufficient to cause direct thermal ablation of bound target cells or to enhance the natural killing ability of CTL.

868-TCR transduced CTL were employed directly as a means to target the latent viral reservoir by the 'kick and kill' hypothesis. Latent HIV-1 was reactivated in a variety of latent cell line models, but inconclusively in a primary model. 868-TCR transduced CTL killing of latently infected cells was observed. However, reactivation of latent virus did not necessarily relate to increased antigenicity of latent cells, perhaps in part due to anti-latency drug-induced alterations in MHC class I expression.

Despite recent and sustained interest in HIV-1 cure strategies, the prospect still remains elusive. Only through the development of sensitive assays that measure the HIV-1 reservoir and their application to novel and innovative cure strategies will HIV-1 ever be functionally cured or eradicated in patients on a large scale.

Acknowledgements

I would firstly like to thank my supervisors Dr John Frater and Professor Rodney Phillips for allowing me to work in their laboratories in the Peter Medawar Building for Pathogen Research. I would also like to thank the Nuffield Department of Medicine for awarding me the NDM prize studentship, thus enabling me to pursue my scientific career. I would also like to thank all members of the Frater and Goulder group, both past and present for all their help and support (and fun!) over the years. A special thanks to Jacob Hurst, Nicola Robinson and Matthew Pace.

I would also like to thank Una O'Doherty for teaching me her integrated HIV-1 DNA Q-PCR assay in her laboratory, and for providing me with her integration standard.

I would also like to thank Andy Sewell and Adaptimmune, especially Andrew Gerry and Bent Jakobsen for providing me with 868-TCR transduced Cytotoxic T cells and Adaptimmune again for providing me with the HLA-A2 and B2M lentiviral vector. Without this generous donation of reagent, none of this work would have been possible. I would also like to thank the Medical Research Council Clinical Trials Unit for their permission to undertake this project. I would like to thank the organizers of SPARTAC and the participants themselves.

I'd especially like to thank Carmella Elan-Gaston, Louise Durning and Quentin Sattentau for all their advice and support.

However, above all, I would most like to thank my wonderful mother, father, brother and grandmother for their unreserved support, for listening, for advising and for caring. I really could not have done this without you. Oh, and Saffy too.

Contributions to this thesis

All of these projects have depended on the supervision, advice and input from my supervisors Dr John Frater and Professor Rodney Phillips.

Chapter 1

The introduction formed the basis of a book chapter published by InTech with the co-authors Williams J, Fidler S and Frater J (Williams, Fidler et al. 2011) and a review article published in the Quarterly Journal of Medicine with the co-authors Williams J, and Frater J (Williams and Frater 2012).

Chapter 3

Kathleen Gaertner designed the HIV-1 LTR AS/OS primers and probes. Una O'Doherty (University of Pennsylvania) provided the integration standard and taught me how to run the integration assay. Mathew Jones performed all ddPCR experiments (Figure 3.14). Jacob Hurst designed the 'definetherain' software.

The total and albumin assays have been published in the Journal of virology with the following co-authors: Duncan, C. J. Williams, J. P. Schiffner, T. Gartner, K. Ochsenbauer, C. Kappes, J. Russell, R. A. Frater, J. Sattentau, Q. J. (Duncan, Williams et al. 2013). The total and Albumin assays have also been published in the journal of virological methods with the following coauthors: Williams, J. Jones, M. Gartner, K. Phillips, R. Hurst, J. Frater, J, where myself and Mathew Jones contributed equally (Williams, Jones et al. 2014).

The integration assay has been submitted as a manuscript with the following co-authors: Williams, J.P. Hurst, J. Koelsch, K. Stohr, W. Robinson, N. Brown, H. Fisher,

M. Kinloch, S. Cooper, D. Schechter, M. Tambussi, G. Fidler, S. Carrington, M. Babiker, A. Weber, J. Kelleher, A. Phillips, R.E. Frater, J.

Chapter 4

I thank the participants of SPARTAC and the SPARTAC Trial Investigators: Trial Steering Committee: Independent Members- A Breckenridge (Chair), P Clayden, C Conlon, F Conradie, J Kaldor*, F Maggiolo, F Ssali, Country Principal Investigators- DA Cooper, P Kaleebu, G Ramjee, M Schechter, G Tambussi, JM Miro, J Weber. Trial Physician: S Fidler. Trial Statistician: A Babiker. Data and Safety Monitoring Committee (DSMC): T Peto (Chair), A McLaren (in memoriam), V Beral, G Chene, J Hakim. Co-ordinating Trial Centre: Medical Research Council Clinical Trials Unit, London (A Babiker, K Porter, M Thomason, F Ewings, M Gabriel, D Johnson, K Thompson, A Cursley*, K Donegan*, E Fossey*, P Kelleher*, K Lee*, B Murphy*, D Nock*). Central Immunology Laboratories and Repositories: The Peter Medawar Building for Pathogen Research, University of Oxford, UK (R Phillips, J Frater, L Ohm Laursen*, N Robinson, P Goulder, H Brown). Central Virology Laboratories and Repositories: Jefferiss Trust Laboratories, Imperial College, London, UK (M McClure, D Bonsall*, O Erlwein*, A Helander*, S Kaye, M Robinson, L Cook*, G Adcock*, P Ahmed*). Clinical Endpoint Review Committee: N Paton, S Fidler. Investigators and Staff at Participating Sites: Australia: St Vincents Hospital, Sydney (A Kelleher), Northside Clinic, Melbourne (R Moore), East Sydney Doctors, Sydney (R McFarlane), Prahran Market Clinic, Melbourne (N Roth), Taylor Square Private Clinic, Sydney (R Finlayson), The Centre Clinic, Melbourne (B Kiem Tee), Sexual Health Centre, Melbourne (T Read), AIDS Medical Unit, Brisbane (M Kelly), Burwood Rd Practice, Sydney (N Doong), Holdsworth House Medical Practice,

Sydney (M Bloch), Aids Research Initiative, Sydney (C Workman). Coordinating Centre in Australia: Kirby Institute University of New South Wales, Sydney (P Grey, DA Cooper, A Kelleher, M Law). Brazil: Projeto Praca Onze, Hospital Escola Sao Francisco de Assis, Universidade federal do Rio de Janeiro, Rio de Janeiro (M Schechter, P Gama, M Mercon*, M Barbosa de Souza, C Beppu Yoshida, JR Grangeiro da Silva, A Sampaio Amaral, D Fernandes de Aguiar, M de Fatima Melo, R Quaresma Garrido). Italy: Ospedale San Raffaele, Milan (G Tambussi, S Nozza, M Pogliaghi, S Chiappetta, L Della Torre, E Gasparotto), Ospedale Lazzaro Spallanzani, Roma (G DOffizi, C Vlassi, A Corpolongo). South Africa: Cape Town: Desmond Tutu HIV-1 Centre, Institute of Infectious Diseases, Cape Town (R Wood, J Pitt, C Orrell, F Cilliers, R Croxford, K Middelkoop, LG Bekker, C Heiberg, J Aploon, N Killa, E Fielder, T Buhler). Johannesburg: The Wits Reproductive Health and HIV-1 Institute, University of Witswatersrand, Hillbrow Health Precinct, Johannesburg (H Rees, F Venter, T Palanee), Contract Laboratory Services, Johannesburg Hospital, Johannesburg (W Stevens, C Ingram, M Majam, M Papathanasopoulos). Kwazulu-Natal: HIV-1 Prevention Unit, Medical Research Council, Durban (G Ramjee, S Gappoo, J Moodley, A Premrajh, L Zako). Uganda: Medical Research Council/Uganda Virus Research Institute, Entebbe (H Grosskurth, A Kamali, P Kaleebu, U Bahemuka, J Mugisha*, HF Njaj*). Spain: Hospital Clinic-IDIBAPS, University of Barcelona, Barcelona (JM Miro, M Lopez-Dieiguez*, C Manzardo, JA Arnaiz, T Pumarola, M Plana, M Tuset, MC Ligerro, MT Garca, T Gallart, JM Gatell). UK and Ireland: Royal Sussex County Hospital, Brighton (M Fisher, K Hobbs, N Perry, D Pao, D Maitland, L Heald), St James' Hospital, Dublin (F Mulcahy, G Courtney, S ODea, D Reidy), Regional Infectious Diseases Unit, Western General Hospital and Genitourinary Dept, Royal Infirmary of Edinburgh, Edinburgh (C

Leen, G Scott, L Ellis, S Morris, P Simmonds), Chelsea and Westminster Hospital, London (B Gazzard, D Hawkins, C Higgs), Homerton Hospital, London (J Anderson, S Mguni), Mortimer Market Centre, London (I Williams, N De Esteban, P Pellegrino, A Arenas-Pinto, D Cornforth*, J Turner*), North Middlesex Hospital (J Ainsworth, A Waters), Royal Free Hospital, London (M Johnson, S Kinloch, A Carroll, P Byrne, Z Cuthbertson), Barts & the London NHS Trust, London (C Orkin, J Hand, C De Souza), St Marys Hospital, London (J Weber, S Fidler, E Hamlyn, E Thomson*, J Fox*, K Legg, S Mullaney*, A Winston, S Wilson, P Ambrose), Birmingham Heartlands Hospital, Birmingham (S Taylor, G Gilleran). Imperial College Trial Secretariat: S Keeling, A Becker. Imperial College DSMC Secretariat: C Boocock.

* Left the study team before the trial ended.

The SPARTAC trial was funded by a grant from the Wellcome Trust (069598/Z/02/Z).

Nicola Robinson extracted the majority of HIV-1 DNA from CD4+ T cell enriched SPARTAC PBMCs and also ran some total and integrated HIV-1 DNA assays. Jacob Hurst provided invaluable advice on statistical methodology and independently verified the results presented in accordance with the wishes of the Medical Research Council Clinical Trials Unit. Wolfgang Stohr and Abdel Babiker (MRC CTU, London) also provided advice on statistical methodology as well as providing all the necessary information including HLA-type, viral clade, viral load, CD4+ T cell count, estimated time since seroconversion at enrolment, time to primary endpoint, time on antiretroviral treatment, and time to viral rebound to 50 and 400 RNA copies / mL plasma.

Cellular activation and exhaustion marker analysis was performed by Stephen Hickling. CD4 and CD8+ T cell ELISpots were performed by Nicola Robinson and Helen Brown. D-dimer and IL-6 concentration in plasma were determined by Elizabeth Hamlyn. Mary Carrington provided the datasets needed for Figure 4.12. HLA typing was performed by the Oxford Transplant Centre, Churchill Hospital, University of Oxford and University of Oklahoma by Steven Cate.

This chapter formed the basis for work submitted as a manuscript with the following co-authors: Williams, J.P. Hurst, J. Stohr, W. Robinson, N. Brown, H. Fisher, M. Kinloch, S. Cooper, D. Schechter, M. Tambussi, G. Fidler, S. Carrington, M. Babiker, A. Weber, J. Koelsch, K. Kelleher, A. Phillips, R.E. Frater, J.

Work from this chapter also formed the basis of late breaker abstract (WELBA04), (Williams et al., 2013a) presented orally by myself at IAS 2013, the pre-conference symposium, press conference and filmed interview all in Kuala Lumpur. Co-authors were Williams, J.P. Hurst, J. Robinson, N. Fidler, S. Weber, J. Babiker, A. Phillips, R.E. Koelsch, K. Kelleher, T. Frater J.

Work from this chapter was also submitted as a late breaker poster (403LB) (Williams et al., 2014b), presented at CROI 2014 (Hurst, Williams et al 2014). Co-authors included Hurst, J. Williams, J.P, Hamlyn, E. Koelsch, K. Fidler, S. Babiker, A. Weber, J. Kelleher, T. Phillips, R.E and Frater, J.

Chapter 5

James Ussher, Chris Willberg and Nicole Yager provided valuable advice for FACS analysis. Saman Ghannadzadeh (Department of Physics, University of Oxford) assisted with SQUID experiment analysis. Helen Fryer (Department of Zoology)

provided statistical advice. Helen Christian (Department of Physiology, Anatomy and Genetics) assisted with TEM sample preparation. Anya Lissina and Andy Sewell (Cardiff University) provided CTLs transduced with the 868-TCR. Quentin Pankhurst and Paul Southern (Department of Physics, Royal Institute, UCL) provided essential advice on nanotechnology and provided the MACH system. Experiments identifying the IL-P of Feraspin-R (Figure 5.2) and uptake of other SPIONs into macrophages (Figure 5.13) were performed by Paul Southern.

This chapter has formed the basis of a publication in the International Journal of Nanomedicine with the following co-authors: Williams, J. P. Southern, P. Lissina, A. Christian, H. C. Sewell, A. K. Phillips, R. Pankhurst, Q. Frater, J. (Williams, Southern et al. 2013)

Chapter 6

Una O'Doherty and Matthew Pace assisted in teaching me the primary model of latency. Matthew Pace also performed some experiments whereby MHC class I downregulation was investigated in human CD4⁺ T cells (Figure 6.11) and helped with general experimental and analytical advice. Andrew Gerry and Bent Jakobsen from Adaptimmune provided 868-TCR transduced CTLs from multiple donors and a lentiviral vector encoding HLA-A2 and B2M.

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Abbreviations

Ac	Acetylated
ACTG	AIDS Clinical Trials Group
AIDS	Acquired Immunodeficiency Syndrome
Alu	Arthrobacter lutherus
ALU	Specific amplification of Alu-gag integrated HIV-1 DNA
APC (cy7)	Allophycocyanin (cytochrome 7)
ART	Antiretroviral Therapy
ART-12	SPARTAC arm, where patients received 12 weeks of HAART, before TI
ART-48	SPARTAC arm, where patients received 48 weeks of HAART, before TI
AVOVA	Analysis of Variance
BCL-2	B Cell Lymphoma-2
BHQ	Black Hole Quencher
BLT	Bone marrow, liver and thymus
Cas	CRISPR associated
CASCADE	Concerted Action on SeroConversion to AIDS and Death in Europe
CCR	Chemokine c-c motif receptor
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CHERUB	Collaborative HIV-1 Eradication of viral Reservoirs: UK BRC
CL	Confidence Limits
CMV	Cytomegalovirus
CNS	Central Nervous System
CRISPR	Clustered regularly interspaced short palindromic repeat
Ct	Cycle Threshold
CTL	Cytotoxic T cell
CTO	Cell Tracker Orange
CTV	Cell Tracker Violet

CXCR	Chemokine c-x-c motif receptor
ddPCR	Droplet digital PCR
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
<i>E. Coli</i>	Escherichia coli
E:T	Effector:Target
EC	Elite Controller
ECD	Phycoerythrin-Texas Red conjugate (energy coupled dye)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent Assay
ELISpot	Enzyme-linked Immunosorbent spot assay
emu	Electromagnetic units
<i>env</i>	Envelope glycoproteins (gp120 and gp41)
ER	Endoplasmic reticulum
ETS	Estimated time since seroconversion at enrolment
FACS	Fluorescence Activated Cell Sorting
FAM	Carboxyfluorescein
FATAL	Fluorometric assessment of T cell activated Lysis
FCS	Foetal calf serum
FDA	US Food and Drug Administration
FITC	Fluorescein Isothiocyanate
FL1	Fluorochrome 1
FMO	Fluorescence minus one
FSC	Forward Scatter
GAG	Background gag-only HIV-1 DNA
<i>gag</i>	Group-specific Antigen (Capsid proteins)
GALT	Gut Associated Lymphoid Tissue
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase

GFP	Green Fluorescent Protein
gp	Glycoprotein
GPR	Gag Positive Reservoir
HAART	Highly Active Antiretroviral Therapy
HAT	Histone Acetyl Transferase
HBT	Human Breast Tumour
HDACi	Histone Deacetylase Inhibitors
HEXIM	Hexamethylene bisacetamide inducible molecule
HIV-1(-1)	Human Immunodeficiency Virus (1)
HLA	Human Leukocyte Antigen
HMBA	Hexamethylene bisacetamide
HPTN	HIV-1 Prevention Trials Network
HR	Hazard ratio
hTERT	Human telomerase reverse transcriptase
IFN	Interferon
IL	Interleukin
IO	Ionomycin
IQR	Interquartile range
IR	Infra red
IS	Integration Standard
IU	International Units
IUPM	Infectious Units per Million input cells
I κ B α	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
kA	Kiloampere
LAG	Lymphocyte activation gene
LB	Luria-Bertani Medium
LC	LightCycler
LCMV	Lymphocytic choriomeningitis
LMP	Latent Membrane Protein

Ln	Natural Logarithm
Log ₁₀	Logarithm to the base of 10
LTNP	Long Term Non Progressor
LTR	Long Terminal Repeat, containing regulatory elements
Itx	SPARTAC participants who subsequently re-started long term HAART after reaching primary endpoint
MACH	Magnetic Alternating Current Hyperthermia
MFH	Magnetic Fluid Hyperthermia
MFI	Median Fluorescence Intensity
MgCl ₂	Magnesium Chloride
MHC	Major Histocompatibility Complex
MHz	Megahertz
min	Minutes
MOI	Multiplicity of Infection
MRI	Magnetic resonance Imaging
mRNA	Messenger RNA
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
<i>nef</i>	Negative Regulatory Factor Protein
NFAT	Nuclear Factor of Activated T-cells
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIH	National Institute of health
NOD	Non Obese Diabetic
OD	Optical Density
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD	Programmed Death Receptor
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll
PHI	Primary HIV-1 Infection

PI3-K	Phosphatidylinositol-4-5-bisphosphate 3-kinase
PKC	Protein Kinase C
PMA	Phorbol 12-myristate 13-acetate
<i>pol</i>	Viral enzymes (protease, reverse transcriptase and integrase)
PTC	Post Treatment Controller
pTEFB	Positive Transcription Elongation Factor B
PTEN	Phosphatase and Tensin homologue
ptx	SPARTAC participants who received 48-weeks of HAART after randomization
Q-PCR	Quantitative PCR
Qdot	Quantum Dot
<i>rev</i>	Regulator of Expression of Virion Proteins
RNA	Ribonucleic Acid
RNAP	RNA Polymerase
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
s	Seconds
S.D	Standard deviation
SAHA	Suberoylanilide Hydroxamic Acid; Vorinostat
SCID-hu	Severe Combined Immunodeficient mice implanted with Human Foetal Tissues
SEB	Staphylococcus Enterotoxin B
SIV	Simian Immunodeficiency Virus
SL9	SLYNTVATL
SMART	Strategies for Management of Antiretroviral Therapy
SOC	Standard of Care SPARTAC trial arm.
SPARTAC	Short Pulse Anti-Retroviral Therapy at Seroconversion
SPION	Superparamagnetic Iron Oxide Nanoparticles
SQUID	Superconducting Quantum Interference Device
SSC	Side Scatter

TAP	Tapasin Associated Protein
TAR	Transactivating region
<i>tat</i>	Trans-activator of HIV-1 gene expression
TC	Tissue Culture
T _{CM}	Central memory T cell
TCR	T cell Receptor
T _{EM}	Effector memory T cell
TEM	Transmission Electron Microscopy
TET	Tetramer
TH	T-Helper
TI	Treatment Interruption
TIM	T cell Immunoglobulin and Mucin protein
TNF	Tumour Necrosis Factor
T _{SCM}	T cell with stem cell like properties
T _{TM}	Transitional memory T cell
U	Units
<i>vif</i>	Viral Infectivity Factor
VISCONTI	Viro-Immunological Sustained Control after Treatment Interruption
VPA	Valproic Acid
<i>vpr</i>	Viral Protein R
<i>vpu</i>	Viral Protein U
WHO	World Health Organization
wk	Week
WT	Wild type

WORD COUNT: 40723

1. Introduction

This chapter formed the basis of a review article (Williams and Frater, 2012) and 'Characterisation, Evaluation and Clinical Significance of Latent HIV-1 Reservoirs and Therapeutic Strategies for HIV-1 Eradication' – Chapter 3 of the InTech published book entitled 'Recent Translational research in HIV-1/AIDS' (Williams et al., TANG, P. Y.-W., 2011).

1.1 Human Immunodeficiency Virus-1

Human Immunodeficiency Virus-1 (HIV-1) is a Lentivirus and member of the *Retroviridae* family of viruses. When HIV-1 infects a cell, its genome is reverse transcribed into cDNA, which integrates into the host cell genome. In productive infection, transcription and translation of the integrated HIV-1 proviral DNA ensues, creating the proteins needed for the formation of new infectious virions.

1.1.1 Structure of HIV-1

The HIV-1 genome consists of two single-strand, positive sense RNA copies that are bound to nucleocapsid proteins and the viral enzymes integrase and reverse transcriptase. The viral genome and accessory and regulatory proteins Nef, Vpr and Vif are surrounded by the viral protein p24 core, which itself is surrounded by the protein p17 matrix layer. A host-derived lipid bilayer surrounds the matrix layer and contains the viral envelope glycoproteins gp120 and gp41. The envelope enables HIV to bind to receptors expressed on target cells, thus leading to membrane fusion and infection (all reviewed in (Stevenson, 2003)) (structure summarised in Figure 1.1).

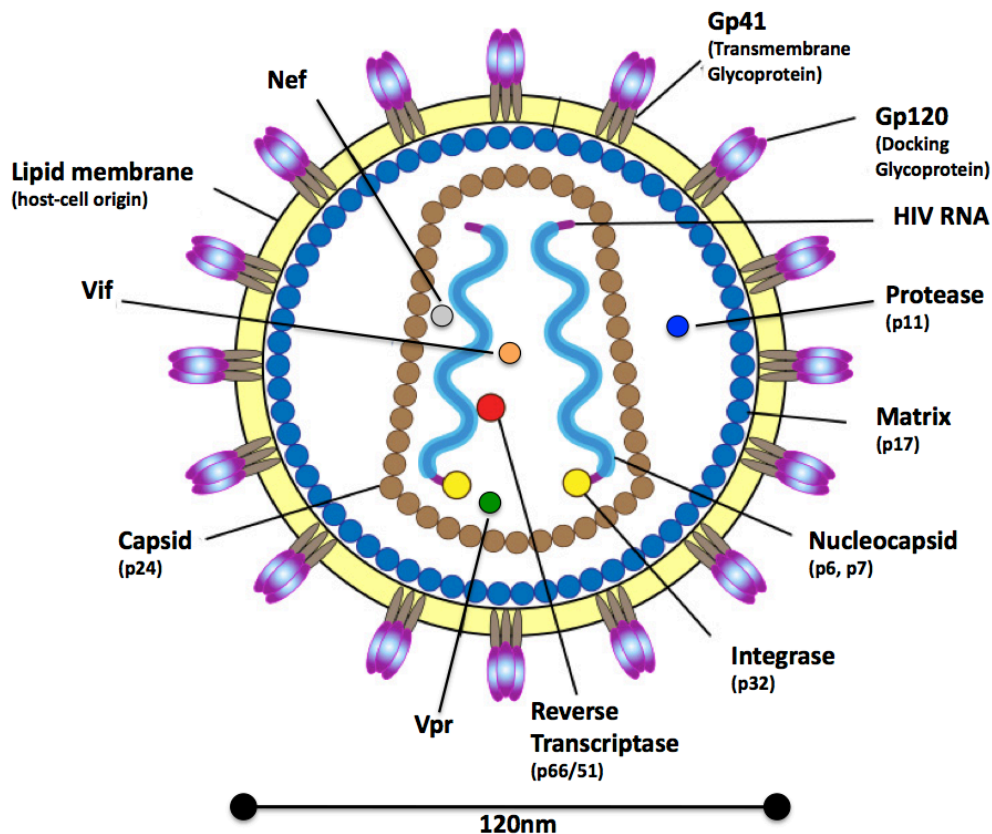


Figure 1.1. Schematic structure of an HIV-1 virion. (Adapted from http://commons.wikimedia.org/wiki/File:HIV-1_Virion.svg)

1.1.2 The HIV-1 genome (summarized in Fig 1.1 and 1.2)

The HIV-1 genome is approximately 9.7kB in length. The *gag* gene encodes the core structural proteins, p6, p7, p17 and p24. *Pol* encodes protease, reverse transcriptase and integrase enzymes, which are necessary for productive infection and viral release. *Env* encodes the envelope glycoprotein gp160, which is cleaved into gp120 and gp41; glycoproteins used for target cell binding and fusion. HIV-1 contains 6 additional accessory genes: *nef*, *rev*, *tat*, *vif*, *vpr* and *vpu*, which increase viral transcription and infectivity, cell-to-cell transmission and also modulate host immune responses. The viral promoter or long terminal repeat (LTR) sequences control subsequent viral production through host and viral *tat* mediated mechanisms (all reviewed in (Greene and Peterlin, 2002)) (summarised in Figure 1.2).

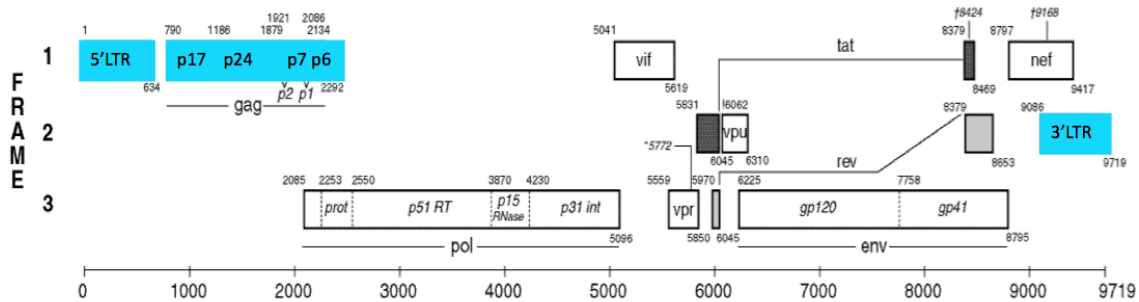


Figure 1.2. HIV-1 Genome. (Adapted from Los Alamos National Library: <http://www.HIV-1.lanl.gov/content/sequence/HIV-1/MAP/landmark.html>). Gene regions coloured blue are of particular relevance to this thesis.

1.1.3 The HIV-1 replication cycle

HIV-1 infects CD4+ T-cells (preferentially HIV-1-specific CD4+ T cells (Douek et al., 2002)), macrophages (Embretson et al., 1993) microglial cells and astrocytes (Trillo-Pazos et al., 2003) and possibly CD34+ haematopoietic stem cell progenitor cells (McNamara et al., 2012, Carter et al., 2010). HIV-1 gp120 binding to the cell surface receptor CD4 induces conformational changes, which enhance chemokine c-c motif receptor 5 (CCR5) (Alkhatib et al., 1996, Deng et al., 1996) or chemokine c-x-c motif receptor 4 (CXCR4) co-receptor binding (Kwong et al., 1998). After fusion and entry, the HIV-1 RNA genome is reverse transcribed into cDNA, imported into the nucleus and integrates into the host cell DNA (reviewed in (Greene and Peterlin, 2002, Blankson et al., 2002)), preferentially into the introns of actively expressed genes (Schroder et al., 2002). The earliest transcription of HIV-1 genes is Tat independent. However, Tat leads to trans-activation and further transcription of the viral genome. Nef leads to CD4, CXCR4/CCR5 and Major Histocompatibility Complex (MHC) Class 1 downregulation, protecting the infected cell from host immune responses and superinfection. Rev aids nuclear export of the unspliced transcripts encoding structural and enzymatic proteins as well as the full length HIV-1 RNA genome, (all reviewed in (Greene and Peterlin, 2002)).

Packaging of the HIV-1 virus ensues after viral transcription and translation is completed. Functional viral proteins are created through protease-mediated cleavage of polypeptide chains. Mature infectious virions then bud from the host cell membrane. Infected cells are thought to die due to CD8+ T cell cytolysis, or viral cytopathic effects (all reviewed in (Greene and Peterlin, 2002)). Some studies suggest macrophages are more resilient to the viral cytopathic effect (Cobos-Jimenez et al., 2011). The lifecycle of HIV-1 is summarised in (Rambaut et al., 2004) and Figure 1.3.

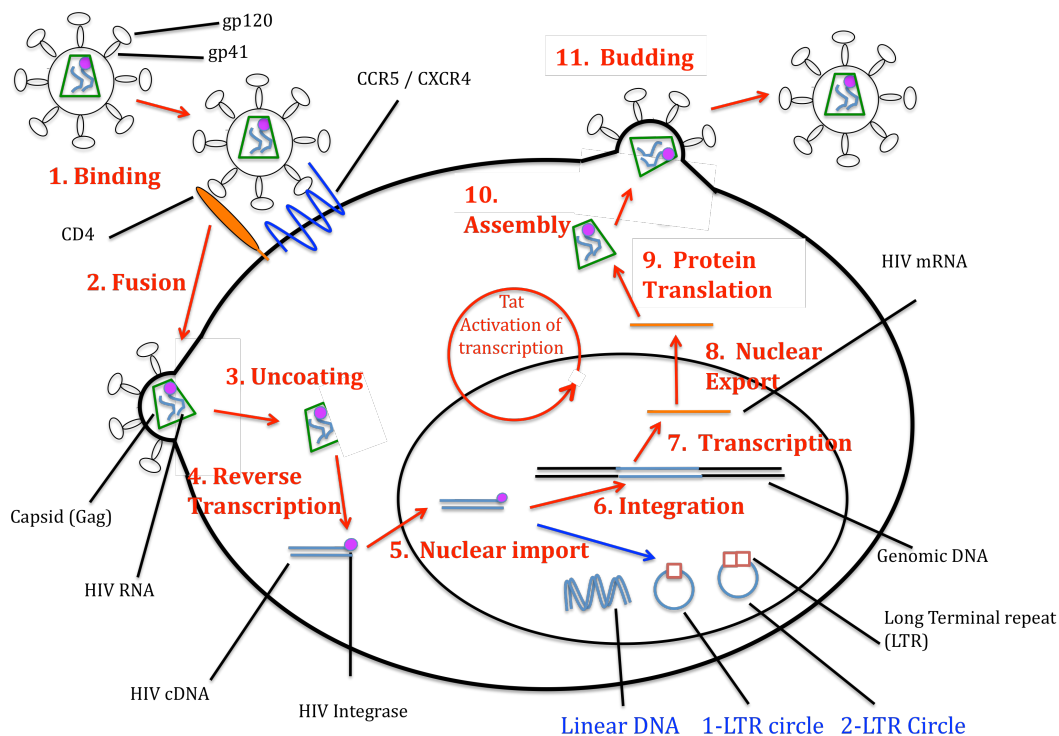


Figure 1.3. HIV-1 replication cycle. Unintegrated DNA products are represented in blue text. Loosely based on (Rambaut et al., 2004) Figure 1.

1.1.4 HIV-1 disease progression

HIV-1 is transmitted through the exchange of infected bodily fluids, sexually, intravenously or from mother-to-child. Studies of SIV in macaques have demonstrated partially activated genital mucosa T cells to be the earliest target cells supportive of productive SIV replication (Zhang et al., 1999). Similar studies suggested SIV then disseminates through draining lymph nodes and into the Gut Associated Lymphoid Tissue (GALT) where extensive memory CD4⁺ T cell depletion occurs (Li et al., 2005, Mattapallil et al., 2005).

The acute phase of HIV-1 infection occurs two to four weeks post HIV-1 transmission. It is thought that in the majority of patients, HIV-1 infection starts from one single founder virus (Keele et al., 2008). Patients may experience flu-like symptoms, lymphadenopathy and fever (Gurunathan et al., 2009) with viral loads as high as 10^7 copies per mL of plasma and a subsequent decrease in CD4⁺ T cell count (Little et al., 1999, Kaufmann et al., 1998), predominantly in the GALT (Brenchley et al., 2004) (Figure 1.4). The Fiebig stages of acute infection can be distinguished using laboratory tests (Fiebig et al., 2003) (Figure 1.4). After peak viremia, virus levels decrease to a set point, concomitantly with the appearance of CD8⁺ T cell responses in the chronic phase of HIV-1 infection (Borrow et al., 1994, Koup et al., 1994). This viral set point is strongly predictive of rate of progression to AIDS (Mellors et al., 1996). CD4⁺ T cell counts then partially recover during the 'clinical latency' phase, where patients are mostly asymptomatic.

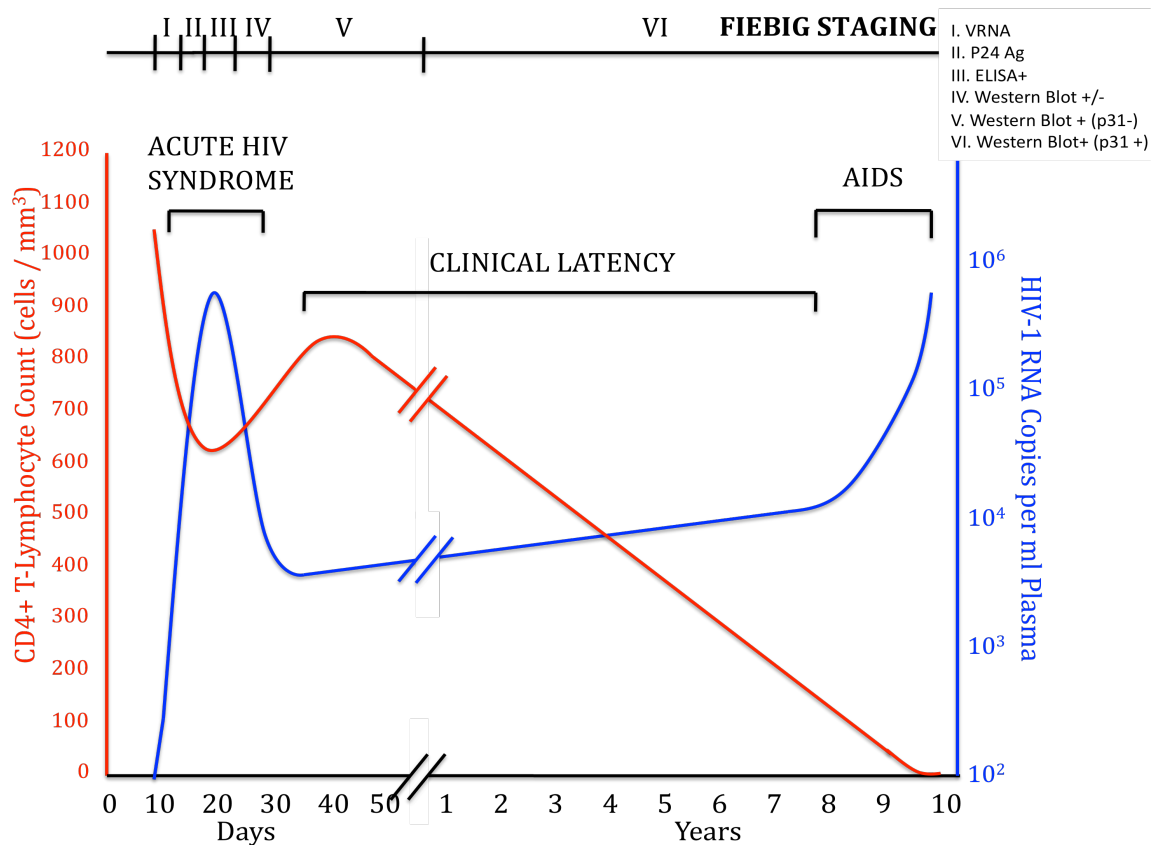


Figure 1.4 Cartoon depicting a typical course of HIV-1 infection with Fiebig staging, determining the stage of acute infection.

Adapted from <http://commons.wikimedia.org/wiki/File:HIV-1-timecourse.png>.

Shortly after the initiation of CD8+ T cell responses during acute infection, HIV mutates in response to selective immune pressure (Fratr et al., 2007). This mutational propensity of HIV-1 enables it to avoid host immune responses (Goulder et al., 1997a, Phillips et al., 1991). In the chronic phase of HIV-1 infection, the CD4+ T cell count continues to decline and the immune dysfunction associated with late-stage HIV-1 allows opportunistic infections eventually to claim the lives of HIV-1 infected patients, in AIDS (Poli et al., 1993) (Figure 1.4).

1.2. HIV-1 specific T cell Immune Responses

1.2.1 The Role of CD8+ T cells in HIV-1 control

CTLs are thought to play a pivotal role in control of HIV-1 viremia. HIV-1-specific CTLs recognise HIV-1-infected cells that express MHC class I alleles containing processed peptides (8-11 amino acids in length) of viral origin in their binding grooves (Zinkernagel and Doherty, 1997). CTL were first demonstrated to mount peptide specific responses against HIV-1 in 1988 (Nixon et al., 1988). In acute HIV-1 infection, the appearance of HIV-1-specific CD8+ CTL responses correlates with the decline in peak viremia (Figure 1.4), implicating a role for CTLs in control of HIV-1 replication (Borrow et al., 1994, Koup et al., 1994). The administration of anti-CD8 monoclonal antibodies in SIV-infected macaques inhibited the decline of peak viremia (Matano et al., 1998) and in chronic SIV infection, increased viral load (Schmitz et al., 1999). Also, the presence of more HIV-specific CTLs resulted in lower viral loads (Ogg, Jin et al. 1998) and a recent study showed that strong Gag specific CD8+ T cell responses were associated with a lower viral load (Kiepiela et al., 2007).

There are three major MHC class I alleles: Human Leukocyte Antigen (HLA)-A, B and C. These are highly polymorphic and lead to a very diverse repertoire of presented peptide. MHC class I allele expression associates strongly with HIV-1 disease outcome. For example, HLA-B*35:02/03 expression associates with rapid disease progression and high viral load set points (Gao et al., 2001, Carrington et al., 1999). Conversely, HLA-B*27:05 and HLA-B*57:01/02/03 expression associates with slow disease progression and low viral load set points (O'Brien et al., 2001, Migueles et al., 2000, Kaslow et al., 1996). Viraemic control was observed

in HLA-B*27 positive patients who made strong CTL responses against the immunodominant gag epitope KRWIILGLNK (KK10) (Goulder et al., 1997b, Ammaranond et al., 2005). Also, simultaneous expression of multiple protective MHC class I alleles may result in even better viral control (Leslie et al., 2010).

The CTL selection pressure can be weakened if viral peptide sequences mutate enough so that they are no longer recognised by CTL. This can lead directly to immune escape, a hallmark of chronic phase HIV-1 infection (Phillips et al., 1991). However, such escape mutations may be associated with high viral fitness costs (Feeney et al., 2004, Goulder et al., 1997b, Martinez-Picado et al., 2006). There is also evidence that certain escape mutations revert back to a wild type sequence if transmitted to an HLA mismatched host, presumably because CTL selection pressure has been relieved (Leslie et al., 2004). Incidentally, CD8+ T cell responses against Gag are deemed to be the most protective and are most commonly associated with long term virological control (Kiepiela et al., 2007), perhaps due to their immunogenic and conserved nature (reviewed in (Goulder and Watkins, 2008)).

Such protective HLA alleles are enriched in patients who naturally progress to AIDS at much slower rates, termed 'Long Term Non Progressors' (LTNP) (Kaslow et al., 1996, Carrington and O'Brien, 2003, Hendel et al., 1999). CTL from LTNPs and elite controllers (ECs), a subset of LTNPs who maintain viral loads below the level of detection of clinical assays, may bind to HLA molecules with higher avidity, may be more polyfunctional, and have a higher clonal turnover (Almeida et al., 2007, Betts et al., 2006, Critchfield et al., 2008, Horton et al., 2006), express more Perforin (Migueles et al., 2002) and have better lytic granule loading perhaps

because of more efficient nuclear factor of activated T cell (NFAT) translocation (Migueles et al., 2008). Conversely, HLA homozygosity, which causes decreased peptide diversity, is associated with more rapid disease progression (Carrington et al., 1999).

However, the majority of patients, even those expressing protective HLA-alleles, still progress to AIDS (Hay et al., 1999, Draenert et al., 2004). Despite immunodominant CTL gag responses in HLA-B*27/B*57 positive controllers and progressors being quantitatively similar, controllers may be more able to inhibit viral replication by expressing more cross-reactive T cell receptor (TCR) clonotypes against epitope variants. This could explain the differences in outcome of HLA-B*27 and B*57 expressing patients (Chen et al., 2012).

Together, these data indicates that CTL pressure mediated through protective HLA Class 1 epitope expression is an important factor for virological control.

1.2.2 CD8+ T cell dysfunction in HIV-1 infection

Despite CD8+ T-cells partially limiting HIV-1 replication (Schmitz et al., 1999, Hersperger et al., 2011, Walker et al., 1987), HIV-1 specific CTLs lose their polyfunctional effector functions hierarchically over time in response to chronic antigen exposure (Wherry et al., 2003). Usually IL-2 release, proliferative and killing capacities are the first to be lost, followed by TNF α and the IFN γ response the last to cease. HIV-1-specific CD8+ T cells isolated from humans that lack any *ex vivo* effector function are associated with low CD4+ T cell counts and high viral loads (Wherry and Ahmed, 2004). The final stage of CTL exhaustion is deletion of that cell (Zajac et al., 1998).

CTL dysfunction can be regulated by the cellular expression of inhibitory receptors or 'exhaustion markers' (Virgin et al., 2009) such as Programmed Death Receptor-1 (PD-1) (Trautmann et al., 2006, Day et al., 2006, Petrovas et al., 2006), whose function is thought to dampen down the immune system and prevent over-activation. Inhibition of PD-1 in chronically HIV-1-infected humanized mice (Seung et al., 2013) and SIV-infected macaques (Velu et al., 2009) lead to lower viral loads and improved CD8⁺ T-cell responses. Other inhibitory receptors such as Lymphocyte Activation Gene (LAG-3) (Blackburn et al., 2009) and T cell Immunoglobulin and Mucin domain-3 (TIM-3) (Jones et al., 2008) have also been assigned roles in T cell exhaustion. Similarly, impaired CD4⁺ T cell function is a hallmark of progression to AIDS (Oxenius et al., 2002).

These 'exhausted' cells prevent optimal immune control of chronic HIV-1 infection as normal cellular survival or proliferative signal transduction pathways and cytokine expression profiles are dysfunctional (reviewed in (Wherry, 2011)).

1.2.3 The Role of CD4⁺ T cells in HIV-1 control

Progression to AIDS is slowed in patients with strong CD4⁺ gag ELISpot responses (Boaz et al., 2002, Soghoian et al., 2012) and CD4⁺ T cell responses were dominant over CD8⁺ responses in determining time taken to primary endpoint in the SPARTAC trial (Frater et al., 2013). In other studies, proliferative gag-specific CD4⁺ T cell responses perhaps relate to lower viral loads, (Rosenberg et al., 1997), although this observation is disputed (Betts et al., 2001, Dalod et al., 1999, Gea-Banacloche et al., 2000). Recently the presence of strong CD4⁺ T cell responses was linked to virological control in acute infection (Schieffer et al., 2014). HIV-1-

specific cytolytic CD4⁺ T cell frequency was also associated with lower set point viral loads (Soghoian et al., 2012). Together, these data indicate CD4⁺ T cells also have an important role in controlling HIV-1 infection.

1.3. Highly Active Antiretroviral Therapy

Highly active antiretroviral therapy (HAART) consists of a combination of at least three antiretroviral drugs; typically a protease or non-nucleoside reverse transcriptase inhibitor and two nucleoside/nucleotide analogue reverse transcription inhibitors (Bartlett, 2001, Hammer et al., 2006). There are currently 22 licensed antiretroviral drugs (Shen and Siliciano, 2008) which can inhibit HIV-1 entry, reverse transcription, integration and virion maturation.

HAART was introduced in 1996 and led to a significant decline in morbidity and mortality in HIV-1 infected individuals in the developed world (Gulick et al., 1997). For example, mortality in 1255 studied patients fell from 29.4 to 8.8 per 100 person years between 1995 and 1997 (Palella et al., 1998). HAART significantly reconstitutes CD4⁺ T cell levels (Mocroft et al., 2007), which results in decreased progression to AIDS and death (Zwahlen et al., 2009, Ray et al., 2010). However, despite life expectancy of asymptomatic early HAART-treated patients being very similar to uninfected individuals (van Sighem et al., 2010), HAART is not curative, so life-long administration must occur.

Despite the successes of HAART, 35.3 million people are HIV-infected worldwide and 1.6 million died from AIDS in 2012 (WHO, 2012) with the majority of infected people still not on therapy (Piot and Quinn, 2013). Administration of life-long HAART has caveats, including social stigma, drug toxicity and the risk of drug resistance through non-adherence. Also, dementia and cardiovascular disease are associated with HAART (Volberding and Deeks, 2010). Additionally, HAART also fails to reinstate the dysfunctional immune cell response (Migueles et al., 2009, Kalams et al., 1999).

Interestingly, the HPTN052 study (Cohen et al., 2011) showed that HAART administration to HIV-1 infected individuals reduced the chance of viral transmission by 96% meaning HAART may also be useful for prevention of infection.

1.3.1 Clinical Impact of HAART

When HAART is initiated, four plasma virus decay phases occur. A rapid decline in plasma virus first ensues, with a half-life of approximately 2 days (Perelson et al., 1997, Cavert et al., 1997, Perelson et al., 1996, Wei et al., 1995). Concomitantly, CD4+ T cell counts recover (Ho et al., 1995) (Figure 1.4). This initial decline is thought to represent the clearance of short-lived productively HIV-1-infected activated CD4+ T cells by the immune system or by the viral cytopathic effect (Trono et al., 2010).

A subsequent viral decay phase, with a half life of a few weeks, is thought to be due to the destruction of HIV-infected cells in anatomical reservoir sites, and infected macrophages, which are known to be more resistant to the cytopathic effect of HIV (Perelson et al., 1997, Trono et al., 2010).

Using the 'Single Copy Assay' (Palmer, Wiegand et al. 2003) that measures viral load, two more decay phases have been observed (Palmer, Maldarelli et al. 2008). The third decay phase corresponded to a cell with a half-life of 39 weeks, and could represent HIV-1 bound to Follicular Dendritic Cells (Zhang and Perelson, 2013). The half-life for the final phase of decay is likely to represent that of the 'latent HIV-1 reservoir' (Section 1.4). Estimates for this decay rate range from 44 months (Siliciano et al., 2003, Finzi et al., 1999) to an infinite length of time, meaning that

patients will never be cured by HAART (Hatano et al., 2010). Other studies have shown that if patients are treated early, HIV-1 DNA levels may decline with a half-life of 5 months (Chun et al., 2007) (Section 1.4.3). However, it is not known to what level the HIV-1 reservoir must deplete for a person to be cured.

1.4. HIV-1 Reservoirs and Latency

1.4.1 Formation of latently HIV-1-infected Reservoirs

Viral re-expression does not always occur immediately after integration. In rare cases, proviral DNA re-expression is silenced, forming a pool of latently infected cells. This latent reservoir is established early in acute infection (Chun et al., 1998, Ananworanich et al., 2012).

Current theory suggests latency occurs when productively infected, 'activated' CD4⁺ T-cells with integrated provirus revert to a resting, quiescent G₀ state that is not conducive for viral replication (Hermankova et al., 2003, Chun et al., 2003). Since latent and uninfected CD4⁺ T cells are phenotypically identical (Brooks and Zack, 2002), they cannot be targeted by host immune mechanisms for elimination. Some evidence suggests that memory CD4⁺ T cells (which include the majority of cell population) proliferates homeostatically, thus stabilizing the size of the latent reservoir (Chomont et al., 2009). If homeostatic proliferation of latent cells occurs, this means that viral persistence is ensured and that lifelong HAART by itself may never eradicate HIV (see 1.4.2).

Limiting concentrations of certain host transcription factors including Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Nuclear Factor of Activated T-cells (NFAT) and Positive Transcription Elongation Factor B (pTEFB), as well as viral Tat, are involved in maintenance and breaking of latency. This is generally achieved through epigenetic modulation of histone DNA binding proteins, which leads to a repressive DNA state, not conducive for DNA polymerase

binding, viral transcription and infectious viral release (summarised in (Karn, 2011)) and discussed in detail in section 1.5.

Clonal proliferation of latently infected haematopoietic stem cells and CD4⁺ T cells with stem cell-like properties (T_{SCM}) could theoretically lead to latent provirus being contained in all differentiated progeny cells (Buzon et al., 2014), with the homeostatic proliferation not having caused latent viral reactivation (Bosque et al., 2011). Transcriptional interference, whereby transcription of a host gene by an upstream promoter inhibits LTR-driven transcription of HIV-1, could potentially act as a mechanism that maintains a latent phenotype, although the contribution of this is disputed (Duverger et al., 2009). DNA itself, particularly CpG island methylation, may also contribute to the maintenance of the latent viral reservoir (Blazkova et al., 2009).

However, *in vitro* latency cell model studies involving spinoculation as a means to increase infection rates without enhancing integration efficiency, show that HIV-1 directly infects resting CD4⁺ T cells, although at reduced rates (Dai et al., 2009, Swiggard et al., 2005, Agosto et al., 2007, Lassen et al., 2012, O'Doherty et al., 2000). Thus, direct infection of resting CD4⁺ T cells may be another potential mechanism of latency formation as infectious virus was not produced from these cells *in vitro* (Pace et al., 2012).

1.4.2. Location of the HIV-1 reservoir

The largest cellular reservoir of latent, inducible HIV-1 is resting CD4⁺ T-cells (Chun et al., 1997b, Finzi et al., 1997, Wong et al., 1997), despite some estimates suggesting only one in a million are latently infected in some HAART-treated

patients (Finzi et al., 1997). Most provirus is located in memory (CD45RO+) CD4+ T-cells (Chun et al., 1997a).

Latently infected central memory (T_{CM}) and transitional memory CD4+ T cells (T_{TM}) may act as separate cellular reservoirs. In aviremic HAART-treated patients that have had good CD4+ T-cell reconstitution, most integrated HIV-1 DNA is located in T_{CM} cells. T_{CM} reservoir persistence was hypothesised to ensue through proliferation driven by the presence of low-level antigen and T cell survival, thus this pool decreases over time. However, in aviremic HAART patients who have poor CD4+ T cell reconstitution, most integrated provirus is located in T_{TM} cells. The persistence of the reservoir is guaranteed through IL-7 driven homeostatic proliferation (Chomont et al., 2009). HIV-1 provirus has also been discovered in haematopoietic stem cells (McNamara et al., 2012, Carter et al., 2010) and mast cells may also be a distinct cellular HIV-1 reservoir (Sundstrom et al., 2007).

However, since the brain, GALT, and genitourinary tract can all contain latently HIV-1-infected cells, they can act as distinct anatomical HIV-1 reservoirs (Trono et al., 2010). Biopsy studies have located HIV-1 DNA and RNA in anatomical reservoirs such as the ileum, duodenum, colon and rectum, with HIV-1 DNA concentrations decreasing from rectum to duodenum (Yukl et al., 2010a). The GALT may act as a separate anatomical reservoir to PBMCs (Mowat and Viney, 1997) as sequences found in the plasma did not match those found in the GALT (Lerner et al., 2011). Additionally, gp120 HIV-1 sequences found in genital secretions and blood plasma from 5 acute HIV-1 seroconverters were non-matching, suggesting the urogenital tract and resting CD4+ T-cells to be distinct

anatomical reservoirs (Zhu et al., 1996). HIV-1 was also detected in semen from HAART-treated patients (Craig et al., 2004, Nunnari et al., 2002).

HIV-1 also persists in cerebrospinal fluid cells and nervous tissue cells such as astrocytes and microglia (Kramer-Hammerle et al., 2005, Lambotte et al., 2005, Bell, 2004) and PBMC HIV-1 sequences were also different to those sequenced from the central nervous system (CNS) (Cheng-Mayer et al., 1989).

HIV-1 sequences from tubular epithelial kidney cells (Bruggeman et al., 2000), in HAART-suppressed patients (Marras et al., 2002, Winston et al., 2001) were again distinct to those found in PBMCs, suggesting the kidney could act as a separate anatomical reservoir.

1.4.3 Clinical significance of the latent HIV-1 reservoir.

1.4.3.1 Residual and rebound viraemia

The vast majority of patients exhibit residual detectable plasma HIV-1, even after long-term HAART (Palmer et al., 2003, Palmer et al., 2008, Maldarelli et al., 2007) and viral recrudescence occurs to a set-point level, equivalent to that measured before therapy, when HAART is interrupted (Harrigan et al., 1999, Davey et al., 1999a). Despite HAART treatment decreasing T cell activation status, levels are still consistently higher than normal (Hunt et al., 2003) unless early HAART is administered, in which case the T cell activation set point during treatment may be decreased (Jain et al., 2013). The cause of persistent immune activation is elusive and may be due to a number of factors including presence of residual viremia (Mavigner et al., 2009) or microbial translocation in the gut (Brenchley et al., 2006). This persistent immune activation also associates with progression to AIDS

(Hazenbergh et al., 2003, Kuller et al., 2008). The actual HAART regime does not impact residual viremia (Maldarelli et al., 2007). Since progression to AIDS continues even in patients treated 10 days after seroconversion (Chun et al., 2010), the HIV-1 reservoir must be formed very early in primary HIV-1 infection.

It is not fully known if residual viremia is replication competent and contributes to viral rebound and clinical progression on treatment interruption (TI) (Trono et al., 2010). Three independent landmark studies demonstrated that latent cells contained inducible, replication-competent virus (Finzi et al., 1997, Chun et al., 1997b, Wong et al., 1997). However viable virus was discovered in the plasma of one HAART treated patient (Sahu et al., 2010).

Residual virus could be caused by low-level viral replication in regions of the body termed 'sanctuary sites', where HAART drug penetration is poor, such as the GALT (Cohen, 2011). Suboptimal HAART penetration into the lymphatic tissue associated with higher levels of persistent viraemia (Fletcher et al., 2014). Indeed, there is evidence for on-going residual HIV-1 replication in the CNS (Lambotte et al., 2005) and ileum, with plasma viral rebound sequences differing from those found in the GALT (Lerner et al., 2011, Yukl et al., 2010b, Chun et al., 2008).

However, in the majority of fully suppressed HAART-treated patients, no viral evolution is observed (Bailey et al., 2006, Joos et al., 2008, Josefsson et al., 2013). Also residual viral sequence data can be very homogenous, indicating single clonal reactivation (Bailey et al., 2006, Josefsson et al., 2013). In addition, intensified HAART does not lead to decreased residual viremia as one might expect were HAART not to be fully efficacious (Archin et al., 2010, Dinoso et al., 2009, McMahon et al., 2010) at least in the peripheral blood.

Supporting the existence of continuing viral replication, transient increases in circularised and unintegrated episomal HIV-1 DNA (Figure 1.3) were observed on intensification of HAART with the integrase inhibitor Raltegravir and d-dimer levels also decreased in a subsequent study (Buzon et al., 2010, Hatano et al., 2013b). Indeed, episomal HIV-1 DNA sequences clustered with rebound viral sequences, suggesting continued viral replication is responsible for post-TI rebound viraemia (Sharkey et al., 2011).

However, at the time of writing, there is more evidence to suggest that residual HIV-1 viremia is caused by sporadic release of reactivated reservoir virus from CD4+ T cells (Brennan et al., 2009, Sahu et al., 2009, Joos et al., 2008), but the possibility that residual replication occurs in some patients cannot be discounted.

1.4.3.2 HIV-1 DNA level predicts clinical outcome

Currently, the only validated surrogate markers for disease progression are viral load and CD4+ T cell count (Mellors et al., 1996, Mellors et al., 1997). Viral load associates with HIV-1 DNA level in untreated (Verhofstede et al., 1994) and HAART treated patients (Chun et al., 2011, Zhao et al., 2002). Also CD4+ T cell count associates negatively with HIV-1 DNA level (Parisi et al., 2012). Since productive HIV-1 infection leads to CD4+ T cell death, patients with a low viral load, and thus low HIV-1 DNA levels are perhaps more likely to have higher CD4+ T cell counts.

HIV-1 DNA levels also associate significantly with immunological markers of disease progression. PD-1 expression associated significantly with HIV-1 DNA levels in chronically infected HAART treated patients (Hatano et al., 2013a). However, HIV-1 DNA levels only correlated with immune activation in viraemic,

not aviraemic patients enrolled in the ANRS116 SALTO study (Weiss et al., 2013). Similarly, proviral HIV-1 DNA level did not correlate with the level of immune activation in 10-year HAART-suppressed HIV infected patients (Poizot-Martin et al., 2013). Another study has shown weak associations between cell associated HIV-1 RNA, proviral HIV-1 DNA level (but not HIV-1 plasma RNA level) and T cell activation in HAART treated patients (Hatano et al., 2012).

HIV-1 DNA levels are also independently predictive of clinical outcome. Higher PBMC-associated HIV-1 DNA levels associated strongly with faster clinical progression in chronically HIV-1-infected HAART-treated patients, sampled at the point of TI (Piketty et al., 2010) and in untreated acute infection (Goujard et al., 2006, Minga et al., 2008). HIV-1 DNA levels in patients who received ART in chronic infection also independently predicted survival after TI (Tierney et al., 2003), although as these patients were receiving limited analogue suppression therapy, it is unknown how fully suppressed they were. Also in chronic infection, PBMC HIV-1 DNA levels similarly predicted disease progression independently of viral load or CD4 count (Rouzioux et al., 2005).

Pre-HAART HIV-1 DNA levels predicted residual viremia during HAART in 100 chronically infected patients (Havlir et al., 2005) and the magnitude of rebound virus and treatment failure in chronically infected patients (Yerly et al., 2004). In a small study, low proviral DNA levels at the point of TI in acutely HIV-1-infected patients predicted those who maintained viral rebound set points below 5000 copies/mL (Lafeuillade et al., 2003) although hydroxyurea and IL-2 treatment complicated the results. HIV-1 DNA levels also associated strongly with pre-HAART viral load (Fourati et al., 2013).

1.4.4 Targeting of the latent HIV-1 reservoir

Since the latent viral reservoir is most likely causative of viral rebound on TI, individuals can never stop HAART. If the latent reservoir could be targeted it would mean patients could undergo TI without fear of viral rebound and continued progression to AIDS. However, no known cell-surface phenotypic differences exist between latently infected and uninfected resting CD4⁺ T cells (Brooks and Zack, 2002), thus the immune system cannot specifically clear latent cells. This provides a major barrier to eradication efforts.

However, recent data have identified direct HIV-1 infection of resting CD4⁺ T cells leads to integration and Gag protein expression without efficient viral spread, with maintenance of a resting cellular phenotype, which challenges the traditional idea that latent cell HIV-1 transcription has been completely silenced (Pace et al., 2012). In this study, viral spread was enhanced on IL-7 treatment, which correlated with elevated Env translation (Pace et al., 2012). This agrees with observations where HIV-1 RNA was found *in vivo* in resting CD4⁺ T cells (Lassen et al., 2004, Lassen et al., 2006, Li et al., 2005, Zhang et al., 1999) and PBMCs (Fischer et al., 2008, Pasternak et al., 2009, Hockett et al., 1999).

Since some HIV-1 in infected resting cells is silent, some is transcribed and some HIV-1 gag is translated without spreading infection, latency may be a 'spectrum', rather than just transcriptionally silenced proviral DNA (Pace, Graf et al. 2012). If some resting latently infected cells express Gag protein, it may therefore be possible to target these cells with CTLs, depending on whether and/or which antigens are being presented on MHC class I molecules. It was hypothesised that ECs have lower HIV-1 reservoir levels because strong CTL responses can be made

against cells expressing very small amounts of Gag (Graf et al., 2011). These Gag positive latently infected resting CD4⁺ T cells are targetable by EC CTLs and have been isolated *in vivo* (Graf et al., 2013). A part of the latent viral reservoir might therefore be 'targetable'.

1.5. Therapies aimed at HIV-1 cure

HIV-1 cure is a major issue that needs to be addressed. This is especially true since recent the recent STEP prophylactic vaccine trial (Buchbinder et al., 2008) did not significantly protect patients from HIV-1 infection. Also, the Thai trial (RV144) (Rerks-Ngarm et al., 2009), suggested those that were vaccinated had a 31.2% lower HIV-1 infection rate compared to those receiving placebo ($p=0.04$). Since HIV-1 preferentially infects HIV-1-specific CD4⁺ T cells (Douek et al., 2002), vaccines stimulating CD4⁺ T cells could result in higher viral replication and higher reservoirs perhaps explaining the higher HIV-1 acquisition rate observed in the STEP trial (Buchbinder et al., 2008).

However, T cell based CMV-vector therapeutic vaccines have demonstrated control of SIV infection (Hansen et al., 2011, Hansen et al., 2009). Immune-mediated progressive loss and even complete eradication of SIV-RNA and DNA was reported in a subset of vaccinated macaques that controlled SIVmac239 infection post challenge (Hansen et al., 2013). These results have not yet been observed in clinical trials.

However, autologous dendritic cells pulsed with attenuated HIV-1 transiently reduced HIV-1 viral load set point 12 and 24 but not 48 weeks post TI (Garcia et al., 2013). Additionally, HIV Gag-specific humoral responses at seroconversion have also been linked with clinical outcome (Cheingsong-Popov et al., 1991). Despite broadly neutralizing antibodies being present isolated *in vivo* (Walker et al., 2011), they have little impact on long-term clinical progression (Euler et al., 2010, Piantadosi et al., 2009).

The ability of HIV-1 to mutate creates immense intra and inter-patient viral diversity, which makes targeting all viral variants with CTL or antibody inducing vaccines very challenging. Anti-latency therapies, in the presence of HAART, have been devised to remove latent HIV-1 from its reservoirs, thus curing a patient. These drugs may perhaps allow patients to interrupt HAART, with either partial or total virological remission. In this sense, HIV-1 may be thought of in terms of cancer.

HIV-1 eradication and HIV-1 cure are not mutually exclusive. Eradication means no HIV-1 can be detected in a patient, thus a sterilising cure has been achieved. However, there is increasing evidence for a benefit of early HAART treatment in the context of 'functional cure', which has been achieved in the VISCONTI patients, despite HIV-1 DNA being detectable (Saez-Cirion et al., 2013). In these 14 patients, early and prolonged HAART initiated in PHI has led to low and even declining HIV-DNA levels in the absence of therapy, which is independent of protective HLA-type or the presence of strong CTL responses. In either case, it is crucial for the latent HIV-1 reservoir to be depleted by some mechanism.

There is an increasing number of case studies over the last few years that suggest HIV-1 eradication or functional cure is and has been made possible (summarised in (Richman et al., 2009)).

1.5.1 *CCR5 modulation*

HIV-1 has potentially been eradicated in one individual. CD4⁺ T cells homozygous for a 32 base pair ($\Delta 32/\Delta 32$) mutation in the *CCR5* gene are not permissive for infection by CCR5-tropic strains of HIV-1 (Liu et al., 1996). Progression to AIDS is

slowed even in patients heterozygous for this mutation (Eugen-Olsen et al., 1997, Cohen et al., 1997). A bone marrow transplant was performed in an HIV-1-infected leukaemia patient, but using a donor carrying this rare mutation. All the newly generated CD4⁺ T-cells thus carried this mutation after engraftment. This patient has been off HAART for over 5 years with no convincing viral rebound or detectable proviral HIV-1 DNA or RNA in the bone marrow, blood or rectal mucosa (Allers et al., 2011, Hutter et al., 2009, Yukl et al., 2013). However, it is not known whether radiotherapy, chemotherapy, anti-thymocyte globulin, graft versus host disease or a combination of all 4 factors were implicated in the mechanism of cure.

However, in two CCR5 $\Delta 32$ heterozygous HIV-1-infected patients, a bone marrow transplant from a wild type donor resulted in undetectable HIV-1 DNA or RNA, possibly due to graft versus host disease clearing the reservoir (Henrich et al., 2013). However, virus rebounded in both patients 12 and 32 weeks post treatment cessation, perhaps from non-sampled anatomical reservoirs (Henrich et al., 2014).

As bone marrow transplants carry significant health risks and CCR5 $\Delta 32$ donors are rare, ways have been devised to mimic this phenotype using gene therapy. Gene editing of *CCR5* in CD4⁺ T cells using zinc finger nucleases targeted to a target site within *CCR5* was performed in pre-clinical tests. The resulting cells were functionally normal but were resistant to HIV-1 infection and HIV-1 RNA levels decreased on infusion of these cells in a murine model (Perez et al., 2008).

This technology was assessed for safety in a recent clinical trial. Adoptive transfer of expanded autologous CD4⁺ T cells with gene-edited *CCR5* into chronically infected patients on HAART was safe. Engraftment of *CCR5* edited cells was observed and these cells carried a survival advantage compared to native CD4⁺ T

cells (Tebas, Stein et al. 2014). The concern that mutating only CCR5 would still allow CXCR4 or dual-tropic viruses to infect cells may be resolved with a dual receptor ZFN, recently reported in a humanised murine model (Didigu et al., 2014).

1.5.2 Early HAART treatment

There is contradictory evidence to suggest benefits of early HAART onset after initial HIV-1 infection. Latent reservoir formation was not inhibited if HAART was administered in primary infection, despite control of plasma viremia (Chun et al., 1998). However, patients who are treated with HAART in primary HIV-1 infection do generally have lower HIV-1 reservoir levels than those treated during chronic infection (Eriksson et al., 2013, Koelsch et al., 2011).

A small subset of patients enrolled in the VISCONTI trial, who received prolonged HAART in primary HIV-1 infection have remained aviremic for long periods of time post TI. These 14 patients had no genetic traits commonly found in ECs, so were defined as ‘post treatment controllers’ (PTCs). PTC viral reservoir levels were very low and in some cases, decreased over time (Hocqueloux et al., 2010, Saez-Cirion et al., 2013). Data from this cohort suggest that very early and prolonged therapy could limit the size of the viral reservoir, perhaps to a point where it can be controlled by host immunity in the absence of HAART (Saez-Cirion, Bacchus et al. 2013).

Also, the results from the SPARTAC trial showed that 48 weeks of HAART during primary HIV-1 infection could significantly delay disease progression (Fidler et al., 2013) (Figure 1.5A) and delay viral rebound (Stohr et al., 2013) (Figure 1.5B)

although no PTCs was observed. Participants who were randomized within an estimated 12 weeks of therapy had slower disease progression than those who had a longer interval between seroconversion and randomization, as observed previously (Porter et al., 2003). Low CD4+ T cell responses also predicted a more rapid time to primary endpoint (Frater et al., 2013).

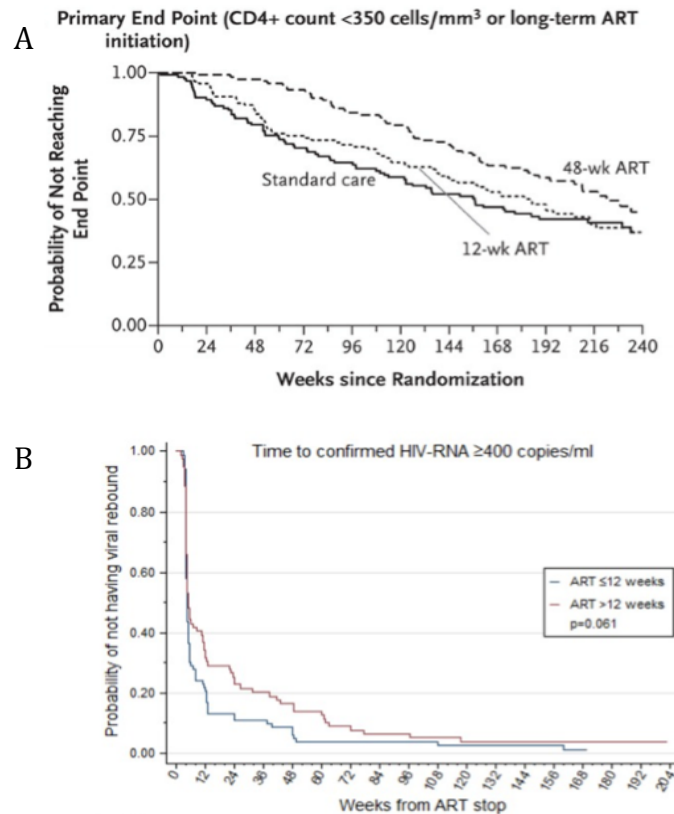


Figure 1.5 Published data from the SPARTAC trial. A) 48 but not 12 or 0 weeks of HAART significantly delayed disease progression. Reproduced with permission from (Fidler, Porter et al. 2013)), Copyright Massachusetts Medical Society. B) Longer HAART duration delayed viral rebound time. Reproduced with permission from (Stohr, Fidler et al. 2013) Figure 1A.

HAART treatment in acute infection depleted the viral reservoir in another study (Strain et al., 2005). Similarly, ECs, who control HIV-1 replication in the absence of HAART, have particularly low HIV-1 reservoir levels (Graf et al., 2011). Most notably, extremely early HAART treatment of the Mississippi baby within 31 hours of birth may have resulted in the second case of HIV-1 functional cure (Persaud et al., 2013), as perhaps the HIV-1 reservoir was not yet seeded (Chun et al., 2010, Ananworanich et al., 2012). There is also an immunological benefit of HAART administration in primary HIV infection (PHI), as T cell function is preserved (Oxenius et al., 2002).

1.5.3 T cell Activation

Some anti-latency drugs work by activating resting latent CD4⁺ T cells, which induces HIV-1-LTR transcription and viral expression. Virus could then be cleared or 'purged' from latently infected cells by the viral cytopathic effect or by immune targeting (Bowman et al., 2009). Any released virus would not re-infect cells because patients would still be receiving HAART.

IL-2 and the anti-CD3 antibody OKT3 both activate CD4⁺ T cells through multiple signalling pathways. The activation signals could induce acetylation of histone proteins that bind the integrated HIV-1 DNA site, loosening the HIV-1 binding and making the environment more conducive for viral transcription. IL-2 therapy increased CD4⁺ T cell levels by 50% and induced transcription of HIV-1 in 40% of patients tested (Kovacs et al., 1995). CD4⁺ T cell counts 6 months post high-level IL-2 therapy were 94.8% higher than baseline (Davey et al., 1999b).

The amount of latently infected cells in patients receiving both IL-2 therapy and HAART was lower than in patients just receiving HAART (Chun et al., 1999) although a subsequent study disputed this (Dybul et al., 2002).

However, studies attempting viral ‘purging’ with global T cell activators induced cytokine storms and severe associated toxicities (Prins et al., 1999). In one study, CD4⁺ T cell levels never fully recovered post-OKT3 treatment (van Praag et al., 2001). Even intensification of IL-2 and OKT-3 treatment with Didanosine and Hydroxyurea in aviremic patients failed to inhibit rebound viremia (Geeraert et al., 2008) and did not eradicate HIV-1 reservoirs *in vivo* (Kulkosky et al., 2002).

IL-7 was thought to be a promising candidate for viral eradication as IL-7 therapy in the SCID-hu mouse model reactivated latent virus in CD4⁺ T cells and thymocytes, with negligible impact on cell phenotype (Scripture-Adams et al., 2002). Similarly, IL-7 therapy stimulated reactivation of latent virus in some HAART-treated patient CD4⁺ T-cells *ex vivo* (Lehrman et al., 2004). However, since IL-7 promotes latent cell homeostatic proliferation, doubts have been raised with regard to the clinical usefulness of this therapy (Chomont et al., 2009). Furthermore, IL-7 mediated homeostatic proliferation was shown not to reactivate latent provirus in a primary latency cell model (Bosque et al., 2011).

1.5.4 Protein Kinase C agonists

Prostratin is a phorbol ester derived from *Homalanthus nutans* tree bark. Prostratin is a Protein Kinase C (PKC) agonist and induces proviral transcription and infectious viral release (Kulkosky et al., 2001) by activating the NF- κ B pathway (Biancotto et al., 2004). Specifically, Prostratin stimulates PKC phosphorylation of the inhibitor of nuclear factor of kappa light polypeptide gene enhancer in B-cells, alpha (I κ B α), causing its degradation. The transcription factor NF- κ B is released and thus transcription of latent provirus is activated (Figure 1.6) (Williams et al., 2004).

Hexamethylene bisacetamide (HMBA) can also stimulate viral re-expression by modulating the Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-K) signalling cascade (Contreras et al., 2007). Similarly to Prostratin, CD4 down-regulation occurs concomitantly, thus also potentially inhibiting *de novo* PBMC infection (Klichko et al., 2006). HMBA was well tolerated in phase II cancer trials (Andreeff et al., 1992).

P-TEFb recruitment to the viral promoter is necessary to overcome abortive HIV-1 transcription events that occur in the absence of viral Tat. The hexamethylene bisacetamide inducible molecule (HEXIM) complex binds to and inhibits P-TEFb. However, HMBA inhibits the HEXIM complex, liberating P-TEFb, which is subsequently recruited to the HIV-1 promoter where transcripts can be elongated (Choudhary et al., 2008). The presence of Tat bypasses this control mechanism and directly recruits P-TEFb to the HIV-1-promoter (Zhu et al., 1997) (summarised in (Barton et al., 2013, Bowman et al., 2009) and Fig. 1.6).

However, since HIV-1 preferentially infects activated T-cells, Prostratin and HMBA could theoretically increase the number of cells permissive to HIV-1 infection increasing the latent reservoir size and this would also be the case for the global T cell activators IL-2 and IL-7 (Chomont et al., 2009, Bowman et al., 2009). The PKC pathway modulator Bryostatin reactivates latent HIV-1 without global T cell activation (Mehla et al., 2010). CD4 is also downregulated on drug treatment resulting in less potential targets for HIV-1 re-infection (Boto et al., 1991). However, in an ovarian cancer trial, Bryostatin caused severe myalgia in all patients (Morgan et al., 2012).

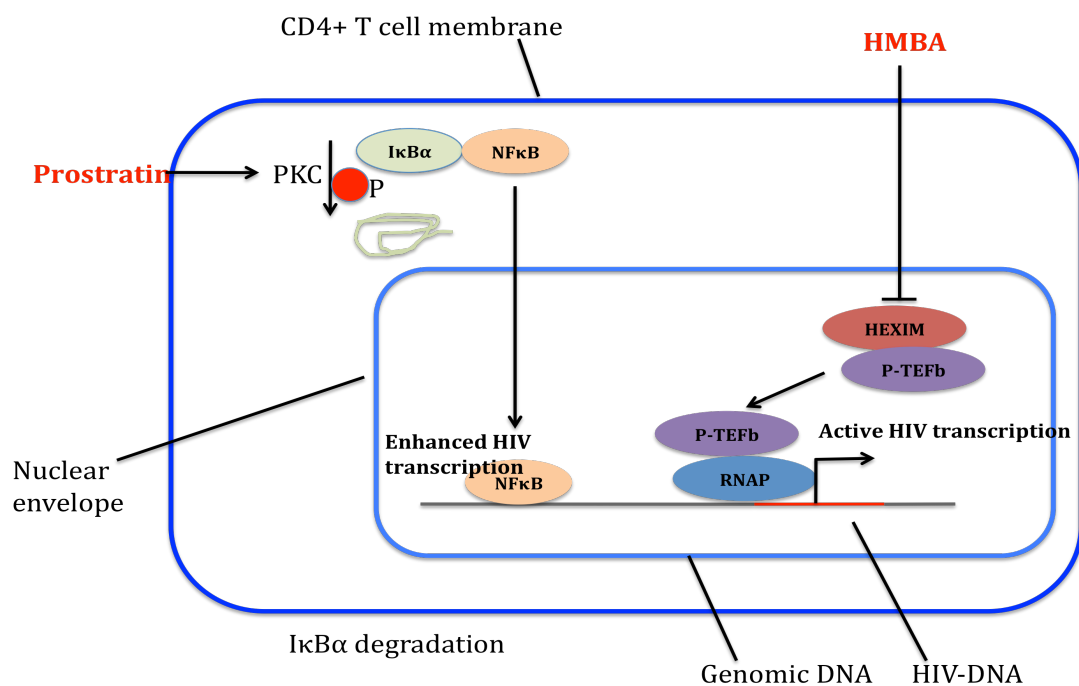


Figure 1.6. Latent cell activation by Prostratin and HMBA. P = phosphorylation event. Loosely based on (Barton et al., 2013) Figure 1.

1.5.5 Histone Deacetylase Inhibitors

Histone deacetylase inhibitors (HDACi) reactivate latent virus by de-repressing the restrictive chromatin environment that surrounds integrated latent provirus (Figure 1.7). The FDA-approved drug Valproic Acid (VPA) reactivates HIV-1 expression from latently infected CD4+ T cells from patients on HAART (Ylisastigui et al., 2004) and in combination with intensified HAART, accelerated clearance of HIV-1 from the latent CD4+ T-cell reservoir in 3 out of 4 patients (Lehrman et al., 2005). This observation has not been reproduced in more recent clinical trials (Siliciano et al., 2007, Archin et al., 2010, Sagot-Lerolle et al., 2008, Routy et al., 2012). Class 1 HDAC proteins are most commonly associated with maintenance of latency (Choudhary and Margolis, 2011).

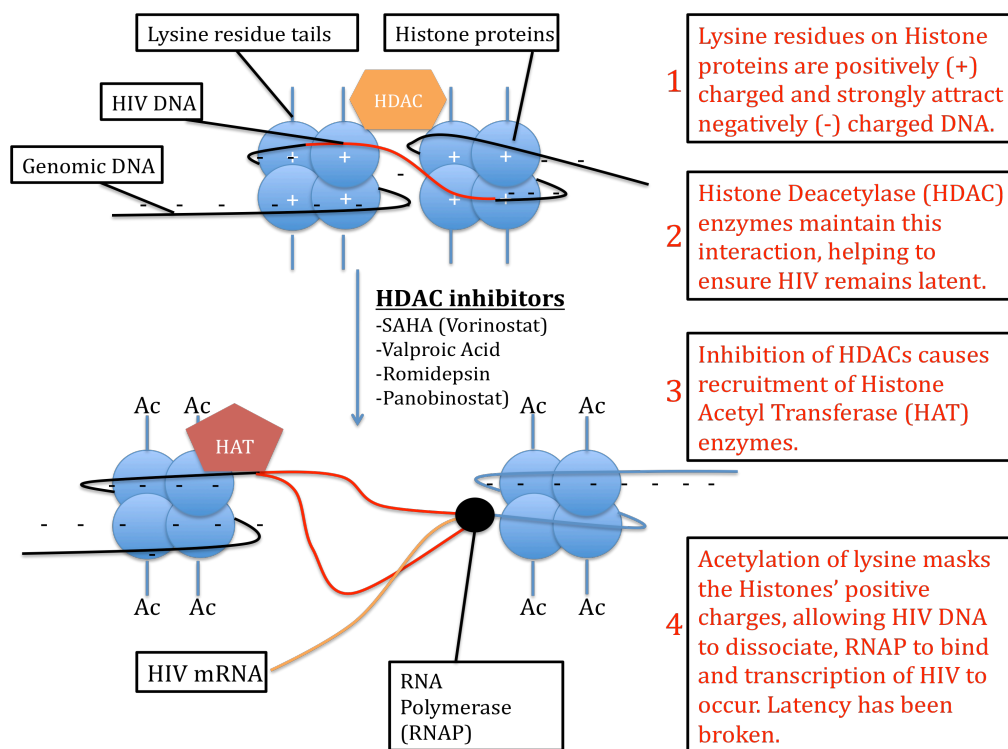


Figure 1.7. Mechanism of action for Histone Deacetylase Inhibitors

Suberoylanilide hydroxamic acid (SAHA; Vorinostat) is an FDA-approved drug for the treatment of cutaneous T cell lymphoma. It is a more potent and selective HDACi than VPA and stimulates HIV-1 expression in patient cells *ex vivo* (Archin et al., 2009, Contreras et al., 2009). Other even more potent HDACi include Panobinostat (Rasmussen et al., 2013) and the American FDA-approved Romidepsin, which demonstrates anti-tumour activity in the nanomolar range (Bolden et al., 2006).

1.5.6 The 'Kick and kill' hypothesis

There is increasing awareness in the HIV-1 field that HDACi or other anti-latency drug treatment might not be sufficient for HIV-1 cure on its own. The first *in vivo* evidence of reservoir disruption was gleaned when one 400mg dose of SAHA stimulated full-length HIV-1 RNA in latently infected CD4+ T cells (Archin et al., 2012). The results of a subsequent clinical trial, where HIV-1 patients are given multiple doses of SAHA show similar results, with no significant increases in plasma viremia or a reduction in the number of latently infected cells (Elliott et al., 2013). Disconcertingly, drug tolerance occurred in a subsequent multi-dose study, with SAHA quickly losing potency on repeat treatment (Archin et al., 2014).

Similarly there also evidence suggesting HDACi treatment of latently infected cells induces read-through transcription of proviral DNA, without actually specifically stimulating latent viral mRNA transcription (Bullen et al., 2014). This would help to explain why *in vivo* dosing of SAHA resulted in increased HIV-RNA detection, but not any concomitant increases in plasma viral load (Archin et al., 2012). However, 12 doses of Panobinostat were given to 15 men on HAART over 8 weeks and 60% of participants expressed detectable HIV-1 RNA in their blood, compared with only

28% before treatment. This indicates that HDACi might stimulate HIV-1 transcription *in vivo*, although this study has not yet been published (Tolstrup, 2013).

Disulfiram is a metal-chelator in the dithiocarbamate family and is FDA approved for use in deterring alcoholism (Conticello et al., 2012). It is thought to act via the phosphatase and tensin homologue (PTEN) pathway (Doyon et al., 2013), which is important for cell proliferation and survival. Despite Disulfiram not activating latent HIV-1 in all *in vitro* models of latency (Xing et al., 2011), Disulfiram caused an unexpected increase in plasma viremia after a 14-day administration *in vivo* (Spivak et al., 2014) although the data validity has been challenged. Similarly to the SAHA trials, no changes in the amount of latently infected cells could be observed. A new dose-escalation clinical trial is planned to address these concerns (ClinicalTrials.gov, 2014).

However, a landmark study showed that re-expression of virus from latently infected cells was not sufficient to kill the infected cell as previously thought (Shan et al., 2012). Instead CTL were required to eliminate latently infected cells. Therefore, HDACi could be used to 'kick' or activate latent HIV-1, leading to transcription, translation and antigen presentation. The newly antigenic cells could then be targeted 'killed' by host CD8+ T cell responses (Figure 1.8).

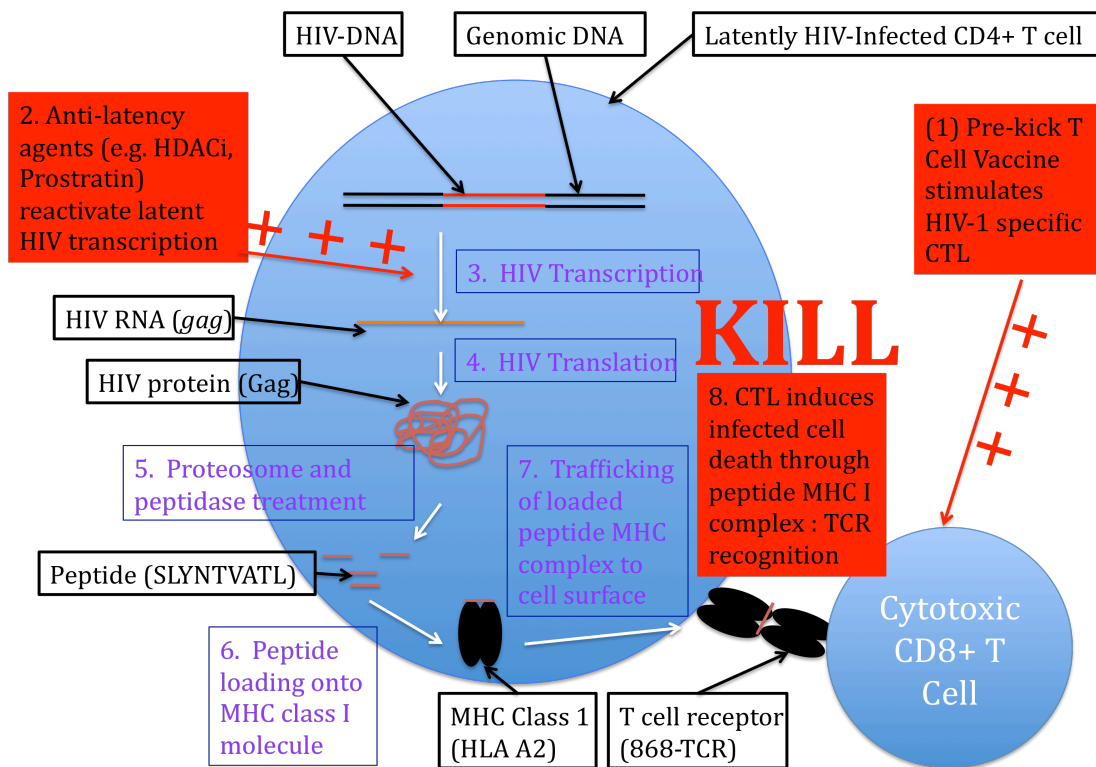


Figure 1.8. A summary of the ‘Kick and Kill’ hypothesis, in context for chapters 5 and 6 of this thesis

However, due to the defective CD8+ T cell response in HAART treated patients, only CTLs that were pre-stimulated with gag peptides and IL-2, or CTLs from ECs, who have strong CTL responses (and highly functional central memory responses despite undetectable viremia (Ndhlovu et al., 2012)), could target and kill cells in which latent HIV-1 had been reactivated (Shan et al., 2012).

The lack of available HIV-1 antigen after long-term HAART administration may mean low levels of HIV-1-specific CTL are present at the time of viral ‘kick’ (Casazza et al., 2001). Ways to boost CTL numbers and HIV-1-specific responses are therefore needed so the reservoir can be cleared. One such example would be therapeutic vaccination. A recent study in macaques demonstrated immune mediated clearance of SIVmac239 in some animals that were vaccinated prior to challenge. The authors concluded that such a vaccine could also exert strong and

broad immune pressure on reactivated latent cells, controlling viral rebound levels (Hansen et al., 2013). However, as yet, no vaccine efficacy trials designed to stimulate CD8⁺ T cell responses have demonstrated any benefit (McMichael and Koff, 2014).

Also, killing of HIV-1 RNA expressing cells, not cleared by HAART in the bone marrow, liver and thymus (BLT) mouse model was observed by co-administering the immunotoxin 3b3-PE38 (Denton et al., 2014), which targets the conserved CD4 binding site on gp120. This technology could be applied to increase killing of reactivated latent cells.

Another example to assist with the 'kill' part of the kick and kill hypothesis, is the utilization of TCR genetic strategies. The high affinity wild type 868-TCR is the strongest natural binding TCR ever measured (Varela-Rohena et al., 2008). HIV-1-infected patient 868, from which this TCR was derived, maintained a strong and sustained response against the HLA-A2-restricted immunodominant and conserved epitope SLYNTVATL (Varela-Rohena et al., 2008). If CTL could be transduced with this TCR, improved viral control through killing of infected cells might be established in a wide number of patients, as HLA-A2 is the most common HLA Class 1 allele in western populations. Indeed, 868-TCR transduced CTL targeted HIV-1-infected cells *ex vivo* (Varela-Rohena et al., 2008) and the results from a recent clinical trial NCT00991224 assessing safety and tolerability of 868-TCR transduced CTLs infused into HLA-A2 positive patients with HIV-1 (ClinicalTrials.gov, 2014) are eagerly awaited. TCRs can be mutated for even higher specificities, although there is concern about cross-reactivity of the adapted 868-TCR (personal communication with Andrew Gerry, Adaptimmune).

1.6 The future

The field at present is exploring the 'kick and kill' approach for eradication of HIV-1. Administration of potent anti-latency agents could make the HIV-1 reservoir visible to patients own CTLs. Alternatively, patients could be given a CD8+ T cell vaccine, or be infused with CTLs with high affinity TCRs to improve killing of the infected cells (Figure 1.7).

Interestingly, recent data suggests that the majority of HIV-1 DNA after long term HAART represents non-inducible, perhaps defective virus. Also, repeated rounds of HDACi treatment might be necessary to induce all inducible latent viruses present (Ho et al., 2013). Supposing all inducible 'functional' viruses have been removed, the non-inducible virus will still be measurable. HIV-1 won't have been eradicated, but patients might have been functionally cured (Saez-Cirion et al., 2013).

Alternatively to the kick and kill approach, clinical studies are being planned to target persistent immune activation which occurs despite the presence of HAART. For example, anti PD-1 ligand antibodies (BMS-936559; NCT02028403), or Interferon-alpha (NCT01295515) therapy may help to purge viral reservoirs (ClinicalTrials.gov, 2014). Indeed, pegylated interferon- α 2b therapy decreased integrated HIV-1 DNA levels in patients on HAART (Azzoni et al., 2013).

There is also hope surrounding a new technology involving site-specific gene editing techniques such as clustered regularly interspaced short palindromic repeat (CRISPR) systems and CRISPR-associated (Cas) proteins. The human Cas9 nuclease can form protein-DNA interactions and digest any target sequence of DNA

that matches that of the associated guide RNA (Mali et al., 2013). This system significantly reduced latent viral reactivation after cell stimulation, demonstrating the usefulness of this technology in the quest for an HIV-1 cure (Ebina et al., 2013).

However, in order to more fully characterise the HIV-1 reservoir and assess the potential for viral eradication, improved methods for measuring the reservoir identifying biomarkers that can predict post treatment control in future studies and novel strategies that enhance CTL function and recognition of the latent viral reservoir for the kick and kill approach to a viral cure need to be further developed and investigated.

1.7 Thesis Aims

In this thesis I intend to:

1. Develop and apply sensitive assays to measure total and integrated HIV-1 DNA reservoir levels in CD4+ T cells.
2. Explore the impact of HIV-1 DNA on clinical progression. I apply these newly developed assays to subtype B patients enrolled in the SPARTAC trial. The HIV-1 reservoir will be quantified in each of these patients and the impact of the reservoir level will be investigated specifically with regard to clinical progression, time to viral rebound and level of HIV-1 DNA rebound on cessation of HAART. Relationships between immunological and virological markers of progression and HIV-1 DNA levels in untreated patients will also be investigated.
3. Explore novel approaches to targeting the HIV-1 reservoir.
 - 3.1 Investigate a novel nanotechnology approach to improve the killing ability of CTLs through thermotherapy. This technology could act as an adjunctive therapy in the kick and kill approach for targeting the HIV-1 reservoir.
 - 3.2 Assess the potential for eradication of HIV-1 by studying 868-TCR transduced CTL targeting of the latent reservoir. A range of anti-latency agents will be used to stimulate a range of latent cell line models and a primary model of latency.

2. Materials and Methods

The total HIV-1 DNA and adapted Albumin Q-PCR methods have since been published in the Journal of Virology (Duncan et al., 2013) and the Journal of Virological Methods (Williams et al., 2014a). The integrated HIV-1 DNA assay has been submitted as a manuscript. Methods presented have also been published in the International Journal of Nanomedicine (Williams et al., 2013b). It is clear in the subheadings where methods have been published.

2.1 Clinical samples

2.1.1 The SPARTAC trial. (Chapter 4)

The **Short Pulse AntiRetroviral Treatment At HIV-1 seroConversion** (SPARTAC trial) (Fidler et al., 2013); is the largest randomised clinical trial investigating short-course HAART compared with no HAART in primary HIV-1 infection. Participants were recruited from Australia, Brazil, Italy, South Africa, Spain, Uganda and the UK and Ireland. 60% were male and 40% female. All participants were recruited within an estimated time since seroconversion (ETS) at enrollment of 6 months.

Primary HIV-1 infection was determined by a positive antibody test within 6 months of a negative result, a negative HIV-1-antibody test with positive HIV-1 RNA PCR, a low level of HIV-1 antibodies ($OD < 0.6$), an increase in antibody titer over a two-week period, or presence of symptomatic acute HIV-1 seroconversion syndrome supported by antigen positivity and less than 4 bands on a western blot.

429 participants were screened, of which 371 underwent randomization into three trial arms (Figure 2.1). The 58 patients that were excluded did not meet the criteria for primary infection, met other protocol exclusion criteria or there were

other reasons for exclusion. These included inappropriate age of consent for participating in a clinical study, inability or unwillingness to give written informed consent, pregnancy, those unlikely to adhere to protocol or therapeutic regimen, those likely to use narcotics during the study period and where HAART was indicated or contraindicated. All eligible participants were fully informed and provided consent in accord with the Helsinki agreement.

124 participants, at baseline, were assigned to receive no HAART (Standard of care / SOC), 123 received 12-weeks of short course standard HAART followed by cessation of therapy (ART-12) and 124 received 48-weeks of short course standard HAART followed by cessation of therapy (ART-48). 123 SOC, 120 ART-12 and 123 ART-48 participants were included in the analysis after 5 further participants were excluded (Figure 2.1). Samples were stored in liquid nitrogen at a concentration of 1 to 5×10^6 cells per vial in freezing media (40% RPMI, 50% FCS and 10% DMSO v/v). The samples utilized are summarised in section 4.3.1.

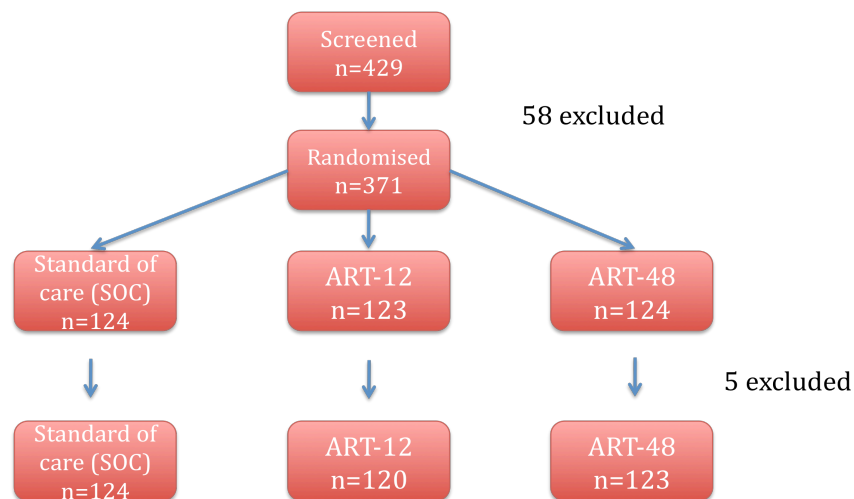


Figure 2.1 SPARTAC Trial Design.

Any further information can be found on the Medical Research Council website:

http://www.ctu.mrc.ac.uk/research_areas/study_details.aspx?s=32

Or the SPARTAC trial website:

http://www1.imperial.ac.uk/departmentsofmedicine/divisions/infectiousdiseases/infectious_diseases/HIV-1_trials/HIV-1_treatment/spartac/

2.1.2 SPARTAC Immunology

2.1.2.1 CD4+ and CD8+ T cell ELISpot data

Nicola Robinson and Helen Brown acquired this data. The methods are described elsewhere (Kiepiela et al., 2007, Frater et al., 2013, Fidler et al., 2002). Briefly, CD4 T-cell interferon gamma ELISpot assays were carried out in real time only in UK subtype B participants on fresh blood samples post CD8+ T cell depletion. HIV-1 specific CD4+ T cell responses were detected by ELISpot against subtype B p24 produced using a Baculovirus Expression Vector System. Samples were all analysed in duplicate. Results were expressed as Spot forming units per million CD8+ T cell depleted PBMCs.

CD8+ T cell responses were determined by interferon gamma ELISpot to subtype B consensus HIV-1 Gag overlapping peptides (123 15mers overlapping by 11 amino acids; AIDS Reagent and Reference Program, NIH). Negative controls were performed in duplicate, other assays in singlicate. Results were expressed as spot forming units per million cells.

2.1.2.2 CD4+ and CD8+ T cell exhaustion and activation markers

Stephen Hickling acquired this data. The method is described elsewhere (Hamlyn et al., 2012). Briefly, Frozen PBMCs were thawed and incubated with live/dead

stain (pacific blue; Invitrogen), CD3-pacific orange, CD4-Alexa 700 and CD8-PECy5 (all BD). Cells were also stained with PD-1 (APC), LAG-3 (FITC) and TIM-3 (PE) antibodies or HLA-DR-PE-Cy7 (all eBioscience) and CD38-Qdot 605 (Invitrogen). Staining was measured using an LSR-II flow cytometer (BD). Positive staining was assured through the use of isotype controls.

2.1.2.3 IL-6 and d-dimer plasma concentrations

Elizabeth Hamlyn acquired this data. The method is described elsewhere (Hamlyn et al., 2013). Briefly, Plasma IL-6 and D-dimer concentration was analysed at baseline using the Quantikine HS IL-6 immunoassay kit (R&D Systems) and Innovance D-dimer (Siemens Healthcare Diagnostics) from 229 patients recruited in Italy, Australia, UK and Brazil.

2.1.2.4 HLA-typing

HLA typing was performed by the Churchill Hospital transplant centre, Oxford and by Steven Cate, at the University of Oklahoma. Reverse sequence-specific Dynal RELITM oligonucleotide kits for HLA A, B and C loci were used (Dynal Biotech, UK).

2.1.3 Statistical analysis for Chapter 4

Linear regression and Spearman's rank correlations to investigate associations between DNA levels and other covariates were used as appropriate. Pearson correlations were used to investigate the relationship between total and integrated HIV-1 DNA levels. DNA levels have been compared between various groups using t, Mann-Whitney or Kruskal-Wallis tests, as appropriate. Where data were paired, paired T tests were performed. However, due to the lack of sample pairing across time points, unpaired tests were generally performed unless specifically noted.

Multiple testing was accounted for by applying Bonferroni's correction. The association between HIV-1 DNA and time from randomization to SPARTAC primary endpoint was investigated using Kaplan-Meier plots and univariate Cox models. Where participants received HAART, the time from treatment interruption to primary endpoint was analysed. Association with time to rebound was investigated using Kaplan-Meier plots, univariate Cox regression models and Spearman correlations. There was no censoring within the groups prior to rebound. All statistics and graphs were calculated using Prism version 5.0. Cox Regression Models were performed using IBM SPSS Statistics version 22.

2.2 HIV-1 DNA preparation and analysis

2.2.1 HIV-1 DNA preparation from SPARTAC samples. (Chapter 4)

CD4⁺ T cells were enriched from bulk frozen SPARTAC PBMCs using the Dynabeads Untouched human CD4⁺ T cell enrichment kit (Invitrogen) as per the manufacturers instructions. HIV-1 DNA was extracted from CD4⁺ T cells using the QiaAMP blood mini kit (Qiagen) using the manufacturers instructions. HIV-1 DNA was eluted in a minimum of 50µL of distilled water, depending on cell number, and was stored at 4°C.

2.2.2 Q-PCR Standard preparation (Chapters 3 and 4)

8e5 cells and ACH2 cells, that reportedly contain one integrated copy of HIV-1 per cell, were used in duplicate as Q-PCR standards for cell number and HIV-1 copy number. Cells were grown in R10 media (see section 2.4.1). DNA was extracted using a Qiagen blood mini kit, as per the manufacturers instructions. DNA for Q-

PCR standards were serially diluted in distilled water with cell and HIV-1 copy numbers ranging from 1×10^5 to 1×10^0 DNA copies per PCR reaction.

2.2.3 Measurement of cell number and total HIV-1 DNA in SPARTAC samples

Chapters 3 & 4 (Duncan et al., 2013, Williams et al., 2014a)

Cell copy number was quantified in triplicate at two dilutions using a slightly adapted albumin Q-PCR assay (Mexas et al., 2012). The master mix contained 2x Lightcycler 480 Probes Master Mix (Roche), 200nM Probe (FAM- CCT GTC ATG CCC ACA CAA ATC TCT CC – BHQ-1) and 250nM of each primer (Forward: GCT GTC ATC TCT TGT GGG CTG T, Reverse: AAA CTC ATG GGA GCT GCT GGT T) with 10 μ l DNA sample in a total volume of 25 μ l.

25,000 input cells per 10 μ l were assayed in triplicate for 'total' HIV-1 using 500nM Probe (FAM – AGT RGT GTG TGC CCG TCT GTT G – BHQ-1), 500nM LTR_OS primer (GRA ACC CAC TGC TTA ASS CTC AA) and 500nM LTR_AS primer (TGT TCG GGC GCC ACT GCT AGA GA) (MWG) and 2x Roche LightCycler 480 probes master mix, in a total volume of 25 μ l. Both Q-PCR amplifications were performed using the following program: one cycle of 95°C for 10 minutes; 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. The data was analysed using the Roche LightCycler software. Kathleen Gaertner developed the 'total' HIV-1 primers and probe.

2.2.4 Measurement of integrated HIV-1 DNA Chapters 3 & 4 (Williams, Hurst et al 2014)

Integrated HIV-1 was measured using an assay based on that published by (Liszewski et al., 2009) but with some minor modifications. 40 repeated integration measurements were run per patient. Alu_F (GCC TCC CAA AGT GCT

GGG ATT ACA G) and Gag Reverse (GTT CCT GCT ATG TCA CTT CC) primers were used for the first round PCR reaction along with 5 PCR reactions to which no Alu_F primer was added (gag-only), to serve as a background control for determination of sample positivity. The first round master mix was identical, except 1.5U platinum taq was used per 50µl reaction. The second round Q-PCR reaction was the same as the total reaction described above, but with 10µl of first round product being the input DNA. 7500 input cells were assayed per repeat well, of which there were 40.

The integration standard (IS) used was a kind gift from Una O'Doherty. One standard curve was generated by plotting the average cycle threshold (Ct) value across the 40 replicates at each IS dilution (ranging from 70 – 0.2 copies per well), so long as at least one integration signal was significantly different (2.s.d) to the average gag-only background signal. All standards were diluted in 2µg/ml PBMC DNA (equivalent of 7500 cells) to maintain the same number of Alu sites per well. Ln(Copy number) was plotted versus Ln(average Ct) and each point on the standard curve was repeated in duplicate. The standard curve fitted extremely well to a line of best fit ($r^2 = 0.987$), which was then used to calculate copy numbers in patient samples.

The copy numbers for each of the 40 replicates was quantified using the standard curve, generating a mean value and associated error. Plate to plate variation was assessed using quadruplicate replicates of 8e5 cells, which have 1 copy of HIV-1 per cell, diluted to 10 copies per well as a first round PCR DNA input.

A table of all primers and probe sequences used can be found in Table 2.1.

Table 2.1 Primer and Probe Sequences

Primer	Sequence (nucleotides)	HXB2 nucleotide position	Melting temperature/ °C
Albumin_F	GCT GTC ATC TCT TGT GGG CTG T (22)	N/A	62.1
Albumin_R	AAA CTC ATG GGA GCT GCT GGT T (22)	N/A	60.3
LTR_OS	GRA ACC CAC TGC TTA ASS CTC AA (23)	509-528	66
LTR_AS	TGT TCG GGC GCC ACT GCT AGA GA (23)	645-626	61.5
Alu_F	GCC TCC CAA AGT GCT GGG ATT ACA G (25)	N/A	66.3
Gag_Reverse	GTT CCT GCT ATG TCA CTT CC (20)	1505-1486	57.3
Probe			
Albumin	FAM- CCT GTC ATG CCC ACA CAA ATC TCT CC – BHQ-1 (26)	N/A	66.4
Total LTR	FAM – AGT RGT GTG TGC CCG TCT GTT G – BHQ-1 (22)	555-573	63.1

2.3 CD8⁺ T cells (Chapter 5 and 6)

2.3.1 868-TCR lentiviral transduction of CD8⁺ T-Cells

2.3.1.1 CTLs were kindly supplied already transduced with the 868-TCR by Andy Sewell (University of Cardiff) for use in Chapter 5 (Williams et al., 2013b). Described is the protocol performed by Anya Lissina and Andy Sewell (Cardiff University). Information provided by Anya Lissina.

Prior to transduction, primary CD8⁺ T-cells were isolated from human peripheral blood mononuclear cells (PBMC) using anti-CD8⁺ MACS microbeads (Miltenyi Biotech), were applied to a 24-well tissue culture plate at 10⁶ CD8⁺ cells/well in CK cell culture media (R10 supplemented with 2.5% Cellkines (Helvetica Healthcare), 200 IU/ml IL-2 and 25 ng/ml IL-15 (Peprotech)) and stimulated to divide by addition of anti-CD3/anti-CD28 antibody-coated microbeads (Invitrogen) in a 6:1 beads:cells ratio, for 24 hours at 37°C and 5% CO₂. Following the incubation period, half of the cell culture media was removed and replaced by concentrated lentiviral supernatant encoding the 868-TCR. Efficiency of transduction of CD8⁺ T-cells (typically >80%) was assessed by flow cytometry using anti-CD8 antibody and cognate SL9/HLA A2 tetramer staining, following a 72-hour exposure to the lentivirus. If required, the transduced CTLs were further tetramer-sorted to obtain a clonal 868-TCR-expressing CTL line.

The lentivirus was generated by transduction of the packaging 293T cell line with the 868-TCR-encoding lentiviral vector and the packaging plasmids pΔ8.91 (coding for matrix and transcription proteins) and pMD2G (coding for envelope proteins),

in the presence of Express-In transfection reagent (Thermo Scientific). The original self-inactivating Lenti-SxW lentiviral vector (full name pHR'SINcPPT-SFFV-x-WPRE) used in this study, was developed (Demaision et al., 2002) and kindly donated by Waseem Qasim (Molecular Immunology Unit, University College London). The 868-TCR construct, comprising the 868-TCR alpha and beta chains separated by an SGSg spacer sequence and 18-amino acid 2A fragment (Yang et al., 2008) was initially assembled in a pUC19 shuttle vector, before being transferred to the Lenti-SxW lentiviral vector for transduction of primary CD8⁺ T-cells.

2.3.1.2 CTLs were kindly supplied already transduced with the 868-TCR by Andrew Gerry (Adaptimmune) for use in Chapter 6). Provided is the method performed by Andrew Gerry (Adaptimmune Ltd.). Information provided by Andrew Gerry.

A T150 flask of confluent HEK293T cells was transduced with 15 µg of Lentiviral vector encoding the HIV-1 gag SL9 868-TCR (Varela-Rohena et al., 2008) along with a total of 43 µg of 3 packaging plasmids, using Turbofect transduction reagent (ThermoFisher). Supernatants collected at 24 h and 48 h were concentrated by centrifugation at 10,000 x g for 16 h at 4°C. Cell pellets were resuspended in R10 and snap frozen on dry ice until required.

PBMC were isolated from fresh venous blood from healthy human volunteers. CD8⁺ and CD4⁺ T cells were separated from the PBMC using untouched CD8⁺ and CD4⁺ isolation kits (Invitrogen) as described in the manufacturer's instructions, and incubated separately overnight in R10 culture medium containing 100 IU/ml human recombinant IL-2 (Proleukin, Novartis) and 3 Dynabeads CTS

antiCD3/CD28 beads (Invitrogen) per cell (1×10^6 cells per ml, 1 ml per well of a 24 well plate). Following overnight stimulation, CD8⁺ T cells were transduced by addition of 1 mL crude lentiviral supernatant and fed with CD4⁺ T cell-conditioned medium. 3 days post transduction, CD4⁺ T cells were added to the transduced CD8⁺ T cell wells and fed by addition of fresh medium (with IL-2) every other day, with bead removal at day 5. After 12 days cells were cryopreserved at 2.5×10^6 cells/ml until required.

2.3.2 CTL culture. (*Chapters 5 and 6*) (*Williams et al., 2013b*)

The 868-TCR-transduced primary CD8⁺ T cells received in collaboration with Andy Sewell, University of Cardiff required pre-expansion for up to 3 weeks after initial stimulation, as previously described (Varela-Rohena et al., 2008). Briefly, CTL were defrosted, and incubated in 25mL cytokine media (R10 supplemented with 5% Cellkines, 200IU/mL IL-2, 25ng/mL and 100µg/mL PHA) containing 1 million irradiated feeder cells from 3 donors for 5 days in a tilted TC25 flask. After 5 days, 15mL more cytokine media was added, but this time with no PHA. Cells were then washed and cultured in IL-2 media (R10 supplemented with 100IU/mL IL-2 and 1µg/mL IL-15).

The 868-TCR transduced CD8⁺ T cells required for chapter 6 were defrosted and cultured at 1×10^6 cells/mL in R10 supplemented with 100IU/mL IL-2 overnight before use in killing assays.

2.3.3 CTL treatment with Feraspin-R (Chapter 5) (Williams et al., 2013b)

Feraspin-R (stock concentration 10 mg/mL) was custom made by Miltenyi Biotech, UK. 3×10^5 CTLs per 100 μ L were cultured at 37°C, 5% CO₂ in IL-2 media (RPMI 1640 media (Sigma) supplemented with 25 ng/mL IL-15 (Miltenyi Biotechnology), 100IU/mL IL-2 (Roche Diagnostics), 10% heat inactivated FCS, 2mM glutamine, 0.1 mg/mL streptomycin and 100 U/mL penicillin (all Sigma)), with Feraspin-R concentrations of 0-1mg/mL for 24 hours, unless otherwise stated. Feraspin-R was sonicated before re-suspension in the cell culture medium. For controls, untreated CTLs were cultured identically, except distilled water was added instead of Feraspin-R. After incubation, with or without Feraspin-R, all cells were washed by centrifugation five times in PBS at 350g. An anti-dextran antibody conjugated to FITC, as previously reported by Beer et al (Beer et al., 2008) was used to stain the Feraspin-R treated CTLs intracellularly and extracellularly (section 2.7).

2.3.4 Improving uptake of Feraspin-R. (Chapter 5) (Williams et al., 2013b)

To improve nanoparticle uptake, CTLs were starved for two hours in PBS, washed and cultured in R10 media prior to incubation with nanoparticles. Other studies have demonstrated the transfection reagents Protamine Sulphate (Sigma) and Lipofectamine (Invitrogen) increase cellular SPION uptake (Beer et al., 2008, Arbab et al., 2005). The experiments were performed as published. Briefly, a 1mM stock concentration of protamine sulphate was made and was added to cell culture medium at 5 μ g/mL. 3.75 μ L of Lipofectamine was added to 96.25 μ L of R10 media and was mixed for 20 minutes. 50 μ L of 10mg/mL Feraspin-R was added and the

solution was mixed constantly for 20 minutes to load the Lipofectamine. The solution was then made up in R10 to a final concentration of 1mg/mL Feraspin-R. This was then added to the CTL in culture.

2.3.5 Microtomy and preparation for Transmission Electron Microscopy (TEM) *(Chapter 5) (Williams et al., 2013b)*

A minimum of 10^6 Feraspin R-‘loaded’ or ‘unloaded’ CTLs were fixed in 3% w/v paraformaldehyde, 0.05% v/v glutaraldehyde in PBS (pH 7.2) for 5 min at room temperature. Cells were embedded in LR Gold resin for electron microscopy by standard methods. Briefly, cells were stained with uranyl acetate (2% w/v in distilled water), dehydrated through increasing concentrations of ethanol (70-100%) and embedded in LR Gold (London Resin Company, Reading UK). Resin blocks were trimmed and then cut using a Reichart-Jung Ultracut ultramicrotome with glass knife prepared using a LKB knifemaker. Semi-thin (0.5 μ m) sections were mounted onto glass slides, stained with 1% toluidine (with 1% borax) and examined to ensure the cells were in section. Ultrathin sections (50-80 nm) were mounted on nickel grids (Agar Scientific Ltd., UK). Grids were then double stained at room temperature with 2% UA in methanol followed by lead citrate. The latter was done in a carbon dioxide-free environment preventing the formation of a lead-based precipitate. Sections were rinsed briefly in distilled water and then dried thoroughly prior to examination using a JEOL 1010 transmission electron microscope.

2.3.6 Feraspin-R quantification (Chapter 5) (Williams et al., 2013b)

Magnetic susceptibility measurements were performed using a Superconducting QUantum Interference Device (SQUID) (Quantum Design). A change in sample position leads to a change in coil flux and thus circuit current, which can be used to infer the magnetic moment of the sample. Washed Feraspin-R-loaded and unloaded CTLs (minimum of 3×10^5 cells per sample) were left to dry overnight at 37°C and 5% CO₂. Once dried, samples were mounted in a gel capsule placed inside a plastic straw. SQUID voltages at different sample positions are averaged and taken at varying field strengths, up to 7 Tesla. By fitting the data to software models, the magnetic moment of Feraspin-R loaded CTLs can be calculated. The total content of iron per cell was calculated by comparing experimentally determined 'emu' signals with the known emu/g value for Feraspin-R and by correcting for the amount of input cells.

2.3.7 Thermotherapy studies (Chapter 5) (Williams et al., 2013b)

A Magnetic Alternating Current Hyperthermia (MACH) system was used to expose SPION to a 1MHz alternating magnetic field with peak amplitude of 12kA/m, thus generating localised heat (Figure 2.2). To assess heat-related CTL death, CTLs incubated with 1mg/ml Feraspin-R for 24 hours, treated with or without transfection agents or starvation, were exposed to the alternating magnetic field for 1 hour. The solution temperature was kept constant at 37°C using an air warmer, to control for any bath heating effect. Cell death was measured 1 hour and 24 hours post incubation, to detect immediate and delayed cell death, using

the toxicity protocol described above. The experiments were controlled using identically pre-treated CTLs, which were incubated at 37°C for 1 hour, but not exposed to the alternating magnetic field. A further control assessed immediate and delayed cell death of Feraspin-R loaded CTLs by incubating the cells at known temperatures on a PCR gradient (Bio-Rad) for 20 minutes.

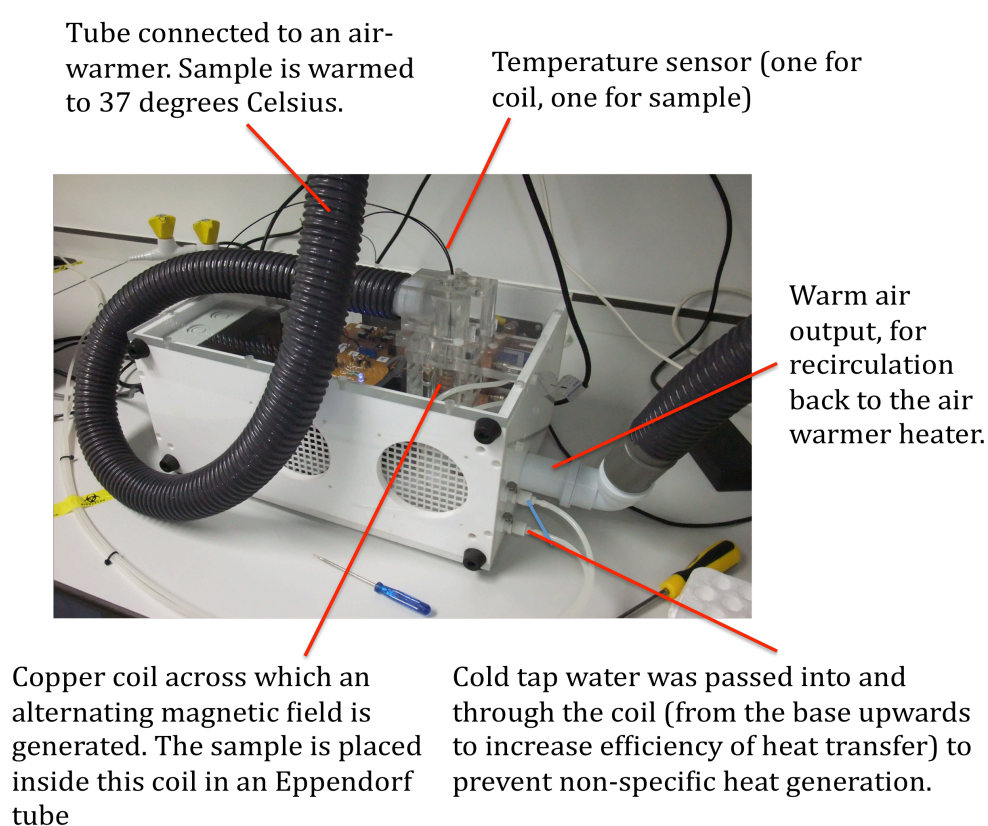


Figure 2.2 Apparatus used for exposing Feraspin-R labeled CTLs to an alternating magnetic field for use in thermotherapy studies.

2.4 Cell lines (Chapters 3, 4, 5, 6)

2.4.1 Cell line culture

8e5, CEM-GXR, U1, ACH2, J-Lat6.3, J-Lat 8.4 and T2 cell lines were all sourced from the NIH AIDS reagent program and cultured in R10 media, unless otherwise stated. Briefly, RPMI-1640 medium (Sigma) was supplemented with L-Glutamine (146mg/L), Penicillin G (100IU/mL) and Streptomycin Sulfate (100µg/mL). Fetal calf serum was inactivated by incubation at 56°C for 60 minutes and was added to a concentration of 10% by volume.

2.4.2 Preparation of anti-latency drugs

Prostratin (Sigma) was diluted in DMSO to a final concentration of 500µM. Suberoylanilide hydroxamic acid (SAHA; Vorinostat), Panobinostat, Romidepsin (all Selleckchem) and Valproic Acid (VPA) (Sigma) were diluted to stock concentrations of 10mM, 10mM, 1M and 1mM, respectively, all in distilled water. Aliquots were discarded if defrosted more than twice.

2.4.3 Stimulation of latent cell lines

U1, ACH2 and J-Lat6.3 and 8.4 cell lines (all from NIH AIDS reagent program) were cultured at 1 million cells/mL in R10 and the presence of drug for 24 or 48 hours, as required in triplicate TC96 plate wells. Unstimulated cells were cultured in the same amount of distilled water or DMSO, depending on drug used.

2.4.4 HLA-A2 transduction of latent cell lines

U1, ACH2 or J-Lat 6.3/8.4 cells were split 1:1 24 hours before transduction. 1 million cells from each cell line were cultured in 0.5mL R10 in a 24 well plate. 1mL of the lentiviral supernatant provided by Adaptimmune Ltd. was then added. After 72 hours, cells were washed twice and HLA-A2 expression was tested (section 2.7.4).

2.5 NL4-3 viral stock manufacture (Chapter 6)

2.5.1 Viral Plasmid Expansion

Plasmid NL4-3 was obtained through the NIH AIDS Reagent Program. It is a full-length replication competent strain of subtype B HIV-1. NL4-3 plasmid was expanded in TOP10 chemically competent *E-Coli* (Invitrogen). 50mL of LB broth was reconstituted from tablets (Sigma) and autoclaved before culturing with E-coli and NL4-3 overnight at 37°C in a shaking incubator. E-coli was then pelleted and the NL4-3 plasmid was extracted using the Qiagen maxi-prep kit, as per manufacturers instructions. The concentration of plasmid DNA was quantified using a nanodrop spectrophotometer. MJ-4 is a replication competent strain of subtype C HIV-1 and was obtained from the NIH AIDS Reagent Program. It was used for subtype C Q-PCR standards. The protocol for amplification and extraction was the same as for NL4-3.

2.5.2 Manufacture of NL4-3 virus

4×10^6 CEM-GXR cells were electroporated with 5 μ g of NL4-3 plasmid (300V, 500 μ F) in 300 μ l R10 media as previously described (Wright et al., 2010, Miura et al., 2009). The cells were then added to 10mL of pre-warmed R10 already containing 1×10^6 CEM-GXR cells and 10 μ L polybrene. 5mL R10 was added after 5 days of culture. The percentage of GFP expression was measured daily with a maximum of 57.4% CEM-GXR cells infected 8 days after electroporation (Figure 2.3). Viral supernatants were harvested at 7 and 8 days after electroporation and kept at -80°C.

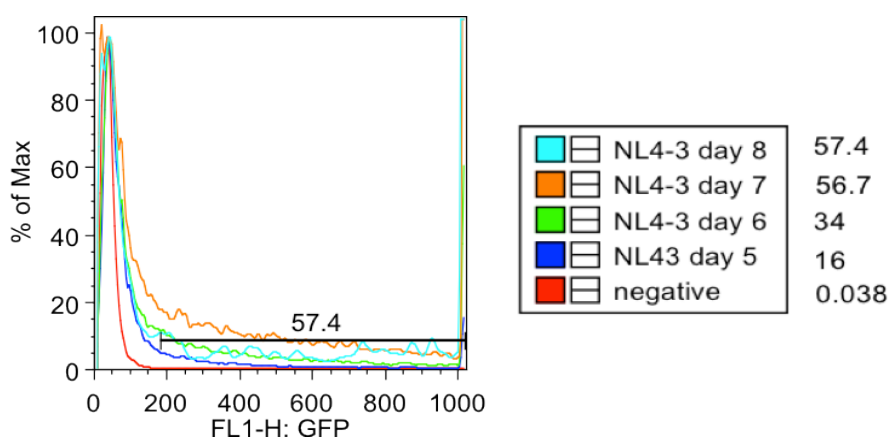


Figure 2.3. Infection rates of NL4-3 plasmid-transfected CEM-GXR cells after 5, 6, 7 and 8 days of culture in R10 media. GFP expression was measured by Flow Cytometry. Numbers represent the percentage of infected cells. Plots gated on uninfected control (negative).

2.5.3 Titering of NL4-3 viral stocks

The day before viral exposure, CEM-GXR cells were split 1:1. CEM-GXR cells were counted and re-suspend at 1×10^6 cells/100 μ l for each sample, including negative control. In a 24 well plate, 400 μ L of defrosted viral supernatant, or media for the

negative control, was added to 100 μ L of the CEM-GXR cells making a total volume of 500 μ L per well. The next day, 1mL of media was added to each of the wells. The following day, the cells were pelleted and the samples were fixed in 10% formaldehyde. The GFP+ fraction, gated on the negative control, was measured on a FACS calibur machine (BD) (Figure 2.4). The titer of the virus was calculated using the following equation:

$$\text{Volume needed for the MOI wanted} = \frac{(\text{MOI wanted} / \% \text{GFP positive cells}) \times 400\mu\text{l}}{(1 \times 10^6 \text{ cells} / \text{number of cells to be infected})}$$

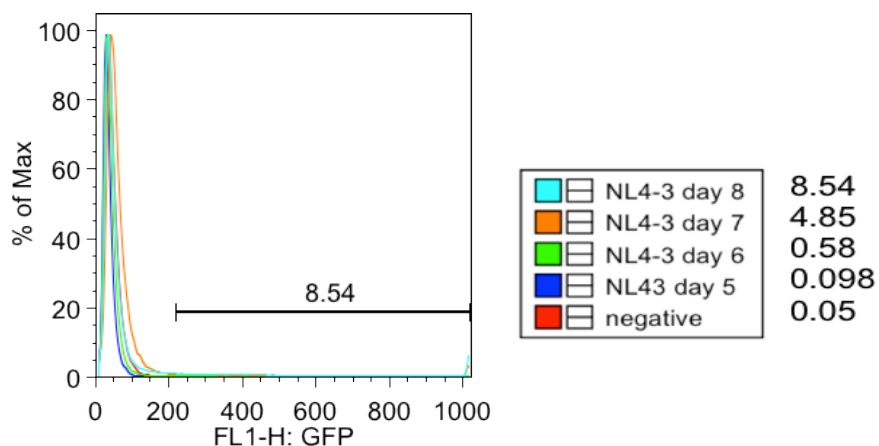


Figure 2.4. Infection rates of uninfected CEM-GXR cells with NL4-3 plasmid-transfected CEM-GXR cell supernatants after 5, 6, 7 and 8 days of culture in R10 media. GFP expression was measured by Flow Cytometry. Numbers represent the percentage of infected GXR cells. Plots gated on control whereby CEM-GXR cells were incubated with supernatant from uninfected GXR cell cultures (negative gxr).

2.6 CD4 + T cells (Chapter 6).

2.6.1 Activated CD4+ T cell generation for infection

250ng/mL PMA and 500ng/mL Ionomycin were added to 1e5 CD4+ T cells in R20 media for at least 5 hours at 37°C, 5% CO₂.

2.6.2 Generation of latently infected cells by spinoculation

The spinoculation protocol has been adapted from (Pace et al., 2012). CD4+ T cells were isolated from uninfected PBMCs using the EasySep CD4+ T cell negative selection kit (Stemcell technologies). Cells were resuspended in R20 media (R10 plus an additional 10% of FCS to minimize cell death) to a concentration of 1x10⁷ cells per mL, and pNL4-3 virus was added an MOI of 3 or 1.5, depending on cell numbers. Negative isolation ensured CD4+ T cells remained untouched, limiting the possibility of activation of these cells. Cells were spun at 1200g for 2 hours at room temperature as previously described (Swiggard et al., 2005). Cells were washed 3 times in R20, and stained immediately, or after at least 2 days of culture in the presence or absence of 1.25µM Saquinavir (AIDS Reference Reagent Program) to inhibit spreading infection at an optimal concentration (Pace et al., 2012) or 30µM Raltegravir (AIDS Reference reagent Program) (Lassen et al., 2012) to prevent integration.

Cells were then stained for activation markers. Cell supernatants from spinoculated resting cells and spinoculated pre-activated cells were spinoculated with 500,000 GXR cells, as above to determine infectious viral release and the percentage of GFP+ cells was measured 2 days post spinoculation.

For reactivation studies, cells were treated with Raltegravir 48 hours after spinoculation and were cultured in the presence of ART and anti-latency drug for 24 or 48 hours, after which p24 and activation marker expression was assessed by FACS (section 2.7.2 and 2.7.4).

Reactivation through spreading infection was assayed 48 hours after spinoculation in cell populations not treated with Saquinavir, or treated with Saquinavir. Untreated were split into two further populations and were either treated with Saquinavir for 24 or 48 hours or left untreated after stimulation with 2 CD3/CD28 beads per cell. If cells are latent, the percentage difference between Saquinavir treated and untreated populations (in terms of intracellular p24 expression) should be greater post stimulation, as spreading infection will have occurred.

For killing assay studies, cells were stimulated for 24 hours with respective anti-latency drugs, 48 hours post spinoculation (MOI 1.5) and in the presence of 1.25 μ M Saquinavir.

2.7 Antibody staining for FACS analysis (All results Chapters)

2.7.1 Extracellular staining

At least 20,000 cells were required for staining per replicate. All experiments required the use of a Live/Dead Near IR fixable dead cell stain (APC-Cy7). Each well of cells was stained with 50 μ L of the live dead stain and the required antibodies, diluted appropriately in PBS. For nanoparticle toxicity measurements,

an annexin V antibody (Miltenyi) was used, and this required the antibody master mix to contain 1x Annexin V binding buffer (BD).

2.7.2 Intracellular staining

For intracellular staining in Chapter 5, cells were resuspended in 200µl of Cytofix/Cytoperm Fixation/permeabilisation solution (BD) for 25 minutes, washed, resuspended in 200µl of Perm/Wash buffer (BD), and washed again twice. Antibody cocktails were added in 10% perm/wash solution, diluted in PBS) for 25 minutes at room temperature. The cells were then washed twice more in Perm/Wash.

For intracellular staining in Chapter 6, the protocol was adapted to give better resolution between negative and positive p24 populations (Graf et al., 2013). Post surface staining, cells were treated with 0.5% paraformaldehyde for 30 minutes and were washed twice in FACS buffer (PBS with 10% FCS by volume and 1mM EDTA (Sigma)). Cells were stained in 50µL of appropriately diluted antibody mixes in FACS buffer and 0.5mg/mL saponin (Sigma) for 40 minutes. Cells were then washed twice in FACS buffer. All cells were finally fixed in 2% formaldehyde (Sigma) for at least 20 minutes and were stored at 4 degrees for no longer than 48 hours before running on an LSR-II FACS machine (Becton Dickinson).

2.7.3 Tetramer folding.

The tube containing monomer was wrapped in foil and 0.2mg/mL extravidin-PE conjugate (Sigma) was added in 10 aliquots over 20 minutes to form a 1:20 molar

ratio of streptavidin conjugate:monomer. Samples were continually mixed on a roller rig at 4°C. Sample was subsequently stored at 4°C for 2-3 months in the dark.

2.7.4 FACS panels

CTL characterisation: 1:50 CD8-PerCP (BD), 1:50 Live/Dead Fixable near Infra Red (IR) dead cell stain kit APC-Cy7 (Invitrogen), 1:100 CD14 (Pacific Blue), 1:50 CD3 (ECD) (Beckman Coulter) and 1:25 SLYNTVATL-tetramer (PE) (2.7.3), all diluted in PBS (Invitrogen). Gates were defined by unstained controls.

Stimulation of latent HIV-1 from cell line models: Extracellular: 1:50 Live/Dead (APC-Cy7), Intracellular: 1:200 p24-RD1 (Becton Coulter). Gates were based on unstimulated cells.

Spinoculation of CD4+ T cells: Extracellular: 1:50 Live/Dead (APC-Cy7) and Intracellular: 1:200, p24-KC57 or RD1 (Beckman Coulter), (optionally 1:100 CD4 (AlexaFluor700) (BD) if PBMCs were spinoculated). Gates were based on uninfected cells

CD4+ T cell activation panel: 1:50 Live/Dead (APC-Cy7), 1:100 CD4 AlexaFluor700 and either 1:50 HLA-DR (Pacific Blue), 1:50 CD25 (APC) and 1:50 CD69 (FITC) (all ebioscience), or 1:50 CD69, HLA-DR and CD25, all on FITC (all ebioscience). Gates were based on FMO controls.

Staining MHC Class One. 1:50 Live/Dead (APC-Cy7), 1:100 HLA-A2 (PE or APC) (ebioscience) or 1:100 HLA-ABC (FITC (ABD serotech) or APC (ebioscience)). Gates were based unstained cells.

Staining dextran coated nanoparticles: 1:50 Live dead (APC-Cy7), 1:25 Dextran-FITC (either intracellularly or extracellularly stained) (Stemcell Technologies). Dextran negative cells were gated on untreated CTLs.

Nanoparticle toxicity/thermoablation: 1:50 Live/Dead (APC-Cy7), 1:25 Annexin V-FITC (Miltenyi). Annexin-V⁺ Dead⁺ gates were defined using 50mM Camptothecin (Sigma) treated cells for 4 hours, as a positive control.

2.8 FATAL killing assay (Chapters 5 and 6)

This assay is adapted from the FATAL (Fluorometric assessment of T lymphocyte antigen specific lysis) assay, as first described by (Sheehy et al., 2001).

2.8.1 Killing of peptide-pulsed cells (Chapters 5 & 6). (Williams et al., 2013b) (CTL provided by Andy Sewell).

T2 (TAP-deficient) 'target' cells or HLA-A2 transduced U1 or J-Lat 6.3 were incubated (pulsed) with the HIV-1 Gag peptide, SLYNTVATL or A2 non-target peptide FLGKIWPSYK (both NIH AIDS Reagent Program) at the required concentrations for 1 hour at 37°C, and then washed three times in PBS. The peptide-pulsed T2 cells were stained with 5mM carboxyfluorescein succinimidyl ester (CFSE) stock (Invitrogen), diluted to 0.1µM, for 10 minutes, and washed twice in PBS. 50,000 peptide-pulsed cells were added per well in 50µl R10 media. 50,000 unpulsed T2 cells were stained with 10mM CellTracker Orange (CTO) diluted to 5µM (Invitrogen) for 20 minutes, washed twice in R10 and added to each well at the same concentration and volume. 868-TCR-transduced CTLs were

added at the specified Effector:Target ratio to a total volume of 150µl. FATAL assays were performed at least in duplicate.

After incubation, the cells were washed and stained with CD8-PerCP (BD) and the Live/Dead Fixable near Infra-Red dead cell stain (Invitrogen) in PBS for 25 minutes at 4°C. The cells were washed twice, resuspended in PBS and stored in the dark at 4°C until use. CFSE (FITC) versus CTO (PE) FACS plots were gated on Live, CD8 negative cells (Figure 2.5). The percentage of CFSE+ target cells (relative to CTO+ non target cells) in the presence of CTL for each experimental replicate was divided by the mean (of at least two experiments) percentage of CFSE+ target cells (relative to PE+ non target cells) in the absence of CTL. This value was multiplied by 100 and was subtracted from 100 to form % cytotoxicity as a percentage of controls. E.g. For 10⁻⁷M peptide pulsed cells below, % cytotoxicity (% control) = 100-((19.6/52)×100) = 62.3.

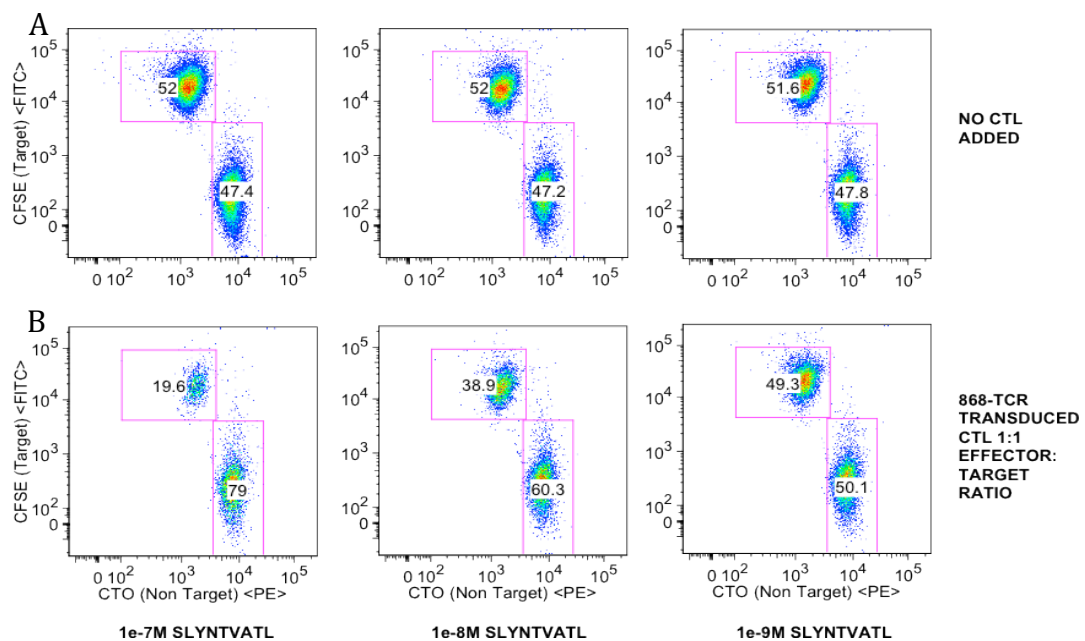


Figure 2.5 FATAL killing assay. A) Target (CFSE-stained) and Non-Target (CTO-stained) T2 cells were incubated in a 1:1 ratio. Target cells were pre-incubated with the stated SLYNTVATL peptide concentration. B) CTLs were added at a 1:1 Effector:Target ratio. Changes in the CFSE+ population can be seen on addition of CTLs. Killing is dependant on dose of peptide used to pulse target cells. All plots are pre-gated on live, cd8- cells.

2.8.2 Impact of temperature on killing assay (Chapter 5) (Williams et al., 2013b)

(CTL provided by Andy Sewell).

To assess the impact of temperature of CTL killing ability, identical FATAL assays using peptide-pulsed target cells were either incubated at 37°C or 40°C, both at 5% CO₂ and were incubated for varying amounts of time.

2.8.3 Impact of Feraspin-R loading on killing assay (Chapter 5) (Williams et al., 2013b)

(CTL provided by Andy Sewell).

To assess the impact of Feraspin-R loading on CTL killing ability, independent FATAL assays were conducted as above, but using CTLs treated with increasing doses (0-1 mg/mL) of Feraspin-R for 24 hours. CTLs were added at varying E:T ratios to the FATAL assay (0:1, 1:1 and 1:10). Target cells were peptide pulsed with a range of SLYNTVATL concentrations.

2.8.4 Impact of thermotherapy on killing assay (Chapter 5) (Williams et al., 2013b)

(CTL provided by Andy Sewell).

To assess the effect of Feraspin-R thermotherapy on the enhancement of Feraspin-R loaded CTL killing, FATAL assays were incubated with and without alternating magnetic field exposure for 5 hours at 37°C. Experiments were performed in duplicate. Target cells were pulsed with 1x10⁻⁷M SLYNTVATL.

2.8.5 Killing of latently infected cell lines (Chapter 6)

The above assay was adapted when using J-Lat6.3 and J-lat 8.4 cells because these cells naturally express GFP, meaning CFSE could not be used to stain target cells. Target cells were instead stained with cell tracker violet (CTV) (Invitrogen).

Since J-Lat 6.3 and U1 cells are infected with latent HIV-1, peptide pulsing is not necessarily required for them to be targeted by CTL. Input cells were either left unstimulated or were stimulated with the required concentrations of anti-latency agents for 24 hours or 48 hours for J-lat6.3 and U1 cell lines respectively. Target cells were those transduced with HLA-A2; non-target cells were those expressing a native MHC class I repertoire. However, both target and non-target cells were cultured and stimulated identically. CTLs, provided by Adaptimmune Ltd. were added at a 1:1 E:T ratio unless otherwise specified. Killing assays were run overnight or for 5 hours, to limit bystander killing, as specified.

2.8.6 Killing of latently infected primary cells (Chapter 6)

A combination FATAL assay with p24 intracellular stain was developed. The target cells were spinoculated CD4⁺ T cells from an HLA-A2⁺ donor and were stained with CFSE. The non-target cells were spinoculated non-infected CD4⁺ T cells from the same donor and were stained with CTV. CTLs provided by Adaptimmune were added at the described E:T ratio. Cells were then stained after overnight incubation with 1:100 CD8 (AlexaFluor700) (BD), 1:50 Live/Dead (APC-Cy7) (Invitrogen), 1:100 HLA-A2 (APC) (ebioscience). Cells were also stained intracellularly with p24-RD1 (PE) (Beckman Coulter) (Figure 6.18).

3. Method Development of Q-PCR assays for quantification of the HIV-1 Reservoir.

The total HIV-1 DNA and adapted Albumin Q-PCR methods have since been published in the Journal of Virology (Duncan et al., 2013) and the Journal of Virological Methods (Williams et al., 2014a). The integrated HIV-1 DNA assay has submitted as a manuscript.

3.1 Introduction

Quantitative methods that measure HIV-1 DNA levels are becoming ever more important for monitoring disease progression. In order to detect the HIV-1 reservoir and monitor the impact of viral eradication strategies, towards a functional or sterilizing cure, there is a need for sensitive methods to quantify patient HIV-1 DNA copy numbers in clinical trials aimed at characterizing the HIV-1 reservoir and assessing the potential for viral eradication.

Quantitative real-time PCR (Q-PCR) is frequently used to quantify HIV-1 DNA levels. HIV-1 can exist in multiple forms in infected cells (Sloan and Wainberg, 2011), either integrated into the host genome or unintegrated linear and circular (1-LTR and 2-LTR) HIV-1 DNA (Figure 1.3). Q-PCR can assess the relative contributions of these HIV-1 DNA states to the HIV-1 reservoir by using specific primers and probes. Q-PCRs using ‘Total’ HIV-1 primers will identify all forms of HIV-1 present in the cell. If a patient is on suppressive HAART, there should be no residual viral replication and total and integrated HIV-1 DNA levels should converge over time since 2-LTR circles and unintegrated HIV-1 DNA are labile *in vivo* (Sharkey et al., 2005, Chavez et al., 2007). This assumption was made in the

VISCONTI trial, where 'total' not integrated HIV-1 DNA was measured (Saez-Cirion et al., 2013).

However, other studies suggest that 2-LTR circles are much more long-lived than previously thought (Pierson et al., 2002, Butler et al., 2002) and supposing there is some residual replication of HIV-1 on non-suppressive HAART, not all reverse transcripts necessarily integrate (Chun et al., 1997a). Indeed, studies have shown that there often is an abundance of total HIV-1 DNA levels over integrated DNA even after a year of HAART meaning total HIV-1 DNA levels cannot act as a surrogate marker for integrated HIV-1 DNA (Agosto et al., 2011).

There are a number of methods to specifically measure integrated HIV-1 DNA levels (summarised in (Liszewski et al., 2009)). Gel separation can separate genomic DNA from shorter, unintegrated DNA species, but this process is laborious for large samples and has debatable purity (Lehrman et al., 2005, Koelsch et al., 2008). Inverse and linker PCRs (Chun et al., 1997a, Chun et al., 1995) lack precise integration standards and the high mutation rate of HIV-1 creates issues in the DNA digestion steps, common to both these assays (Liszewski et al., 2009).

Most research groups nowadays quantify integrated HIV-1 using Alu-PCR methods. Alu elements are around 300bp long and occur randomly every 5000bp through the genome (Jelinek and Schmid, 1982, Mighell et al., 1997). There is an 'up-front' first round PCR using one genomic and one HIV-1 specific primer. This biases the subsequent second round HIV-1 specific Q-PCR step to amplify integrated rather than unintegrated HIV-1 DNA. Alternatively, a 'tag' can be attached to the first

round HIV-1-specific primer, which can then bind a second round HIV-1-specific primer (Brussel and Sonigo, 2003, Yamamoto et al., 2006). This ensures that only first round product is amplified in the second round.

In this chapter, two methods that measure integrated HIV-1 DNA were tested. One method involves the use of a tagged first round primer (Yamamoto et al., 2006), and the other is a very rigorous and controlled method for quantifying HIV-1 DNA (Liszewski et al., 2009). Advantages and disadvantages of both methods are uncovered and discussed, and the eventual method (Liszewski et al., 2009) chosen to quantify the SPARTAC trial was adapted.

3.2. Aims and Objectives:

3.2.1 Aims

I aimed to develop sensitive assays that measure total and integrated HIV-1 DNA and to apply these assays to the measurement of the HIV-1 reservoir in acutely HIV-1-infected patients, recruited to the SPARTAC trial.

3.2.2 Objectives

1. Cell number will be quantified using a housekeeping gene Q-PCR assay
2. Total HIV-1 DNA levels will be measured by Q-PCR.
3. Integrated HIV-1 DNA levels will be measured by nested Alu Q-PCR.

3.3 Results

3.3.1 Q-PCR Standards

Absolute quantification of DNA copy number by Q-PCR requires a standard curve. 8e5 and ACH2 cell lines reportedly contain one integrated provirus per cell. DNA from 8e5 and ACH2 cells was extracted and cell number was quantified using a nanodrop spectrophotometer (2.2.2). The standard curve generated was then verified using an integration standard (IS) of known cell and HIV-1 copy number (1 copy of integrated HIV-1 per cell), donated by Una O'Doherty. 8e5 (Ghosh et al., 2003, Desire et al., 2001, Kabamba-Mukadi et al., 2005, Legoff et al., 2006) and ACH2 cells (Chomont et al., 2009) have previously been used as Q-PCR standards. However, unlike for 8e5 cells, ACH2 cells encode a replication competent, inducible provirus (Duh et al., 1989).

Using 8e5 cells as standards, ACH2 cells contained more than one copy of HIV-1 DNA per cell (Figure 3.1) suggesting that some HIV-1 replication was occurring. This was backed up by the presence of constitutive p24 expression by intracellular FACS analysis (Figure 3.1B). Therefore, 8e5 cells were chosen for use in the standard curve for both quantification of cell number and also total HIV-1 copy number.

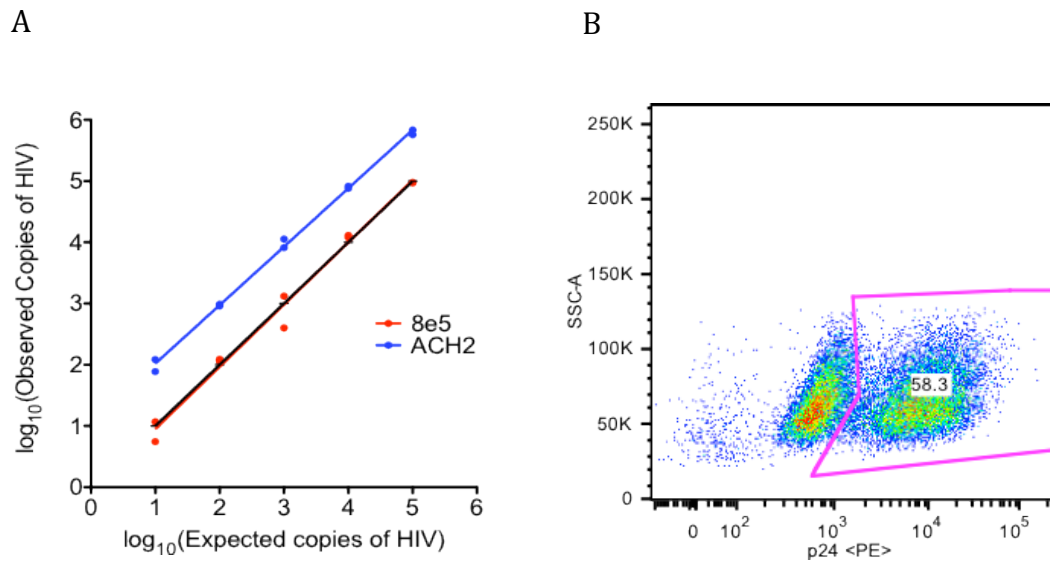


Figure 3.1 Copies of HIV-1 per cell of standard. A) HIV-1 Copy numbers per cell were calculated for 8e5 and ACH2 cells in duplicate. The black line represents the expected values if 1 copy of HIV-1 per cell was present. B) A representative experiment for intracellular p24 staining in live, unstimulated ACH2 cells.

3.3.2 Housekeeping gene Q-PCR assays

The housekeeping gene Q-PCR assay is necessary for expressing copies of HIV-1 DNA per cell equivalent. The genomic target genes Glyderaldehyde-3-Phosphate Dehydrogenase (GAPDH) (Barber et al., 2005) and Albumin (Mexas et al., 2012) were amplified using serial dilutions of 8e5 cell standards ranging from 100,000 to 1 copy. The GAPDH Q-PCR was performed in a total volume of 25 μ l, containing 1 μ l H₂O, 500nM Probe (Roche #60), 500nM GAPDH_F (AGC CAC ATC GCT CAG ACA C), 500nM GAPDH_R (AGG CTC GTA GAC GCG GTT C), 10 μ l Roche Master mix and 10 μ l of sample. Q-PCR amplification was performed using the following program: one cycle of 95°C for 10 minutes; 45 cycles of 95°C for 15 seconds and 58°C for 1 minute. The albumin assay is described in 2.2.3.

Consistently, the Albumin Q-PCR results gave standard curves with lower error and efficiencies nearer to 2 and higher fluorescence values than the GAPDH assay (Figure 3.2), so the Albumin assay was chosen for normalising HIV-1 copy number to the number of input cells.

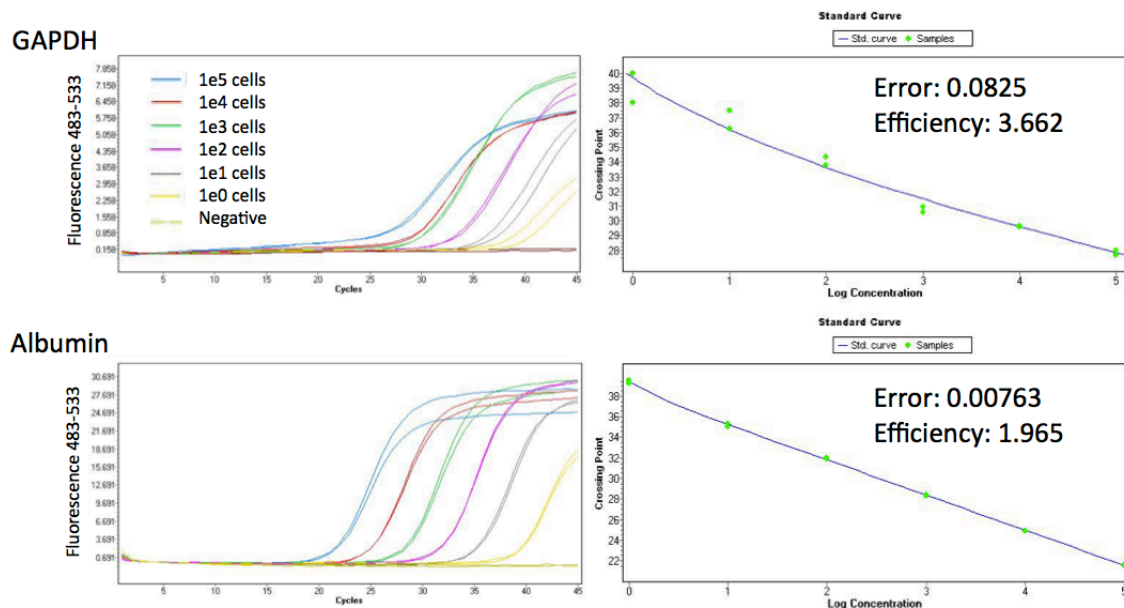


Figure 3.2 Albumin and GAPDH housekeeping Q-PCR assays. Typical Q-PCR readouts using the Roche LC480 software. Amplification curves (left) and standard curves (right) are shown using A) GAPDH and B) Albumin primer/probe sets. All assays were performed in duplicate with the same sample.

3.3.3 Total HIV-1 DNA Q-PCR assays (2.2.3)

The total HIV-1 Q-PCR assay quantifies all HIV-1 DNA in a sample. The DNA can be unintegrated in a circular (1-LTR or 2-LTR circles) or linear form, or integrated into the host genome. A number of different primer and probe sets were compared in terms of sensitivity and reproducibility (Figure 3.3). The LTR_AS/OS primer and probe sets (as designed by Kathleen Gaertner) (method in 2.2.3) and RU5 (Swiggard et al., 2005) primer and probe sets, were the most sensitive, but the RU5 primer/probe set (Forward: 5'TTAAGCCTCAATAAAGCTTGCC-3', Reverse:

5'GTTTCGGGCGCCACTGCTAGA3', Probe 5'FAM-CCAGAGTCACACAACAGACGGGCACA-3') was unable to consistently appropriately amplify the 1 HIV copy standard. The Total_LTR primer and probe sets (Forward: CCGTCTGTTGTG TGACTCTG, Reverse: GAGTCCTGCGTCGAGAGA and Probe: FAM-TAGTCAGTGTGGA -BHQ-1) (Suzuki et al., 2003) consistently resulted in standard curves with unacceptable Q-PCR error and efficiency readouts. Standards were run in duplicate, for each standard dilution. All PCR reactions used the same concentrations of primer, probe and master mix, with the same PCR cycling conditions as detailed for LTR_AS/OS primer and probe sets in 2.2.3.

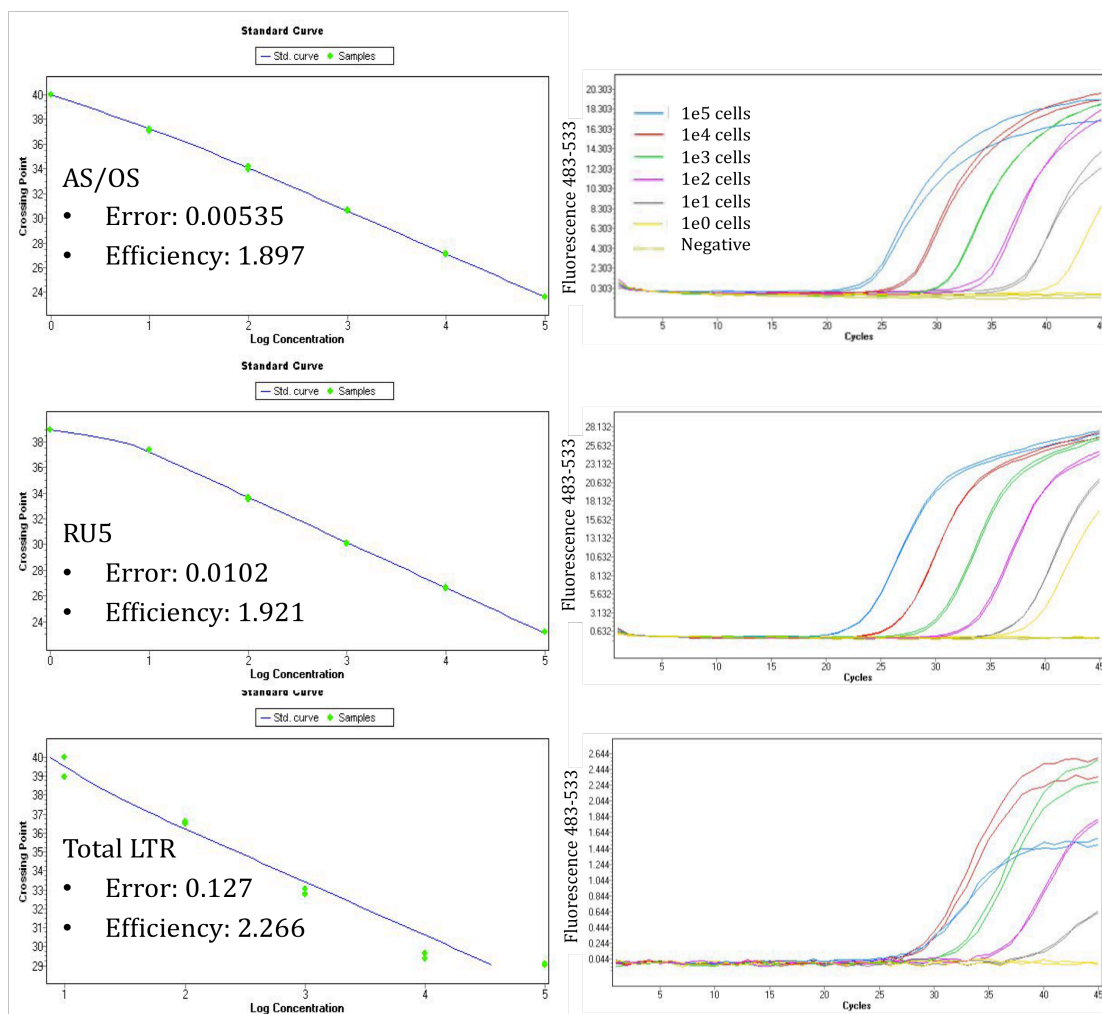


Figure 3.3 'Total' Q-PCR assays. Representative Q-PCR readouts. Shown are calculated standard curves (left) and amplification curves (right) using A) LTR_AS/OS, B) RU5 and C) Total_LTR primer/probe sets. All assays were performed in duplicate.

Patients enrolled in the SPARTAC trial were predominantly infected with Subtype B and C HIV-1. The LTR_AS/OS and RU5 primer and probe sets could both amplify Subtype B virus (Figure 3.3). To determine HIV-1 copy numbers equally across all samples in the trial, primers and probes needed to be cross-clade compatible. The LTR_AS/OS primers and probes were specifically designed by Kathleen Gartner to amplify multi-clade virus.

The LTR_AS/OS and RU5 primer and probe sets were tested for their ability to amplify subtype C sequences using serial dilutions of the subtype C plasmid, MJ4. LTR_AS/OS could amplify this sequence down to 10 copies (Figure 3.4A), whereas RU5 primers and probes could not (Figure 3.4B). The variation between replicates and lack of resolution between 10 and 1 copy samples for LTR_AS/OS is likely down to inaccuracies in dilution of neat plasmid from very high stocks.

The LTR_AS/OS primer and probe set was tested for equally efficient subtype B and C amplification. MJ4 was diluted to the same copy number as the 8e5 subtype B DNA and was assayed in duplicate at 5 dilutions. There was no significant difference across the standard curve Cycle Threshold (Ct) values when either plasmid or cellular DNA was used, by 2-way ANOVA ($p = 0.92$) (Figure 3.4C).

The LTR_AS/OS primer and probe sets were then tested for their ability to amplify subtype B and C SPARTAC samples. It was possible to quantify the amount of HIV-1 DNA per million PBMCs in all 8 samples tested, regardless of HIV-1 clade (Figure 3.4 D).

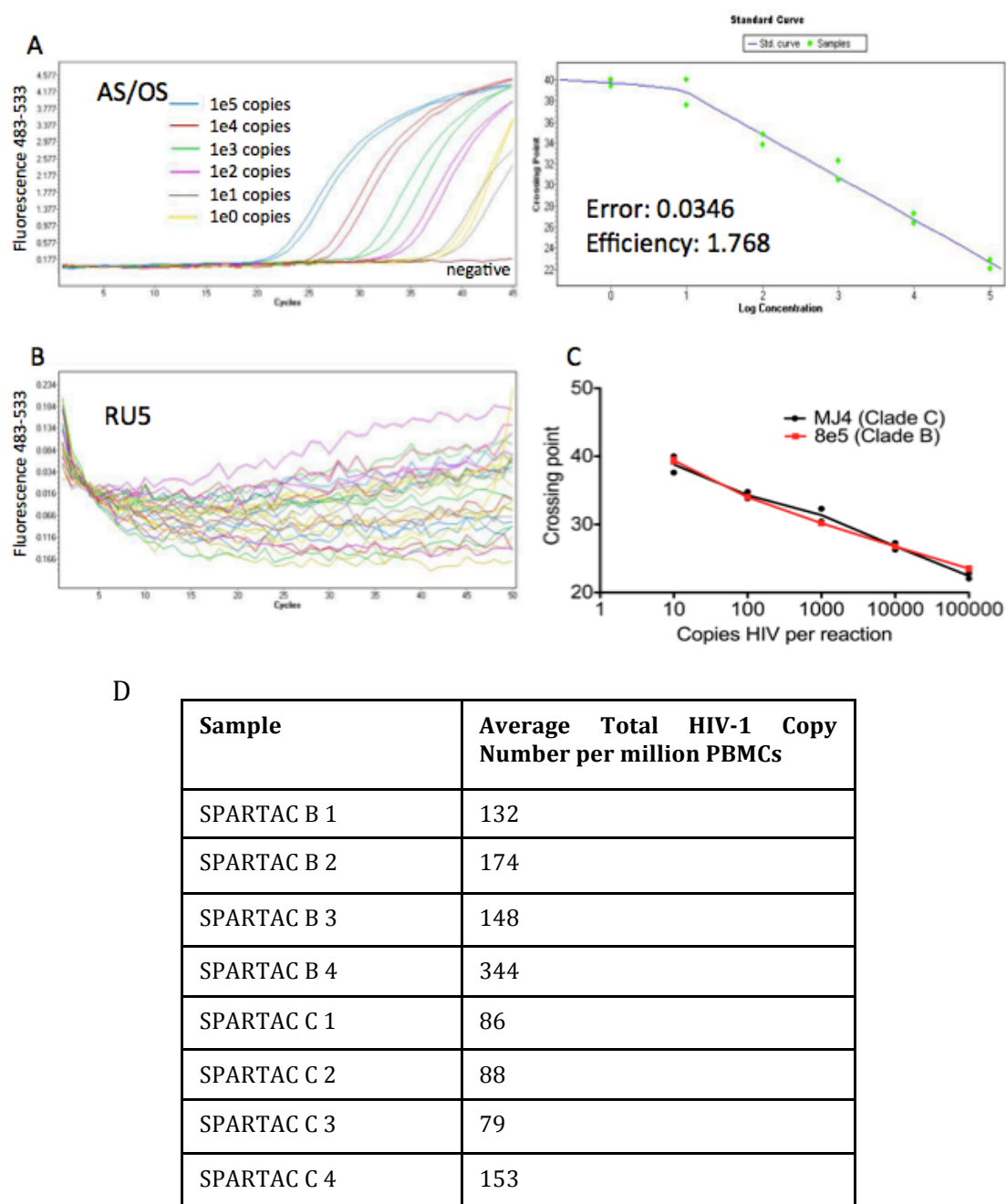


Figure 3.4. Multi-Clade application of LTR_AS/OS ‘total’ assay. A) Q-PCR readouts (left panel) and standard curve (right panel) for LTR_AS/OS primers and probes amplifying the subtype C standard MJ4. B) Q-PCR readouts for RU5 primers and probes amplifying MJ4 standards. C) Cycle threshold (Ct) values for each standard dilution using both clade standards were plotted against input HIV-1 copy number. All assays performed in duplicate. D) HIV-1 DNA copy numbers were calculated using 4 SPARTAC subtype B and 4 SPARTAC subtype C patient samples, each in triplicate.

The LTR_AS/OS primer/probe set was tested for appropriate amplification of a standard with 'known' HIV-1 copy number (Integration Standard (IS); 3.3.5) (Liszewski et al., 2009) alongside the RU5 primer/probe sets. The 'actual' copy number of HIV-1 per unit volume was independently determined in the O'Doherty lab. The LTR_AS/OS primer and probe set gave an HIV-1 copy number, closer to the expected value than the RU5 primer and probe set (Figure. 3.5). The RU5 primer/probe sets appeared to overestimate HIV-1 DNA copy number. Thus, the 8e5 'total' standards were able to accurately quantify the IS 'integration' standards meaning standards for both assays were calibrated.

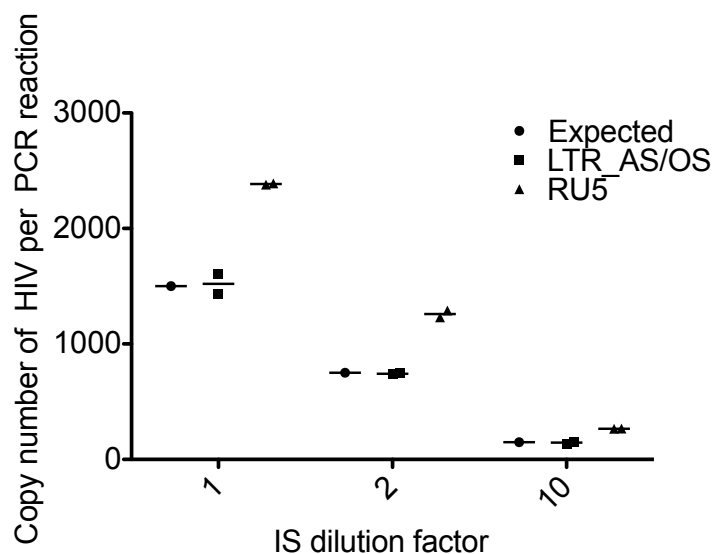


Figure 3.5. Amplification of HIV-1-infected cells of known HIV-1 copy number

The average copy numbers of diluted 'known' IS standard HIV-1 DNA samples were calculated using both LTR_AS/OS and RU5 primer/probe sets, in duplicate.

Due to these observations, the LTR_AS/OS primers and probes were chosen to measure total HIV-1 DNA in all SPARTAC samples.

3.3.4 Integrated HIV-1 DNA Q-PCR assays

Current opinion suggests unintegrated HIV-1 DNA is a labile marker of on-going replication with questionable function (Sharkey et al., 2005, Chavez et al., 2007). In order to more accurately quantify the latent HIV-1 reservoir, some laboratories choose to specifically measure integrated HIV-1 DNA levels.

3.3.4.1 Tagged Alu PCR assay

The first integration assay to be trialled was published by (Yamamoto et al., 2006) (summarised in Figure 3.6). This was chosen because the system ensures only the first round PCR product is amplified in the second round Q-PCR step. Forward (AGCCTCCCGAGTAGCTGGGA) and reverse (TTACAGGCATGAGCCACCG) sense Alu primers, which account for proviral orientation relative to Alu repeats (Brussel et al., 2005), and a gag-specific first round primer (CAATATCATACGCCGAGAGTGCGCGCTTCAGCAAG) selectively amplify HIV-1 Alu-gag DNA amplicons, which are used as template for the second round Q-PCR reaction. The first round gag primer has an artificial non-binding 'tag' (underlined), which acts as the target for the second round 'gag-tag' primer (CAATATCATACGCCGAGAGTGC). The other second round primer targets the HIV-1-LTR region (TTGTTACACCCTATGAGCCAGC).

For the first round PCR, 1/10 of total cell DNA was used as an input in a 25 µL reaction mixture comprising 1xTaqman buffer A, 300 nM primers, 3.5 mM MgCl₂, 200 µM dNTP, and 0.025 U/µL AmpliTaq Gold. Cycling conditions were as follows: 10 minutes at 95°C and 12 cycles of (95°C for 15 s, 60°C for 30 s, 72°C for 5 min).

For the second-round Q-PCR, 1/25 of the first-round PCR product was used as an input in a 25 μ L reaction mixture comprising 1xTaqman buffer A, 3.5 mM MgCl₂, 300 nM primers, 600 nM probe, 200 μ M dNTP, and 0.025 U/ μ L AmpliTaq Gold. Cycling conditions were as follows: 95°C for 10 min, followed by 45 cycles of amplification (95°C for 15s, 60°C for 60s).

The probe (FAM-CGCTTCAGCAAGCCGAGTCCTGC-BHQ) binds to the same sequence as the first round gag primer, but with higher affinity. This stops amplification of the first round product by the first round primer, which may still be present, ensuring only the second round reaction can take place. Because two Alu primers are used, high levels of alu-alu products are formed, which do not act as targets for the second round Q-PCR step. Input DNA was derived from 8e5 cells.

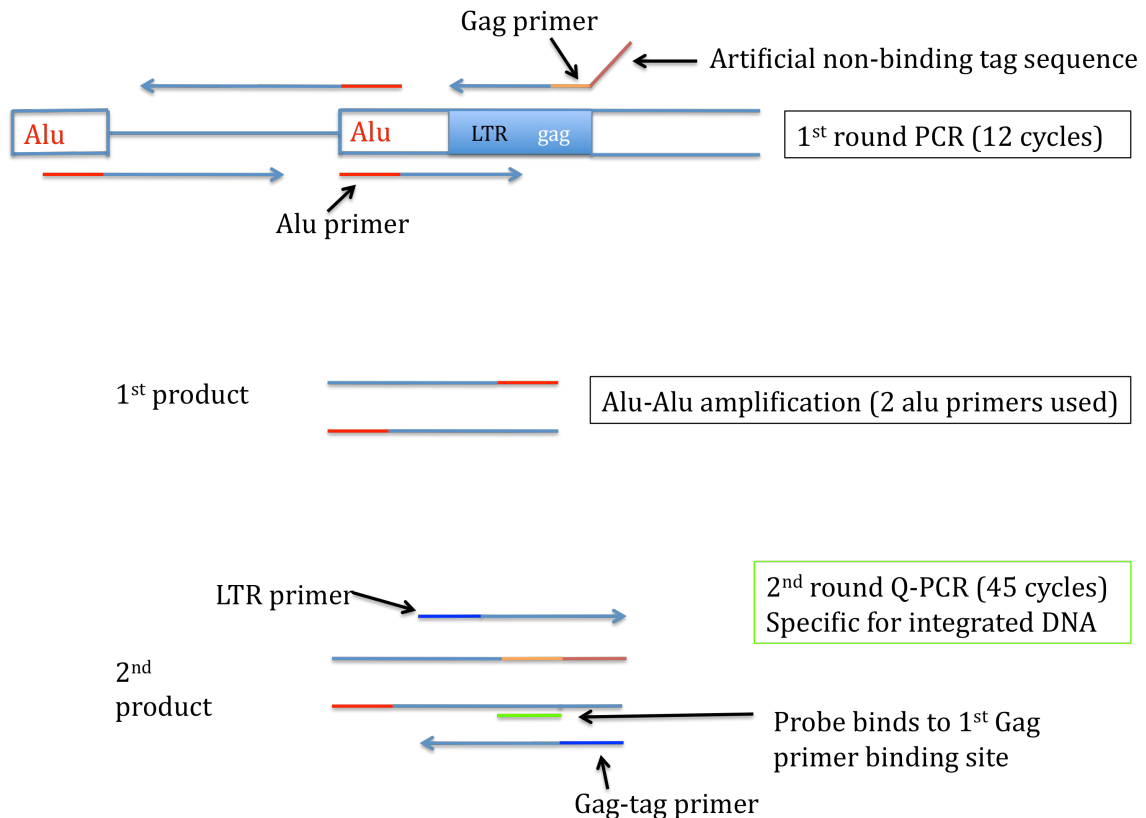


Figure 3.6 Summary of Alu-PCR by (Yamamoto, Tanaka *et al*, 2006).

The method was unable to amplify less than 10,000 copies of HIV-1 (Figure 3.7A). The 2nd round Q-PCR protocol was thus adapted to use the Roche LC480 2x master mix instead of Taqman buffer and Taq, as this was recommended for use with the LC-480 Q-PCR machine. This adaption enabled 1000 copies of HIV-1 to be detected (Figure 3.7B). Due to the sensitivity of the LTR_AS/OS assay, the forward primer was changed to LTR_OS (Figure 3.7C). This resulted in improved fluorescence readings. Addition of 5x more template DNA enabled 100 copies of HIV-1 to be quantified (Figure 3.7D).

This lack of sensitivity may be due to the fact that two alu primers (sense and antisense) were incorporated together to take account of proviral orientation. As there would be so much alu-alu priming, the amount of actual alu-gag priming might be limited, resulting in high background and low sensitivity. Therefore the same reactions were set up using both primers (Figure 3.7E), or reactions in which either primer was added, followed by post-amplification mixing (Figure 3.7F).

However, the assay could still not discriminate below 100 copies of HIV-1. This sensitivity, despite optimisation, was not deemed sufficient. Also, when the first round PCR product negative control (where water, but no HIV-1 DNA was added) was assayed as template in the second round Q-PCR step, there was consistent DNA amplification at levels similar to 10 copies of HIV-1. This non-specific amplification was described in the original paper (Yamamoto et al., 2006), but it amplified later than their lowest standard and was deemed not a problem. For these reasons, another integration assay needed to be attempted.

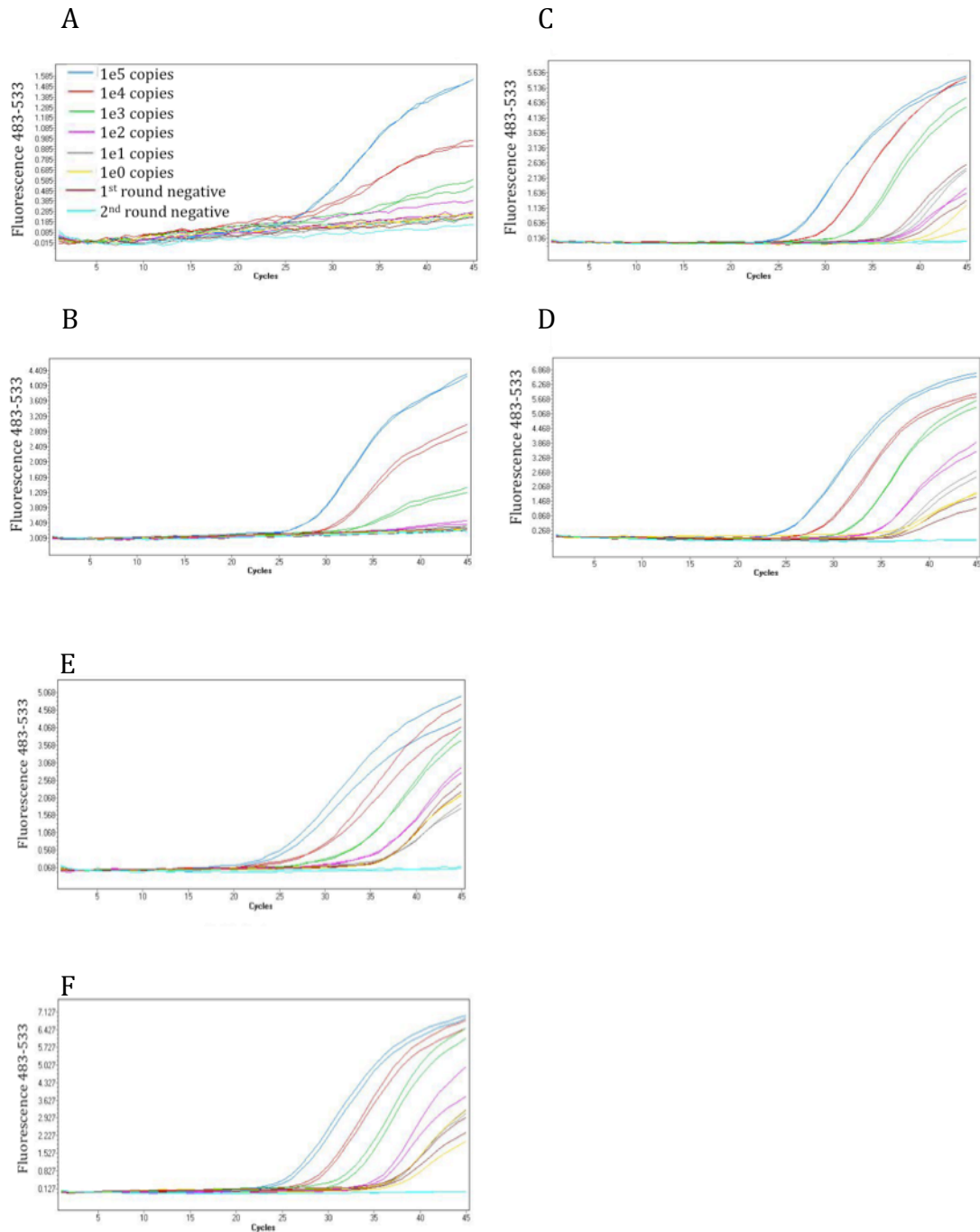


Figure 3.7. Optimisation of tagged Alu-PCR method. Q-PCR readouts showing amplification curves for assays that were performed A) as published, or with modified B) Master mix C) LTR Forward primer, D) input DNA amount. E) Both Alu primers were used in the same first-round PCR reaction before second-round Q-PCR analysis, F) Each Alu primer was used separately in two independent first-round PCR amplifications, and the products were mixed post-amplification for the second-round Q-PCR step. All assays performed in duplicate.

3.3.4.2 Repetitive sampling Alu-PCR assay

This assay is strongly based on that of (Liszewski et al., 2009), and I am very grateful to Una O'Doherty for teaching me this assay at her laboratory at the University of Pennsylvania. It is a rigorous and sensitive assay and was used in a recent collaborative study (Eriksson et al., 2013), where different methods used to measure the HIV-1 reservoir were compared to the gold standard viral outgrowth assay (VOA) (Siliciano and Siliciano, 2005). This assay quantifies replication competent virus that has been stimulated out of latently infected cells. Of all reservoir quantification techniques tested, the integration assay correlated best with the VOA.

3.3.5 Explanation of the original method (Summarised in Figure 3.8. Modified (see 3.3.6) method presented in 2.2.4.

The IS is used to construct the standard curve. This standard reflects *in vivo* integration site heterogeneity, which is crucial since amplification occurs more efficiently when HIV-1 integrates closer to an Alu site. Briefly, the IS was prepared by infecting 293T cells with a replication defective retroviral construct (HIV-1 env was replaced with a green fluorescent protein (GFP) gene). Thus, unintegrated DNA will dilute to undetectable levels as the cells double, whereas integrated HIV-1 DNA remains stably associated (Liszewski et al., 2009). Approximately 1 copy of HIV-1 DNA per GFP expressing cell was calculated, and southern blots demonstrated variable amplicon lengths after PCR, indicating integration site

heterogeneity. The presence of a representative standard for *in vivo* infection also negates the need for two alu primers.

Multiple IS dilutions were used to construct the standard curve. Since each dilution for the standard curve requires 40 PCR replicates with additional controls, the standard curve cannot be run alongside patient samples on the same plate. Instead it must be set up initially and monitored over time for deviation, using IS controls diluted to known copy numbers. For each point on the standard curve, the IS is diluted in 2µg/ml PBMC-DNA and this 'known' copy number is added to each of 40 PCR wells for first round PCR amplification. The IS is spiked into PBMC-DNA unlike for the total assay because the first round PCR assay primes genomic DNA, so the same amount of Alu sites must be present. 2 µg/ml PBMC-DNA is used as this corresponds to the addition of 7500 cell equivalents per replicate – the number of patient cells that are quantified per well. So few cells are added per well because of issues with background Alu-Alu amplifications. By using a small cell number and repeating many times, the sensitivity can be vastly improved. The first round PCR reaction involves a genomic Alu forward primer and a multi-clade reverse primer in gag (Alu-Gag). 40 PCR cycles are performed.

The second round PCR reaction is not specific for integrated HIV-1 DNA. However, the 40 cycles of first round PCR biases the 45-cycle second round Q-PCR to amplify integrated HIV-1 DNA. Because of this non-specificity, it is important to measure the contribution of unintegrated HIV-1 DNA to the output Q-PCR signal. In order to assess this, 40 first round PCRs containing the gag primer only, are incorporated onto the same first round plate and are then assayed identically in the second

round Q-PCR step. The RU5 primer and probe sets are used for the second round Q-PCR (3.3.3).

Three DNA populations are detected by Q-PCR; wells in which there is no HIV-1 DNA (NEG), wells in which there is a background gag-only HIV-1 DNA signal (GAG) and wells where there is specific amplification of Alu-gag integrated HIV-1 DNA (ALU) (Figure 3.9). The ALU reaction Q-PCR Ct values are averaged and if they are significantly different ($p=0.1$) from the GAG reactions, the $\text{Ln}(\text{average ALU Ct})$ is plotted against the $\text{Ln}(\text{copy number added to each well})$ (Figure 3.9, 3.10A). This was performed, at least in duplicate, for each IS dilution. However, if low input HIV-1 DNA is to be quantified, the average ALU and GAG Ct values will not be significantly different. In this case, a distinct standard curve is used. The natural log of the percentage of the 40 wells that have a Ct value significantly (2.s.d) higher than the average GAG signal is plotted against the $\text{Ln}(\text{copy number added to each well})$ to form a 'percent positive' standard curve (Figure 3.10B).

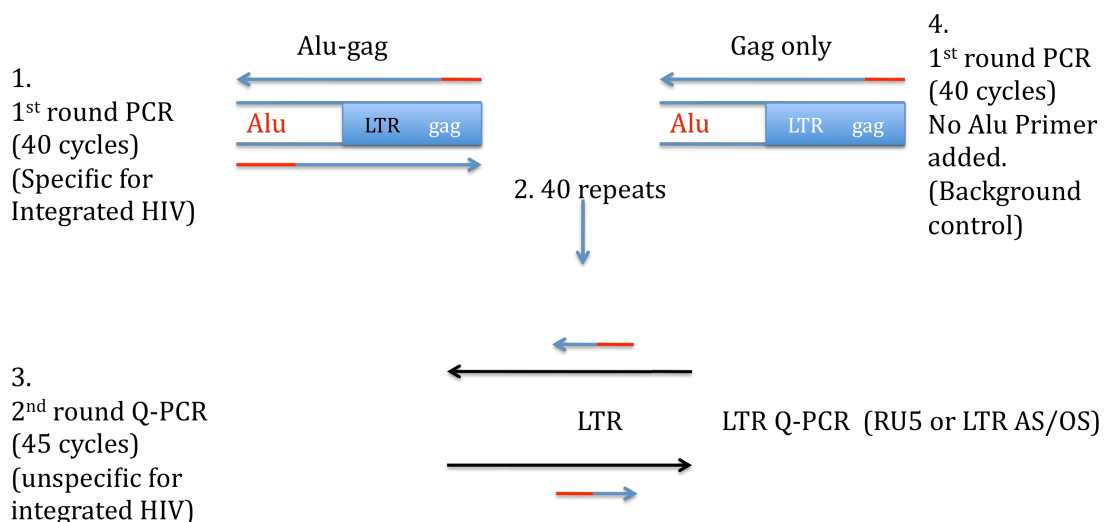


Figure 3.8 Summary of the ALU-PCR assay as published by Liszewski *et al*

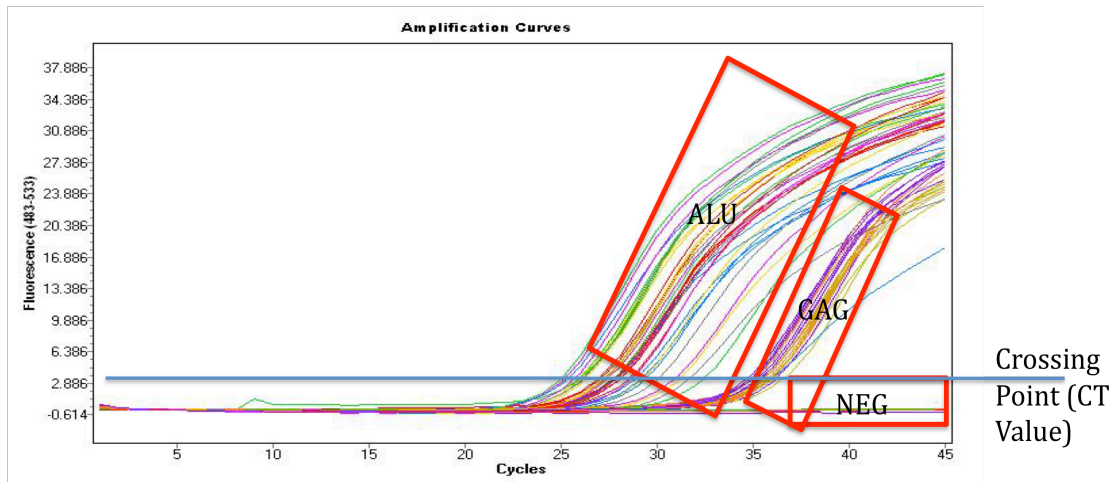


Figure 3.9 Q-PCR amplification curve readout for the integration assay

Three amplified populations are observable: wells in which there was no amplified HIV-1 DNA (NEG), background 'unintegrated' HIV-1 DNA levels (GAG) and integrated HIV-1 DNA levels (ALU), from a successful first round ALU-GAG PCR amplification. The Cycle threshold values were calculated by Roche LC480 software (represented by blue line). 40 ALU and 40 GAG replicates shown. Representative of 40 HIV-1 copies per well.

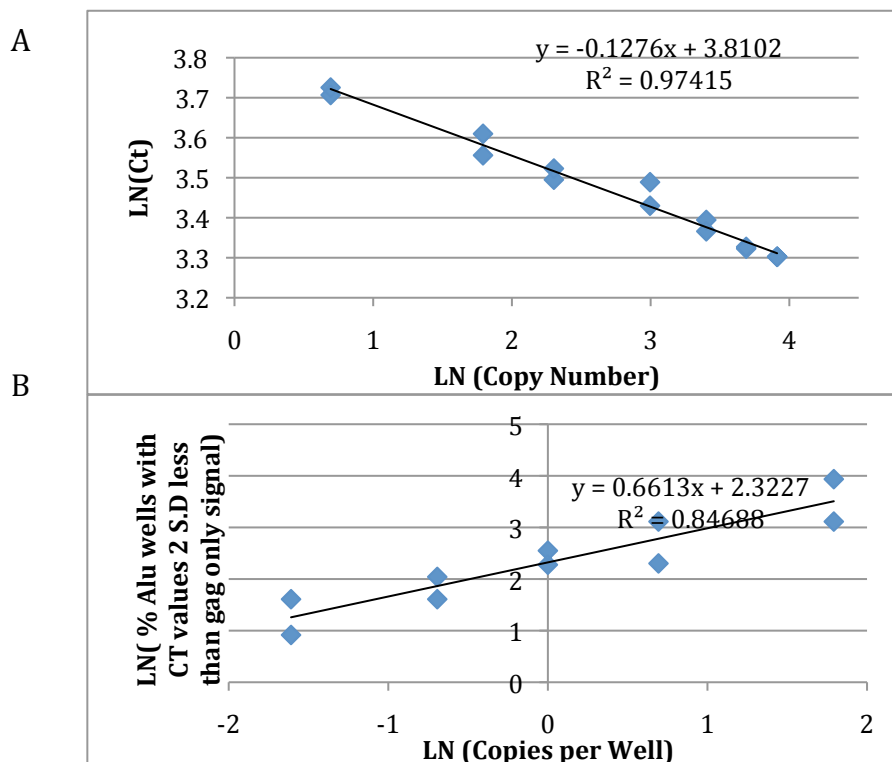


Figure 3.10 Statistical significance between ALU and GAG populations determines the use of the appropriate standard curve. A) When the difference between ALU and GAG is smaller than $p = 0.1$, all ALU Ct values are averaged and plotted against input copy number of IS per well. The data is log-transformed. B) When the difference between ALU and GAG is greater than $p = 0.1$, the percentage of the 40 ALU wells that have Ct values significantly different (2 standard deviations) from the average GAG signal, is plotted against input copy number of IS per well. The data is log-transformed.

3.3.6 Problems and solutions:

1. Optimisation of the integration assay Q-PCR master mix

The Q-PCR master mix was first modified to use 2x Roche master mix, as this was required for optimal performance on the LC480 Q-PCR platform. Since the input DNA from the first round PCR is 10µl, to use the master mix at 2x and to have appropriate primer and probe concentrations, the final volume of the Q-PCR reaction needed to be 25µl rather than 20µl. Also, in order for the integration assay and the total assay to be the most comparable and multi-HIV-1 clade compatible, it was decided that the second round Q-PCR step for the integration assay should be the same as the optimised 'total' assay. The RU5 primers and probe set was not used. The denaturation and extension times for the Q-PCR step were adapted for optimal performance.

2. Optimisation of Taq use.

Taq was titrated down from 2.5x to 1.5x (Figure 3.11A and B respectively). On amplification of Despite the ALU Ct values shifting to the right (i.e. indicating a loss of sensitivity), the ALU and GAG populations were still highly significantly different ($p = 1.5e-15$). Using enriched CD4⁺ T cell DNA (Figure 3.11C) rather than PBMC DNA (Figure 3.11D) resulted in Q-PCR ALU Ct values to be substantially shifted back to the left. This increased sensitivity agrees with a previous study (Eriksson et al., 2013). Dynabeads were used to enrich CD4⁺ T cells to 98.2% purity (Figure 3.11 E and F).

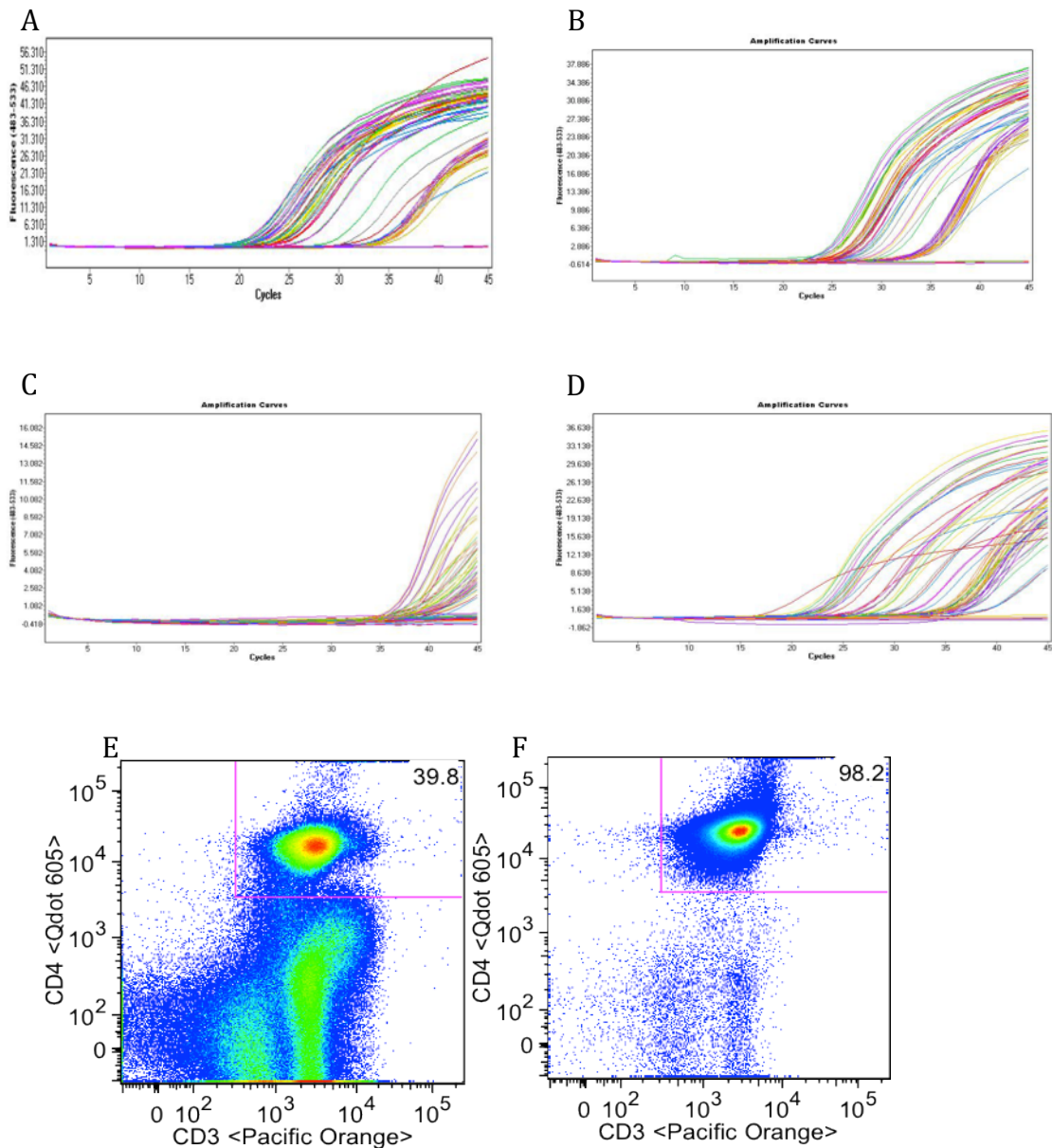


Fig 3.11. Optimisation of the integration assay. Master mix taq concentrations were reduced from A) 2.5x to B) 1.5x. Quantification of HIV-1 DNA from C) PBMC or D) CD4+ T cell-enriched DNA from the same patient. 40 ALU and 40 GAG replicates were performed. Q-PCR plots based on addition of 40 HIV copies per well. Proportion of CD4+ T cells E) before and F) after CD4 enrichment. CD4+ T cell enrichment. Gates determined using PBMC populations. Dead cells have been excluded.

3. Application of a single standard curve to the Alu PCR assay.

A new universal standard curve was generated by averaging all ALU Ct values for each IS dilution, so long as at least one ALU Ct value was 2.s.d lower than the average GAG Ct signal (Figure 3.12). Since the GAG wells were only present to distinguish the potential usage of the two standard curves for sample quantification, the number of repeats was decreased to 5. All points on the graph fitted extremely well to a line of best fit ($r^2 = 0.99$; polynomial regression), which was then used to calculate copy numbers in patient samples.

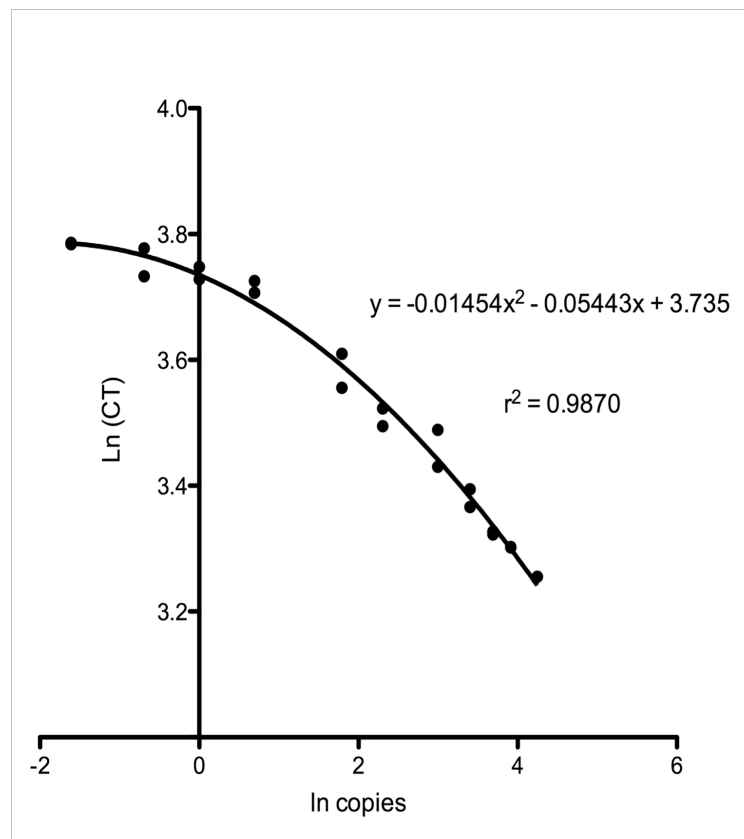


Figure 3.12. A universal standard curve for calculating integration levels
All ALU Ct values for each IS dilution, so long as at least one ALU Ct value was 2 standard deviations lower than the average GAG Ct signal, were averaged. A polynomial line of best fit was fitted to the data. Each point represents an average of 40 replicates. The entire standard curve was repeated in duplicate.

4. Estimating error in the integration assay

Each ALU crossing point value was inserted into the standard curve, rather than just the average of all 40 replicates. PBMCs from 47 HIV-1 infected patients enrolled in the SPARTAC trial, who received 48 weeks of HAART, had undetectable viral loads and were sampled at the point of treatment interruption (demographics in Table 4.2) were enriched for CD4+ T cells by negative selection and all DNA was extracted (2.2.1). When median total and integrated HIV-1 DNA levels (1814 and 992 copies of HIV-1 DNA per million CD4+ T cells, respectively) were calculated, the results were comparable with what has previously been reported (Figure 3.13 and Table 3.1).

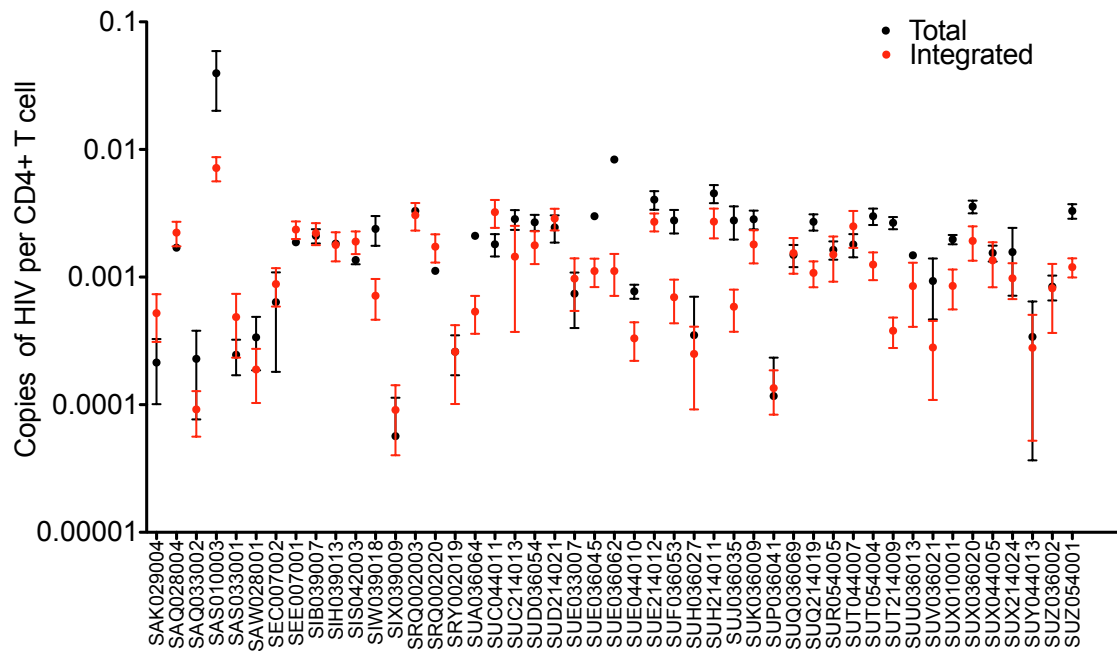


Figure 3.13. Comparison of SPARTAC patient total and integrated HIV-1 DNA levels. Total and integrated HIV-1 DNA levels calculated with 1-year HAART suppressed SPARTAC patients are broadly similar to expected levels published elsewhere (Agosto, Liszewski et al. 2011) using the assays developed in this chapter. For each patient sample, 40 replicates were performed for the integration assay and 3 for the total assay.

Table 3.1. Comparison of published HIV-DNA levels per million CD4+ T cells, for patients treated with one year of HAART, in PHI or chronic infection.

Study	'PHI'	'Chronic' infection
(Koelsch et al., 2008) ^a	Total:494 Integrated: 50	Total: 2488 Integrated: 788
(Graf et al., 2013)		Total: 660 ^b Integrated:199 ^b
(Eriksson et al., 2012) ^c (Per million CD4 T cells)	Total ^d :47 ^b (84 ^e) Integrated :84 ^b (400 ^e)	Total ^d : 93 ^b (286 ^e) Integrated : 286 ^b (850 ^e)
(Besson et al., 2014)		Total: 1054
(Ibanez et al., 1999)		Total: 500 ^b Integrated:110 ^b

- a) Raltegravir was included in the HAART regime.
b) Per million PBMCs not CD4+ T cells
c) Acute and chronic patients treated with HAART for 5 and 8 years, respectively
d) A Droplet Digital Total PCR assay was used (total level was lower than integrated level).
e) Estimates of CD4+ T cell infection, from graph (as raw data is not presented)

5. Assessment of plate-to-plate variation

To assess plate-to-plate variation, 8e5 cells, which have a fixed integration site, were assayed in quadruplicate after dilution to 100 copies per well (Figure 3.14). The plate-to-plate coefficient of variation was 8.31% (average of 72 plates). Additionally, these internal standards demonstrate concordance between the integration and total assay because approximately 1 HIV-1 copy per 8e5 cell was calculated, using the integration assay (Figure 3.14). This is expected since 8e5 cells encode replication incompetent virus and total and integrated HIV-1 DNA levels should be the same.

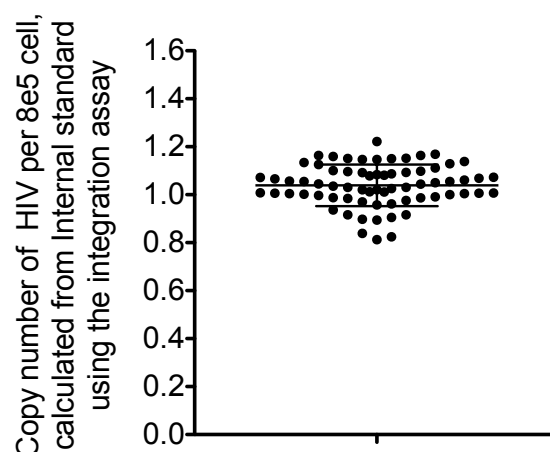


Figure 3.14. Assessment of plate-to-plate variation in the Integration assay. 100 8e5 cells were assayed for HIV-1 copy number in quadruplicate on each integration plate. The data was normalized to HIV-1 copy per cell.

3.4 Discussion

There is a need to identify the most informative assays to quantify the HIV-1 reservoir. Measuring the HIV-1 reservoir is problematic, with multiple available assays ranging from simple Q-PCR estimation of cell-associated HIV-1 DNA to expensive and labour intensive viral outgrowth assays (Liszewski et al., 2009, Avettand-Fenoel et al., 2009, Eriksson et al., 2013, Siliciano and Siliciano, 2005).

The viral outgrowth assay (VOA) is considered to be the gold standard assay for measuring the HIV-1 reservoir (Siliciano and Siliciano, 2005) and was used as a benchmark measure of the latent reservoir in a recent collaborative study comparing the methods available (Eriksson et al., 2013). This assay detects replication competent virus after latently infected patient cells are stimulated with anti-latency agents *ex vivo*. Despite the VOA having been simplified in a recent publication (Laird et al., 2013), it is still a complex procedure and required much more starting material than was available from the SPARTAC trial. Also, because not all inducible proviruses are stimulated out of latency, this method underestimates the latent viral reservoir size (Ho et al., 2013) and could explain why the reservoir levels calculated by this method were so much lower compared to the HIV-1 DNA readouts in a recent collaborative study (Eriksson et al., 2013).

Conversely, defective and thus 'non-inducible' proviruses also exist. These cannot contribute to viral rebound and clinical progression on HAART interruption. Q-PCR methods cannot discriminate between inducible and defective proviral sequences, so latent reservoir size may be overestimated (Ho et al., 2013, Eriksson et al., 2013). Strictly speaking, for the purposes of true HIV-1 eradication, there must be no detectable HIV-1 DNA present whether it is functional or not. However, defective HIV-1 DNA may still be present in those individuals who have been cured. Additionally, extremely low and even undetectable reservoir levels after years of HAART are not necessarily indicative for the potential of viral recrudescence on treatment interruption (Chun et al., 2010, Henrich et al., 2014).

However, it must be noted that patients who demonstrate long-term virological control have low or undetectable HIV-1 DNA levels as measured by Q-PCR. Also, the estimated size of the HIV reservoir as measured by the integration Q-PCR assay correlated best with the VOA assay in the collaborative study by (Eriksson et al., 2013). Therefore, there is still a need to quantify the latent reservoir by Q-PCR.

Three Q-PCR assays are presented to quantify HIV-1 DNA levels in the SPARTAC trial. The albumin Q-PCR method, which was slightly adapted for use on the Q-PCR LC480 instrument (Mexas et al., 2012) (Figure 3.2), and the total assay (Figure 3.3, 3.4, 3.5) have since been published in the Journal of Virology (Duncan et al., 2013) and the Journal of Virological Methods (Williams et al., 2014a). The integration assay was adapted from the previously published original method (Liszewski et al., 2009) (Figure 3.11, 3.12).

As in other studies, the 8e5 standards used for 'total' HIV-1 DNA quantification were diluted in water (Desire et al., 2001, Koelsch et al., 2008), not a background of PBMC-DNA. This may affect 'total' assay sensitivity, however the effect is likely to be small since total and integrated HIV-1 DNA levels were so similar in patients who received 48 weeks of HAART, as they theoretically should be assuming no continued viral replication. Also, there is no genomic priming in the total assay, as in the integration assay where dilution of IS in PBMC-DNA is crucial.

There are caveats associated with the integration assay, primarily the lack of a standard curve on each Q-PCR plate. This meant that the standards could not control for the 8.31% plate-to-plate variation. However, the assay is based on one that was previously published (Liszewski et al., 2009) and plate-to-plate variation, was minimised by using the same reagents, where possible. There was little variation in the standard curve (Figure 3.12) as an r^2 value of 0.99 was achieved, even when two separate Q-PCR plates were run per IS dilution.

The 8e5 cell line, which has one copy of HIV-1 DNA per cell, was used as standards for the total assay (Figure 3.1), and integration standards (IS) were provided by Una O'Doherty for use in the integration assay. Unlike the RU5 primers, the LTR_AS/OS primer and probe sets developed by Kathleen Gartner could quantify HIV-1 DNA from subtype B and C infection (Figure 3.4) and amplified samples of known HIV-1 IS copy number to expected levels (Figure 3.5), so these were used in preference.

Since the integration assay uses the same primers and probes for the second round Q-PCR step and has a multi-clade compatible gag reverse 1st round primer, this assay could potentially also amplify HIV-1 DNA levels from the subtype C cohort. However, the IS used in the study contained NL4-3 virus, a subtype B virus. Therefore, for future studies quantifying integrated subtype C HIV-1 DNA levels in the SPARTAC trial, HIV-1 DNA quantification using IS containing a subtype C sequence (for example MJ4) must be compared to IS containing NL4-3, similarly to what was done in with total assay in this chapter.

The integration assay was optimised for Q-PCR master mix and cycling conditions (Figure 3.11). Additionally, a single standard curve was developed (Figure 3.12) to quantify integrated HIV-1 DNA levels over a larger dynamic range. However, HIV-1 DNA levels calculated for SPARTAC patients using these two optimised methods were comparable to that calculated for similar patients (Koelsch et al., 2011, Eriksson et al., 2013, Agosto et al., 2011) (Table 3.1). It is difficult to directly compare these findings with those from other clinical trials because methods of HIV-1 DNA detection, cell type analysed, length of time on HAART, HAART drug regimen and estimates of PHI differed. Absolute HIV-1 DNA levels appeared to be more reminiscent of 'chronic', rather than primary HIV infection in SPARTAC, which may be due to later sampling of patients in SPARTAC, relative to other trials or improved assay sensitivity. However, relative HIV-1 DNA level (high versus low) was to be principally investigated in the next chapter. Therefore, the Q-PCR assays presented in this chapter were deemed to be the most suitable of the methods tested for quantifying HIV-1 DNA levels in the SPARTAC trial.

4. HIV-1 DNA as a biomarker of progression and viral rebound in the SPARTAC trial.

This chapter is based on work submitted as a manuscript. It was presented orally at IAS 2013 (Williams et al., 2013a) and as late breaker poster at CROI (403LB)(Williams et al., 2014b).

4.1 Introduction

The provision of HAART for the treatment of HIV-1 infection significantly improves patient morbidity and mortality, with restoration of life expectancy to near normal levels (Zwahlen et al., 2009, Ray et al., 2010). Nevertheless, HAART needs to be taken for life, there is growing evidence for non-AIDS morbidities such as dementia, cardiovascular disease and increased frailty for those on HAART, and there is stigma around being HIV-1 'positive' (Volberding and Deeks, 2010).

The next chapter in the management of the HIV-1-infected individual is to achieve a cure. Related research has been boosted by well-publicized case reports in adults and children (Henrich et al., 2013, Persaud et al., 2013, Saez-Cirion et al., 2013) and the development of novel interventions aimed at cure (Tebas et al., 2014, Denton et al., 2014).

It is well documented that HIV-1 DNA persists in patients on HAART even when the viral load is undetectable using the most sensitive assays (Saez-Cirion et al., 2013, Palmer et al., 2008). Although much of this detectable HIV-1 DNA may be mutated and replication incompetent (Ho et al., 2013), HIV-1 DNA levels may thus still reflect longer-term prognosis when viral load can no longer be measured.

HIV-1 DNA levels are known to associate with viral load (Verhofstede et al., 1994) and CD4+ T cell count (Parisi, Andreis et al. 2012), the only validated surrogate markers of disease progression. Also, HIV-1 DNA levels associate with immunological markers of disease progression (Hatano, Jain et al. 2013, Weiss, Chevalier et al. 2013). High DNA levels also predict clinical outcome (Piketty, Weiss et al. 2010, Goujard, Bonarek et al. 2006, Minga, Anglaret et al. 2008) and after TI strategies, residual viremia level on TI (Havlir, Koelsch et al. 2005), the magnitude of viral rebound (Yerly, Gunthard et al. 2004) and those who maintain low viral set points (Lafeuillade, Poggi et al. 2013) (1.4.3.2). Low HIV-1 DNA levels are also associated with functional cure (Saez-Cirion et al., 2013), elite control (Graf et al., 2011) and by definition, viral eradication.

Also, since HIV-1 treatment guidelines recommend increasingly early administration of HAART, it is likely that the only way to test the efficacy of any new cure-directed intervention will be to stop HAART and assay for rebound viraemia. This raises a number of concerns. Data from the SMART trial associated therapy cessation in chronic infection with increased morbidity and mortality (El-Sadr et al., 2006). Data from the SPARTAC trial did not identify similar concerns in primary infection (Fidler et al., 2013).

There is, therefore, a need to find biomarkers that can predict clinical progression and virological remission. Thus, it might be possible to identify patients who may, or should not be candidates for a Treatment Interruption (TI) study following an intervention aimed at cure.

4.2 Aims and objectives

4.2.1 Aims

No randomised cohort has been used to investigate the predictive role of HIV-1 DNA in terms of trial endpoint, time for plasma virus to become detectable and its association with markers of HIV-1 disease progression in primary HIV infection (PHI). In this chapter, I aimed to investigate how HIV-1 DNA level associates with validated markers of disease progression, how HIV-1 DNA was impacted by HAART and whether HIV-1 DNA was an independent predictor of progression.

4.2.2 Objectives

1. Associations between HIV-1 DNA and virological and immunological markers of disease progression will be investigated in untreated infection.
2. The impact of HAART will be studied cross-sectionally by trial arm and longitudinally in patients who received HAART and underwent TI.
3. HIV-1 DNA will be used as a biomarker for predicting progression and time to rebound in untreated infection and at the point of TI.

4.2.3 Hypotheses: In the SPARTAC trial:

- HIV-1 DNA associates with immunological and clinical markers of HIV-1 disease progression in untreated infection.
- HIV-1 DNA levels are predictive of clinical progression
- HIV-1 DNA levels predict the time taken for viraemic rebound on stopping HAART, advocating its role in future treatment interruption protocols.

4.3 Results

4.3.1 SPARTAC patient selection and demographics

In this chapter, participants for this sub-study of SPARTAC were those infected with subtype B HIV-1 and for whom adequate samples were available (Table 4.1). The SPARTAC trial is described in more detail in section 2.1. CD4+ T cells were sampled for HIV-1 DNA in all available participants at baseline, regardless of trial arm. Participants randomised to the ART-48 arm were sampled at week 48 as they stopped HAART and at a further 4, 12 and 60 weeks post HAART interruption (52, 60 and 108 weeks post-HAART initiation). Participants, viraemic at the point of interruption of 48 weeks of HAART (>50 copies/mL), were excluded. Participants randomized to ART-12 were additionally sampled at week 12, just prior to TI. The majority of patients at this time point had detectable viraemia.

The baseline demographics of all sampled participants, regardless of trial arm (left column), those randomised to receive no HAART (SOC) at baseline (middle column) and those randomised to the ART-48 arm sampled at the point of TI (right column), are all summarised in Table 4.2. 17 patients randomized to the ART-48 arm had sample available at all time points. Table 2.3 summarises the number of patients sampled for subsequent baseline HLA analyses (4.3.5; Figure 4.10 and 4.11).

Table 4.1 Number of patient samples available, where there were sufficient sample for total and integration assays, at each time point, for each trial arm

Time post randomization (weeks)	SOC		ART-12		ART-48	
	Total (n)	Integrated (n)	Total (n)	Integrated (n)	Total (n)	Integrated (n)
0	51	38	51	38	52	35
12	-	-	34	34	-	-
48	-	-	-	-	47	47
52	-	-	-	-	46	39
60	-	-	-	-	32	27
108	-	-	-	-	48	31

Table 4.2 Patient demographics. * sampled just before TI of 48 weeks of HAART

	All sampled baseline participants (marker association study)	SOC participants at baseline (baseline survival study)	ART-48 participants (reservoir survival and rebound study)
N (Total)	154	51	47
N (Integrated)	111	38	47
Log ₁₀ Total copies/million CD4+ T cells	3.88 (3.42-4.24)	4.01 (3.49-4.29)	3.25 (2.90-3.44)*
Log ₁₀ Integrated copies/million CD4+ T cells	3.6 (3.26-3.79)	3.6 (3.3-3.8)	3.0 (2.7-3.2)*
Time since seroconversion (days)	73.82 (49.2-95.8)	73 (49-93)	76.5 (52.0-96.9)
Log ₁₀ Viral Load copies/mL	4.62 (3.95-5.25)	4.72 (4.14-5.26)	<50*
CD4 Cell count (cells/μL)	558 (428-680.9)	550 (400-691)	848 (655-980)*
Country of recruitment	Australia 21 (13.6%) Italy 18 (12%) Brazil 13 (8.4%) UK 102 (66.2%)	Australia 8 (15.6%) Italy 7 (13.7%) Brazil 5 (9.8%) UK 31 (60.8%)	Australia 5 (10.6%) Italy 5 (10.6%) Brazil 3 (6.4%) UK 34 (72.3%)
Viral Subtype	B (100%)	B (100%)	B (100%)
Sex	Female 4 (3%) Male 150 (97%)	Female 2 (4%) Male 49 (96%)	Female 1 (2%) Male 46 (98%)

Table 2.3. Number of patients sampled at baseline for 2-digit HLA analysis

HLA A	n	HLA B	n	HLA B	n	HLA C	n
A_01	45	B_07	32	B_51	11	C_01	14
A_02	81	B_08	28	B_52	7	C_02	18
A_03	26	B_13	5	B_53	5	C_03	36
A_11	17	B_14	10	B_55	6	C_04	32
A_23	3	B_15	31	B_57	11	C_05	21
A_24	32	B_18	8	B_58	2	C_06	32
A_26	12	B_27	16			C_07	69
A_29	13	B_35	20			C_08	11
A_30	9	B_37	10			C_12	22
A_31	13	B_38	9			C_13	1
A_32	9	B_39	10			C_14	2
A_33	4	B_40	17			C_15	3
A_36	1	B_41	2			C_16	15
A_66	2	B_44	39			C_17	2
A_68	9	B_45	5				
A_69	1	B_49	2				
A_74	2	B_50	4				

4.3.2 Total and integrated HIV-1 DNA levels correlate.

First, the relationship between total (2.2.3) and integrated (2.2.4) HIV-1 DNA (2.2.1) levels in untreated patients was investigated at baseline. Participants in this study are outlined in the first column of Table 4.2. HIV-1 DNA levels were log-transformed and expressed per million CD4+ T cells (Figure 4.1A). Both total and integrated measurements correlated strongly as expected in untreated patients (Pearson correlation, $r=0.846$, $p<0.001$), with total HIV-1 DNA levels being of higher magnitude than integrated HIV-1 DNA levels (Figure 4.2B). Median DNA (IQR) was 7707 (2477–18187) and 3830 (1563–6325) copies of HIV-1 per million CD4+ T cells, for total and integrated assays, respectively.

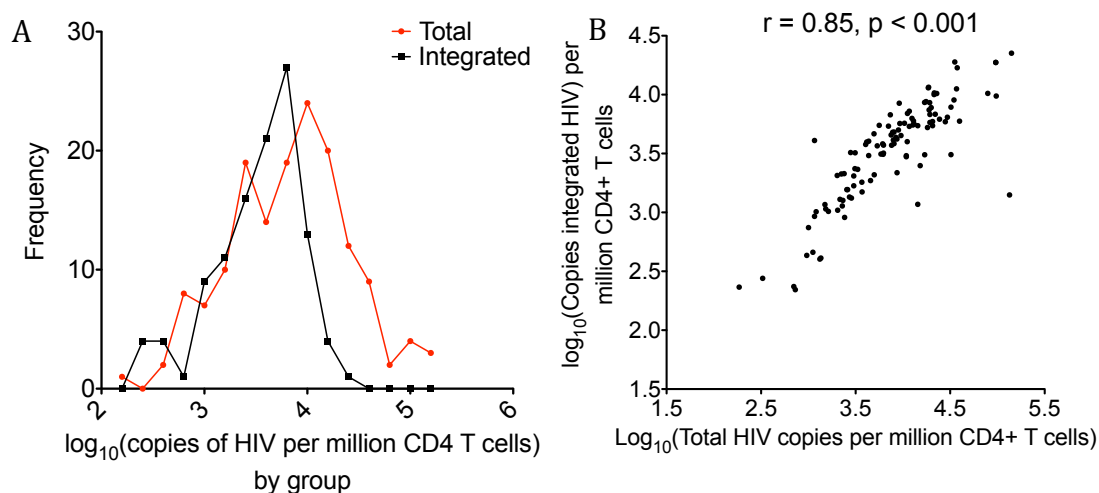


Figure 4.1 The relationship between $\log_{10}$ total and integrated HIV-1 DNA levels in untreated patients at baseline. A) Frequency Histograms for total and integrated HIV-1 DNA. B) Pearson correlation for total and integrated HIV-1 DNA levels. Total $n=154$. Integrated $n=111$.

4.3.3 HIV-1 DNA level associates with viral load and CD4+ T cell count

Baseline HIV-1 plasma viral load (Mellors et al., 1996) and CD4+ T cell count (Frater et al., 2013) are strong predictors of HIV-1 clinical progression. The relationship between these markers of progression and the HIV-1 DNA levels present in untreated PHI were thus investigated, from the same time-point of data acquisition. Both these measures of HIV-1 DNA associated positively with plasma viral load (total and integrated, both $p < 0.001$; $r^2 = 0.48$ and 0.64 , respectively), and inversely with CD4+ T cell count (total and integrated $p < 0.001$; $r^2 = 0.2$ and 0.27 , respectively), as expected (Figure 4.2).

The relationship between HIV-1 DNA levels and the ETS was explored. Unlike in other studies, (Eriksson et al., 2013, Koelsch et al., 2008, Ananworanich et al., 2012) a relationship between ETS and HIV-1 DNA levels was not evident (Figure 4.3). Since the HIV-1 reservoir is formed very early after initial infection (Chun et

al., 1998), HIV-1 DNA levels may have stabilized by the time most SPARTAC patients had been recruited, despite all having been recruited within an ETS of 6 months.

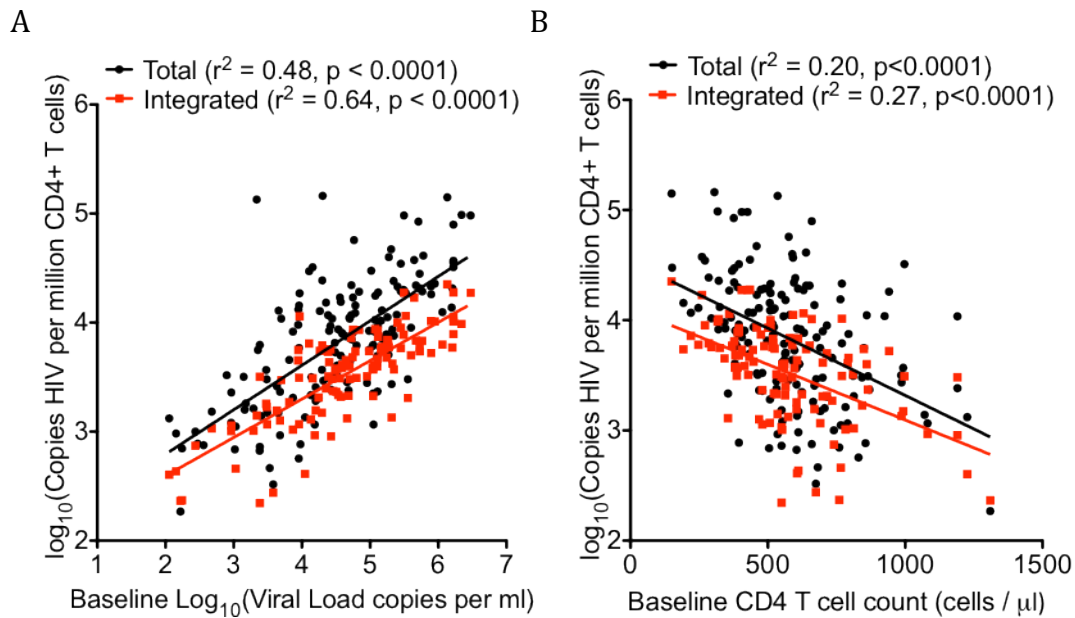


Figure 4.2. Linear regressions between HIV-1 DNA and A) viral load and B) CD4+ T cell count (total n=154, integrated n=111).

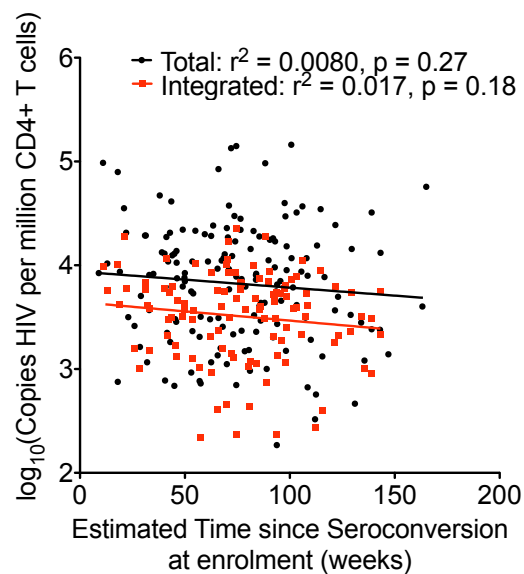


Figure 4.3 Linear Regression between Estimated time since seroconversion (ETS) and total and integrated HIV-1 DNA levels (total n=154, integrated n=109).

4.3.4 Immunological markers of HIV-1 disease progression correlate with baseline HIV-1 DNA levels

Due to the close associations between HIV-1 DNA levels and clinical diagnostic markers of HIV-1 disease progression, it was hypothesised that HIV-1 DNA levels might correlate with immunological markers of HIV-1 disease progression. First, relationships between HIV-1 DNA and immunological markers were investigated in untreated patients at baseline. D-dimer, a soluble fibrin degradation product and blood coagulation and immune activation marker was more significantly associated with total and integrated HIV-1 DNA levels (Total $p < 0.0001$, Integrated $p = 0.0003$) than IL-6, a pro-inflammatory cytokine (Figure 4.4).

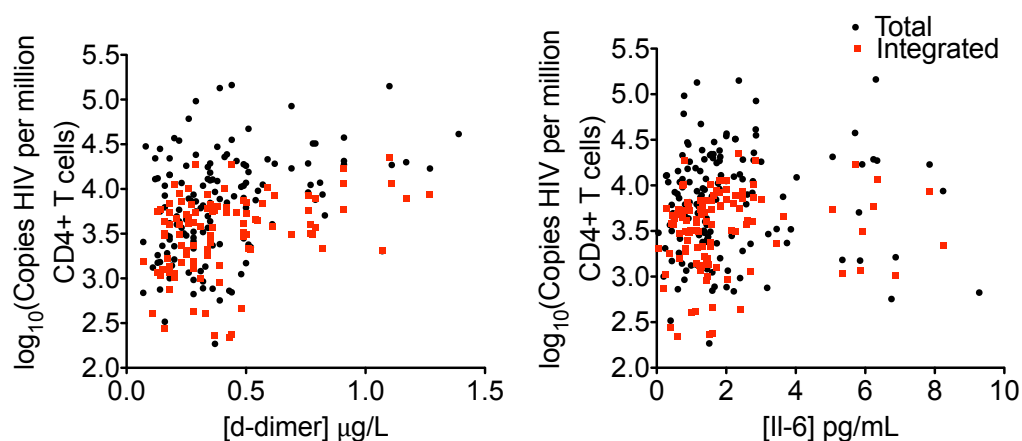


Figure 4.4 Spearman's rank correlations between HIV-1 DNA and D-dimer or IL-6 concentration. Total $n=144$, Integrated $n=106$.

There were also significant correlations between expression of the activation markers HLA-DR (Total $p = 0.014$, Integrated $p = 0.0067$), CD38 (Total and Integrated $p < 0.00001$) and HLA-DR+CD38+ (Total and Integrated $p < 0.0001$) expression on CD8+ T cells with baseline HIV-1 DNA levels (Figure 4.5).

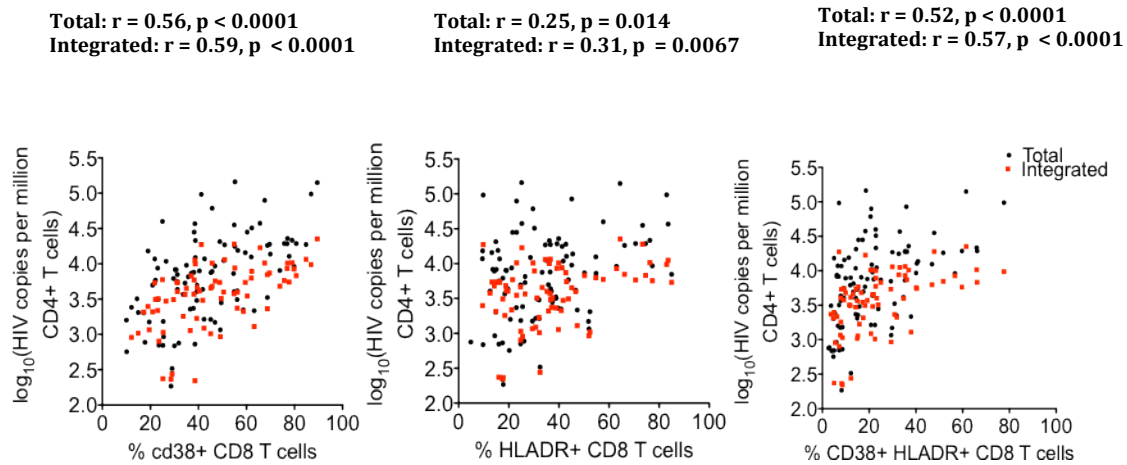


Figure 4.5. Spearman's rank correlations between HIV-1 DNA and CD8+ T cell activation markers. Total n=93, Integrated n=74

CD38 (cyclic ADP ribose hydrolase) is a glycoprotein present on T cells, which has downstream signal transduction and calcium signalling effector functions. HLA-DR is an MHC class II receptor. Both are upregulated on activated T cells. HIV-1 DNA levels as measured by both assays correlated less strongly with activation marker expression on CD4+ T cells than CD8+ T cells. Correlations between total and integrated HIV-1 DNA and CD38+ (Total $p = 0.027$, Integrated $p = 0.024$) and HLA-DR+CD38+ (Total $p = 0.0001$, Integrated $p < 0.0001$) expression on CD4+ T cells were significant, and were close to significance for HLA-DR expression on CD4+ T cells (Total $p = 0.09$, Integrated $p = 0.06$) (Figure. 4.6).

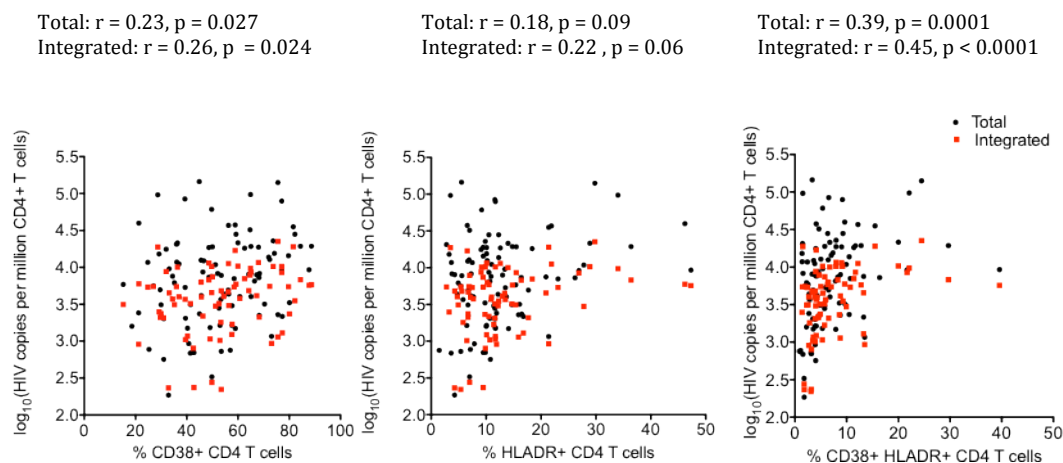


Figure 4.6. Spearman's rank correlations between HIV-1 DNA and CD4+ T cell activation markers. Total n=94, Integrated n=74

There were also significant associations between HIV-1 DNA levels and the percentage of CD8+ T cells expressing the exhaustion markers PD-1 (Total $p = 0.0005$, Integrated $p = 0.0027$), Lag-3 (Total $p = 0.0004$, Integrated $p = 0.0022$) but not Tim-3 (Total $p = 0.94$, Integrated $p = 0.61$) (Figure 4.7). These three T cell surface receptors are involved in the negative regulation or ‘dampening down’ of the immune response to avoid over-activation of the immune system.

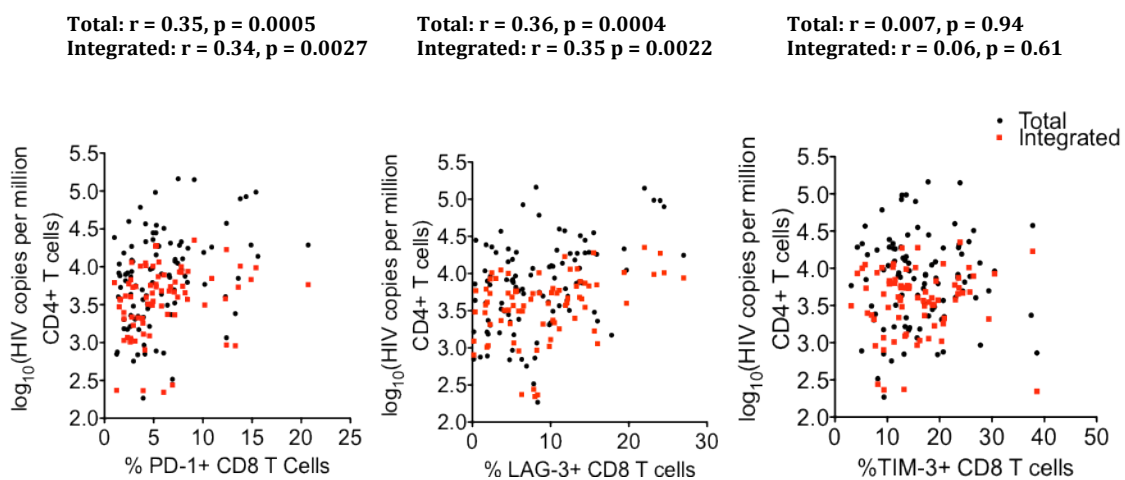


Figure 4.7. Spearman's rank correlations between HIV-1 DNA and CD8+ T cell exhaustion markers. Total n=95, Integrated n=76

However no significant correlations were observed for HIV-1 DNA levels and CD4+ T cells exhaustion marker expression, apart for LAG-3 (total $p = 0.028$, integrated $p = 0.042$) (Figure. 4.8) although PD-1 expression was close to significance. However, less is known about exhaustion on CD4+ T cells and mechanisms of CTL exhaustion might not be the same as in CD4+ T cells. This makes interpretation of these findings challenging and would be interesting to investigate in future studies.

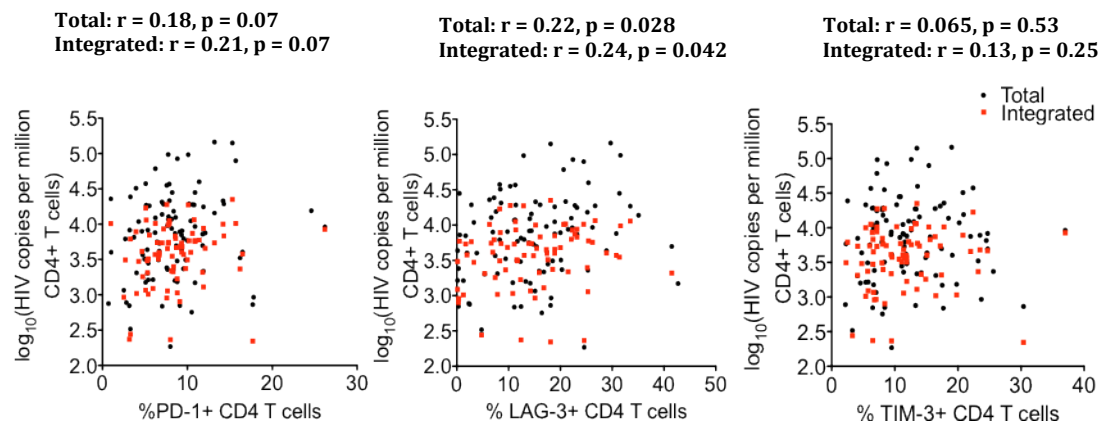


Figure 4.8 Spearman's rank correlations between HIV-1 DNA and CD4+ T cell exhaustion markers. Total n=96, Integrated n=76

As viral load associated closely with HIV-1 DNA levels (Figure 4.2) and is also documented to associate with HIV-1 Gag-directed CD8+ (Kiepiela et al., 2007, Goulder and Walker, 2012), and CD4+ T cell responses (Schieffer et al., 2014), it was hypothesised that T cell immunity might determine HIV-1 DNA levels. Participants who made three or more CD8+ T cell responses to Gag epitopes had significantly lower HIV-1 DNA levels than those who made no response (Total $p = 0.02$, Integrated $p = 0.008$; Mann-Whitney) (Figure. 4.9A). However, the association was no longer significant for total HIV-1 DNA when all 4 populations were compared in a Kruskal-Wallis test (total; $p = 0.11$, integrated; $p = 0.032$). Similarly, participants who made CD4+ T cell responses had lower HIV-1 DNA levels compared to those that did not make a response (Total $p = 0.037$, Integrated $p = 0.012$; Mann-Whitney) (Figure 4.9B).

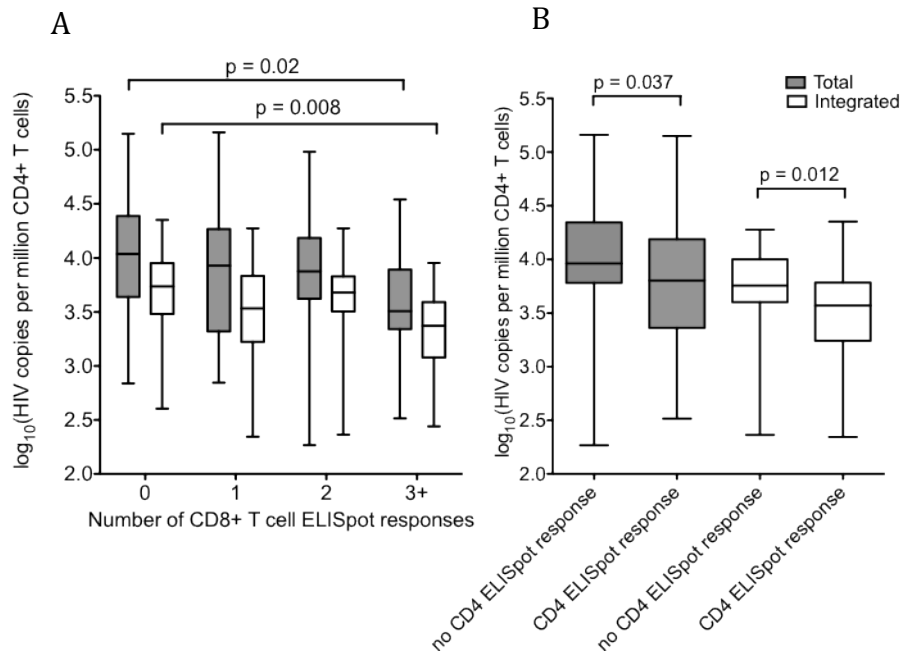


Figure 4.9 Impact of CD4 and CD8+ T cell responses on HIV-1 DNA level in untreated patients at baseline. A) HIV-1 DNA levels presented according to whether the participant made a CD4 interferon gamma ELISPOT response to HIV-1 Gag. Significance determined using Mann-Whitney tests. (B.) HIV-1 DNA levels presented according to the number of HIV-1 Gag OLPs recognised in CD8+ T cell interferon gamma ELISpots. Significance determined using Mann-Whitney tests. Patient numbers; for 'No CD4 response' and 'CD4 response' for total (n=33 and 60) and for integrated (n=27 and 45). For 0, 1, 2, 3+ CD8+ T cell responses for total (n=22, 40, 25 and 20) and integrated (n=22, 30, 23 and 17).

4.3.5 HIV-1 DNA levels associate with HLA-Type

Patient HLA type is a well-documented genetically determined marker of patient outcome conferred through HIV-1-specific immunity. For example, HLA-B*27:05 and B*57:01 expression associates with viral control and HLA-B*35:01/02/03 associates with rapid disease progression. Therefore, HLA-type may impact on the HIV-1 DNA level, which could then lead to control or progression of disease. HLA-types were arranged in order of the median HIV-1 DNA levels, recorded in untreated participants at baseline for where there was sample available. HLA-type was categorised as protective (red) or associated with rapid progression (blue)

based on consistent 2-digit HLA associations with disease progression (Lazaryan et al., 2011, Kaslow et al., 1996, Goulder and Walker, 2012, O'Brien et al., 2001, Hendel et al., 1999, Carrington et al., 1999, Pereyra et al., 2010, Steel et al., 1988) (Figure 4.10).

The most well known 'favourable' HLA alleles B*27 and B*57 associated significantly with lower total HIV-1 DNA level ($p < 0.001$ ($n=16$), $p=0.0065$ ($n=11$), respectively) as did B*52 and A*30. HLA-B*52:01 was previously shown to be protective (Pereyra et al., 2010), but not HLA-A*30. HLA-B*35 was associated with higher total HIV-1 DNA levels, but only HLA-B*08 and HLA-A*01 associated significantly (Figure 4.10A). HLA-A*01 has inconsistently been associated with faster disease progression (Steel et al., 1988). HLA-B*27 ($p=0.032$ ($n=8$)), HLA-C*12, B*52 and B*39 were associated with significantly lower integrated HIV-1 DNA levels. Only HLA-A*01 was significantly associated with higher integrated HIV-1 DNA levels (Figure 4.10B). HLA-B*39:10 has been linked to slower disease progression (Leslie et al., 2010), but HLA-C*12 has not previously been associated with disease progression. The number of patients in this analysis is summarised in Figure 4.3. 4-digit HLA types were similarly investigated, however patient numbers were too low for a similar analysis (data not shown).

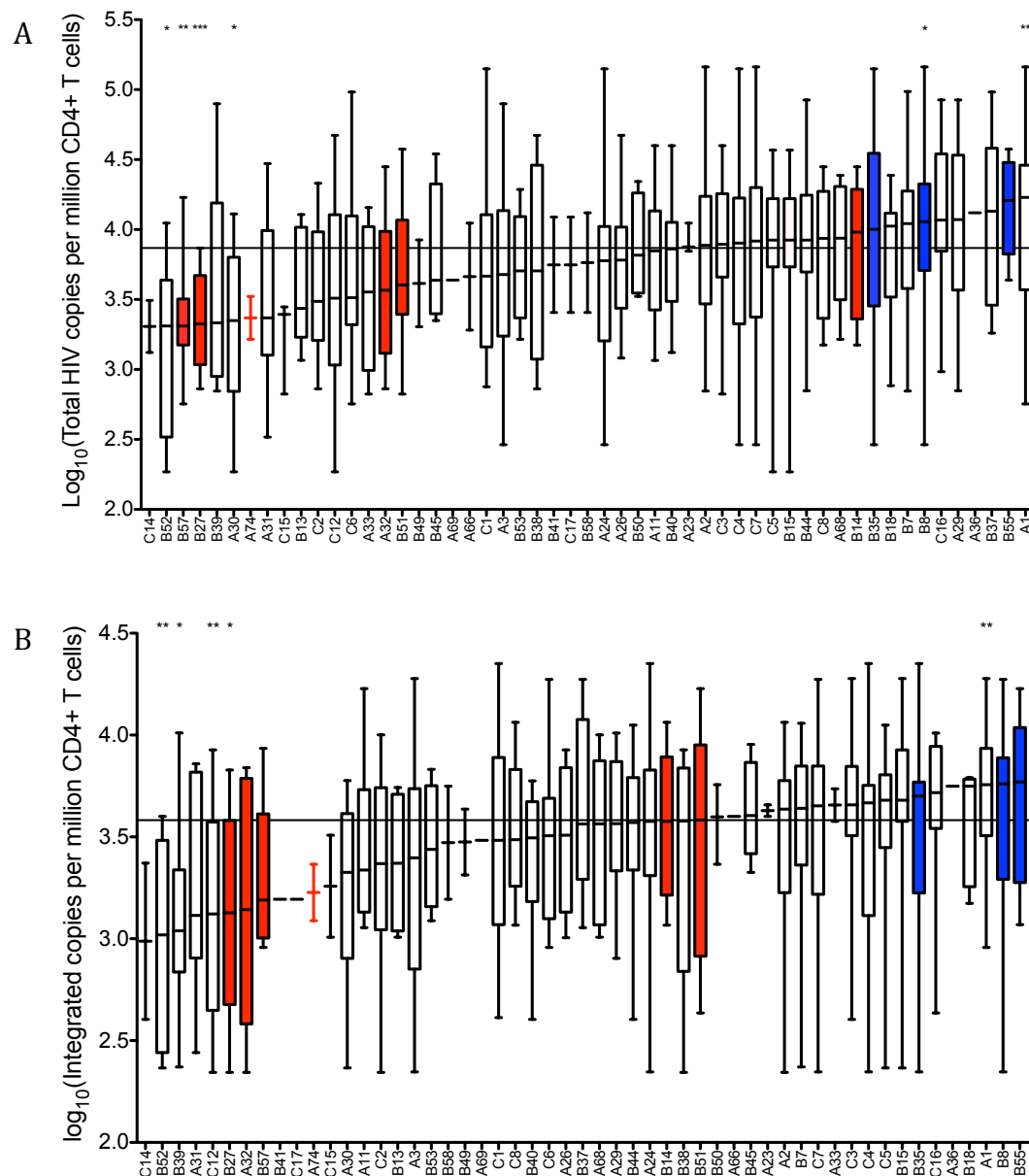


Figure 4.10. 2-digit HLA-type ranked by median A) total and B) integrated HIV-1 DNA level in untreated patients at baseline. Red and blue bars represent alleles that are consistently associated with HIV-1 control and rapid progression, respectively. Significance was determined by comparison of the target population with all remaining samples by Mann Whitney test. The black line represents the median value of the entire cohort. * ($0.01 < p < 0.05$), ** ($0.001 < p < 0.01$), *** $p < 0.0001$. Number of patients for each HLA type detailed in Table 2.3.

Due to the subjective nature of the classification into ‘good’ and ‘bad’ HLA alleles, statistical data was utilised from a demographically similar cohort in which progression to three clinical end-points had been characterised in relation to HLA

Class I: time to death, AIDS (1993 definition) and a CD4+ T cell count ≤ 200 cells/ μ l (Carrington et al., 1999, Gao et al., 2005, Gao et al., 2001, O'Brien et al., 2001). Consistent with the analysis in Figure 4.10, when total and integrated HIV-1 DNA levels were averaged for each HLA-type, they correlated significantly with HLA Class 1-associated relative hazard ratios for all three end-points. HLA types with low hazard ratios had lower HIV-1 DNA levels (Figure 4.11). Integrated levels did not correlate significantly with AIDS93, perhaps due to a lower number of patients sampled, relative to total HIV-1 DNA level measurements.

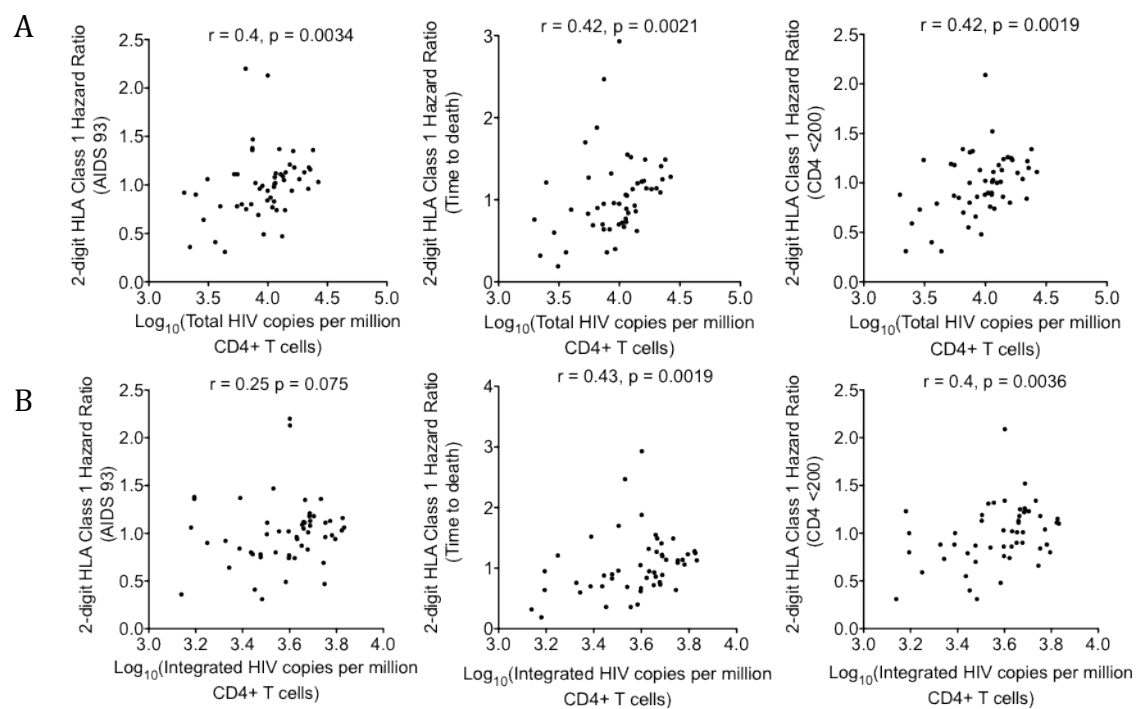


Figure 4.11. Association of HIV-1 DNA with published HLA-type hazard ratios. A) Total and B) Integrated HIV-1 DNA levels were averaged in patients expressing the same HLA-type. HIV-1 DNA levels were plotted against the hazard ratios for each HLA-type, determined in previous publications, time to AIDS, Time to death and time for CD4+ T cells to fall below 200 copies/mL. Spearman's rank correlations were performed. Hazard Ratio data sourced from (Carrington et al., 1999, Gao et al., 2005, Gao et al., 2001, O'Brien et al., 2001) and data was used with permission from Mary Carrington.

4.3.6 HIV-1 DNA levels predict time to primary endpoint at baseline.

HIV-1 DNA levels associated with virological and immunological markers of disease progression. Thus, HIV-1 DNA level was hypothesized to predict primary endpoint of the SPARTAC trial (CD4+ T cell count <350 cells/mL). According to the trial protocol, patients were randomised at baseline into three trial arms: those that remain untreated, and those that receive 12 or 48 weeks of HAART. Patients randomised to the SOC arm and thus who continued to be untreated (total n=51, integrated n=38; Table 4.2 middle column) were chosen for the first analysis. The amount of total and integrated HIV-1 strongly predicted the time to primary endpoint ($p=0.0016$ and 0.0022 , respectively) by Kaplan-Meier log rank test (Figure 4.12) and in univariate Cox proportional hazards analysis ($p<0.0001$ and 0.006 , respectively) (Table 4.4). Baseline viral load ($p=0.011$) and CD4 count ($p<0.0001$) also predicted time to endpoint (Table 4.4).

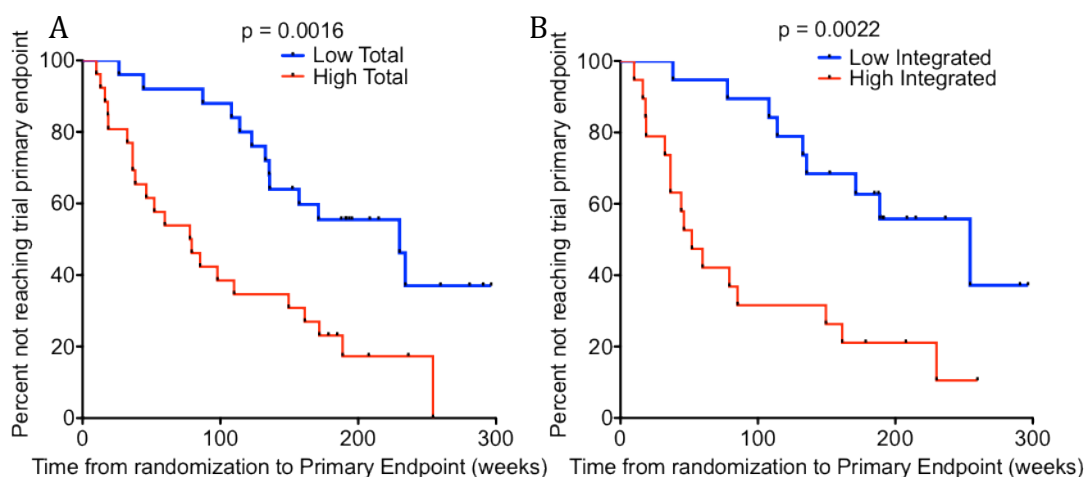


Figure 4.12. HIV-1 DNA in untreated infection predicts clinical progression
A) Total (n=51) and B) Integrated (n=38) HIV-1 DNA levels were split into two 'high' and 'low' groups, by the median HIV-1 DNA value (10471 and 4074 copies of total and integrated HIV-1 DNA, respectively, per million CD4+ T cells). Significance was determined by log rank test. The time from randomization to trial endpoint was measured. Median time to primary endpoint for low and high total HIV-1 DNA levels was 187.0 (IQR 127.0-222.0) and 77.9 (IQR 35.0-172.8) weeks, respectively and for low and high integrated levels were 187.7 (IQR 132.7-214.9) and 52.0 (IQR 32.4-161.3) weeks, respectively.

Table 4.4 : Univariable Cox regression models with log₁₀HIV-1 DNA levels, CD4+ T cell count/100 or log₁₀Viral load as explanatory variables using the SPARTAC trial endpoint (CD4+ T cell count < 350 cells / μ L). HIV-1 DNA levels in untreated patients, who continued to receive no treatment, at baseline. HR, hazard ratio; CL, confidence limits.

Covariate	HR (95% CL)	p value
log ₁₀ total	4.16 (2.09-8.26)	<0.0001
log ₁₀ integrated	5.41 (1.62-18.02)	0.006
Log ₁₀ Viral Load	1.74 (1.13-2.68)	0.011
CD4 + T cell count / 100	0.66 (0.54-0.82)	<0.0001

Table 4.5 : Multivariable Cox regression model with baseline total HIV-1 DNA level, baseline viral load and baseline CD4+ T cell count as covariates, using the SPARTAC trial endpoint (CD4+ T cell count < 350 cells / μ L). HIV-1 DNA levels measured in untreated patients, who continued to receive no treatment. HR, hazard ratio; CL, confidence limits.

Covariate	HR (95% CL)	p value
log ₁₀ Total	3.57 (1.58-8.08)	0.002
log ₁₀ Viral Load	1.25 (0.80-1.95)	0.33
CD4+ T cell count / 100	0.67 (0.53-0.84)	<0.0001

Table 4.6 : Multivariable Cox regression model with baseline integrated HIV-1 DNA level, baseline viral load and baseline CD4+ T cell count as covariates, using the SPARTAC trial endpoint (CD4+ T cell count < 350 cells / μ L). HIV-1 DNA levels measured in untreated patients, who continued to receive no treatment. HR, hazard ratio; CL, confidence limits.

Covariate	HR (95% CL)	p value
log ₁₀ Integrated	2.08 (0.34-12.68)	0.43
log ₁₀ Viral Load	1.22 (0.53-2.81)	0.65
CD4+ T cell count / 100	0.73 (0.59-0.92)	0.007

In multivariable analyses, when the baseline covariates total HIV-1 DNA, viral load, and CD4+ T cell count were included, total HIV-1 DNA but not plasma viral load was predictive of time to primary endpoint (Hazard Ratio (HR) 3.57; $p=0.002$) (Table 4.5). In a separate multivariable analysis employing integrated HIV-1 DNA, baseline viral load and CD4+ T cell count, both baseline plasma viral load and integrated HIV-1 DNA did not significantly predict endpoint (Table 4.6). Despite this, the close relationship between integrated HIV-1 DNA levels and viral load meant that viral load also lost significant power to predict time to primary endpoint. This indicates that integrated HIV-1 DNA levels still possessed some power to predict time to endpoint.

4.3.7 HAART treatment significantly decreases HIV-1 DNA

In order to characterise the HIV-1 reservoir, HAART must be initiated. Two thirds of the participants recruited to SPARTAC were randomised according to the trial protocol to receive either 12 or 48 weeks of HAART before undertaking a treatment interruption. Therefore the impact of HAART on HIV-1 DNA levels (which have been reported in different cohorts (Siliciano et al., 2003, Chun et al., 2007)) and the impact of reservoir size on TI, were investigated.

Firstly, HIV-1 DNA levels were log-transformed (Figure 4.13A). There was a strong correlation between total and integrated HIV-1 DNA levels, similarly to at baseline ($p<0.001$; $r = 0.79$; Pearson correlation) (Figure 4.13B). 8 participants were excluded from this analysis due to non-compliance (Figure 4.13C). Non Subtype-B infected participants were also excluded. The 47 remaining participants (Table 2.2,

right hand column) received a median of 48 weeks of therapy (IQR 47.7-48.7) and had viral loads at the point of TI, which were below 50 copies per mL plasma. Median DNA (IQR) was 1807 (IQR 752–2778) and 986 (IQR 522–1877) copies of HIV-1 per million CD4+ T cells, for total and integrated assays, respectively.

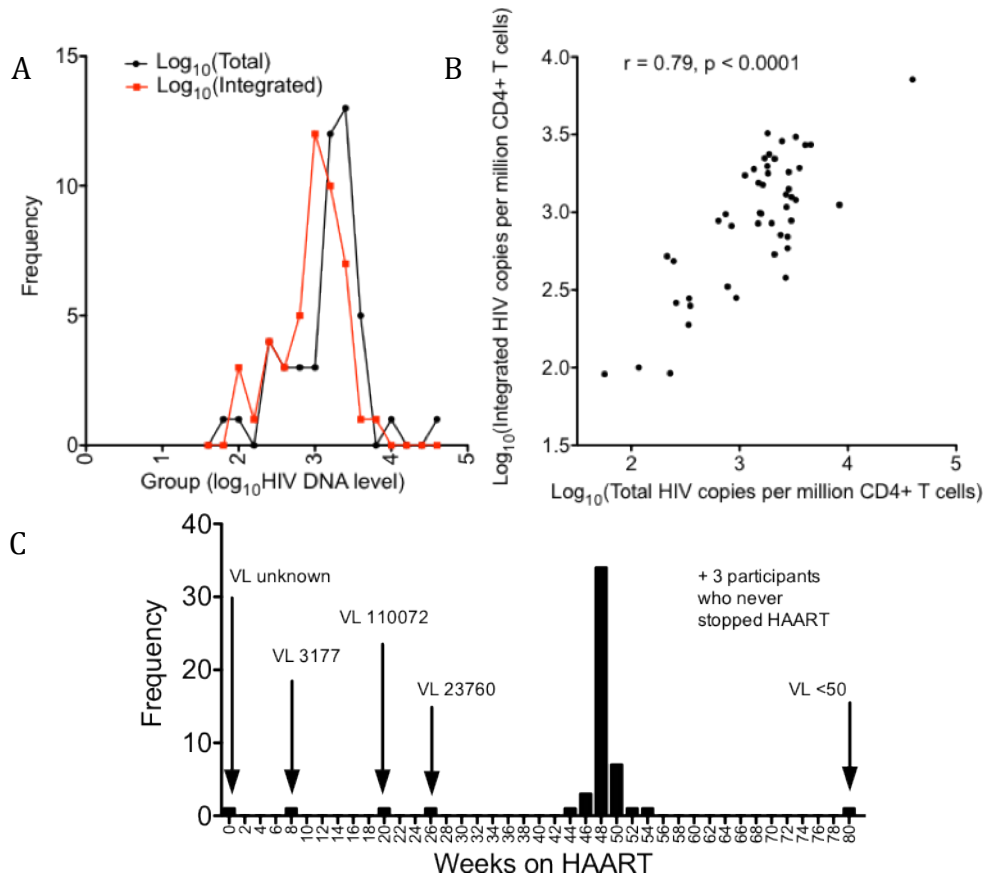


Figure 4.13 Relationship between $\log_{10}$ total and integrated HIV-1 DNA levels in patients treated for 48 weeks with standard HAART. A) Histograms for total (n=47) and integrated (n=47) HIV-1 DNA. B) Pearson's rank correlation for total and integrated HIV-1 DNA levels. C) A histogram of time on HAART for all available subtype B samples randomised to the ART-48 arm. Outliers (those who never started HAART, never stopped HAART or had viral loads (VL) > 50 copies/mL as outlined by blue arrows) were removed from all analyses. Of the remaining compliant 47 samples, the time from TI to primary endpoint was used for subsequent survival analyses.

HIV-1 DNA levels pre and post 48 weeks of HAART, measured by both assays, correlated strongly (total $r=0.79$, $p<0.0001$; integrated $r=0.78$, $p<0.0001$) (Figure 4.14A and B). Before HAART initiation, total and integrated HIV-1 DNA levels were significantly different ($p < 0.0001$). 12 and 48 weeks of HAART significantly decreased the amount of total and integrated HIV-1 DNA (all comparisons $p < 0.0001$). However, levels of total HIV-1 DNA were significantly higher than integrated HIV-1 DNA after 12 or 48 weeks of therapy (week 12; $p = 0.040$, week 48; $p = 0.027$; paired T test) although the differences were small (Figure 4.14C). There was no significant difference between HIV-1 DNA level at wk12 and wk48, which may be due to lack of sample analysed at wk12. Other studies have noticed excesses of unintegrated HIV-1 DNA in some patients after a year of HAART, despite patients having undetectable viremia (Agosto et al., 2011), perhaps due to persistence of 2-LTR circles (Pierson et al., 2002, Butler et al., 2002).

The dynamics of HIV-1 DNA decay on HAART has been previously reported (Siliciano et al., 2003, Chun et al., 2007). Similarly to other studies, most of the HIV-1 DNA decay occurred before 12 weeks of HAART, with a smaller decay occurring between 12 and 48 weeks (Koelsch et al., 2008) (Figure 4.14C). However only 20 out of 38 patients that were randomised to receive 12 weeks of HAART had undetectable plasma viraemia at the point of TI at week 12, so the HIV-1 reservoir may not have been accurately measured at this time point. The higher HIV-1 DNA levels sampled at TI in patients treated with HAART for 12 weeks compared to those treated for 48 weeks may therefore not necessarily be due to a decline in the latent HIV-1 reservoir, as there was evidence for continued viral replication.

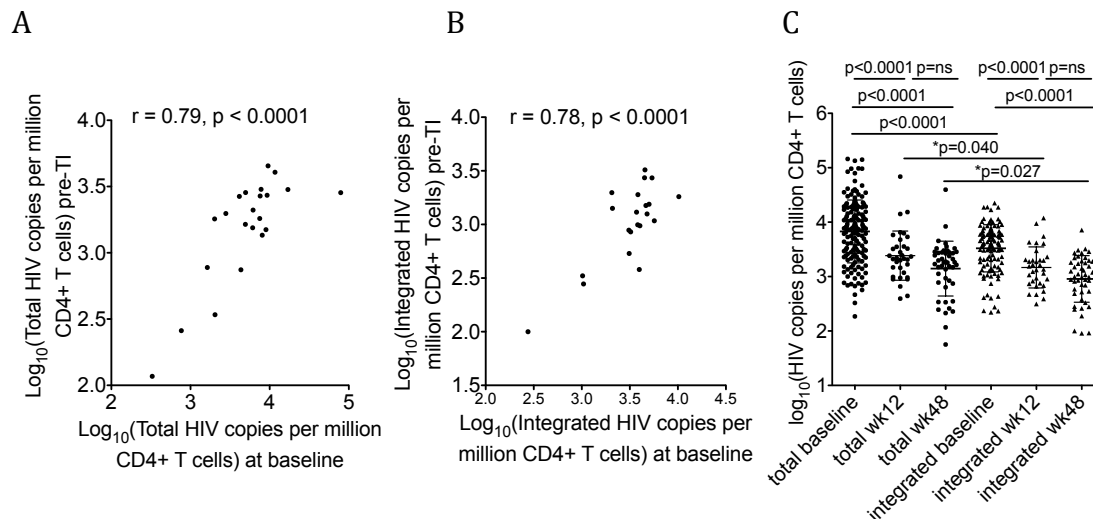


Figure 4.14 Impact of 12 or 48 weeks of HAART on HIV-1 DNA level

Correlations between A) Total (n=22) and B) Integrated (n=21) HIV-1 DNA levels at baseline and after 48 weeks of therapy (at the point of TI) in the same patients. Significance was determined using Pearson's correlations. C) HIV-1 DNA was quantified in untreated patients at baseline (total n=154, integrated n=111) and in those treated for 12 (n=34) or 48 (n=47) weeks, pre-TI. Significance was determined by T test, correcting for multiple comparisons (Bonferroni test). * = paired t tests (excluded from the bonferroni test).

4.3.8 Associations between virological and immunological markers of HIV-1 disease progression and HIV-1 DNA

HIV-1 DNA levels associated significantly with validated virological and other immunological markers of HIV-1 disease progression at baseline. Thus, the same investigations were performed but in patients who received 48 weeks of HAART, where there was available sample just prior to TI. CD4+ T cell counts and HIV-1 DNA reservoir level, both sampled after 48 weeks of therapy, did not associate significantly (Figure 4.15A). Similarly to that observed at baseline, there was no statistically significant association between HIV-1 DNA level after 48 weeks of therapy and estimated time since seroconversion at randomisation (Figure 4.15B). However, total and integrated HIV-1 DNA levels after 48 weeks of therapy still

associated significantly with baseline viral load ($p=0.005$ for both total and integrated assays) (Figure 4.15C), an observation that supports a similar study (Fourati et al., 2013).

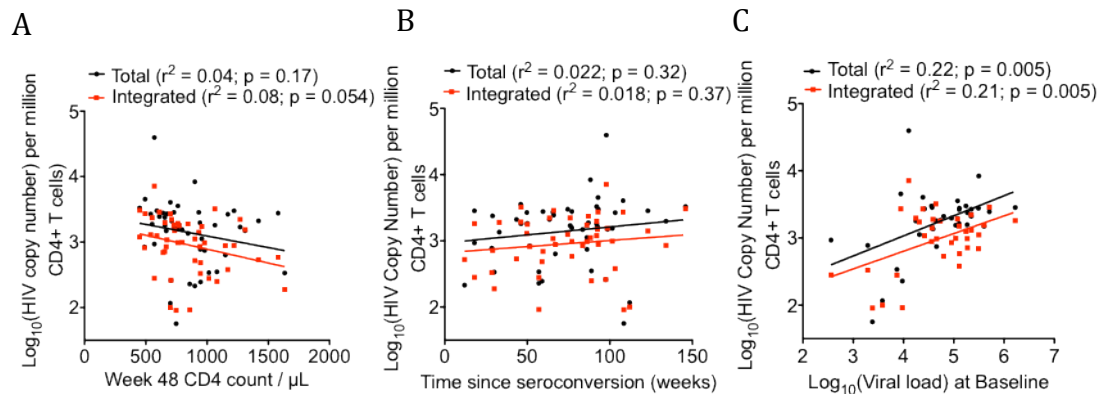


Figure 4.15 Linear Regression analysis between HIV-1 DNA reservoir level after 48 weeks of HAART and markers of disease progression. Total and Integrated HIV-1 DNA level at wk48 was plotted against A) wk48 CD4+ T cell count, B) the estimated time since seroconversion at randomization or C) baseline viral load. N=47 (A and B) N=35 (C).

Unlike at baseline, there were insufficient patient numbers to investigate the impact of individual HLA-type on HIV-1 reservoir level. Instead, total and integrated HIV-1 DNA levels were averaged in those expressing or not expressing the ‘protective’ HLA-B*27 and B*57 alleles. Patients expressing either HLA-B*27 or B*57 did not have a significantly longer time to primary endpoint ($p=0.24$ by log-rank test) (Figure 4.16A). This might be due to the small sample size of those expressing protective alleles ($n=10$). Also, HLA-B*27 and B*57 expressing patients did not have a time to viral rebound that was different from those not expressing the protective HLA alleles (Figure 4.16B). However, patients who expressed HLA-B*27 or B*57 had significantly lower total HIV-1 reservoir levels ($p=0.038$) (Figure

4.16C), and nearly significantly lower integrated reservoir levels ($p=0.07$) (Figure 4.16D) compared to those not expressing the protective HLA alleles.

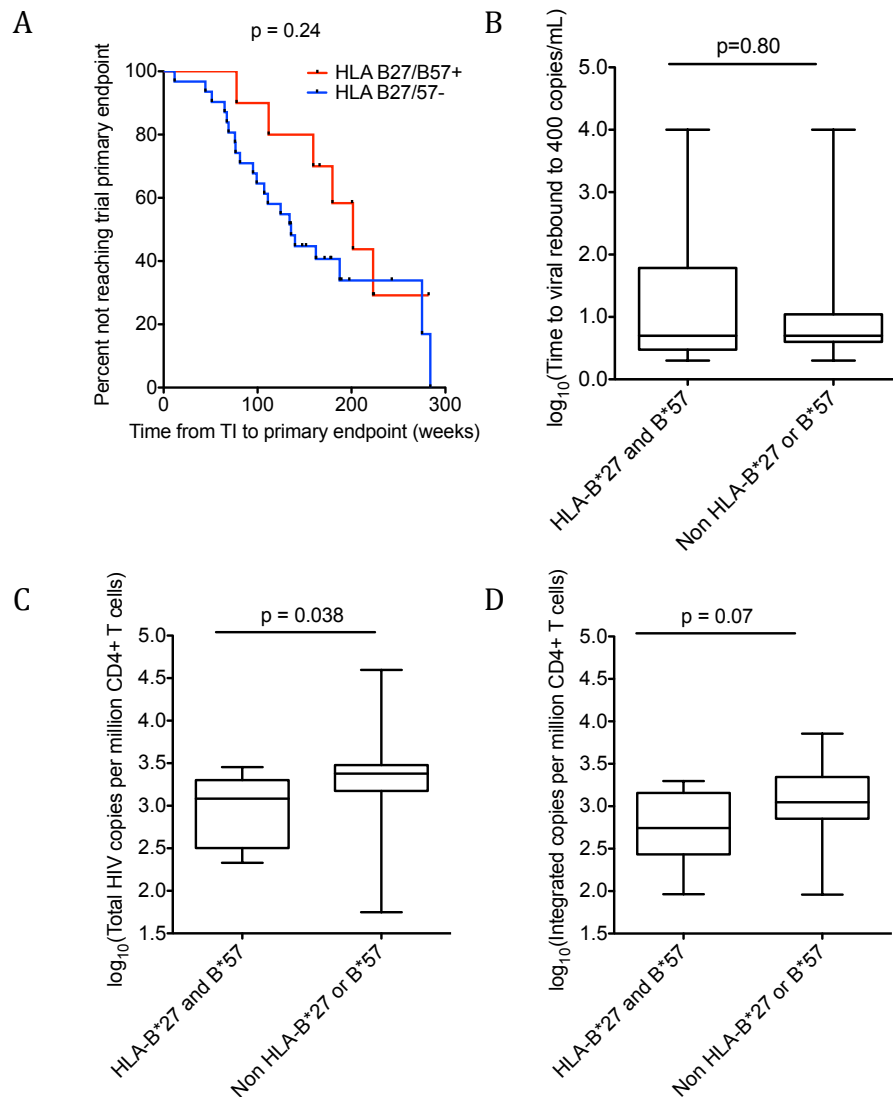


Figure 4.16. Protective HLA-types associate with lower HIV-1 reservoir levels after 48 weeks of HAART. A) Kaplan-Meier plot of time from TI to primary endpoint for patients who did or did not express HLA-B*27/B*57. B) Time to viral rebound to a level of 400 copies/mL was averaged in patients who did or did not express HLA-B*27/B*57. C) Total and D) integrated HIV-1 DNA levels measured after 48 weeks of HAART were averaged in patients who did or did not express HLA-B*27 or B*57 alleles. Significance was determined using Mann-Whitney tests. N=10 and N=31 for patients who did or did not express HLA-B*27/B*57, respectively.

4.3.9 Pre-TI HIV-1 DNA levels predict time to primary endpoint

As HIV-1 DNA levels in untreated acute HIV-1 infection predicted clinical progression in the SPARTAC trial, the HIV-1 reservoir was also tested for its ability to predict primary endpoint. The level of total and integrated HIV-1 DNA reservoir levels after 48-weeks of HAART, sampled at the point of TI, still predicted the clinical endpoint of the SPARTAC trial by Kaplan-Meier analysis ($p=0.039$ and $p=0.031$) (Figure 4.17) and in a univariate Cox regression model ($p=0.013$, and $p=0.023$) (Table 4.7), although less significantly than when DNA was sampled at baseline in untreated infection ($n = 47$; Table 4.2, right hand column).

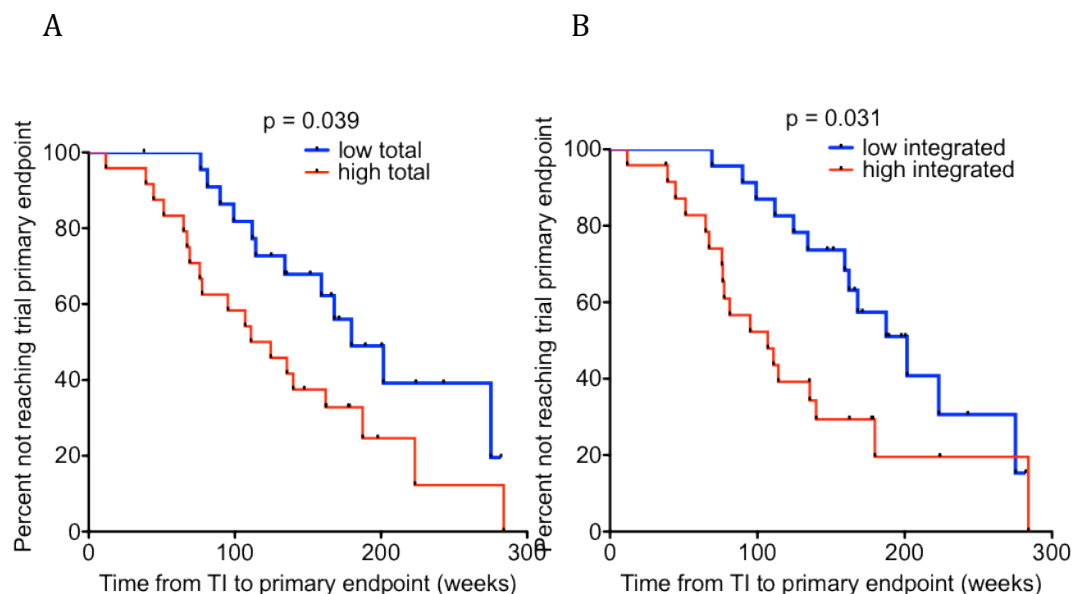


Figure 4.17. HIV-1 DNA after 48 weeks of HAART predicts clinical progression following TI A) Total and B) Integrated HIV-1 DNA levels were split into two 'high' and 'low' groups, by the median HIV-1 DNA value (for Total and Integrated HIV-1 DNA levels, median was 1814 and 992 copies per million CD4+ T cells, respectively). Significance was determined by log rank test. The time from TI to trial endpoint was measured. Median time from TI to primary endpoint for low and high total HIV-1 DNA levels were 159.2 (IQR 111.9-200.6) and 117.8 (IQR 67.8-173.8) weeks, respectively and low and high integrated levels were 166 (IQR 124.9-200.6) and 101.1 (IQR 65.5-156.8) weeks, respectively. ($n=47$ for both total and integrated measurements).

Conversely, in univariable Cox regression models, CD4+ T cell count 48 weeks post therapy induction was not predictive of time to primary endpoint (Table 4.7). Baseline viremia was weakly predictive of endpoint ($p=0.042$). In multivariable Cox regression analyses employing either week 48 total or integrated HIV-1 DNA level and corresponding CD4+ T cell count as a covariate, both HIV-1 DNA measures retained significance when CD4+ T cell count were not significant (Table 4.8 and 4.9). Viral load was not used as a covariate because it was undetectable at the point of TI. Since there was no detectable viremia at TI in these 47 patients, HIV-1 DNA level could be a very useful predictor of clinical progression, especially if pre-HAART viral loads are unknown.

Table 4.7: Univariable Cox regression models with HIV-1 DNA levels, wk48 CD4+ T cell count or baseline viral load as explanatory variables, using the SPARTAC trial endpoint (CD4+ T cell count < 350 cells / μ L). HIV-1 DNA levels were measured in participants just before interruption of 48 weeks of

Covariate	HR (95% CL)	p value
log ₁₀ total	3.44 (1.30-9.09)	0.013
log ₁₀ integrated	3.09 (1.17-8.16)	0.023
CD4+ T cell count / 100	0.94 (0.81-1.08)	0.35
Baseline viral load	1.75 (1.02-3.02)	0.042

Table 4.8 Multivariable Cox regression models with wk48 total HIV-1 DNA levels and CD4+ T cell count

Covariate	HR (95% CL)	p value
log ₁₀ total	3.42 (1.29-9.05)	0.013
CD4+ T cell count / 100	0.94 (0.83-1.08)	0.40

Table 4.9. Multivariable Cox regression models with wk48 integrated HIV-1 DNA levels and CD4+ T cell count.

Covariate	HR (95% CL)	p value
log ₁₀ integrated	2.81 (1.05-7.55)	0.04
CD4+ T cell count / 100	1.04 (0.83-1.11)	0.58

4.3.10 There is a delay in HIV-1 DNA level increase on TI

A concern with TI protocols is that reservoirs may be ‘re-seeded’ in individuals who have low HIV-1 DNA reservoir levels. Thus, the impact of TI on HIV-1 DNA level was investigated. Participant PBMCs were analysed 52, 60 and 108 weeks post randomisation (4, 12 and 60 weeks post-TI), after having received 48-weeks of HAART. HIV-1 DNA levels remained similar and not significantly different up to 12 weeks post-TI, but the difference became significant 1-year post TI in all samples (total $p < 0.0001$, Integrated $P < 0.0001$) (Figure 4.18A) and when samples, paired across all time points, were analysed (Figure 4.18B). There was no significant difference between baseline HIV-1 DNA level and HIV-1 DNA level 60 weeks post-TI for all samples (Figure 4.18A), for those paired across all time points (Figure 4.18B) or for those only paired across the two time points (Figure 4.18C). Unlike HIV-1 DNA, viral load significantly rebounded 4 weeks post-TI ($p = < 0.0001$) (Figure 4.18D). However viral load did not rebound to baseline levels ($p = 0.0003$). Most importantly, however, HIV-DNA level could be measured in patients, when their viral load was undetectable (Figure 4.18E).

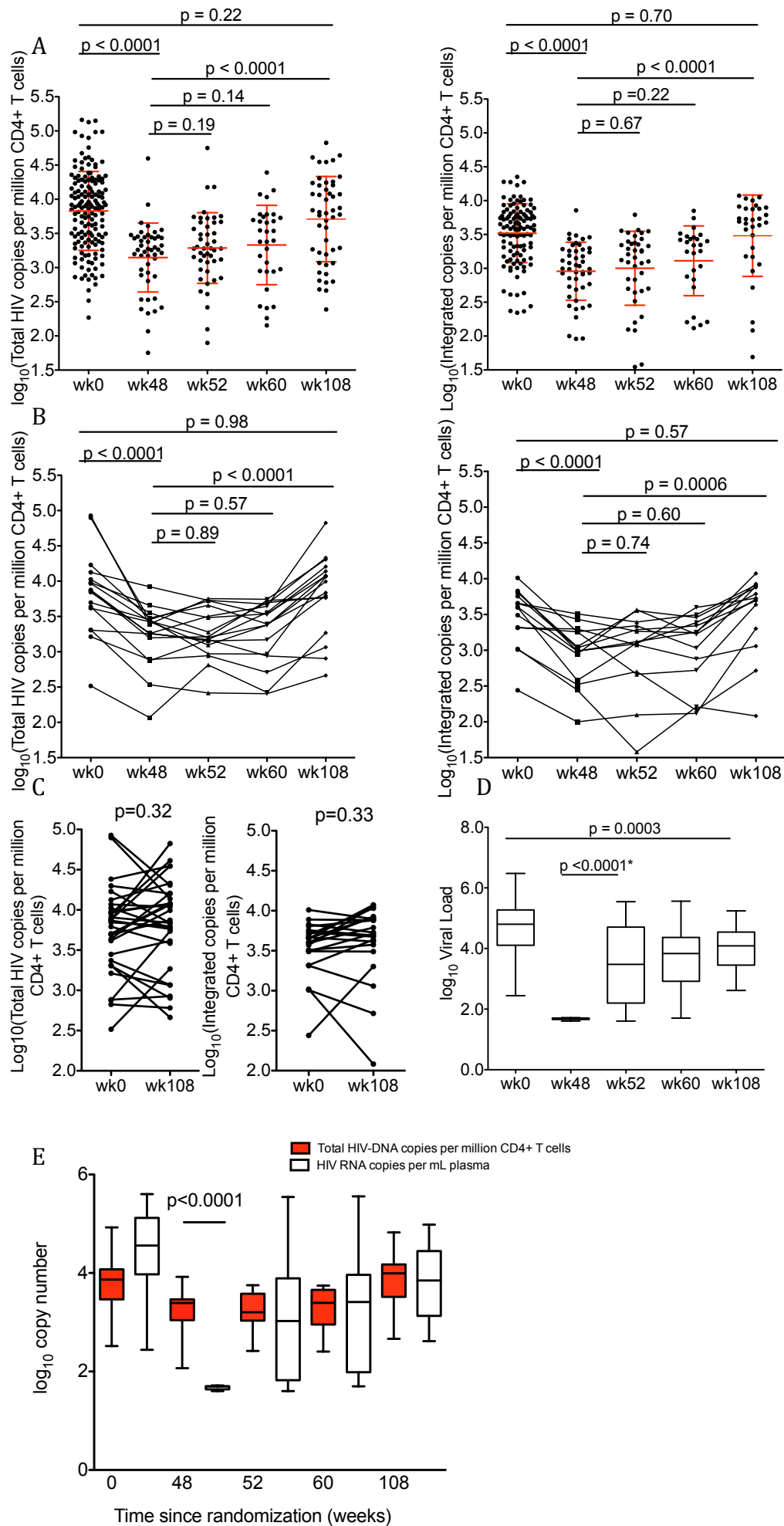


Figure 4.18. Longitudinal analysis of HIV-1 DNA levels post TI. Total and Integrated HIV-1 DNA levels were quantified from A) all measured samples at all time points (wk0, 48, 52, 60, 108: (total n=154, 47, 46 32, 48; integrated n=111, 47, 39, 27, 31)), B) samples paired across all time points (total n=17, integrated n=14) and C) samples paired at baseline and wk108 (total n=31, integrated n=21). D) Longitudinal Box and Whisker plots for viral load at each time point, for participants who were randomised to ART-48, were aviremic at the point of TI and for whom there was a total or integrated HIV-1 DNA measure (wk0, 48, 52; n=47, wk60; n=45, wk108; n=42). E) Total HIV-1 DNA levels and plasma viral load (HIV-1 RNA) were measured in 17 patients with sample across all timepoints. A) Unpaired Student's T test with Bonferroni correction, B) Paired Student's T test with Bonferroni correction, C) paired Student's T-Test and D) Student's T test * = paired, E) paired Student's T test.

4.3.11 Pre-TI HIV-1 DNA levels predict time to detectable viral rebound.

Although almost all participants in SPARTAC experienced VL rebound on stopping HAART, of those who received >12 weeks of therapy, 14% still had undetectable viraemia 12 months later (Stohr et al., 2013). In this different, although overlapping, sub-group of SPARTAC participants, the ability of HIV-1 DNA to predict plasma viraemia rebound post-TI was investigated. In a Kaplan-Meier survival analysis with time to viral rebound to 400 copies as the endpoint, total HIV-1 DNA levels just before TI significantly predicted the time to a viral rebound of 400 copies/mL ($p=0.0038$) with integrated HIV-1 DNA levels almost being significantly predictive ($p=0.060$) (Figure 4.19A). However, when endpoint was defined as the time to a viral rebound of 50 copies/mL, neither total nor integrated HIV-1 DNA levels were predictive ($p = 0.18$ and 0.22 , respectively) (Figure 4.19B).

Univariable Cox regression analyses demonstrated baseline and pre-TI total HIV-1 DNA level to both be predictive of a time to viral rebound of 400 copies/mL ($p=0.032$ and 0.021 respectively). Neither total nor integrated HIV-1 DNA levels sampled at baseline or at wk48 could predict time to a rebound of 50 copies/mL (Table 4.10). Baseline or wk48 CD4+ T cell count and viral load were also not

predictive of time to HIV-1 rebound at either cut-off. This indicates that HIV-1 DNA is potentially a very useful marker for predicting time to viral rebound. In a multivariable Cox regression model with pre-TI total HIV-1 DNA level and CD4+ T cell count as covariates, only total HIV-1 DNA level was predictive of time to viral rebound ($p=0.034$) (Table 4.11). Viral load was not used as a covariate because it was undetectable at the point of TI.

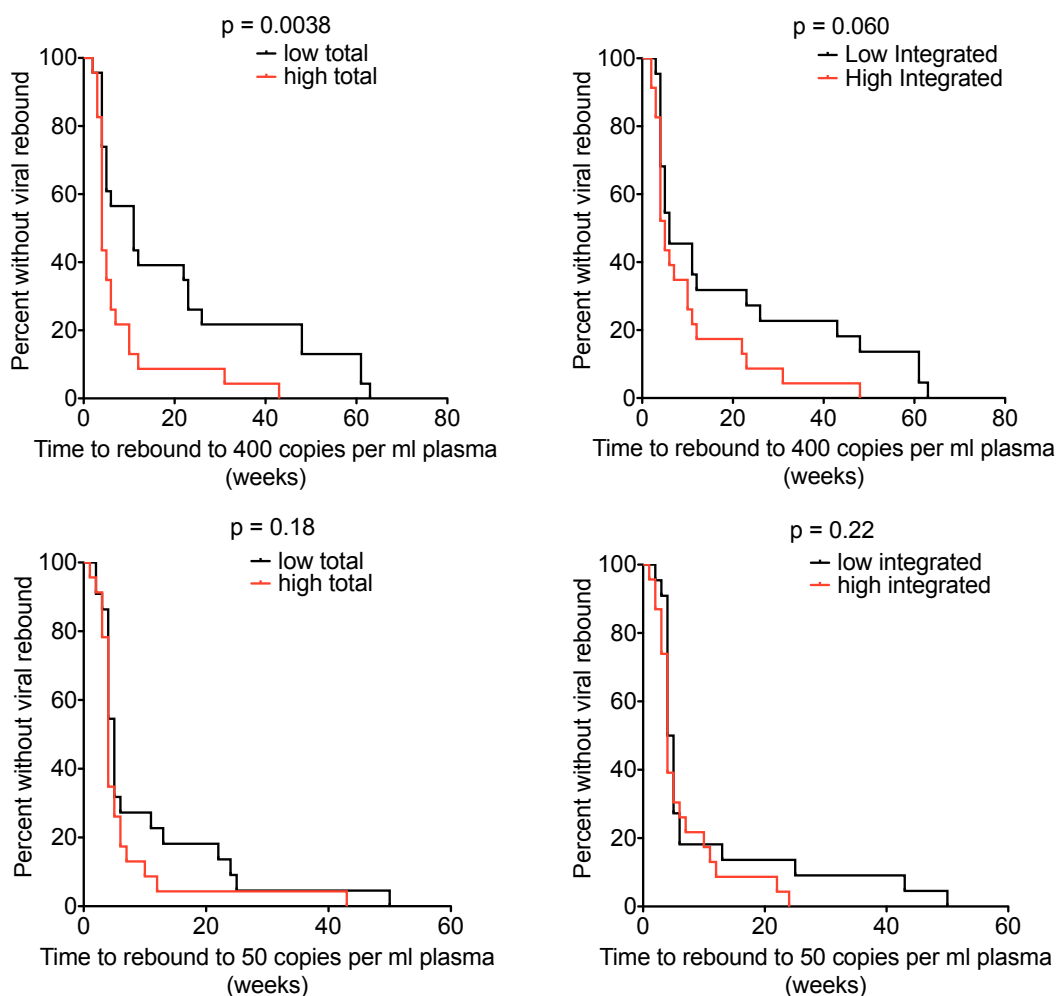


Figure 4.19. Total HIV-1 DNA at TI predicts viral rebound to a level of 400 copies/mL. Total and Integrated HIV-1 DNA levels were split into 'high' and 'low' groups, by the median value. Significance was determined by log rank test. A) Time to a viral rebound of 400 (median Total and Integrated HIV-1 DNA level was 1811 and 985 copies/million CD4+ T cells, respectively; $n = 46$) or B) 50 (median Total and Integrated HIV-1 DNA level was 1814 and 992 copies per million CD4+ T cells, respectively; $n = 45$) copies of HIV-1 per mL plasma was used as the endpoint.

Table 4.10. Univariable Cox regression models with wk-48 HIV-1 DNA levels, baseline or wk-48 CD4+ T cell counts or baseline viral load as the explanatory variables and time to viral rebound as the endpoint. Highlighted in bold are variables that significantly predicted time to rebound.

Covariate	Rebound level (copies/mL)	Hazard Ratio (95% Confidence Limits)	p value
Wk0 log ₁₀ (total)	50	1.53 (0.80-2.91)	0.20
Wk0 log ₁₀ (integrated)	50	1.34 (0.36-5.02)	0.67
Wk0 log₁₀(total)	400	2.08 (1.07-4.06)	0.032
Wk0 log ₁₀ (integrated)	400	2.70 (0.62-11.76)	0.18
Wk48 log ₁₀ (total)	50	1.50 (0.79-2.86)	0.22
Wk48 log ₁₀ (integrated)	50	1.42 (0.70-2.88)	0.34
Wk48 log₁₀(total)	400	2.16 (1.13-4.16)	0.021
Wk48 log ₁₀ (integrated)	400	1.78 (0.86-3.71)	0.12
Wk0 CD4 count/100	50	0.97 (0.82-1.15)	0.27
Wk48 CD4 count/100	50	1.00 (0.89-1.11)	0.96
Wk0 CD4 count/100	400	0.92 (0.78-1.08)	0.32
Wk48 CD4 count/100	400	1.04 (0.92-1.17)	0.53
Wk0 viral load	50	1.30 (0.81-2.08)	0.27
Wk0 viral load	400	1.38 (0.96-1.99)	0.080

Table 4.11. Multivariable Cox regression model with total HIV-1 DNA levels and CD4+ T cell count sampled at the point of TI as the explanatory variables and time to a viral rebound of 400 copies/mL as the endpoint.

Covariate	HR (95% CL)	p value
log ₁₀ total	2.68 (1.31-5.48)	0.0069
CD4+ T cell count / 100	1.06 (0.95-1.20)	0.26

Since all sampled patients had detectable viral rebound, analyses that don't need to take into account censoring of patients are possible. The time taken for a viral rebound of 400 copies was log-transformed and correlated significantly with log-transformed pre-TI total HIV-1 DNA copy number ($r=-0.37$; $p = 0.011$); Spearman's rank test) but not log-transformed integrated HIV-1 DNA copy number (Figure 4.20A). These results support the Kaplan-Meier and Cox regression analyses. Baseline HIV-1 DNA level did not associate significantly with time to viral rebound to 400 copies by Spearman's rank test (Figure 4.20B). However, baseline viral load correlated significantly with time to rebound to 400 copies ($r=-0.44$; $p=0.009$) (Figure 4.20C).

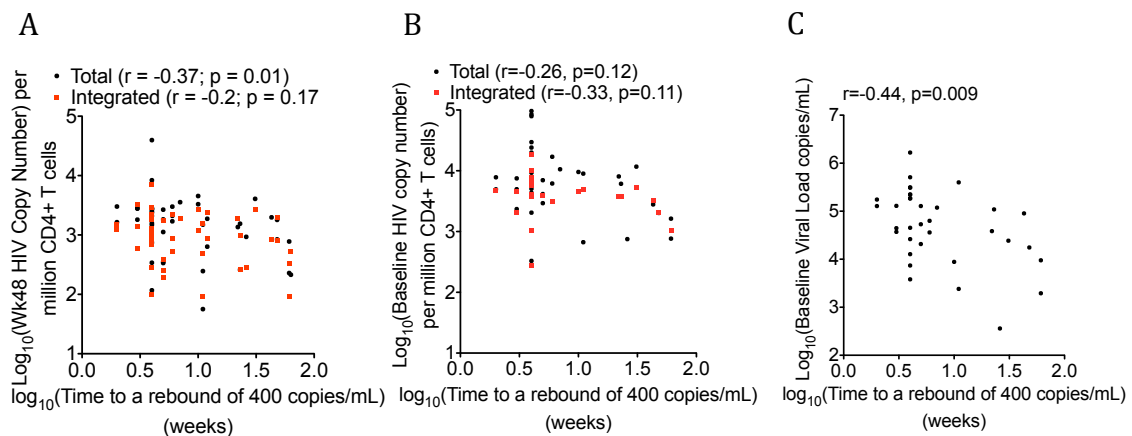


Figure 4.20. Spearman's rank correlations between HIV-1 DNA reservoir level after 48 weeks of HAART (sampled at the point of TI), baseline HIV-1 DNA level or baseline viral load, and time to a viral rebound of 400 copies/mL. A) HIV-1 DNA level at the point of TI ($n=46$), baseline HIV-1 DNA level (total $n=35$, integrated $n=25$) and C) baseline viral load ($n=39$) were plotted against log₁₀(time to viral rebound to 400 copies).

It was unclear why total HIV-1 DNA should predict time to a viral rebound of 400 but not 50 copies/mL in plasma. To explain this, individuals with data available at both cut-offs were studied. Patients with high pre-TI total HIV-1 DNA levels, defined by splitting at the median value, were more likely to rebound straight to

above 400 copies/mL. Patients with lower HIV-1 DNA levels were more likely to initially rebound below 400 but above 50 copies/mL (total $p=0.0074$ and integrated $p = 0.090$; Fisher's exact test) (Table 4.12). However, the association was weaker for integrated HIV-1 DNA levels sampled at the same time point ($p=0.09$; Fisher's exact test) (Table 4.12).

Table 4.12. 2x2 table comparing the number of patients with the same and different times to 50 and 400 copy/mL rebound and their total (above) and integrated (below) pre-TI HIV-1 DNA levels. HIV-1 DNA levels were split into 'high' and 'low' groups by the median value. The proportions are significantly different by Fisher's exact test for total ($p=0.0074$) but not integrated HIV-1 DNA levels ($p=0.091$).

	High Total HIV-1 DNA	Low Total HIV-1 DNA
First detectable VL rebound <400 copies/ml	N=2	N=10
First detectable VL rebound >400 copies/ml	N=21	N=12

	High Integrated HIV-1 DNA	Low Integrated HIV-1 DNA
First detectable VL rebound <400 copies/ml	N=3	N=9
First detectable VL rebound >400 copies/ml	N=19	N=4

Interestingly, the time taken for viraemia to reach greater than 400 copies / mL plasma did not predict the time to primary endpoint (HR 0.99 (Confidence limits (CI) 0.97-1.01), $p=0.37$). This indicates that faster rebound is not necessarily a pre-requisite for faster progression to AIDS.

4.3.12 TI does not impact on reservoir size on HAART re-initiation.

There has been much concern recently about the safety of stopping HAART. Data from the SMART trial associated therapy cessation in chronic infection with increased morbidity and mortality (El-Sadr et al., 2006). There are more theoretical concerns regarding TI, such as the potential for mimicking a seroconversion-type event or replenishing the viral reservoir and thus undoing any advantage that has been gained. The HIV-1 reservoir level could be determined on HAART pre and post TI in a subset of 15 participants who received 48-weeks of HAART (ptx), stopped and then subsequently re-started at least 6 months of long term HAART (ltx) after reaching primary endpoint. There was no significant difference between HIV-1 reservoir size measured at ptx or ltx, by paired students T test (Figure 4.21). Also, the similarity in HIV-1 DNA levels at baseline and 1 year post-TI (Figure 4.18C) supports TI not impacting on the HIV-1 reservoir 'set-point'. There is perhaps less variation in the reservoir at ltx.

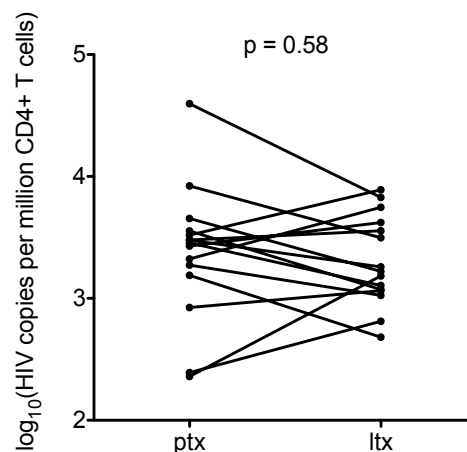


Figure 4.21. Impact of TI on HIV-1 reservoir size

Total HIV-1 DNA levels were calculated in 15 compliant patients after 48 weeks of HAART and sampled before TI (ptx). These patients restarted HAART when primary endpoint was reached. HIV-1 DNA levels at least 6 months post HAART resumption (ltx) were measured. Significance was determined using a paired students T test.

4.4 Discussion

This chapter provides strong evidence to suggest an important role for HIV-1 DNA levels in predicting patient outcome in terms of clinical progression and time to viral rebound on TI.

Pre-therapeutic viral loads are predictive of time to clinical progression (Fourati et al., 2013, Mellors et al., 1996) and are more predictive than CD4+ T cell count (Mellors et al., 1997). However it is not always possible to measure pre-HAART viral load, and viral load during HAART is often below the limit of detection of most clinical assays. Therefore HIV-1 DNA levels present in HAART-treated patients may be a useful and informative parameter for predicting progression to AIDS, viral recrudescence and if it is safe for patients to stop therapy. This is especially pertinent in light of future HIV-1 eradication trials, where TI protocols are crucial.

In this chapter, HIV-1 DNA levels correlated with a variety of immunological and virological parameters that predict clinical progression, as expected and as previously reported. Both markers of inflammation analysed in the study (IL-6 and d-dimer) predicted clinical outcome in chronic HIV-1 infection (Ledwaba et al., 2012, Boulware et al., 2011). However, only d-dimer associated significantly with HIV-1 DNA levels in this chapter (Fig 4.5). The reason for this is unknown and would be interesting to investigate in future studies.

The activation markers CD38 and HLADR are up regulated in HIV-1 infected patients (Ho et al., 1993), with CD38 more strongly predicting disease progression

than HLA-DR or a variety of soluble activation markers (Liu et al., 1997). The exhaustion markers PD-1 (Trautmann et al., 2006, Zhang et al., 2007, Day et al., 2006), LAG-3 (Blackburn et al., 2009) and TIM-3 (Jones et al., 2008) can also be up-regulated in HIV-1 infection. PD-1 is the most studied marker and its expression has been linked with faster clinical progression (Day et al., 2006). However, there is now evidence to suggest increased initial PD-1 expression could be a marker of cell activation (Hong et al., 2013).

In this chapter, baseline PD-1 and Lag-3 expression on CD8⁺ T cells significantly associated with HIV-1 DNA levels from the same time point (Figure 4.7 and 4.8). Indeed, cells with high PD-1 expression have enriched integrated HIV-1 DNA levels (Chomont et al., 2009). TIM-3 expression did not correlate with HIV-1 DNA levels. Evidence from acute LCMV infection suggested that TIM-3 is only transiently expressed in acute infection (Jin et al., 2010), perhaps explaining the lack of association with HIV-1 DNA levels in this chapter. Higher percentages of CD38⁺ and HLA-DR⁺ CD8⁺ and CD4⁺ T cells also correlated with higher HIV-1 DNA levels, which may support more activated T cells being better targets for productive HIV-1 infection (McDougal et al., 1985).

In this chapter, exhaustion and activation markers associated more significantly with HIV-1 DNA level when expressed on CD8⁺, not CD4⁺ T cells (Figure 4.5, 4.6, 4.7 and 4.8). Interestingly, CD8⁺ and not CD4⁺ T cell activation markers were predictive of progression to AIDS in a previous publication (Deeks et al., 2004).

CD8⁺ T cells have an important role in controlling HIV-1 viremia in acute infection (Koup et al., 1994, Borrow et al., 1994), but associations between viral load and HIV-1-specific CD8⁺Tetramer⁺ T cell frequency have been contradictory (Ogg et al., 1998, Betts et al., 2001). However, a recent study showed that strong Gag specific CD8⁺ T cell responses were associated with a lower viral load (Kiepiela et al., 2007).

Similarly, progression to AIDS is slowed in patients with strong CD4⁺ Gag ELISpot responses (Boaz et al., 2002, Soghoian et al., 2012, Frater et al., 2013) and is associated with viral control in acute (Schieffer et al., 2014) and chronic infection (Rosenberg et al., 1997). As seen by ELISpot analysis in this chapter, both CD4⁺ and CD8⁺ T cell responses are significantly associated with lower HIV-1 DNA levels in untreated patients, although the relationships were relatively weak (Figure 4.9).

This study cannot prove that better immunological responses cause a lower reservoir level, as lower reservoir levels may ensure better immunological responses. However, the direction of cause and effect is clearer when investigating the relationship between HIV-1 DNA level and HLA-type, since reservoir levels cannot impact HLA-type.

Indeed, the lower HIV-1 DNA levels quantified in patients expressing 'protective' HLA-B*57 and B*27 alleles in this chapter (Figure 4.10, 4.11 and 4.16) is consistent with elite controllers being enriched for protective HLA alleles, harboring low HIV-1 DNA levels (Graf et al., 2011) and displaying better CD8⁺ T cell responses (Betts et al., 2006, Saez-Cirion et al., 2007).

The relationship between MHC class I and HIV-1 DNA is interesting as the low HIV-1 DNA levels in the VISCONTI PTCs were not associated with stronger CD8+ T cell function or protective HLA type expression. This chapter presents data from a larger cohort analysed prior to HAART. Long term HAART diminishes CTL responses most likely through the loss of antigen (Casazza et al., 2001) and this could help to explain these discrepant results.

Despite low HIV-1 DNA levels predicting a longer time to primary endpoint in untreated infection or post TI, the lines representing 'high' and 'low' HIV-1 DNA populations on Kaplan-Meier graphs became parallel (Figure 4.12 and 4.17), indicating any benefit of having low HIV-1 DNA levels was lost after a period of time. But these results support other clinical trials, whereby low HIV-1 DNA levels associated with a longer time to clinical progression (Goujard et al., 2006, Minga et al., 2008, Piketty et al., 2010).

A potential weakness with the survival analysis is that baseline plasma viral load and not viral load set point (as in other studies) was used to predict clinical progression. This might explain why viral load was less predictive of time to primary endpoint than total HIV-1 DNA levels (Table 4.5). However, despite baseline viral load being higher than viral set point, as expected for acutely infected patients, they correlated very closely (data not shown). This might also explain why baseline viral loads were higher than viral load measurements 1-year post-TI (Figure 4.18D). It is also worth noting that for the greatest chance of post treatment control, individuals must be treated in PHI, before viral set point has been reached. Therefore HIV-1 DNA levels, which are less dynamic (baseline and

wk108 DNA levels were not significantly different (Figure 4.18)), are still potentially a clinically useful predictive biomarker.

48-weeks of HAART treatment decreased the burden of HIV-1 DNA significantly, as reported elsewhere (Koelsch et al., 2008), by 0.5 of a log (Figure 4.14C). However, on TI, there was a period of at least 12 weeks where no significant increases in reservoir level were detected by both assays (Figure 4.18), indicating HAART may have caused some short-term protection, despite viral load rebounding more rapidly (Figure 4.18D). A period of virological remission after TI has also been reported in the Swiss (Gianella et al., 2011), PRIMO-SHM (Grijzen et al., 2012), ANRS CO6 PRIMO (Goujard et al., 2012) and CASCADE (Lodi et al., 2012) cohorts, all studying the impact of HAART in acute infection.

It is perhaps reassuring for future TI studies to note that the presence of rebounded virus did not immediately replenish the HIV-1 reservoir level. Also TI did not impact on reservoir level when HAART was re-initiated (Figure 4.21). However, this post-hoc analysis must be repeated in other similar trials with larger patient numbers.

There was little evidence of long-term post-treatment control (Saez-Cirion et al., 2013, Persaud et al., 2013) in the studied patients as viral load significantly rebounded 4-weeks post-TI and levels of HIV-1 DNA 1-year post therapy interruption were not significantly different to that seen before therapy (Figure 4.18). These patients were perhaps not treated early enough, or for long enough for long-term post-treatment control to be established.

Having a low 'total' HIV-1 reservoir after 48-weeks of therapy resulted in a longer time to a viral rebound of 400 copies/mL and the association was significant by Spearman's rank correlation (Figure 4.20), Kaplan-Meier analysis (Figure 4.19) and in univariable Cox regression models (Table 4.10). The evidence suggesting baseline HIV-1 DNA levels and viral load are predictive of this secondary endpoint was weaker (Table 4.10 and Figure 4.20).

Additionally, patients with high Total HIV-1 DNA levels were more likely to rebound straight to greater than 400 copies/ml, whereas those with lower HIV-1 DNA levels were more likely initially to rebound below 400 but above 50 copies/mL (Table 4.12). Therefore, time to viral rebound in patients with high HIV-1 DNA levels happens not only sooner than in those with low HIV-1 DNA levels, but the rebound kinetics are more rapid as well. The association between high HIV-1 DNA levels and magnitude of rebound might therefore be stronger than for low levels predicting remission. It may thus be possible to predict people who can safely interrupt their treatment during HIV-1 cure strategies.

Interestingly, the time to detectable viral rebound did not predict time to primary endpoint. This could be because immunological factors might better contain and control spontaneous rebound viremia. However, the study presented is underpowered to answer this question. However, faster rebound was more likely in those with high HIV-1 DNA levels, possibly because there is more chance for a spontaneous reactivation event to occur.

It is unknown why integrated HIV-1 DNA level did not predict time to rebound (Table 4.10). The 'total' assay has less intrinsic variability and associated error than the integration assay. It could be hypothesised that continued low levels of *de novo* HIV-1-reverse transcription might be evidence of continued viral replication, the presence of which might lead to faster viral rebound. Also, Tat can be transcribed from unintegrated HIV-1 DNA (Brussel and Sonigo, 2004, Wu and Marsh, 2003), which could make latency reversal and viral recrudescence more likely. However, this study did not address these hypotheses and they would be interesting to address in future studies.

HIV-1 DNA levels did not predict a rebound time to 50 copies/mL (Table 4.10). Since the assay detection limit was 50-copies/mL, viral loads above this threshold could have been random statistical and biological variations (Lee et al., 2006). However, the time to rebound was defined after two consecutive readings above the threshold, making this theory unlikely. Also, operating at the limit of assay detection may be associated with more assay error. Indeed, when viral load cut-offs of 200 rather than 50 copies/mL were used in a recent unpublished ACTG study analysis, the detection of false positive rebound events decreased from 25% to 14% (Riabaudo et al., 2009).

Although baseline viral load correlated with time to viral rebound (Figure 4.20), baseline viral load may not always be known in the clinic and viral load at the point of TI is often undetectable. Total HIV-1 DNA was the only biomarker tested that significantly predicted time to viral rebound and clinical progression, when sampled at baseline and pre-TI (Tables 4.4, 4.5, 4.7, 4.8, 4.10, 4.11 and Figures

4.12, 4.17, 4.19, 4.20). In a multivariable Cox regression model, baseline total HIV-DNA but not viral load was predictive of time to primary endpoint in untreated infection (Table 4.5). In agreement, total HIV-1 DNA level predicted progression to AIDS better than viral load in a recent meta-analytical study (Tsiara et al., 2012). Additionally, the total HIV-1 Q-PCR assay is much easier to set up, cheaper, uses less input cell number and is associated with less plate-to-plate variation than the integration assay.

Total HIV-1 DNA level is thus a very useful biomarker for understanding the HIV-1 reservoir and its clinical implications with regards to control of HIV-1 infection and functional cure, as hypothesised. The findings of this study are thus directly applicable for future clinical trials aimed at eradicating HIV-1 even if baseline viral load is unknown. There is thus much potential to develop an algorithm in future studies combining HIV-1 DNA as a biomarker for predicting progression and post treatment control with other immunological markers, such as those presented in this chapter.

5. Application of Magnetic Fluid Hyperthermia and Super Paramagnetic Iron Oxide Nanoparticles to HIV-1-specific CTL Cytotoxicity.

This work presented in this chapter has been published by Dove Medical Press in the International Journal of Nanomedicine (Williams et al., 2013b).

5.1 Introduction

Super Paramagnetic Iron Oxide Nanoparticles (SPIONs) have been trialled for use in the treatment of cancer by magnetic fluid hyperthermia (MFH). Enough heat can be produced by Brownian motion and Néel relaxation on alternating magnetic field exposure to kill tumour cells by thermal ablation (Pankhurst et al., 2003). On direct injection of SPION, hyperthermic temperatures were achieved in mouse models of breast cancer, leading to improved tumour control (Jordan et al., 1997, Zhai et al., 2009). SPION were well tolerated in human Glioblastoma multiforme patients (Muller, 2009, Maier-Hauff et al., 2007, Jordan and Maier-Hauff, 2007), and thermotherapeutic temperatures were achieved, leading to local tumour control and a doubled survival rate in patients (Maier-Hauff et al., 2007, Maier-Hauff et al., 2010).

In Prostate cancer studies, a 44-51% inhibition in growth of the aggressive MatLyLu tumour variant was observed on MFH treatment, with intratumoural temperatures of up to 70°C, recorded (Johannsen et al., 2005). MFH also induced a 4.5 fold increase in survival time in a rat model of malignant glioma (Jordan et al., 2006). SPION can also be targeted directly to tumours, without direct

intratumoural injection, using SPION-conjugated monoclonal antibodies, specific for cancer antigen. Significant therapeutic responses in mice with hamster blood transfusion (HBT) 3477 human breast cancer xenografts were demonstrated, with tumours taking up to eight times longer to quintuple in size compared to controls (DeNardo et al., 2005).

Cytotoxic T cells (CTLs) can bind to and kill HIV-1-infected cells and limit HIV-1 replication (Schmitz et al., 1999, Hersperger et al., 2011, Walker et al., 1987). However, as disease progresses, HIV-1 specific CTLs lose their effector functions hierarchically, express exhaustion markers and have altered transcriptional profiles, preventing optimal control of infection and these functional defects are not restored by HAART (reviewed in (Wherry, 2011)) (Migueles et al., 2009, Kalams et al., 1999).

CTLs are permissive to SPION uptake (Beer et al., 2008, Iida et al., 2008, Kircher et al., 2003). The application of superparamagnetic iron nanoparticles to HIV-1 has been proposed as a technique for tracking HIV-1-specific CTL (Murthy, 2007, Dodd et al., 2001).

Resovist is a 60 nm carboxydextran coated SPION, with superior cellular uptake properties (Mailander et al., 2008, Metz et al., 2004, Matuszewski et al., 2005) and a higher heating capability, or calculated Intrinsic Loss Power (ILP), than other clinically available SPION (Kallumadil et al., 2009) such as Endorem (sample VII) (Hergt et al., 1998). However, since Resovist is now commercially unavailable, Feraspin-R, a closely related SPION formula with very similar properties to

Resovist (Ludwig et al., 2012), was employed to study the feasibility of using MFH to enhance CTL killing of HIV-1-infected cells.

Theoretically, thermotherapeutic heat generated in Feraspin-R-loaded CTLs through alternating magnetic field exposure could lead to the death of CTL-bound antigen-expressing target cells through direct thermal ablation (summarised in Fig 5.1) (Personal communication with Quentin Pankhurst, Royal Institute, UCL).

A recent study showed that therapeutic intervention of patients' own CTLs, in this instance Gag peptide and IL-2 pre-stimulation, was needed before CTLs could target and clear latently HIV-1-infected cells (Shan et al., 2012). It was hypothesised that thermotherapy could be an alternative adjunctive therapy to enhance CTL targeting of the latent viral reservoir, post viral 'kick'. Autologous functional, exhausted or TCR-transduced patient CD8⁺ T cells could be used for targeted thermotherapy so long as they still bind to their targets through the Human Leukocyte Antigen (HLA) Class I – T cell receptor (TCR) interaction as thermotherapy acts independently of the normal cellular cytotoxic mechanism.

The 868-TCR recognises the immunodominant HLA-A*02:01-restricted, HIV-1 p17 gag-derived epitope SLYNTVATL. Since the leucine residues at p2 and p9 in this epitope are essential for HIV-1 gag trimerization and act as primary anchors for HLA-A2, escape mutants have high fitness costs (Varela-Rohena et al., 2008). Additionally, the soluble recombinant form of the 868-TCR has the highest recorded natural affinity for its cognate antigen ($K_D < 150\text{nM}$) as measured by Surface Plasmon resonance (Varela-Rohena et al., 2008) and would therefore

maximise the length of interaction between target and effector cells for thermotherapy.

Also, for application of MFH to patients, autologous SPION-loaded TCR-transduced CTLs could be adoptively transferred into any patient expressing the relevant HLA allele.

Sensitive methods to assay SPION uptake (Metz et al., 2004, Matuszewski et al., 2005, Mailander et al., 2008, Beer et al., 2008, Iida et al., 2008, Yeh et al., 2010), toxicity (Metz et al., 2004, Matuszewski et al., 2005, Yeh et al., 2010, Mailander et al., 2008, Beer et al., 2008) and effect on 868-TCR-transduced CTL function were developed (Kircher et al., 2003, Beer et al., 2008, Iida et al., 2008) and SPION uptake was optimised using Lipofectamine and FDA-approved Protamine Sulphate.

These optimised assays were then used to test if thermotherapy could impact HIV-1 868-TCR transduced CTL function. No other examples for the application of MFH to an infectious disease (HIV-1) model could be found in the literature at the time of writing.

5.2 Aims and Objectives

5.2.1 Aims

My aim was to determine whether Magnetic Fluid Hyperthermia in Feraspin-R-nanoparticle loaded cells could lead to the death of HIV-1 antigen-expressing target cells through thermal ablation.

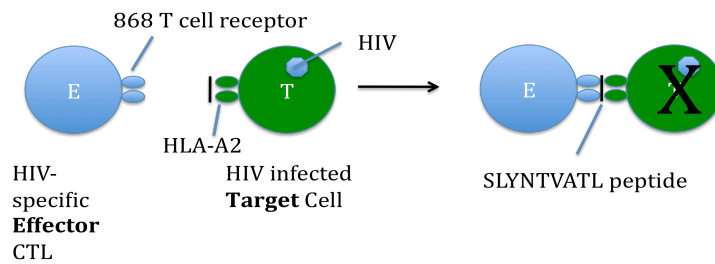
5.2.2 Objectives

1. I will investigate Feraspin-R uptake into CTLs and assay toxicity.
2. I will then assay the impact of Feraspin-R on natural 868-TCR transduced CTL function.
3. An alternating magnetic field, which induces heating of Feraspin-R, will then be applied to assess thermotherapeutic contributions to target cell clearance.

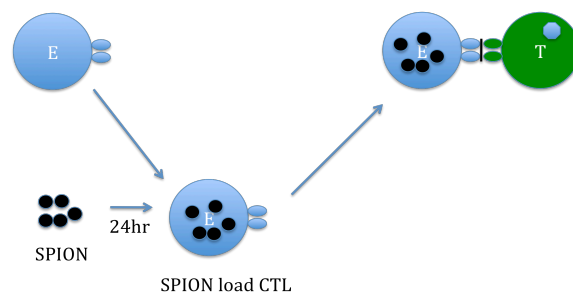
5.2.3 Hypothesis

The thermotherapeutic heat generated in Feraspin-R-loaded 868-TCR transduced CTLs on alternating magnetic field exposure could lead to the death of CTL-bound antigen-expressing target cells by direct thermal ablation.

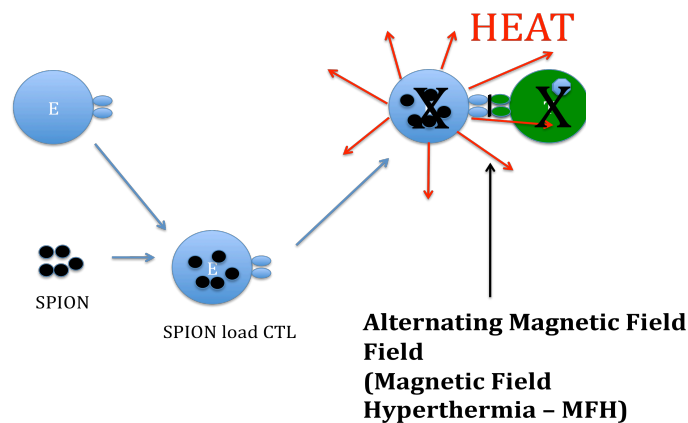
Direct thermal ablation of target cell



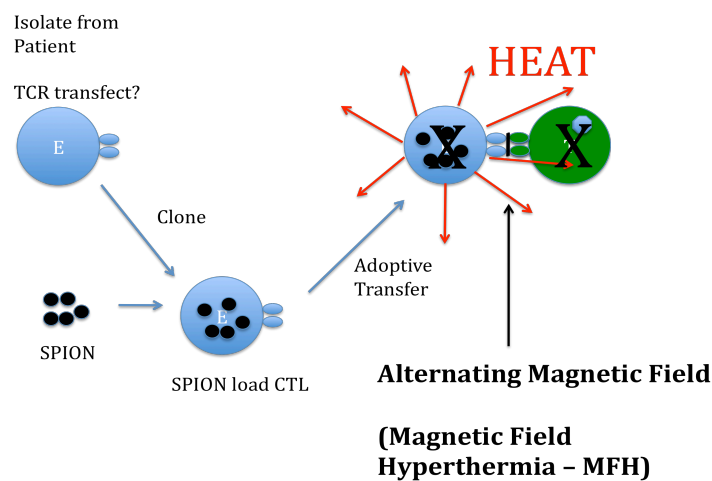
1. Normal CTL induced Lysis



2. Loading of SPION into CTL



3. Thermal ablation of CTL and bound target cell



4. Patient CTL could be isolated, transfected with the 868-TCR, cloned and adoptively transferred prior to thermotherapy

Figure 5.1. The thermotherapeutic heat generated in cloned, Feraspin-R-loaded autologous patient CTLs through alternating magnetic field exposure could lead to the death of CTL-bound antigen-expressing target cells through direct thermal ablation. E: Effector, T: Target.

5.3 Results

5.3.1 Feraspin-R bath heating

560 μ g/ml Feraspin-R ILP was first determined. The temperature was measured and ambient temperature was subtracted. After approximately 30 minutes of incubation, the temperature of the nanoparticle solution had increased by 12°C (Figure 5.2) in an experiment performed by Paul Southern at UCL. To maximise heating, a 1mg/mL maximal concentration of Feraspin-R was chosen for incubation with CTL. In thermotherapy studies, SPION-loaded CTL were incubated for 1 hour to maximise any potential temperature increase. Bath heating is measured here (the heat generated from nanoparticles in an evenly dispersed solution). For this chapter, localised heat-induced toxicity, specific to the Feraspin-R loaded CTL environment was assayed, rather than a more disseminated and potentially toxic effect that would be observed through bath heating of a patient.

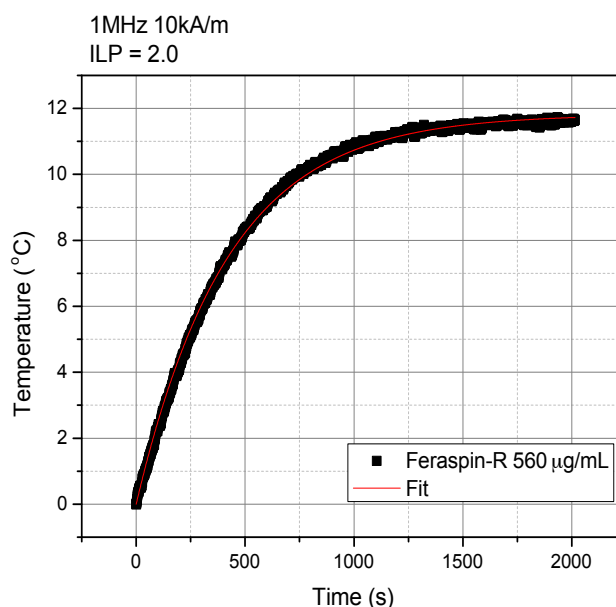


Figure 5.2 Bath Heating potential of Feraspin-R. Experiment performed and image created by Dr Paul Southern, Royal Institute, UCL, London and used with permission. Intrinsic loss power (ILP), Megahertz (MHz)

5.3.2 868-TCR transduced CTL

868-TCR-transduced CTLs (2.3.1.1) were used in this study. It was deemed important to utilise 868-TCR-transduced cells, as it is possible to grow large and pure quantities of TCR-transduced cells, since these can come from many starting precursor cells unlike a monoclonal T cell population. The method used to expand the CTLs (2.3.2) resulted in a population containing 97% CD8+ T cells, with 68.4% CD8+868-TCR+ (Figure 5.3) determined by tetramer (2.7.3) staining (2.7.4). Also, SLYNTVATL peptide is conserved and HLA-A2 is a common HLA-type.

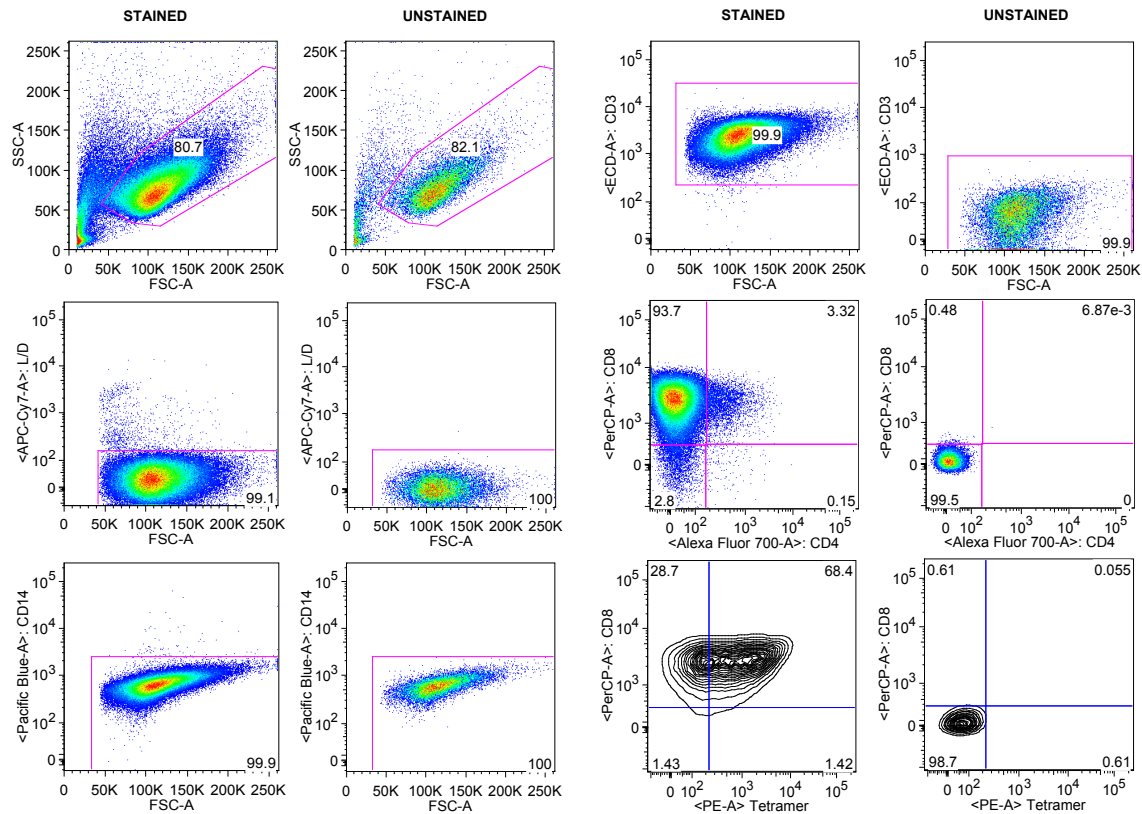


Figure 5.3 Characterisation of the 868-TCR clonal cell line by FACS. The FACS gating strategy is presented. A) CTL gate, B) Live gate, C) CD14- gate, D) CD3+ gate, E) CD8+CD4 gate F) Tetramer+ gate. All gates determined using unstained cells.

5.3.3 Feraspin-R is taken up into 868-TCR CTL

First, it needed to be determined if Feraspin-R could be taken up by CTL. A dose escalation experiment where 868-TCR-transduced CTLs were incubated with 0, 0.1, 0.5 and 1 mg/ml Feraspin-R was performed (2.3.3). The CTLs were viewed under a Transmission Electron Microscope (TEM) (2.3.5). After a 24-hour incubation, Feraspin-R was shown to localise intracellularly (Figure 5.4), in a dose dependent fashion. Even after vigorous washing, Feraspin-R adhered to the cell surface (Figure 5.4B, lower arrow), a likely prerequisite for internalisation. Individual Feraspin-R nanoparticles could also be observed by TEM (Figure 5.4E).

The observation that some Feraspin-R binds to the cell surface has not been reported before, however in previous studies looking at SPION treatment of cells, some surface bound (Matuszewski et al., 2005) and non surface bound SPIONS (Iida et al., 2008) are visible. However, this discovery does not jeopardise the aim of this project, since SPION technically only need to be associated with cells for there to be a thermotherapeutic effect.

The amount of iron associated with the cells was quantified by using a Superconducting Quantum Interference Device (SQUID) (Method summary in 2.3.6). This uptake was time ($p = 0.03$ by 2-way ANOVA) and concentration ($p = <0.001$ by 2-way ANOVA) dependent, with a maximum of 28.1 pg/cell measured after a 24 hour incubation of CTLs with 1mg/ml Feraspin-R (Figure 5.5A). The saturation level of electromagnetic units (emu) at high alternating magnetic fields (measured in Tesla) is proportional to the amount of Superparamagnetic iron

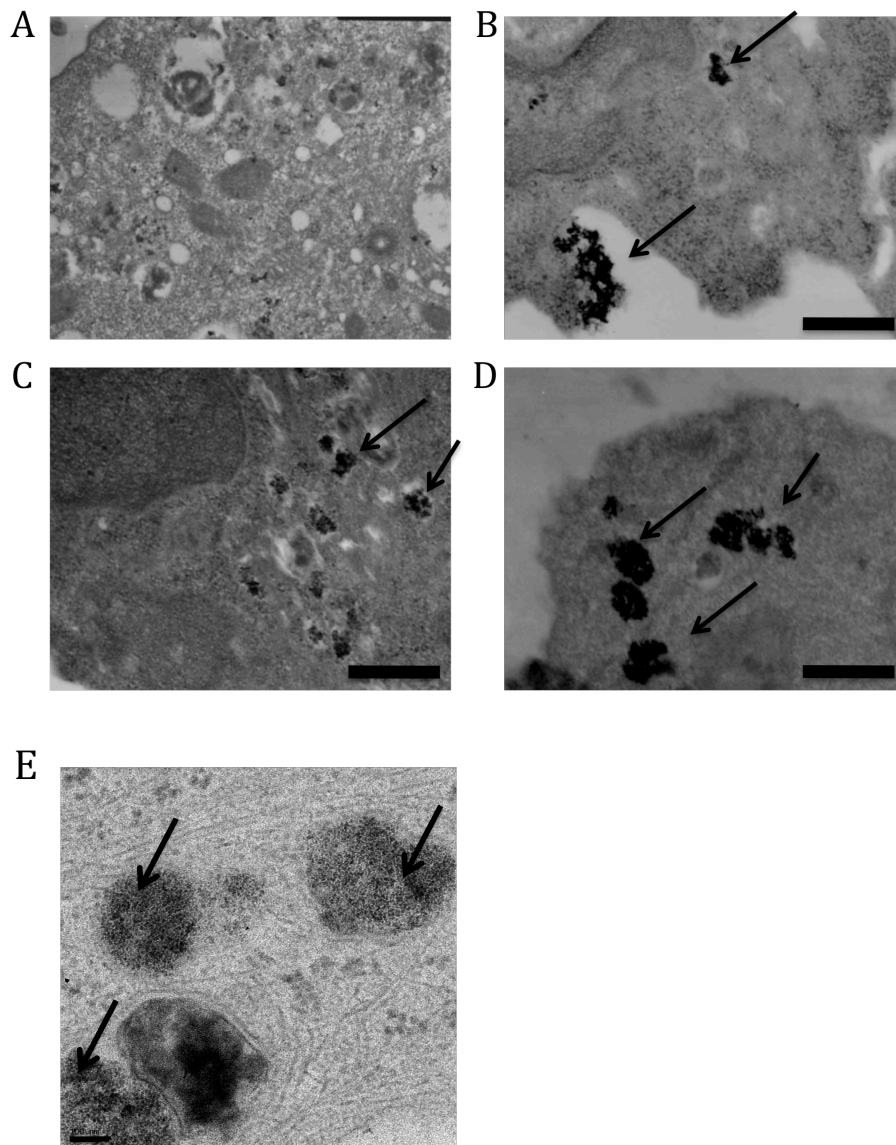


Figure 5.4. Feraspin R is endocytosed dose dependently by 868-TCR-transduced CTLs, and adheres to the cell surface. 868-TCR-transduced CTLs were incubated for up to 24 hours with A) 0mg/ml, B) 0.1mg/ml (upper arrow shows internalized Feraspin-R, lower arrow shows externally bound Feraspin-R), C) 0.5mg/ml and D) 1mg/ml Feraspin R. Uptake was observed by TEM (magnification 20K). E) Individual nanoparticles were observed. Scale bars are 100nm.

oxide present, making quantification by this method possible. To determine the percentage of CTLs that endocytosed Feraspin-R, an anti-dextran antibody conjugated to FITC, as previously reported by Beer et al (Beer et al., 2008) was used to stain the Feraspin-R treated CTLs intracellularly and extracellularly (2.7). The MFI change demonstrated that close to 100% of cells took up Feraspin-R (Figure 5.5B). A generalised linear model statistical test demonstrated this effect to be dose-dependant whether using an intracellular or extracellular stain ($p < 0.001$). The interaction term between stain position (intracellular vs extracellular) and Feraspin-R concentration was also significant ($p < 0.001$) indicating that at high concentrations of Feraspin R, significantly more nanoparticles were located intracellularly than extracellularly, but with the effect not significant at low concentrations.

In order to maximise any potential thermoablative temperature, maximal Feraspin-R uptake needed to be achieved. A trend for enhanced uptake was seen using the transfection reagents Lipofectamine and the FDA approved Protamine sulphate, with a maximum of 53 pg/cell achieved after incubating starved CTLs with a 1mg/ml Feraspin-R/Protamine Sulphate cocktail for 24 hours (Figure 5.5C) (2.3.4). However, starvation did not have a significant effect on uptake over all conditions. This value is similar to that observed in other studies using Resovist analogues, albeit in different cell types and under different conditions (Matuszewski et al., 2005, Metz et al., 2004).

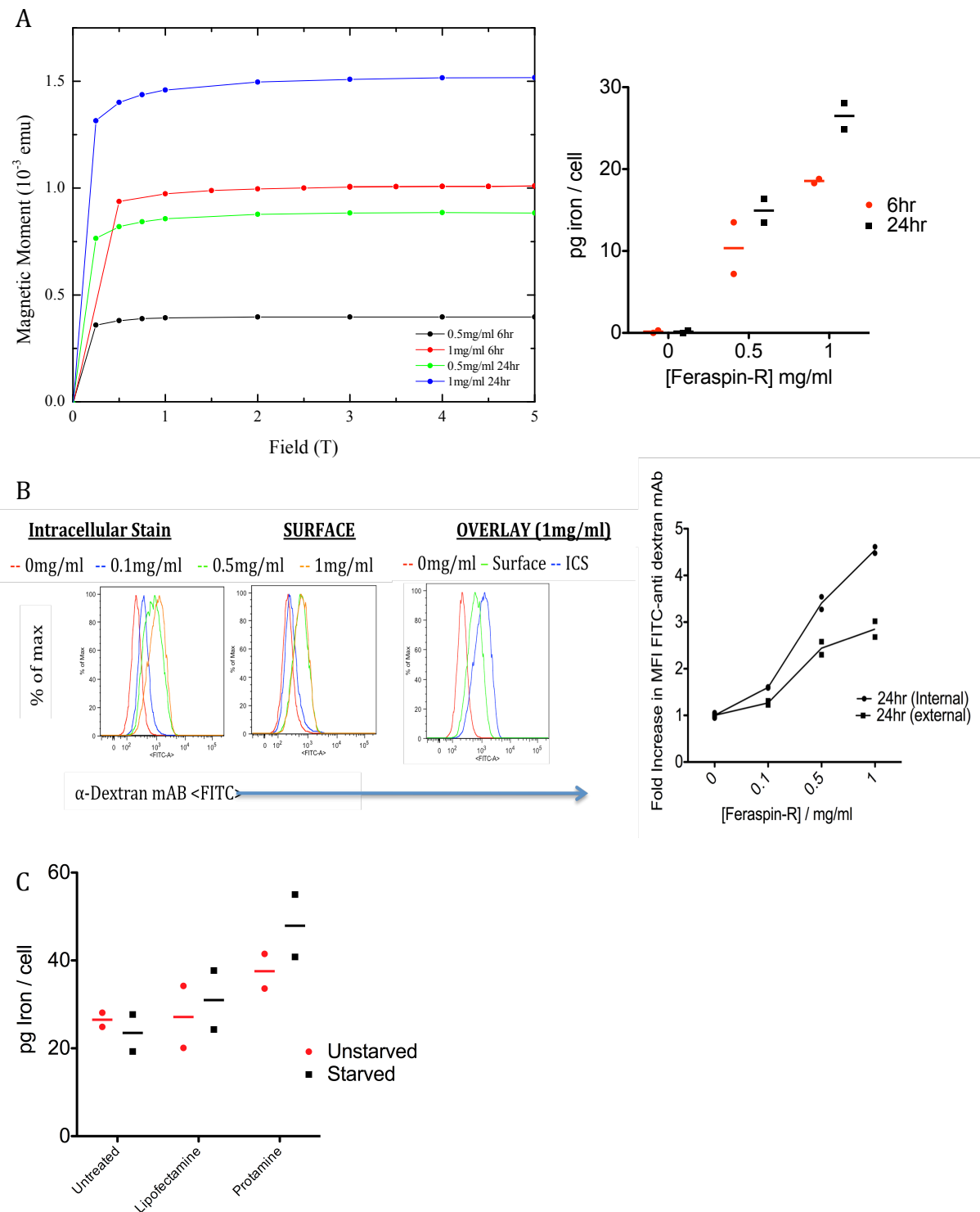


Figure 5.5. Uptake of Feraspin-R into 868-TCR-transduced CTLs. A) CTLs were incubated with varying concentrations of Feraspin-R for 6 or 24 hours. Cell-associated Feraspin-R was quantified by SQUID. Raw SQUID data (left panel) and processed data from duplicate experiments (right panel) are shown. B) Feraspin-R loaded CTLs were stained intracellularly or extracellularly with an anti-dextran antibody-FITC. MFI was calculated (left panel). The fold-increase in MFI for intracellular (circles) and extracellular (squares) Feraspin-R was plotted against the incubation concentration of Feraspin-R (right panel). All experiments were performed in duplicate. (C) Cell culture Feraspin-R media was supplemented with Lipofectamine or Protamine Sulphate for 24 hours. Feraspin-R was quantified by SQUID. Two experiments were performed for each condition. Emu = electromagnetic units, T = Tesla.

5.3.4 Feraspin-R exhibits limited toxicity to 868-TCR CTL

The hypothesis for this project relies on Feraspin-R uptake into CTLs being non-toxic to the CTLs themselves. MTT assays are usually performed to investigate the toxicity of agents through metabolic disruption. However, when the toxicity of Feraspin R was tested using this method with 100,000 CTLs per well, and as described by the manufacturers protocol (ATCC), the cell-associated nanoparticles absorbed at the same wavelength as the MTT reagent (570nm) (Figure 5.6).

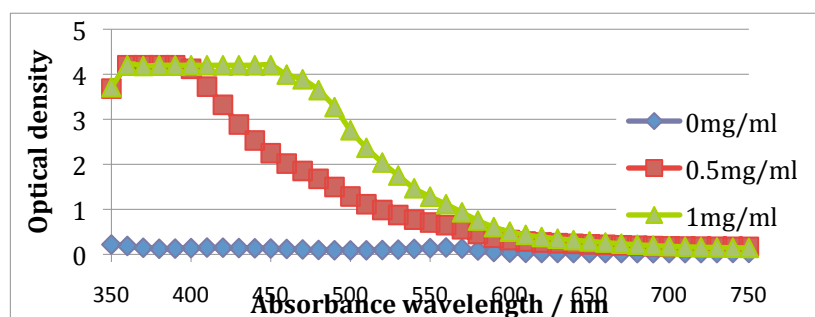


Figure 5.6 MTT reagent and Feraspin-R absorbance spectra

Therefore, another method needed to be devised to measure toxicity of these nanoparticles. Feraspin-R toxicity was measured through a FACS staining protocol (2.7). Annexin V binds to Phosphatidylserine, a phospholipid that is only found on the outer extracellular membrane leaflet after membrane flipping; a hallmark of apoptosis. Camptothecin-treated Feraspin-R-loaded CTLs were used as positive controls to define and gate out dead and apoptotic cells from the live cell population, at each Feraspin-R incubation concentration (Figure. 5.7A). A general linear model statistical test demonstrated significant ($p = 0.042$) toxicity with increasing Feraspin-R dose, after 24 hours, (Figure. 5.7B) although the actual absolute toxicity values are small.

After washing off the nanoparticles and further incubation of CTLs in R10 media, there is a significant interaction between the % of living cells and time after CTL washing ($p = 0.003$; general linear model), with a trend to increasing viability for CTLs over time for both treatment groups. Also, the minor toxic effects of 1mg/ml Feraspin-R were still observed after washing ($p < 0.001$; Generalised linear model) compared to untreated cells, but the trend is for this effect to be lost over time (Figure 5.7C). Transfection reagent treatment, and starvation had a no effect on cell viability (Fig. 5.7D).

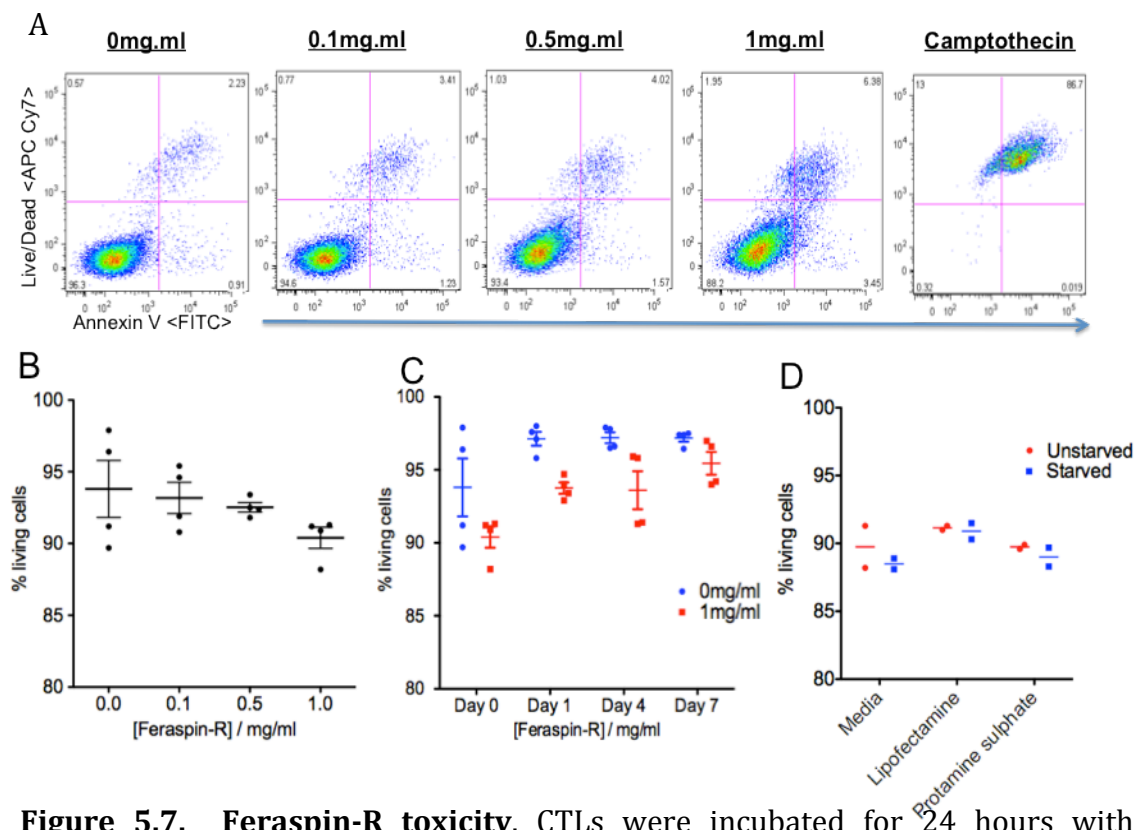


Figure 5.7. Feraspin-R toxicity. CTLs were incubated for 24 hours with increasing doses of Feraspin-R. A) Live CD8⁺ (PerCP) T-cells were gated and Annexin-V⁺ (FITC), Live (APCcy7⁻) cell gates were determined using a Camptothecin-treated control for all doses of Feraspin-R treated CTLs. B) Dead and apoptotic cells were identified and the population of Annexin-V negative, Live cells were calculated as a percentage of total gated CD8⁺ cells (% living cells), and repeated 4 times. C) CTLs incubated for 24 hours with and without 1mg/ml Feraspin-R were washed (Day 0) and cultured for a further week. The % living cells at Day 1,4 and 7 were determined in quadruplicate. This assay was performed at the same time as (B). D) CTLs were incubated with Feraspin-R in the presence of transfection reagents, and the % living cells post 24-hour incubation was determined in duplicate.

5.3.5 Feraspin-R uptake is insufficient for thermal ablation to occur.

As Feraspin-R uptake occurred and only limited toxicity was observed, the impact of heat generation on clearance of target cells was investigated. First, CTLs were incubated with Feraspin-R at 1 mg/ml for 24 hours with and without transfection reagents in an alternating magnetic field for 1 hour (Figure. 5.8 A and B) (2.3.7). Cell death and apoptosis markers were analysed by FACS staining 1 hour and 24 hours after magnetic field exposure. SPION-loaded CTLs were also incubated for the same time and at the same temperature, but were not exposed to an alternating magnetic field. Also, controls were included where Feraspin-R loaded CTLs were incubated at known temperatures. No significant increase and no trend in cell death after alternating magnetic field exposure could be determined by 2-way ANOVA, even after transfection reagent treatment (Figure. 5.10B), despite a 20 minute temperature pulse of above 44.5 degrees being enough to kill 50% of Feraspin-R loaded CTLs 24 hours later (Figure 5.8C).

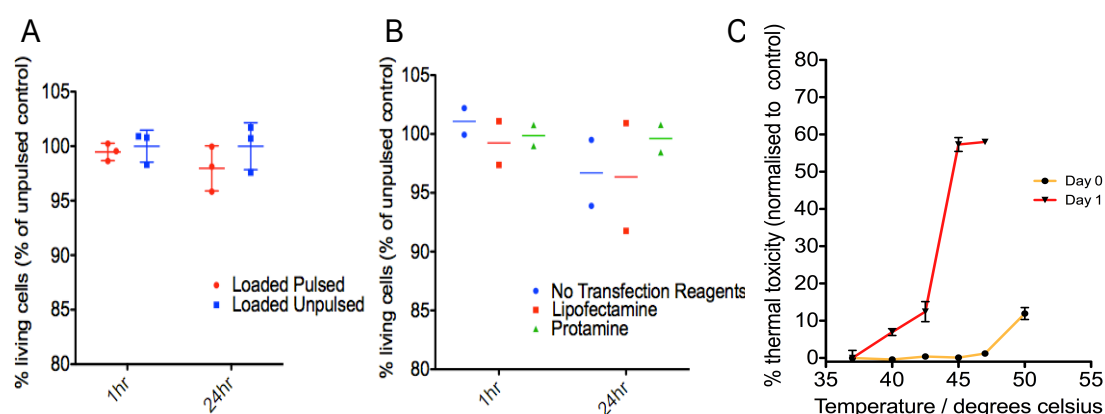


Figure 5.8. Thermal ablation of Feraspin-R loaded CTLs. CTLs treated for 24 hours with 1mg/ml A) in the absence or B) presence of transfection reagents were exposed to an alternating magnetic field for 1 hour. Unmagnetised loaded CTL controls were included. The % of living cells (as in Fig 5.9) was measured 1 and 24 hours post magnetization to detect immediate and delayed cell death. Parts A and B were performed in triplicate and duplicate, respectively. C) Immediate and delayed cell death of Feraspin-R loaded CTLs at various known incubation temperatures, in triplicate.

5.4 Thermotherapeutic enhancement of CTL function

Disappointingly, not enough heat was generated from these SPION loaded CTL for there to be any measurable cell death, by these assays. Interestingly, studies have shown that hyperthermic temperatures enhance CTL cytotoxicity (Smith et al., 1978, Mace et al., 2012) and stimulate CTL interferon-gamma release from CTLs (Mace et al., 2012), possibly in response to pathogen-induced fever. Not enough heat was generated for direct thermoablative cell death, so it was hypothesised that there may be enough heat produced from internalised CTL associated Feraspin-R to enhance CTL function (Figure 5.9).

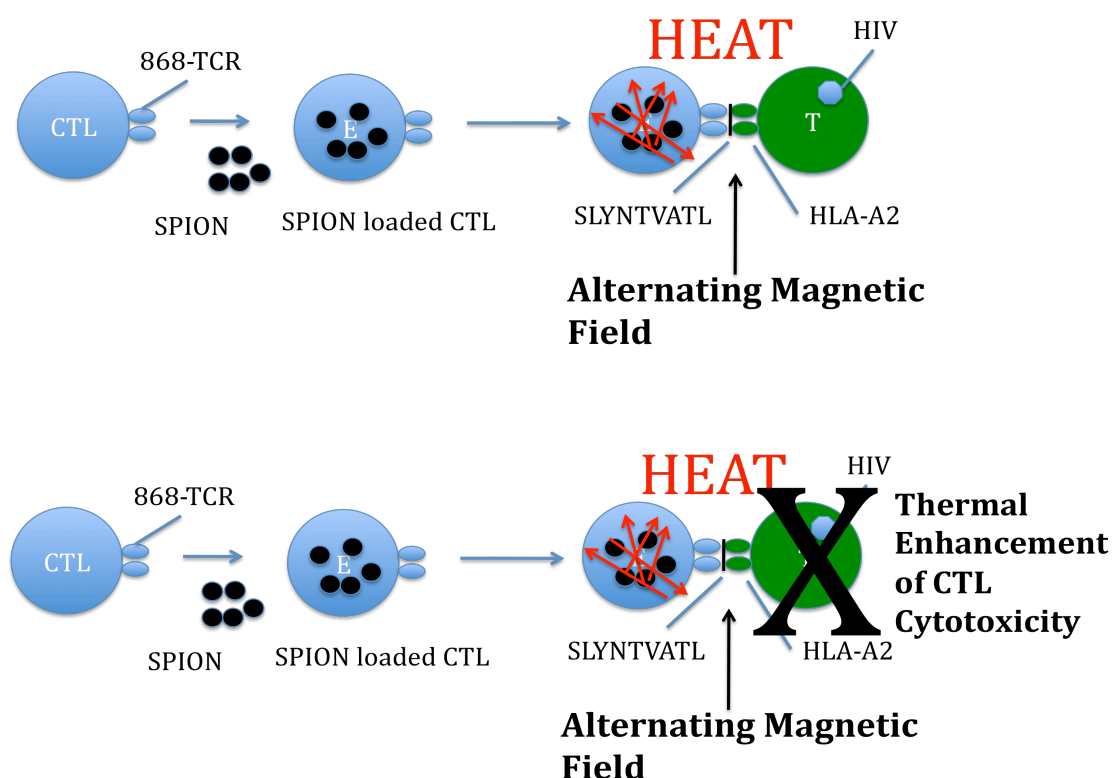


Figure 5.9 Stimulation of CTL function using Magnetic Fluid Hyperthermia.
E: effector, T: Target

5.4.1 868-TCR CTL function is enhanced at 40°C compared to 37°C

868-TCR transduced CTLs were tested for increased killing ability at 40°C. The FATAL assay, as described by (Sheehy et al., 2001) were carried out at 37 and 40°C using two Effector: Target (E:T ratios) of 1:1 and 1:2 (2.8.1 and 2.8.2).

After log transforming time, the generalised linear model demonstrated a statistically significant increase in the % cytotoxicity of CTL at 40, compared to 37°C ($p = 0.017$) across both E:T ratios, and cytotoxicity increased with higher E:T ratios ($p < 0.001$) and log(time) ($p < 0.001$) (Figure. 5.10A). The biggest difference in % cytotoxicity was observed to be after a 5-hour incubation (Figure. 5.10A). The % cytotoxicity of CTLs increased from 48% to 57% ($n = 8$, $p = 0.0003$) after 5 hours of incubation (Figure 5.10B). The increased cytotoxicity at 40°C was not due to direct heat induced target cell death (Figure 5.10C) because T2 cells incubated at both assay temperatures were similarly viable.

5.4.2 Feraspin-R uptake negatively impacts 868-TCR CTL killing ability.

As CTLs better targeted antigen expressing cells at 40°C, it was hypothesised that heat generated from Feraspin-R localised internally in CTLs could be sufficient to increase their killing ability. First, the impact of Feraspin-R loading on natural CTL killing ability was investigated (2.8.3). The killing ability of CTLs decreased by 5-10% on addition of the lowest concentration (0.1 mg/ml) of Feraspin-R across all E:T ratios tested, and then only modestly decreased on addition of more nanoparticles (Figure 5.11A).

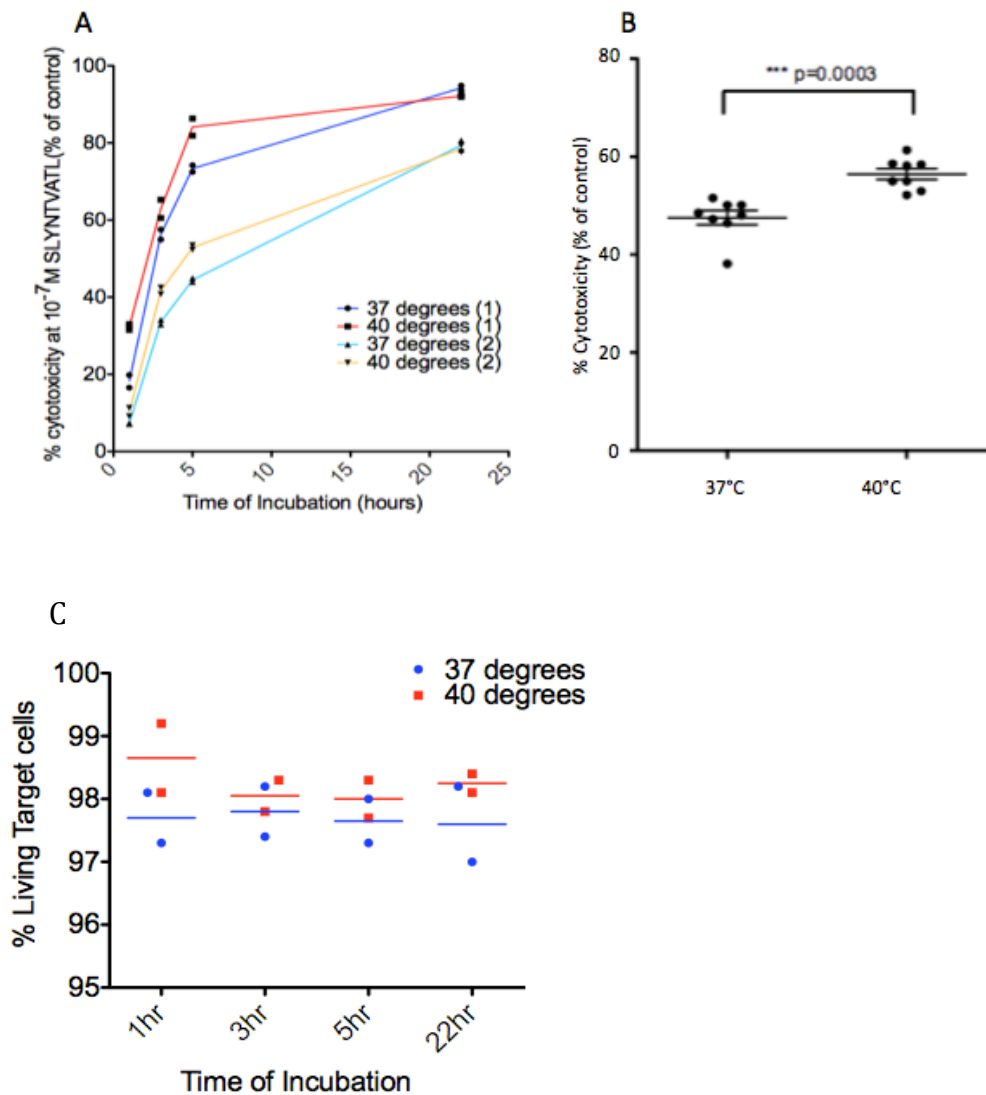


Figure 5.10 Temperature-dependant CTL cytotoxicity. A) Two independent FATAL killing assays were performed and analysed over time using SYLN TVATL-pulsed T2 cells as targets and 868-TCR CTLs as effectors at a 1:1 (1) or 1:2 (2) Effector: Target ratio, either at 37 or 40°C. Decreases in the peptide-pulsed CFSE+ cell population (FITC) were measured, as a percentage of the control pulsed cell population, where no CTLs were added (% cytotoxicity as a % of control). B) % cytotoxicity (as a % of control) using 10⁻⁷M SLYNTVATL pulsed T2 cells was determined after a 5-hour incubation as above, with 8 replicates at 37 or 40°C. (C) The percentage living T2 cells with no CTL addition.

The generalised linear model demonstrated a significant decrease in CTL cytotoxicity on increasing nanoparticle concentration ($\log_{10}(\text{Feraspin-R concentration} + 0.001)$), across all E:T ratios ($p = 0.007$). CTLs loaded for 24 hours with 1mg/ml Feraspin-R were then tested for their ability to kill peptide pulsed T2 cells at different SLYNTVATL peptide concentrations, and EC_{50} values were calculated. There was less than a log difference in killing ability between loaded and unloaded CTLs, with respective EC_{50} values of $1.34 \times 10^{-8} \text{M}$ and $2.59 \times 10^{-9} \text{M}$ (Figure 5.11B). The generalised linear model showed Feraspin-R significantly decreased CTL killing ability across all peptide concentrations ($p = 0.028$) after the % cytotoxicity data was transformed using the following equation ($\logit(5 + \% \text{ cytotoxicity})/100$).

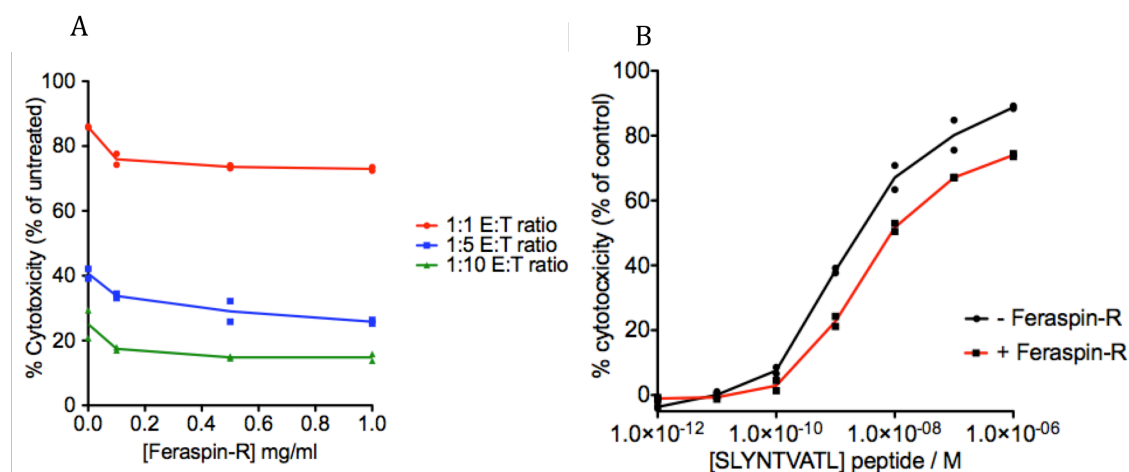


Figure 5.11. Impact of SPION treatment of CTLs and their killing ability. A) Two independent FATAL assays were performed using CTLs pre-treated with various concentrations of Feraspin-R for 24 hours at various E:T ratios. B) Two independent FATAL assays were performed using CTLs treated for 24hr with 1mg/ml Feraspin-R at various concentrations of SLYNTVATL peptide pulsed T2 cells. All FATAL assays were incubated overnight.

5.4.3 868-TCR CTL function is not enhanced by thermotherapy

Finally, the impact of heat generation inside CTL was assayed. FATAL assays using Feraspin-R loaded and unloaded CTLs were performed during 5-hour exposures to an alternating magnetic field (2.8.4). The experiments were performed in duplicate, and were controlled with identical FATAL assays, not exposed to the magnetic field, but incubated at the same bath temperature of 37°C. A decrease in killing ability was seen on nanoparticle addition; however, no significant increase in killing ability of loaded magnetised cells versus loaded un-magnetised cells was observed by 2 way ANOVA (Figure. 5.12). Therefore, not enough heat was generated by the cell-associated Feraspin-R to impact on CTL cytotoxicity, as seen above.

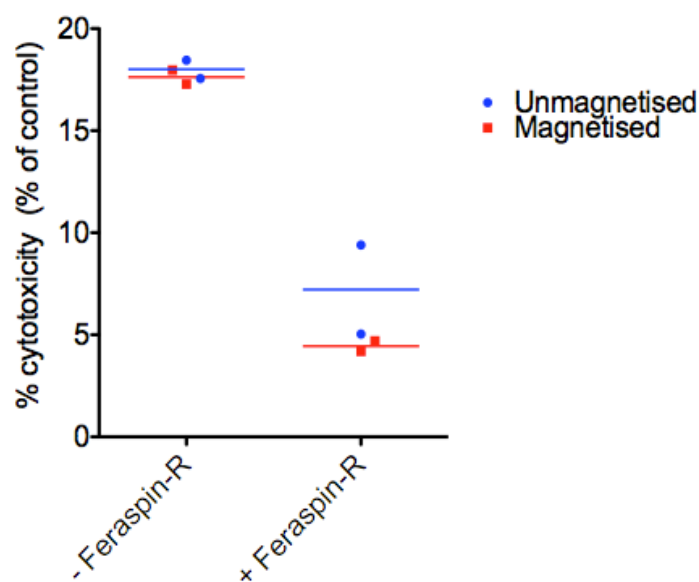


Figure 5.12. Insufficient heat is generated inside 868-TCR-transduced CTLs for enhancement of cytotoxicity. FATAL assays were performed using CTLs either pre-treated with 1mg/mL Feraspin-R or left untreated. CTLs were incubated at a 1:1 E:T ratio with 10^{-7} M SLYNTVATL-treated T2 cells with or without alternating magnetic field exposure for 5 hours. The % cytotoxicity of CTLs (as a % of control) was calculated as before. All experiments were performed in duplicate.

5.5 Discussion

The role of SPIONS and magnetic hyperthermia are exciting potential new therapeutic and diagnostic clinical applications of nanoparticle technology. To date, this has been focused on cancer, but this technology has now been applied to an infectious disease, HIV-1 infection.

Viral 'kick' using anti-latency agents does not necessarily lead to death of the infected cell (Shan, Deng et al. 2012). This may have been because the patient CTLs were defective (reviewed in (Wherry 2011)) or CTL responses had declined due to a lack of available antigen after long term HAART (Casazza, Betts et al. 2001). Novel methods are thus being trialled to enhance targeting and clearance of HIV-1-infected cells pre and post viral reactivation, for example using the immunotoxin 3b3-PE38 (Denton et al., 2014).

SPION technology was applied in a proof-of-concept study to assess whether MFH could enhance HIV-1-specific T cell function. Two hypotheses for enhancing T cell function by thermotherapy were tested. Firstly, thermotherapy might increase the temperature of the CTL and its bound target cell to directly induce cell death. Secondly, raising the temperature of CTLs to the equivalent of physiological fever (40°C) may increase function and cytotoxicity. Nanoparticle toxicity, uptake and effect of cell function is not well characterised for CTLs. In addition, sensitive methods investigating Feraspin-R toxicity, uptake and effect on cell function were tested.

Previous studies have shown CTLs are permissive for SPION uptake, but for the purpose of T-cell tracking through the body by Magnetic Resonance Imaging (MRI) (Beer et al., 2008, Kircher et al., 2003) and targeted CTL mediated thermotherapy in melanoma (Iida et al., 2008). SQUID was chosen as a method for Feraspin-R quantification. It has advantages over other iron quantification methods such as inductive coupled plasma atomic absorption spectroscopy and atomic absorption spectroscopy (Matuszewski et al., 2005), which potentially overestimate iron levels by not discriminating between non-paramagnetic and paramagnetic iron. Additionally, iron concentrations are calculated indirectly in the colorimetric Potassium Thiocyanate method (Iida et al., 2008) and this method assumes that full oxidation or reduction of the sample occurs.

A concentration of 53 pg/cell (Figure 5.5C) was measured in this study, post starvation and on protamine sulphate treatment, a higher value than that observed by (Beer et al., 2008), but similar in studies using Resovist analogues in other cell types (Metz et al., 2004, Mailander et al., 2008, Yeh et al., 2010). A limitation of Lipofectamine is that it is not licensed for use *in vivo*, however Protamine Sulphate is FDA approved for use *in vivo*, and may be useful in future studies where high cellular SPION uptake is crucial.

In this study, Feraspin-R bound to CTLs extracellularly (Figures 5.3 and 5.5B). This is an issue that may not have been addressed in previous studies. Thus, for the purposes of this study, SQUID detected cell-associated Feraspin-R.

No Resovist toxicity to CTLs was described using the trypan blue assay (Beer et al., 2008) but 5% toxicity ($p=0.042$) was observed at 1 mg/ml Feraspin-R (Figures 5.7A and B). The carboxydextran coat of Resovist is degraded in acidic lysosomal cellular compartments and this may lead to reactive oxygen species (ROS) generation by the Fenton reaction (Lunov et al., 2010). ROS cause lipid peroxidation and DNA damage (Yang et al., 2010, Soenen et al., 2010). Toxicity could also have been caused by Ferroptosis (Dixon et al., 2012).

The effect of nanoparticle uptake on CTL killing ability is poorly characterised. IFN- γ secretion ELISAs previously used for this purpose (Iida et al., 2008) do not directly measure CTL killing ability, and chromium release assays (Beer et al., 2008, Kircher et al., 2003) have a number of disadvantages, including a high spontaneous leakage rate, effect of ^{51}Cr on the effector cell population, the fact that certain cell types cannot be labelled properly, insensitivity and safety issues associated with the use of gamma radiation (caveats summarised in (Sheehy et al., 2001)).

Using the FATAL assay, CTL killing ability decreased on Feraspin-R incubation (Figure 5.11). This could potentially be explained by extracellularly bound nanoparticle complexes on the CTL cell surface blocking binding interactions between peptide presenting T2 cells and the 868-TCR, Feraspin-R located in lytic granules interfering or poisoning cytolytic enzymes, or low level Feraspin-R toxicity of CTLs, presumably with the lowest dose of Feraspin-R having the greatest impact on natural killing ability. This would be interesting to investigate in future work.

EC₅₀ values for Feraspin-R loaded (1.34×10^8 M) and unloaded (2.59×10^9 M) CTLs were established for the first time. Previously, no significant differences in CTL killing ability between loaded and unloaded CTLs were described, but this could be due to much lower concentrations of different SPIONs or less sensitive methods being employed for detection (Beer et al., 2008, Kircher et al., 2003, Iida et al., 2008).

Despite it not being necessary for the Feraspin-R-loaded CTLs to retain native killing function in this study, some retention of CTL homing might be desirable. Previous studies have shown through MRI scanning that SPION labelled cancer-specific CTLs traffic to tumours, and are differentially targeted on repeat injection (Kircher et al., 2003). Autoimmune lymphocytes loaded with SPION were also shown to locate to the beta-islet cells of the pancreas in diabetic NOD mice (Moore et al., 2002). This suggests that some CTL homing functions are retained on SPION treatment although this was not addressed in this chapter.

Increased clearance of target cells was not demonstrated using CTL-mediated thermotherapy (Figures 5.8 and 5.12). Despite this, temperatures of above 44.5 degrees for 20 minutes were enough to kill 50% of Feraspin-R loaded CTLs and CTLs were more cytotoxic in killing assays performed at 40°C (Figure 5.8C). Heat in the context of the chapter could thus be therapeutic, however despite transfection reagent treatment and uptake optimisation, insufficient Feraspin-R must have been engulfed per CTL to cause cell death, or adequate thermotherapeutic enhancement of CTL function using the MACH alternating magnetic field system. Future work involving the optimisation of uptake

conditions for clinically available SPIONs may prove useful to fully understand the role of thermotherapy on CTL function.

Since a recent publication demonstrates hyperthermia to increase HIV-1 replication, any future experiments assessing the thermotherapeutic effects of nanoparticles in targeting of HIV-1 infected cells must be performed in the presence of HAART (Roesch et al., 2012). However this study did not assess the potentially beneficial effect of thermotherapy to increase CTL killing of HIV-1 antigen presenting cells.

A recently published fluorescence-based technology for measuring intracellular temperature could be useful in evaluating how much more Feraspin-R per cell is needed to achieve the thermotherapeutic temperatures, how the heat penetrates through the cell membrane and if it is evenly distributed across the cell (Okabe et al., 2012). Recent data suggest that cell cycle stage and cell density are important for maximising nanoparticle uptake (Kim et al., 2011). Although CTLs were treated with Feraspin-R at the same cell concentrations, and CTLs were split 1:1 24 hours before treatment, the above issues were not specifically addressed, and could account for any disparity in repeat SQUID uptake results. However, it is unlikely that these factors would cause sufficient increased SPION uptake for there to be a thermotherapeutic effect and enhancement of CTL function.

Magnetic Resonance Imaging (MRI) has detected the migration of SPION-loaded melanoma-specific CTLs to tumours at a resolution of 3 cells per voxel in mice *in vivo* (Kircher et al., 2003) and trafficking of dendritic cells to melanomas in

patients (de Vries et al., 2005, Kircher et al., 2003). Anti-latency drugs such as SAHA can re-express latent HIV-1 from HAART-treated patient cells (Contreras et al., 2009, Archin et al., 2009). Assuming viral antigens are expressed after treatment, it may be possible to use SPION loaded HIV-1-specific CTLs to identify the elusive and much debated location of the latent HIV-1 viral reservoir.

In summary, HIV-1-specific cytotoxic T cells will bind to and take up SPIONs, and this does affect cell function and cell death. HIV-1-specific CTL kill more efficiently at higher temperatures, equivalent to those induced by a fever. However, in this study, SPION-associated cell death through thermotherapy was not achieved.

As SPION uptake and incubation methods are improved, SPIONs with superior uptake profiles and higher heating capabilities are developed, and the strength of the applied alternating magnetic field is increased, SPION CTL thermotherapy may have significant potential to enhance CTL function and act as an adjunctive therapy in both the imaging and targeting of latently HIV-1-infected cells in future eradication strategies.

6. Potential for 868-TCR transduced CTL to clear the latent HIV-1 reservoir.

6.1 Introduction

6.1.1 'Kick and Kill' hypothesis summary

Gag peptide and IL-2 stimulation of CTL enabled killing of reactivated latent HIV-1 (Shan et al., 2012), suggesting a modified immune response could clear reactivated latent cells. The potential role of CTL in eradication efforts is strengthened by the observation that elite controllers (ECs) harbor strong CTL responses (Porichis and Kaufmann, 2011, Theze et al., 2011) and have low HIV-1 integration levels (Graf et al., 2011).

Studies looking at Gag peptide stimulation and vaccine boosting of the T cell response as an eradication strategy are ongoing. Although the breadth of CTL responses and polyfunctionality were increased through administration of an HIV-1 DNA vaccine (coding for Env [Clade A, B and C], Pol, Nef and Gag [Clade B] followed by an replication deficient adenoviral boost containing all antigens except Nef) the frequency of latently infected cells did not change (Casazza et al., 2013). The therapeutic HIV-1 RNA vaccine AGS-004 improved the HIV-1-specific immune response and reduced the viral load set point with 25% of patients exhibiting decreased integrated HIV-1 DNA levels (Jenabian et al., 2013). Also, increased killing of HIV-1 RNA expressing cells that were not cleared by HAART in the bone marrow, liver and thymus (BLT) mouse model was observed by co-treatment with

immunotoxin 3b3-PE38 (Denton et al., 2014), which targets the conserved CD4 binding site on gp120.

Harnessing the CTL response to clear the reservoir is directly tied to which infected cells can be targeted. This target range may be larger than previously estimated, as a population of latent 'GPR' cells produced p24 in a primary latency model (Pace, Graf et al. 2012) and were detected in low numbers *in vivo* in HAART-suppressed patients (Graf et al., 2013). Also GPR cells were targeted by autologous CTL from ECs (Graf et al., 2013). This supports ECs having lower reservoir levels, as their CTLs may more effectively clear a portion of the reservoir (Graf et al., 2011). Together, this evidence suggests effective CTL can clear latent cells after viral reactivation and a portion of the reservoir without reactivation.

As a proof of principle of an effective immune response clearing latent cells, 868-TCR transduced CTL were employed. This TCR was used to target HIV-1-infected cells *ex vivo* (Varela-Rohena et al., 2008) and *in vivo* in a clinical trial NCT00991224 assessing safety and tolerability of 868-TCR transduced CTLs infused into HLA-A2 positive patients with HIV-1 (ClinicalTrials.gov, 2014).

However, it remains unknown if, similarly to EC CTL, high affinity TCR transduced CTL can also target the latent HIV-1 reservoir. If effective, CTL transduced with high affinity TCRs against conserved viral epitopes could be infused into patients after anti-latency agent treatment to enhance the killing of antigenic latently infected cells, thus clearing the reservoir in the 'kick and kill' approach.

6.1.2 Models of HIV-1 latency

6.1.2.1 Cell lines

U1 cells are chronically infected promonocyte cell line U937 clones, containing two non-replicating copies of proviral HIV-1 DNA (Folks et al., 1987). Suboptimal Tat protein activity ensures a latent phenotype (Cannon et al., 1994, Emiliani et al., 1998). ACH2 cells are chronically HIV-1-infected T cell clones and contain one copy of proviral HIV-1 DNA (Folks et al., 1989, Clouse et al., 1989). A mutation in the Tat binding site on the Trans Activating Region (TAR) (Emiliani et al., 1996) ensures viral latency. J-Lat cell clones (6.3 and 8.4) are derived from Jurkat cells and contain one integrated copy of HIV-1 per cell. Nef is replaced with a Green Fluorescent Protein (GFP) gene (Jordan et al., 2003). Latency is ensured through integration of HIV-1 close to heterochromatic regions (Jordan et al., 2001).

6.1.2.2 Primary cell models

Since primary CD4⁺ T cells are the major site of the HIV-1 reservoir *in vivo*, these cells more likely represent a true model of latency. The primary model chosen for this chapter involves the direct infection of all resting CD4⁺ T cell subsets (Dai et al., 2009), by spinoculation. This technique involves centrifugation of cells with wild-type (WT) NL4-3 virus, to increase viral binding, infection and integration (O'Doherty et al., 2000, Agosto et al., 2007, Swiggard et al., 2005). The inefficiency of resting cell infection is overcome through the use of high viral titers. However, cell numbers are limiting due to the lack of an upfront pre-activation step. The ability to generate latent infection in numerous cell types quickly makes this assay

very desirable. Also, because WT virus is used, this is a more relevant model for studying latent cell antigenicity and CTL targeting of latent cells.

6.2 Aims and objectives

6.2.1 Aims

I aim to investigate, for the first time, to what extent high affinity TCR transduced CTL can target latently infected cells pre-and post cell stimulation.

6.2.2 Objectives

1. Reactivation of latent HIV-1 will be studied in U1, ACH2 and J-Lat 6.3 and 8.4 cells using a panel of PKC and HDACi anti-latency agents.
2. To investigate 868-TCR transduced CTL targeting of these cell lines pre and post latent cell stimulation, they must be transfected with HLA-A2.
3. A primary model of latency will be verified and reactivation of latent HIV-1 will be investigated.
4. The ability of 868-TCR transduced CTL to clear these cells pre and post stimulation will be assayed.

6.2.3 Hypothesis

868-TCR transduced CTLs will be able to target the latent reservoir and will target and clear latent cells treated with anti-latency agents more efficiently.

6.3 Results

HIV-1 reactivation assays were first optimized in U1, ACH2, J-Lat6.3 and J-Lat8.4 chronically infected cell lines (2.4.1). Many prior studies measure reactivation of HIV-1 from latency in these cell lines by quantifying HIV-1 viral p24 by ELISA. This method measures viral release, which represents the most conclusive proof for reactivation of HIV-1 from latency. However, the aim of this study was to investigate antigenicity of latent cells. Quantification of intracellular p24 was deemed a suitable surrogate marker for measuring reactivation, since antigen presentation does not theoretically depend on viral release.

6.3.1 Latent HIV-1 is reactivated in U1 and ACH2 cells

First, in order for latent cells to become targets for 868-TCR CTL, they must present SLYNTVATL peptide on MHC class I receptors expressed on the cell surface. Latent virus needed to be stimulated optimally for there to be the highest chance of CTL targeting. Inducible p24 expression in U1 and ACH2 cells was analysed using PMA (Kalebic et al., 1991) and TNF α (Duh et al., 1989) at previously recommended concentrations, for 48 hours (Kulkosky et al., 2001). Reactivation of latent HIV-1 occurred as the percentage of cells expressing p24 increased (Figure 6.1A) (2.4.3). However, a majority of ACH2 cells were p24 positive without stimulation (Figure 6.1B), which was surprising as only 2% were reported as being HIV-1 RNA positive on first isolation (Folks et al., 1989).

GXR-CEM cells (a T lymphoblastic cell line containing GFP, driven by a Tat dependant promoter) were spinoculated with supernatant from ACH2 cells that were either treated with anti-latency drugs or left untreated. Productive infection, measured by GFP expression, was highest from supernatants of ACH2 cells treated with anti-latency drugs, although virus was also released from unstimulated cells (Figure 6.1C). Thus, reactivation of HIV-1 in U1 was primarily investigated, as these cells produced very little if any background p24 expression.

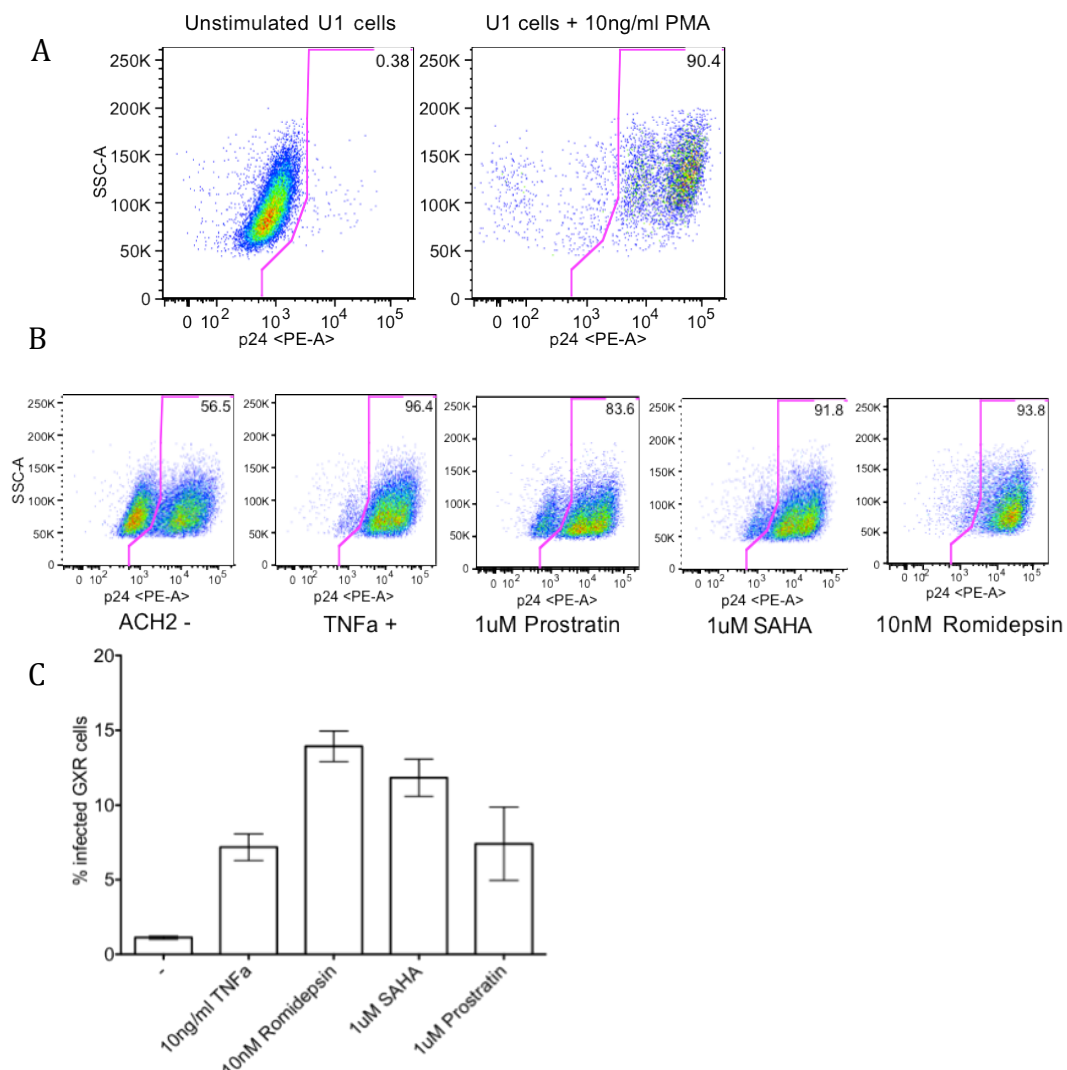


Figure 6.1. Latent HIV-1 reactivation in U1 and ACH2 cells. A) U1 cells were incubated for 48 hours with PMA (right) or were left unstimulated (left). A representative experiment is shown. B) ACH2 cells were incubated for 48 hours with TNF α , Prostratin, SAHA or Romidepsin or were left unstimulated. p24 positive cells were determined by FACS. C) GXR-CEM cells were spinoculated with ACH2 culture supernatants post-48-hour anti-latency drug treatment in three independent experiments. GFP expression was determined 48 hours post spinoculation.

p24 expression could also be induced in U1 cells by the Protein Kinase C (PKC) agonist Prostratin (Figure 6.2), to a level previously observed (Kulkosky et al., 2001). The PMA and Prostratin concentrations (2.4.2) used in this study overlap those used in a previous study (Shan et al., 2013). Also, the HDACi Valproic Acid, SAHA, Panobinostat and Romidepsin reactivated latent HIV-1 in U1 cells (Figure 6.2) although to a lesser extent than the PKC agonists, Prostratin and PMA. The effect of Romidepsin on latent HIV-1 expression in U1 cells has not been reported before. Romidepsin was 1000x more potent than SAHA at reactivating latent HIV-1 in U1 cells (Figure 6.2), which matches previous observations but in a primary cell model of latency (Geleziunas, 2012).

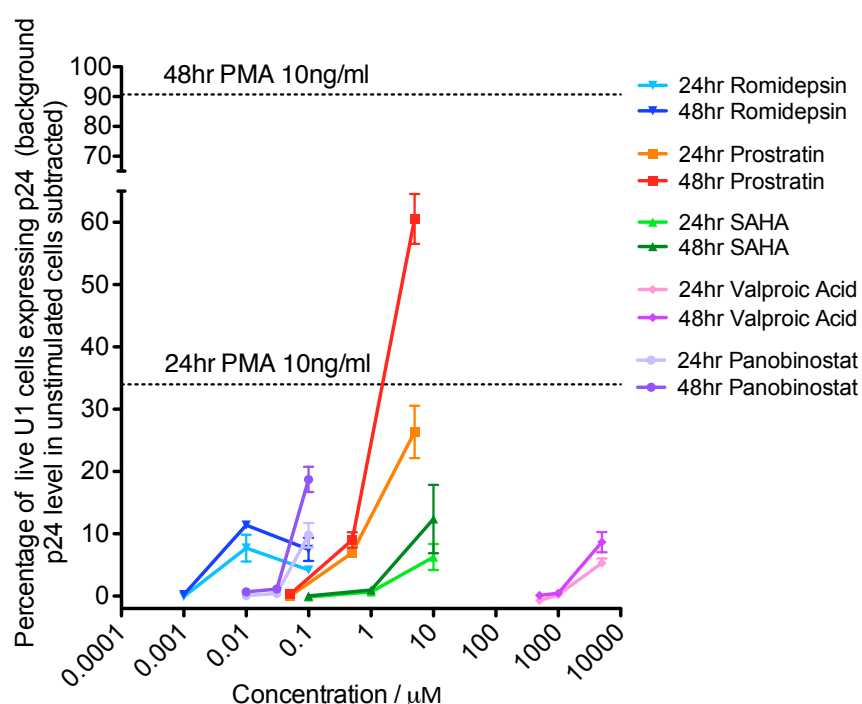


Figure 6.2 Optimal reactivation of HIV-1 in U1 cells. U1 cells were cultured with varying doses of anti-latency drugs for 24 or 48 hours. Intracellular p24 was assayed by FACS in live cells. Background p24 from unstimulated cells was subtracted. The experiment was repeated three times and a further three times (n=6) at the highest (optimal) p24 expression for each drug treatment.

The concentrations of HDACi that induced maximal reactivation matched those determined in other studies, with Valproic Acid being the least potent anti-latency agent, followed by SAHA, then Panobinostat (Rasmussen et al., 2013) (Figure 6.2). A higher expression of latent virus was observed after 48 hours than 24 hours of optimal drug incubation concentration (Figure 6.2) ($p < 0.001$ by 2-Way ANOVA for each optimal drug concentration across the two time points), an observation that matches a previous study using U1 cells and Prostratin (Kulkosky et al., 2001).

6.3.2 Latent HIV-1 is reactivated in J-Lat 6.3 and 8.4 cells

Latent HIV-1 reactivation was investigated in multiple cell line models of latency to more fully understand the targeting capability of 868-TCR transduced CTL. Prostratin reactivated more cells than any other agent tested in J-Lat 6.3 and 8.4 cells (Figure 6.3A). This is in agreement with a recent study where Prostratin was one of the best re-activators of latent HIV-1 in the same cell lines (Spina et al., 2013). Less p24 reactivation occurred, compared to U1 cells, potentially because of the additional copy of HIV-1 present in U1 cells. Maximal intracellular p24 expression was observed after 24 not 48 hours for J-Lat 6.3 cells (Figure 6.3A; $p < 0.001$ at optimal drug reactivation concentration by 2-Way ANOVA between the two time points). A reactivation time of 24 hours was chosen in previous studies employing J-Lat cells (Reuse et al., 2009, Spina et al., 2013). Also, J-Lat 6.3 cells were easier to reactivate than J-Lat 8.4 cells (Figure 6.3A and B) ($p < 0.001$ over each optimal drug concentration by 2-Way ANOVA between cell types), also supporting a recent study (Spina et al., 2013).

A benefit of using J-Lat cells is GFP expression on cell stimulation can be a surrogate marker for viral reactivation (Figure 6.3C). However, despite there being no significant difference in the amount of reactivation by the two markers by 2-way ANOVA, GFP expression does not necessarily provide proof of p24 expression, so both markers were assayed by FACS for subsequent assays.

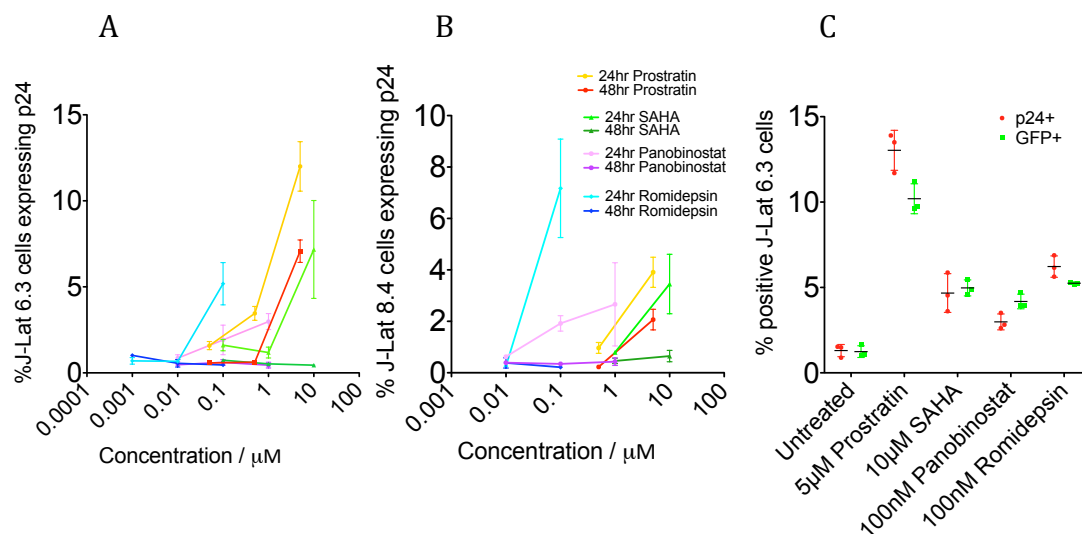


Figure 6.3 Reactivation of HIV-1 in J-Lat 6.3 and J-Lat 8.4 cells. A) J-Lat 6.3 or B) J-Lat 8.4 cells were incubated with varying doses of anti-latency drug for 24 (D1; Day 1) or 48 (D2; Day 2) hours. The percentage of J-Lat cells expressing p24 was assayed by intracellular FACS staining. Background p24 expression from unstimulated cells was subtracted C) Post-reactivation, J-Lat 6.3 cells were stained for both intracellular p24 and GFP (n=3).

6.3.3 Latent cell lines were transduced with HLA-A2

So far, intracellular p24 expression has been induced by a panel of anti-latency agents at recommended concentrations in chronically infected cell lines. However, the aim of this chapter is to determine whether TCR-transduced CTL can target latently infected cells. The CTLs used in this study were transduced with a high

affinity SLYNTVATL restricted 868-TCR at Adaptimmune Ltd. SLYNTVATL is an HLA-A2 restricted peptide. Therefore latent cell lines were transduced with a HLA-A2 and B2M retroviral construct (2.4.4), although the latter gene was superfluous and part of the same viral construct. This is the first time chronically infected cell lines have been manipulated this way for this purpose, to my knowledge.

U1 and ACH2 cells were tested for natural HLA-A2 expression. A T-lymphoblastic cell line (T2) that naturally expresses HLA-A2 acted as positive control. Despite all cells expressing MHC class I molecules, U1 and ACH2 cells expressed very limited, if any HLA-A2 (Figure 6.4).

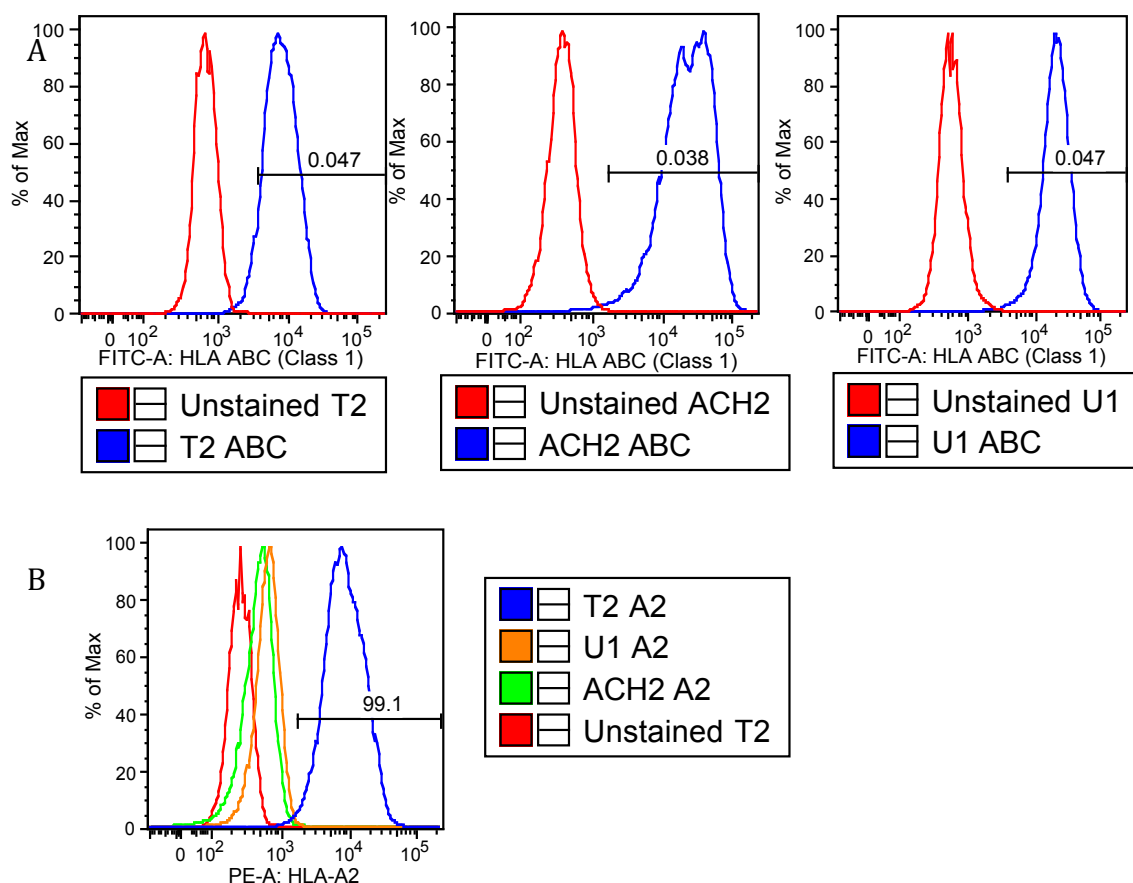


Figure 6.4 Native HLA-A2 expression on ACH2 and U1 cells. A) T2, ACH2 and U1 cells were stained with a non-specific HLA class 1 antibody that stained HLA A, B and C (ABC). Positive populations were gated using unstained cells. B) T2, U1 and ACH2 cells were stained with an HLA-A2 antibody (A2).

HLA-A2 transduction efficiencies varied. 95.5% of ACH2, 99.2% of J-Lat 6.3 and 99.4% of J-Lat 8.4 cells were successfully transduced and expressed HLA-A2 after 3 days of culture (Figure 6.5A). However, only 65.3% of U1 cells were transduced effectively (Figure 6.5A). This may perhaps be due to monocytic U1 cells potentially having different transduction and infection efficiencies compared to the T lymphoblast derived cell lines that were used. Once cells were transduced, HLA-A2 expression was monitored and found to be stable, with no decrease in the number of positive cells detected in U1 cells over at least a month (Figure 6.5B).

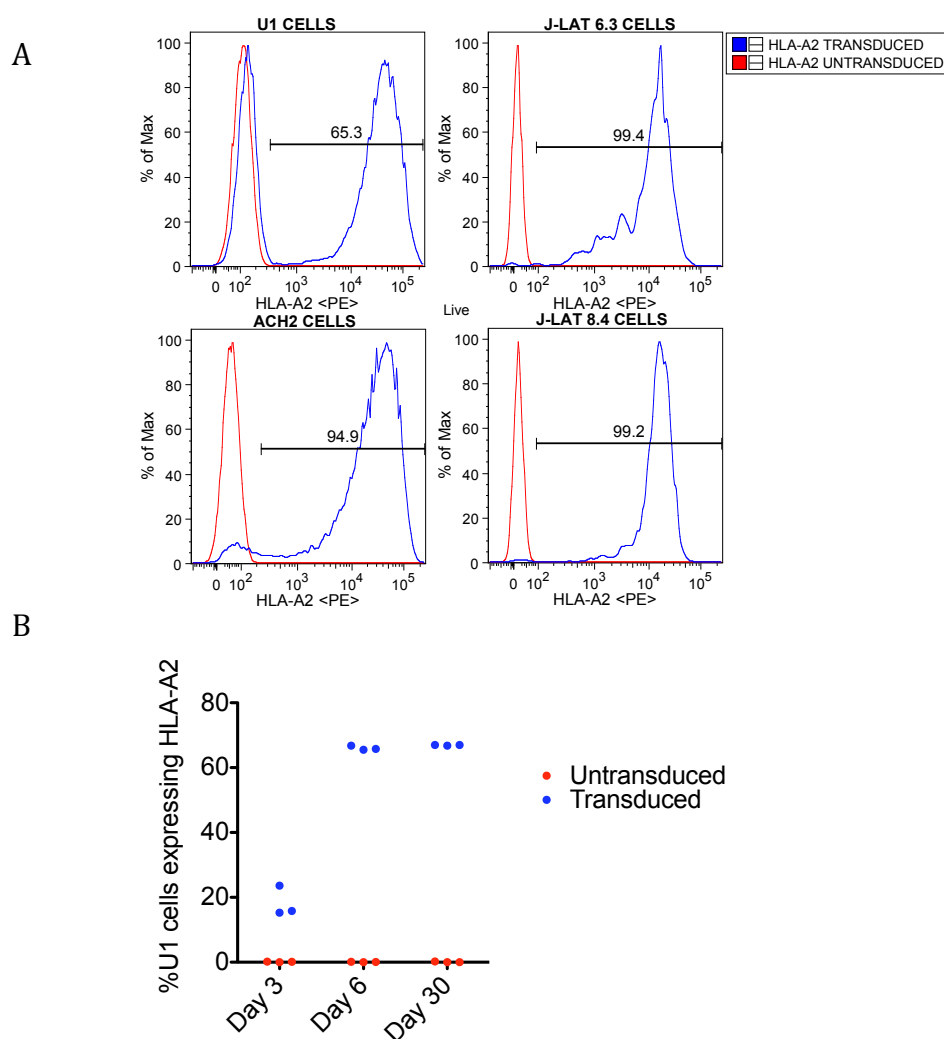


Figure 6.5 HLA-A2 transduction of chronically infected cell lines. A) U1 (top left), ACH2 (bottom left), J-Lat 6.3 (top right) and J-Lat 8.4 (bottom right) cells were infected with a lentiviral HLA-A2 construct. HLA-A2 staining was performed 6 days post infection. B) The percentage of U1 cells expressing HLA-A2 was monitored over time (3, 6 and 30 days), post transfection.

Since the latent cell lines had been manipulated, the impact of HLA-A2 transduction on reactivation of latent HIV-1 was investigated. Drug concentrations optimized for the highest expression level of p24 were re-tested after 1 and 2 days of treatment for U1 cells, and 1 day of treatment for J-Lat 6.3 and J-Lat 8.4 cells (Figure 6.6). There was no significant difference observed in p24 re-expression level between HLA-A2 transduced and non-transduced cells for either cell type (2-Way ANOVA).

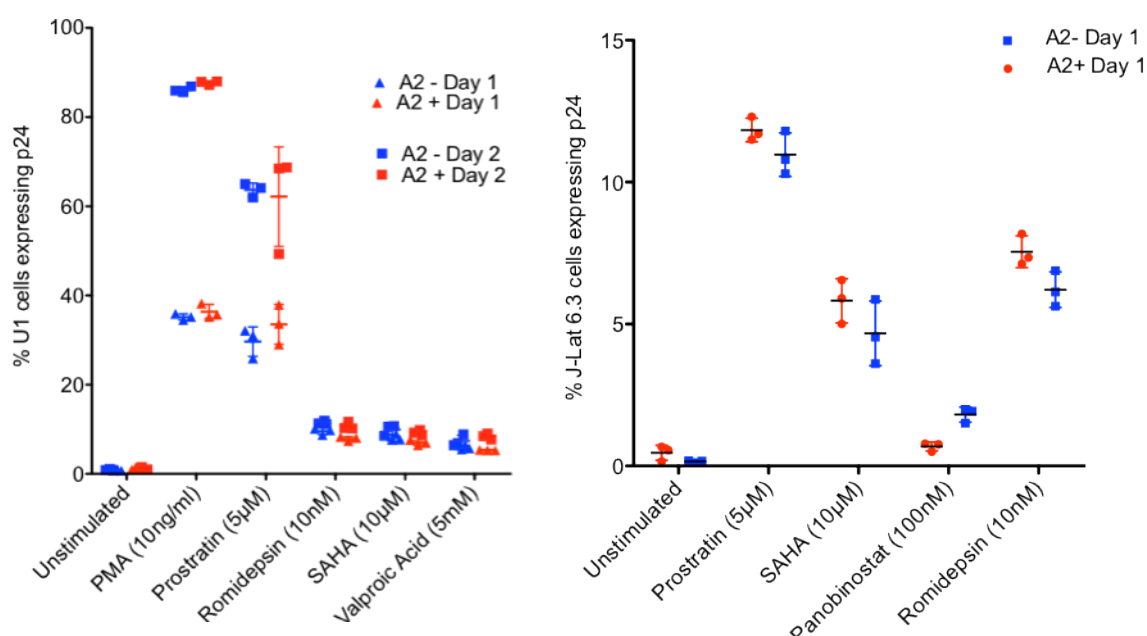


Figure 6.6. Latent HIV-1 reactivation in HLA-A2 transduced and untransduced cells. The percentage of HLA-A2 transduced and untransduced U1 (left) and J-Lat 6.3 (right) cells re-expressing p24 after 24 (Day 1) or 48 (Day 2) hours of drug stimulation.

6.3.4 868-TCR transduced CTL target latent cell lines

Since reactivation of latent virus in HLA-A2-expressing cell line models of latency was achieved, the next step was to observe if 868-TCR transduced CTL (2.3.1.2), could be used to target the latent cells. A variant FATAL killing assay was used to assess SLYNTVATL peptide specific killing of U1 and J-Lat 6.3 cells (2.8.5, Figure 2.7). Target cells (U1 cells transduced with HLA-A2) were stained with Carboxyfluorescein succinimidyl ester (CFSE). Non-target cells (untransduced U1 cells) were stained with Cell Tracker Orange (CTO) or Cell Tracker Violet (CTV). Both target and non-target cells were mixed in a 1:1 ratio. CTLs transduced with the 868-TCR were then added in a 1:1 Effector:Target (E:T) ratio. The percentage of CTL cytotoxicity was calculated as the change in the percentage of target cells incubated with CTL, compared to when no CTL were added.

The same method was performed using J-Lat 6.3 cells rather than J-Lat 8.4 cells, since more p24 reactivation was observed as thus these cells might be better targets for CTL (Figure 6.3A). Since the HLA-A2 transduction efficiency of U1 cells was 66%, any percentage cytotoxicity data was normalized to 100%. Unstimulated U1 and J-Lat 6.3 cells were targeted and killed by CTLs at a 1:1 ratio in co-culture (Figure 6.7A). This was unexpected, as <1% of unstimulated U1 and J-Lat 6.3 cells expressed p24 by FACS analysis. However, it is possible that HIV-1 RNA was transcribed, although this was not specifically looked at. Interestingly, there was no detectable difference in observed killing when U1 or J-Lat 6.3 cells were pulsed with SLYNTVATL. This suggests that the killing seen was optimal (Figure 6.7A).

There was significantly more killing of J-Lat 6.3 than U1 cells ($p = 0.0006$), perhaps because T cells might be more easily cleared.

To confirm whether the observed killing was T cell receptor-specific, J-Lat 6.3 cells were incubated with 868-TCR transduced CTLs and un-transduced CTLs from the same donor (Figure 6.7B). There was significantly lower ($p = 0.0003$) non-specific than specific killing. The small amount of non-specific killing thus could not entirely explain the killing observed using TCR transduced CTLs.

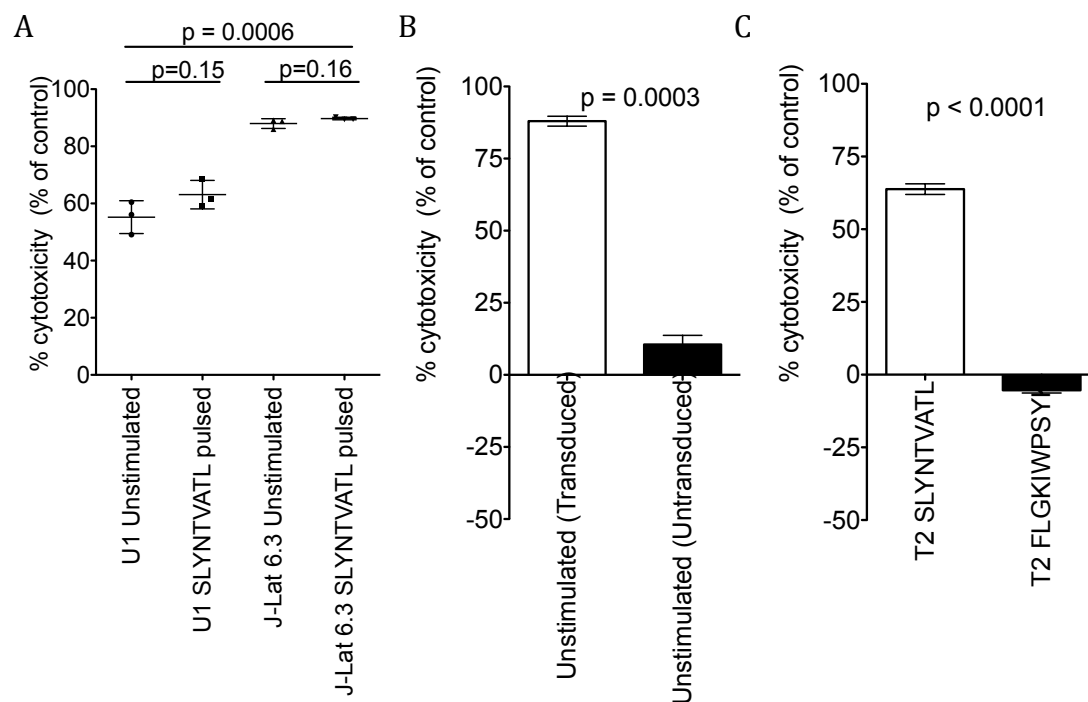


Figure 6.7 868-TCR CTL targeting of unstimulated HLA-A2-transfected U1 and J-Lat 6.3 cells. A) U1 and J-Lat 6.3 cells were incubated with 868-TCR transduced CTLs from 1 donor at 0.6:1 and 1:1 E:T ratios, respectively. U1 and J-Lat 6.3 cells were left unstimulated or were peptide pulsed with 10^{-7} M SLYNTVATL. B) J-Lat 6.3 cells were incubated with 868-TCR-transduced or untransduced CTL from the same donor at a 1:1 E:T ratio. C) T2 cells, which naturally express HLA-A2, were pulsed with a target or non-target HLA-A2-restricted peptide and were incubated with 868-TCR transduced CTL from the same donor at a 0.6:1 E:T ratio. Three independent killing assays were performed per donor. Three killing assays were run for each condition and assays were run overnight. Significance was determined by Student's T test.

T2 cells, a T lymphoblastic cell line that is not latently infected with HIV-1 was pulsed with the target HIV-1 gag peptide, SLYNTVATL, or an HLA-A2 restricted but non-target HIV-1 gag peptide FLGKIWPSYK. Due to low CTL numbers from the same donor, this experiment was performed at a 0.6:1 E:T ratio. Significantly less killing was observed when CTLs specific for SLYNTVATL were incubated with FLGKIWPSYK-pulsed T2 cells (Figure 6.7C) ($p < 0.0001$). Any killing observed was thus specific.

Next, the impact of p24 induction on latent cell line antigenicity was investigated. Target HLA-A2 expressing cells were treated with optimal tested concentrations of anti-latency agents for optimal tested incubation time (Figure 6.2 and 6.3A). Non-target and target cells were treated under identical reactivation conditions. Despite p24 expression increasing on drug treatment, reactivated cells were significantly less antigenic than untreated U1 or J-Lat 6.3 cells (Figure 6.8). For U1 cell killing assays, three experiments were performed using CTL isolated from one donor. For killing assays with J-Lat 6.3 cells as targets, CTL were transduced with the 868-TCR from three donors, and three killing assays were performed per donor. This accounts for donor variability in the system and helps to explain the larger error bars in Figure 6.8B.

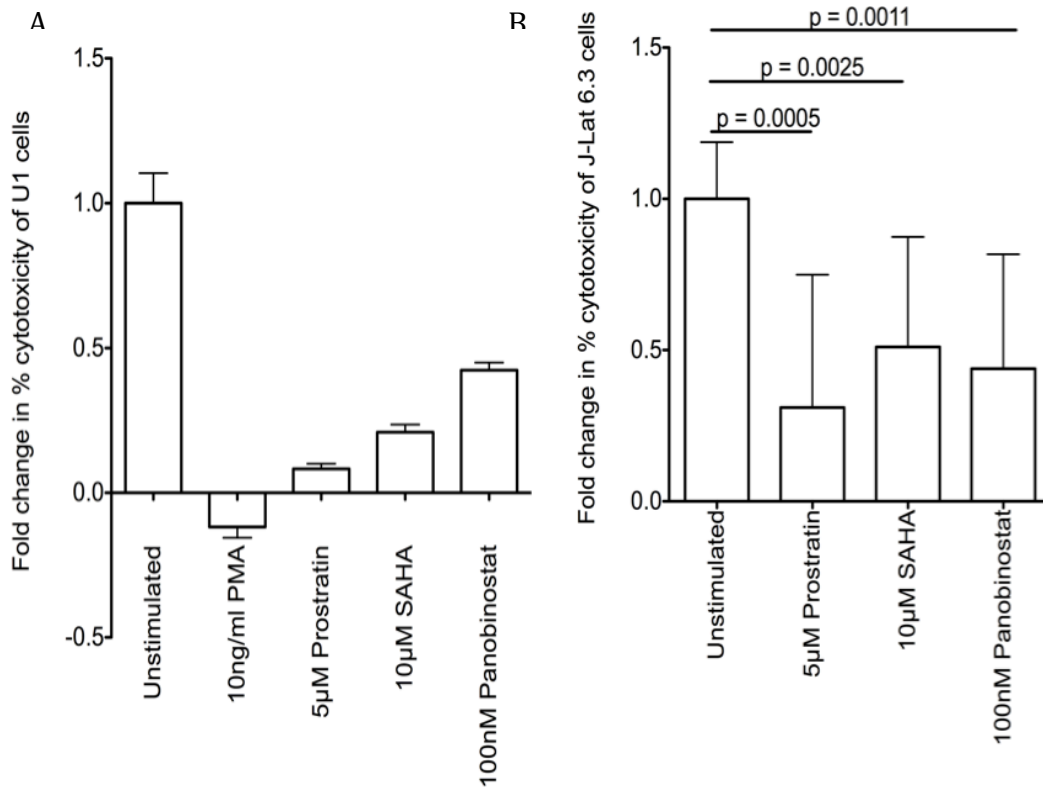


Figure 6.8 868-TCR CTL targeting of stimulated HLA-A2-transduced U1 and J-Lat 6.3 cells. A) U1 and B) J-Lat 6.3 cells were treated with anti-latency drugs under optimal tested reactivation conditions, or were left unstimulated. Three killing assays using U1 cells were performed using 868-TCR transduced CTL isolated from one donor. Three killing assays using J-Lat 6.3 cells were performed with 868-TCR transduced CTL from three donors. To limit bystander killing, effector and target cells were co-cultured for 5 hours. The fold change in percentage cytotoxicity of unstimulated cells (as a percentage of control cells where no CTL were added) was calculated. Significance was determined by Student's T test.

6.3.5 Anti-latency drugs modulate MHC class I expression

The reason why cells expressing increased p24 were less antigenic than unstimulated cells was investigated. It was hypothesized that the anti-latency drugs might cause HLA-A2 downregulation, which in turn leads to fewer or less antigenic 'targets' for the CTL to bind to and kill.

HLA-A2-transduced and non-transduced U1 cells were treated with 'optimal' tested concentrations of anti-latency drugs for 48 hours. Subsequent Median

Fluorescence Intensity of HLA-A2 (on transduced cells) or native HLA Class-1 expression (on un-transduced cells) was recorded by FACS staining (Figure 6.9). Data is expressed as a percentage of HLA-A2 expression on untreated cells.

Both cell lines behaved differently in terms of HLA expression on anti-latency drug treatment. PMA and Prostratin abolished transduced HLA-A2 expression, while up-regulating natively expressed MHC class I molecules in U1 cells (Figure 6.9). In J-Lat 6.3 cells, Prostratin also increased expression of native MHC class I molecules, but did not down-regulate transduced HLA-A2 expression (Figure 6.10). HDACi decreased expression of both transduced and native MHC class I molecules in both cell lines. Unlike in U1 cells, native MHC class I molecule expression was upregulated in J-Lat 6.3 cells with HDACi (Figure 6.10). The results suggest that the anti-latency drugs impacted transduced and native MHC class I expression differently. Thus the reduction in antigenicity of anti-latency drug-treated cells could be artefactual.

To assess this possibility, HLA-A2 expression was recorded in CD4⁺ T-cells post-optimal drug treatment (Figure 6.11). Prostratin increased HLA-A2 MFI in all three donors tested. However, the large range in MFI increase meant the increase was not significant. However, HLA-A2 MFI decreased significantly on Panobinostat ($p=0.042$) and Romidepsin ($p=0.034$) treatment and the decrease was almost significant on SAHA treatment ($p=0.053$), all by paired Student's *t* test. This observation matches unpublished data from Jonathan Karn's laboratory. The HLA-A2 downregulation was not as extreme as seen for U1 or J-Lat 6.3 cells.

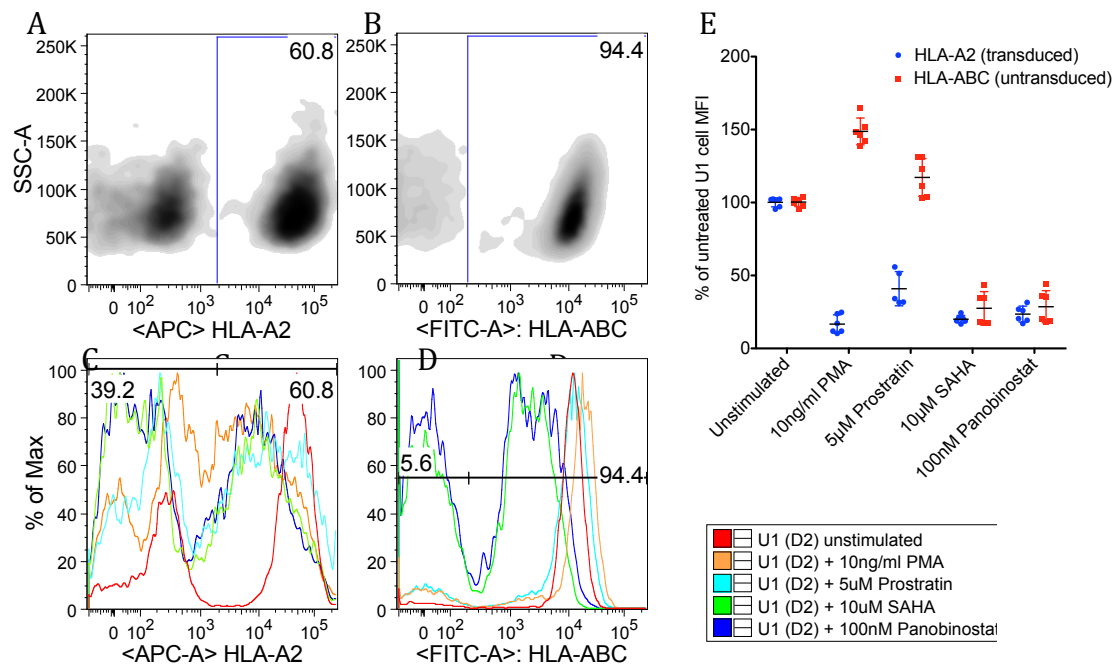


Figure 6.9 Transduced HLA-A2 and native MHC class I expression on U1 cells
 Gating strategies for A) HLA-A2 transfected and B) native MHC-Class 1 expression. C) Transfected and D) Untransfected U1 cells were incubated under previously determined optimal tested conditions for p24 induction (D2 = 48 hours). HLA-A2 or MHC class I was measured by FACS. Gates were defined using unstimulated cells. E) MFI was calculated for each drug concentration and was expressed as a percentage of expression on untreated cells. The experiments were repeated 6 times.

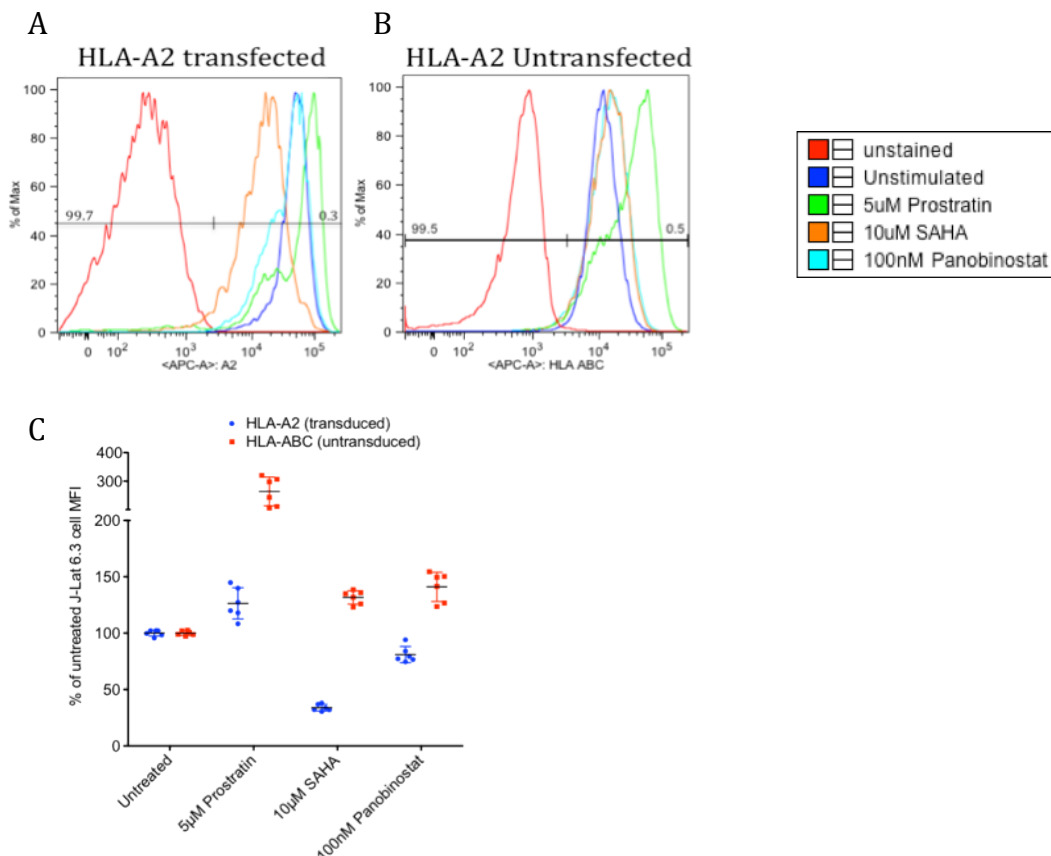


Figure 6.10 Transduced HLA-A2 and native HLA Class 1 expression on J-Lat 6.3 cells. J-Lat 6.3 cells were incubated under previously determined optimal tested conditions for p24 induction. A) Transduced HLA-A2 or B) native MHC class I expression was measured by FACS. C) The MFI was calculated for each drug concentration and was expressed as a percentage of untreated cell MHC class I expression. The experiments were repeated 6 times. Cells were gated using unstained controls.

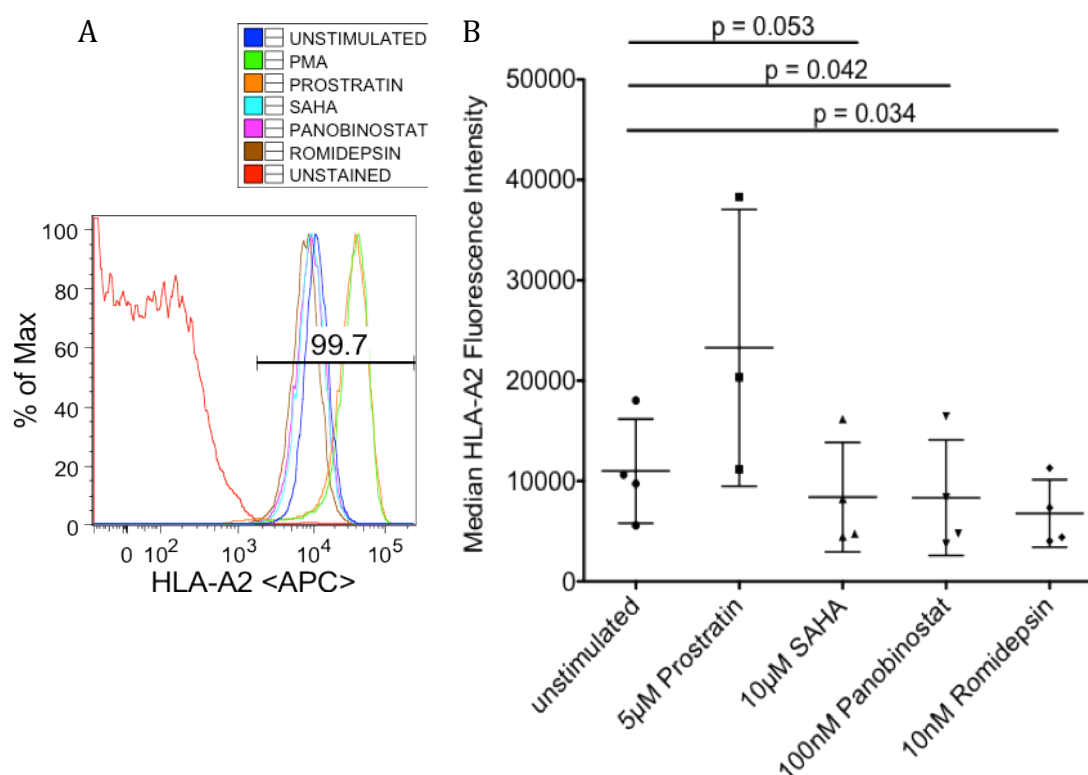


Figure 6.11 MHC class I expression on CD4+ T cells. CD4+ T cells were incubated with a range of concentrations of anti-latency drugs for 24 hours. A) Native MHC class I expression was measured by FACS. B) Single experiments were performed using the MFI of MHC class I allele expression from 3 donors (1 donor replicate performed by Matthew Pace) for Prostratin treatment and 4 donors (2 donor replicates performed by Matthew Pace) for HDACi treatment. Cells were gated using unstained controls. Significance was determined by paired T test.

6.3.6 Development and verification of a primary cell model to investigate targeting of latently infected cells.

So far in this chapter, all observations made for CTL targeting of latently infected cells were performed using latently infected cell lines. Although these models are useful for proof of principle experiments, primary cell models are thought to be a better representation of latency *in vivo*. Also, cells can be isolated from donors that express native HLA-A2 without the added complication of lentiviral transduction.

The assay chosen for development (O'Doherty et al., 2000, Pace et al., 2012, Swiggard et al., 2005, Graf et al., 2013) involves spinoculating resting HLA-A2 expressing CD4⁺ T cells with wild-type NL4-3 virus, culturing for 3 days in the presence of a protease inhibitor to prevent spreading infection, and then staining cells using a p24 antibody to detect Gag Positive Reservoir (GPR) cells. High yields of latent cells can be produced in a few days, rather than weeks or months as in other models. Also, a cell sorter was not needed for this method. This model generates latently infected cells that constitutively transcribe gag and those where anti-latency agents can induce viral release and spreading infection.

NL4-3 plasmid was grown (2.5.1), infectious virus was manufactured in culture (2.5.2) and viral stocks were titered for spinoculation (2.5.3). The spinoculation method increased infection efficiency of resting CD4⁺ T cells (Figure 6.12A). Most input CD4⁺ T cells were CD25 and HLA-DR negative, indicating a resting phenotype (Figure 6.12B). All summarised in section 2.6.

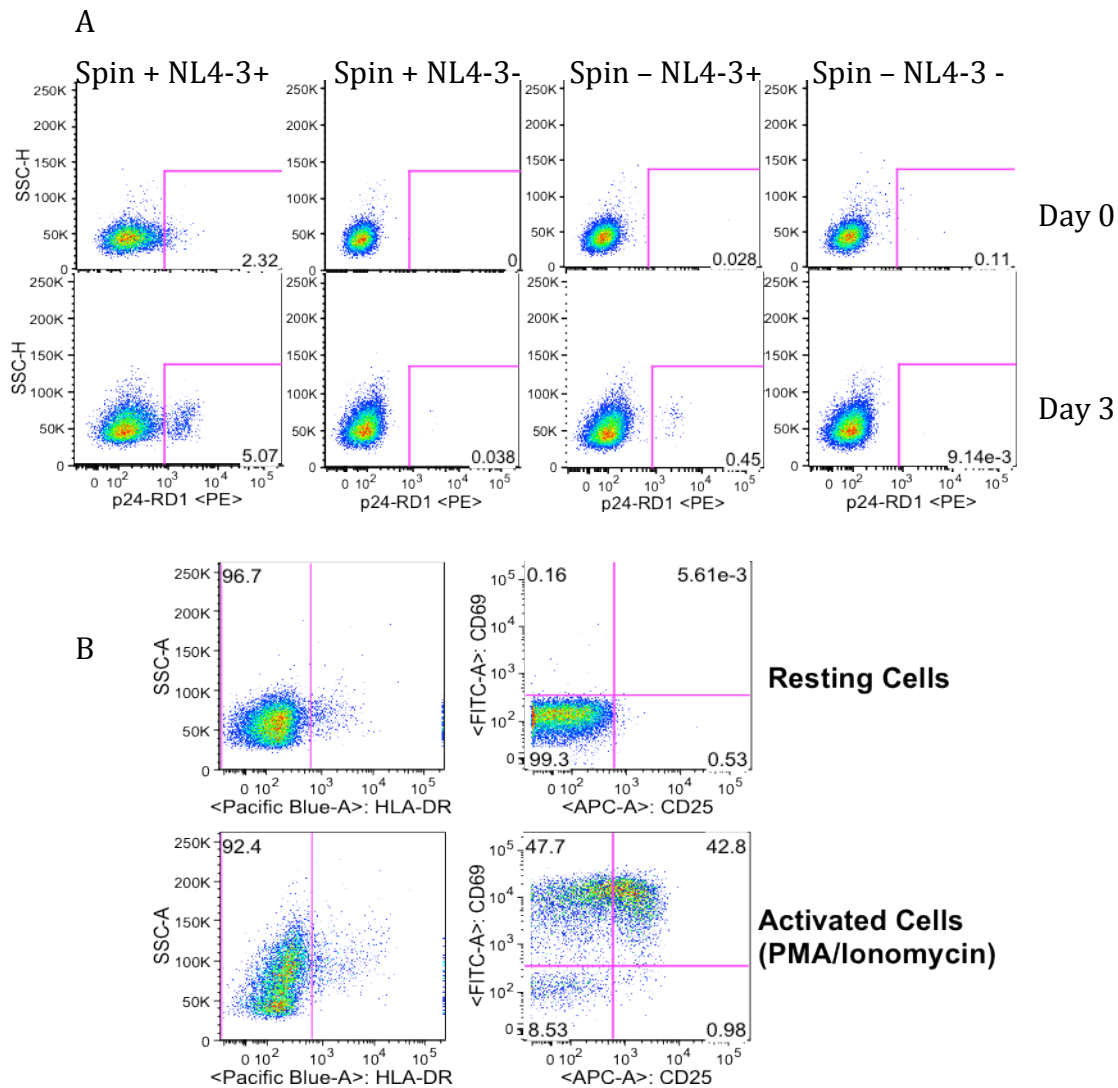


Figure 6.12 Spinoculation of resting CD4+ T cells. A) CD4+ T cells were spinoculated for 2 hours in the presence of media or NL4-3 virus (MOI of 1.5), or were incubated with NL4-3 or media for 2 hours. Cells were washed three times in R10, and were cultured in R20 for 3 days. Intracellular p24 was assayed by FACS. B) Before spinoculation, CD4+ T cells were stained with HLA-DR (left) and CD25 (right) antibodies. Gates were determined by expression level on CD4+ T cells, pre-activated with PMA and Ionomycin.

Spinoculating with an NL4-3 MOI of 3 increased the yield of GPR cells to 14%, which matched the levels seen in the original study (Pace et al., 2012) (Figure 6.13A). GPR cell formation was inhibited by the addition of the integrase inhibitor Raltegravir immediately post spinoculation (Figure 6.13A). The p24+ cells

therefore contained an integrated provirus. Also, the majority of all cells, regardless of whether they were expressing p24 or not were still of a resting phenotype (Pace et al., 2012) (Figure 6.13B).

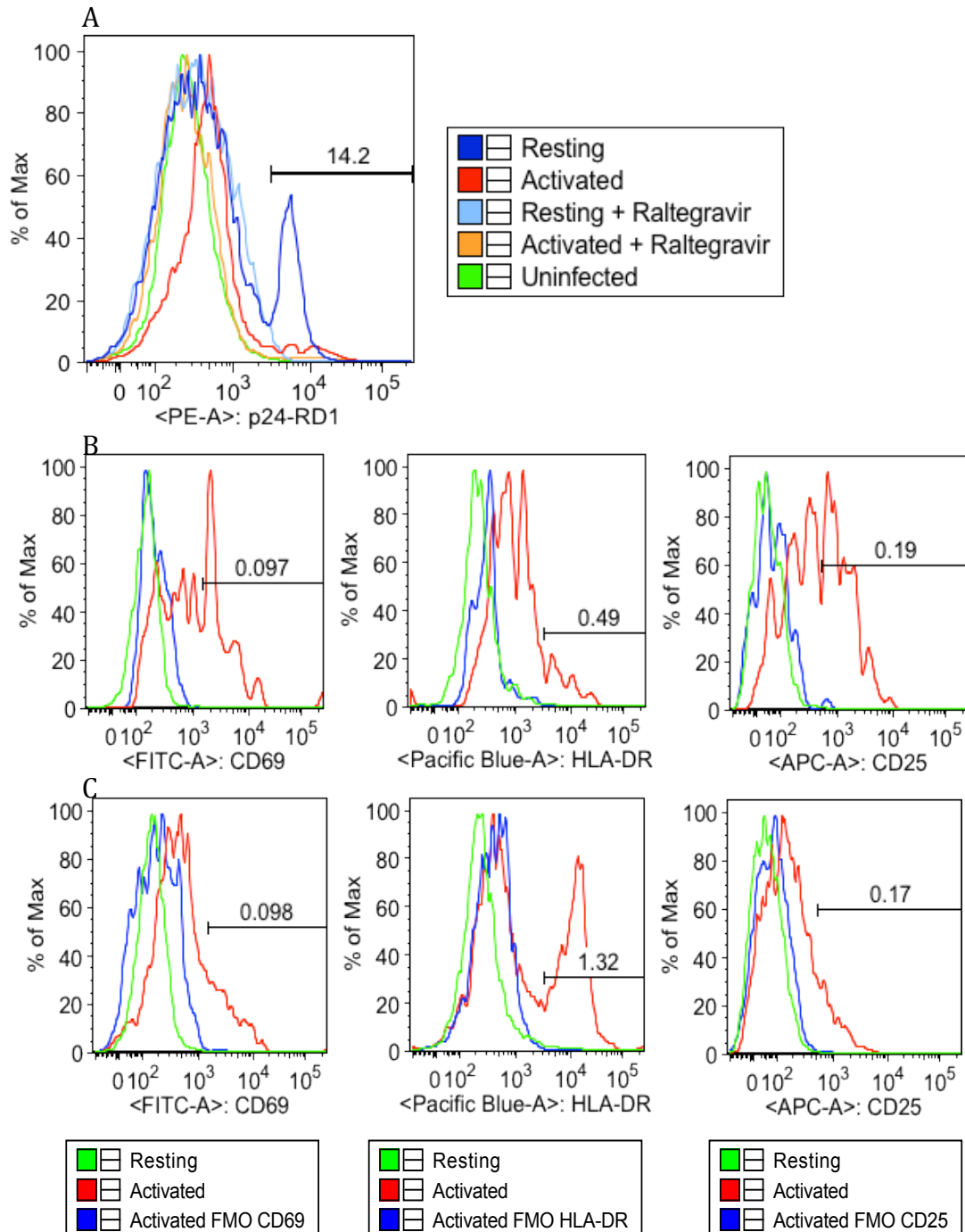


Figure 6.13 Gag positive reservoir cells. A) Intracellular p24 staining of activated or resting CD4⁺ T cells cultured for 3 days post spinoculation with NL4-3 (MOI of 3), in the presence and absence of Raltegravir. B) Only p24⁺ CD4⁺ T cells or C) all CD4⁺ T cells, were stained with anti CD69, HLA-DR or CD25 antibodies. Activation markers expressed in all cells or just p24⁺ cells are plotted. Gates defined by FMO and percentages are marker expression on resting cells.

To confirm that p24+ cells were latently infected, resting and pre-activated (PMA/IO) (2.6.1) CD4+ T cells were spinoculated with NL4-3 and cultured for 3-days in the presence of Raltegravir to stop integration or Saquinavir to prevent viral spread, or no ART. The culture supernatants were spinoculated with GXR-CEM cells. An increase in GXR-CEM GFP production was observed using viral supernatants from activated cells but not resting cells, even in the absence of drug treatment (Figure 6.14). This pilot observation confirms the original study (Pace et al., 2012) and that ART drugs were efficacious at the concentrations used.

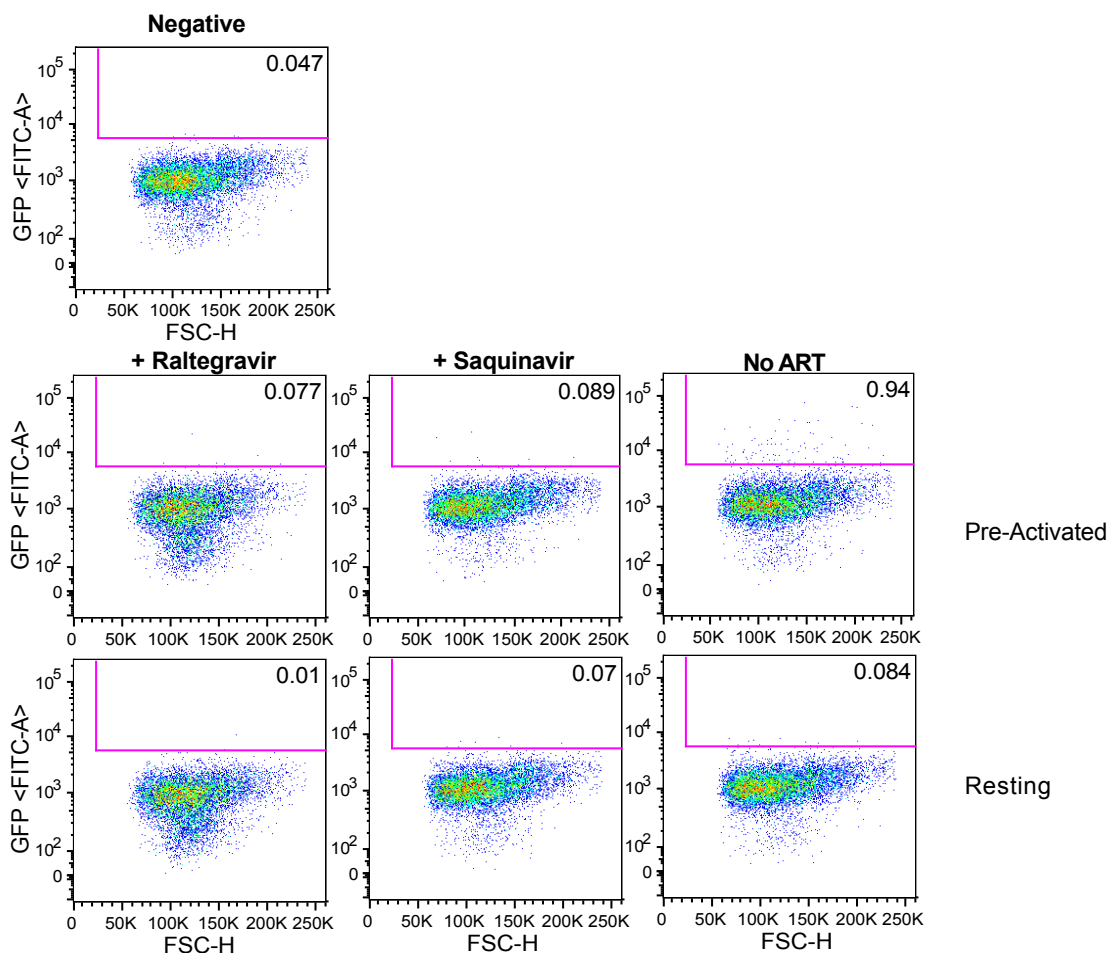


Figure 6.14 p24+ Gag Positive Reservoir cells are latently infected. GXR cells were spinoculated with viral culture supernatants taken from resting and activated CD4+ T cells, 3 days post spinoculation in the presence or absence of ART. GXR cells were subsequently cultured for 3 days and GFP expression was analysed by FACS.

Reactivation of latent virus with anti-latency drug treatment was assayed. Latently infected CD4⁺ T cells were cultured for at least 3-days post spinoculation (MOI of 3) and were stimulated with either CD3/CD28 beads (2 beads per cell) or 100 μ M or 1 μ M SAHA for 48 hours. No convincing increase in p24 expression under any drug treatment condition could be determined (Figure 6.15), even when using a lower MOI, perhaps because too much cell death may have been caused on viral reactivation. Prostratin, PMA, Panobinostat and Romidepsin treatment gave similar negative results. Despite this, treatment of resting cells with PMA or Prostratin altered the activation status of the cells whereas the HDACi did not, observations that agree with the general literature (1.5.3 and 1.5.4) (Figure 6.16).

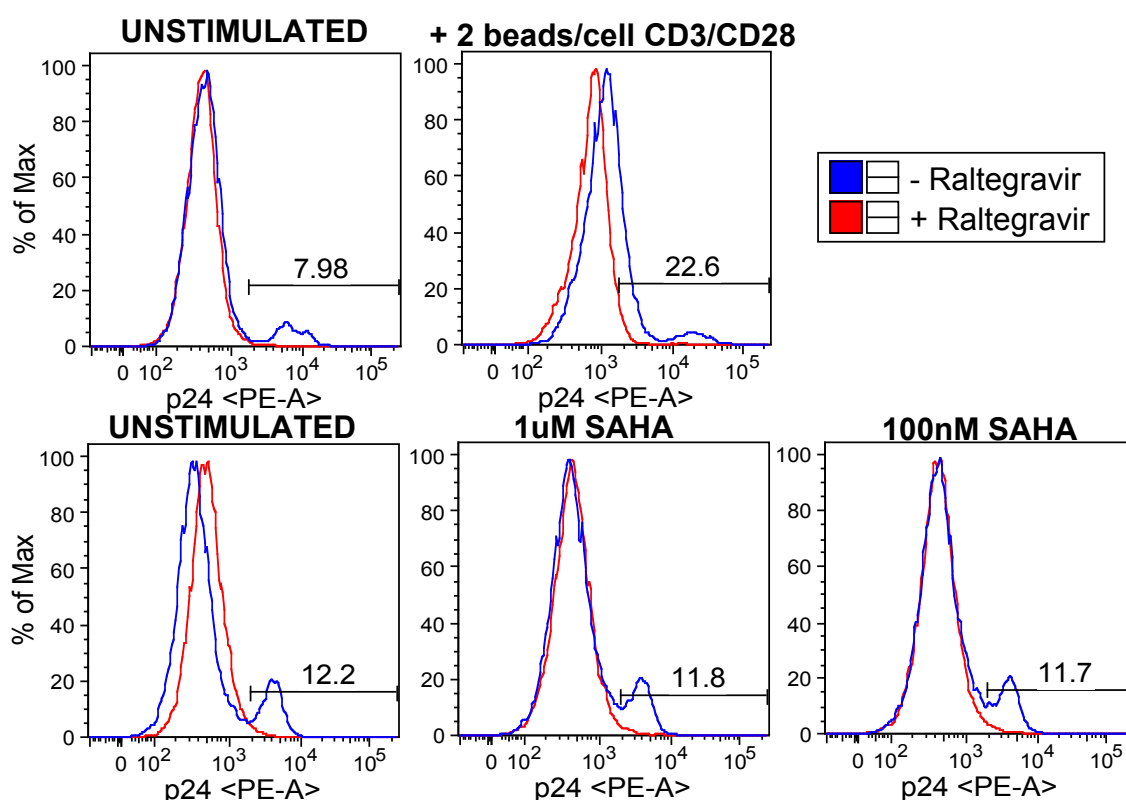


Figure 6.15. Reactivation of latent HIV-1 in a primary cell model. At least 2 days post spinoculation in the presence or absence of Raltegravir, cells were stimulated with A) CD3/CD28 antibodies or B) SAHA at two concentrations for 48 hours, or were left unstimulated. p24 positive cells were determined by Intracellular staining for FACS analysis. Gates determined by Raltegravir-treated control. Separate experiments were performed for CD3/CD28 and SAHA treatment. Representative experiments

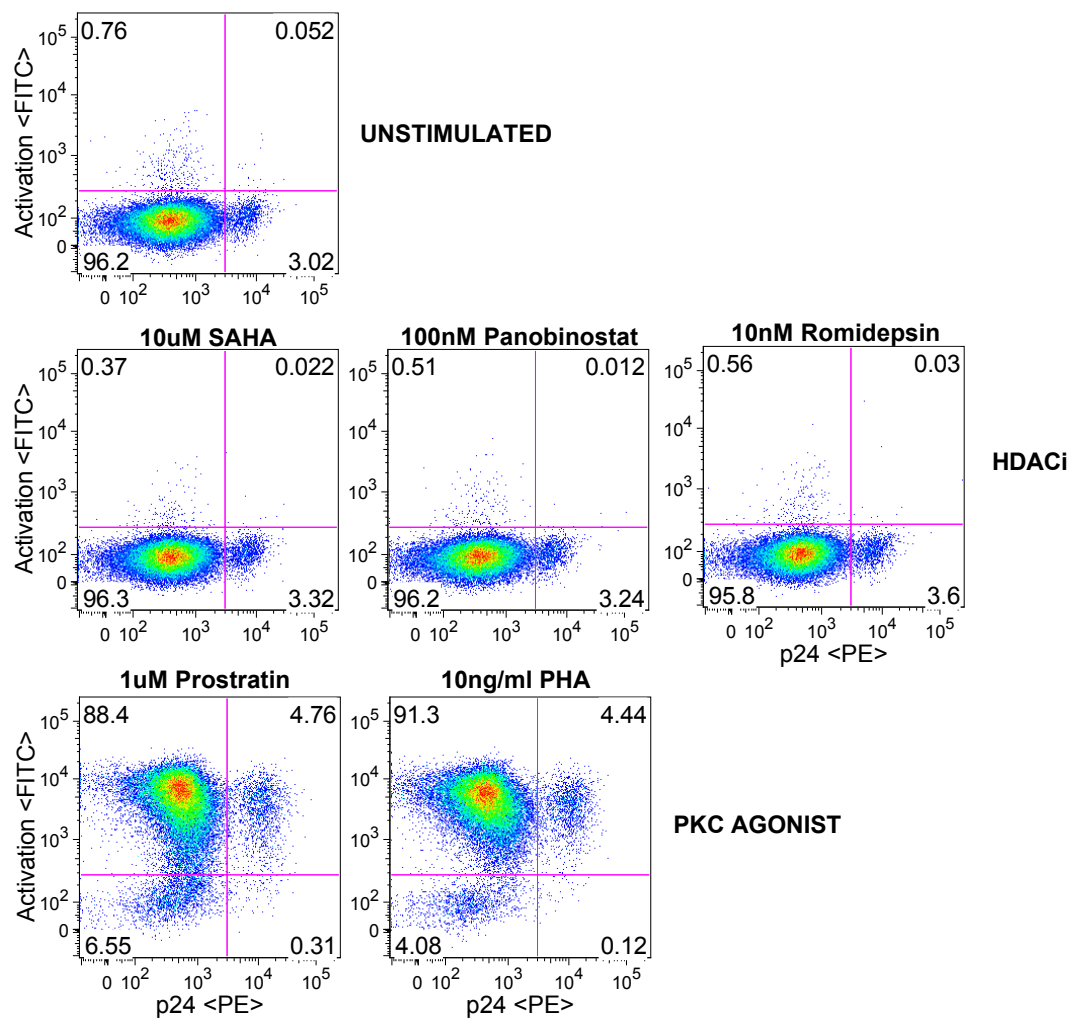


Figure 6.16. PKC treatment disrupts resting cell state. 2 days post spinoculation, cells were stimulated with SAHA, Panobinostat, Romidepsin, Prostratin and PMA. p24 positive cells were determined by Intracellular staining FACS analysis. Activated, FITC+ cells were determined by staining with CD69, CD25 and HLA-DR. Gates were determined by unstimulated control.

To investigate this further, a re-infection assay was performed. CD4⁺ T cells post spinoculation were either incubated in the presence or absence of 1.25 μ M Saquinavir to prevent viral spread. 3-days post spinoculation, these cells were either continually treated with Saquinavir or stopped receiving treatment. The two cultures were both stimulated with anti CD3/28 beads. If latent cells had been

reactivated, a greater p24+ population should be observed in cells cultured without Saquinavir, as infectious virus could potentially have been released, causing re-infection of other uninfected CD4+T cells in the culture. However, after 48 hours of reactivation perhaps a doubling of p24 expression was achieved between two populations of stimulated cells, compared to when the cells were left unstimulated (Figure 6.17). However the shift was not particularly convincing.

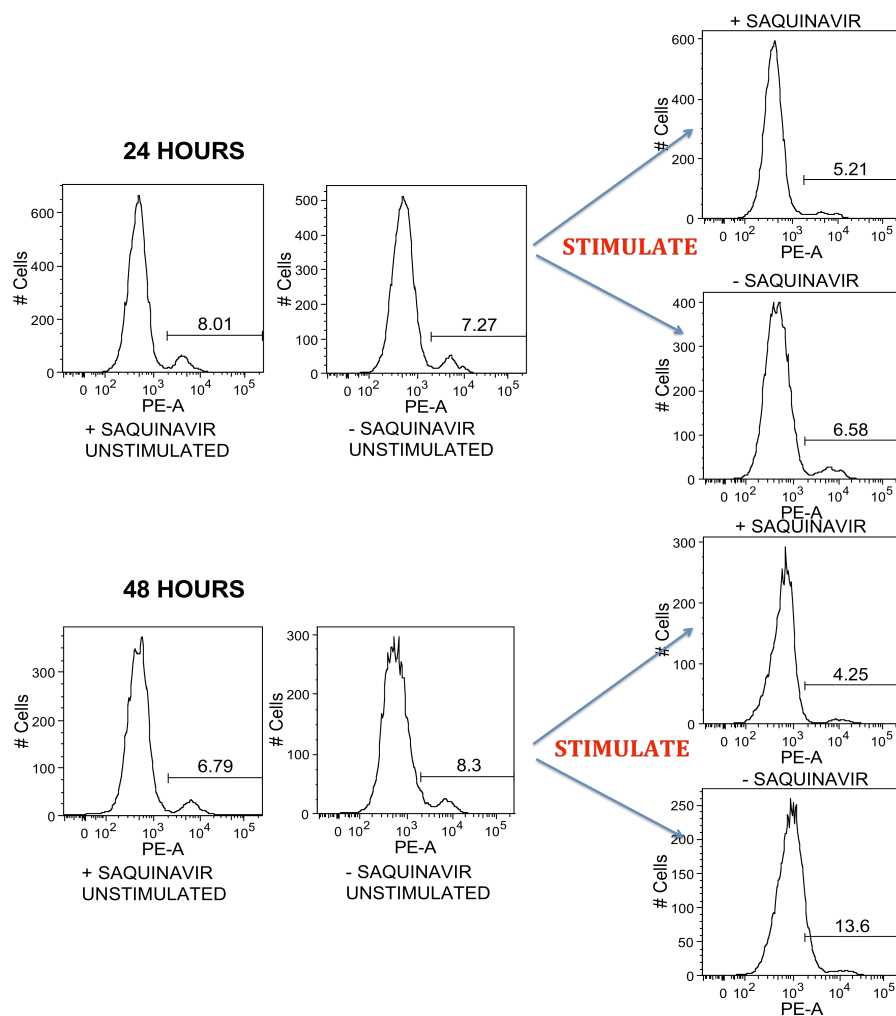


Figure 6.17. Viral release from latent cells in a primary cell model. Saquinavir treated cells were stimulated with anti CD3/CD28 antibodies in the presence or absence of Saquinavir for A) 24 and B) 48 hours, or were left untreated. p24 expression in the four populations was determined by intracellular staining using a PE conjugated antibody.

6.3.7 868-TCR transduced CTL killing of GPR cells

Assuming low-level viral reactivation had occurred, 868-TCR transduced CTLs were then assayed for their ability to target GPR cells post-reactivation with SAHA or Prostratin. Target cells were spinoculated with NL4-3 virus, and non-target cells were spinoculated in R20 media only. Only target cells expressed p24, however both were HLA-A2 positive. The percentage changes in the CFSE stained target populations and p24+ target populations, both relative to controls where no CTLs were added, were determined by FATAL assay (2.8.6, Figure 6.18).

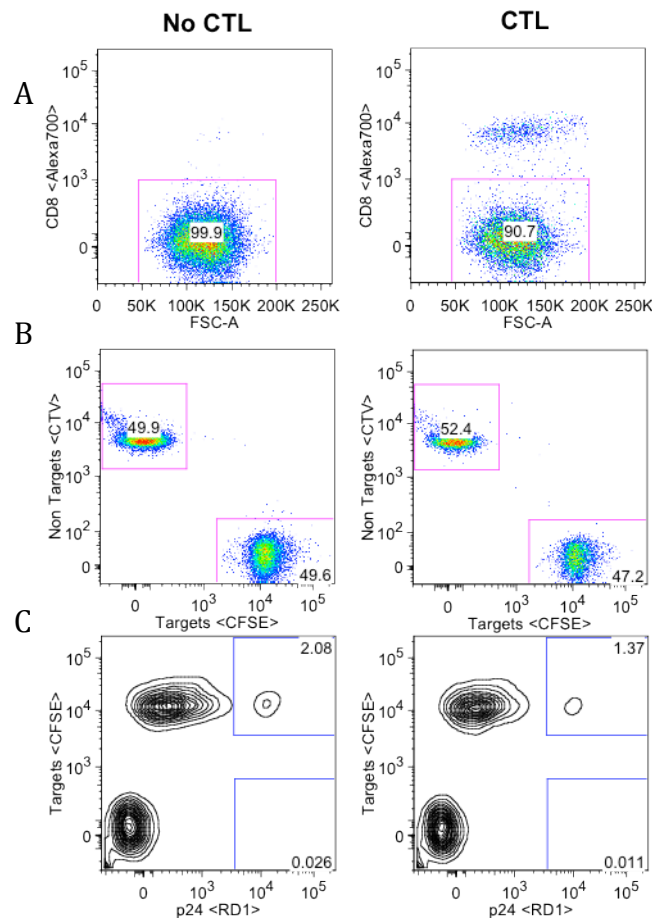


Figure 6.18. Gating strategy for combined FATAL / intracellular p24 killing assay for spinoculated CD4+ T cells treated with 10uM SAHA. Left side: FATAL assay where no CTLs were added. Right side: FATAL assay where CTLs were added. A) CD8+ T cell exclusion. B) Ratios of CTV and CFSE positive populations were 1:1 (left) and some specific target cell death occurred when CTLs were added (right). C) Only CFSE+ cells were p24+. The p24+ population decreased on addition of CTL.

Small amounts of killing of the entire 'target' population was observed (Figure 6.19A), but a more substantial killing of the p24+ population was observed due to the smaller number of these cells. However, the fold change between the two measures of cytotoxicity significantly and concomitantly increased on SAHA treatment (Figure 6.19B).

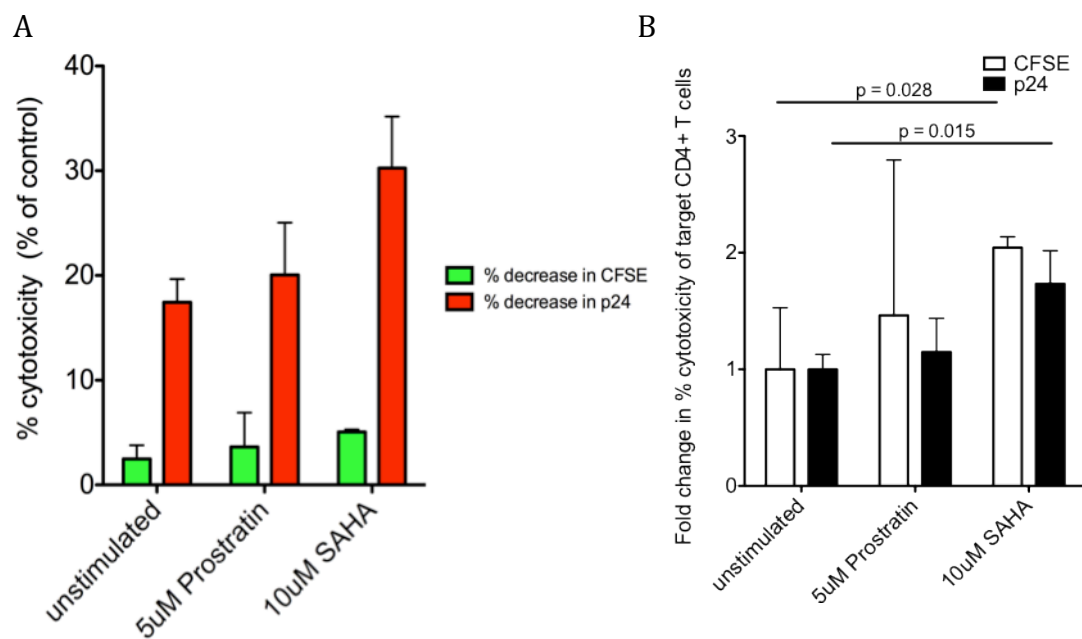


Figure 6.18 868-TCR CTL targeting of latently infected cells in a primary cell model of latency. 868-TCR CTLs from one donor were incubated in three killing assays. GPR cells were generated in a cell model of latency. A 0.6:1 E:T ratio was used. A) The % change in the CFSE+ population was calculated, relative to where no CTLs were added. B) The % change in p24+ CFSE+ cells was calculated, relative to where no CTLs were added. Significance was determined by Student's T Test.

6.4 Discussion

The aims of this project were to investigate the role for 868-TCR transduced CTL in targeting latently infected cells pre and post viral 'kick'. Latent cells were reactivated with anti-latency agents (Figure 6.1, 6.2, 6.3, 6.6), where intracellular p24 protein expression was used as a readout, and then 868-TCR CTL were tested for their ability to target latent cells in a number of latency models (Figure 6.7, 6.8, 6.18). This is the first time, to my knowledge, that CTL transduced with high affinity TCRs have been employed to 'kill' latent cells, post viral 'kick'. Prior studies have only co-cultured reactivated cells with autologous non-specific CTLs (Shan et al., 2012, Graf et al., 2013) or looked at active HIV-1 infection with TCR transduced CTLs (Sewell et al., 1997, Wilson et al., 1998, Varela-Rohena et al., 2008).

The 868-TCR transduced CTLs killed SLYNTVATL pulsed target cells specifically over the native uninfected HLA-A2 peptide repertoire present in uninfected control cells (Figure 6.7). Also, the 868-TCR specifically targeted T2 target cells pulsed with SLYNTVATL and not the HLA-A2 restricted HIV-1 derived peptide FLGKIWPSYK (Figure 6.7). However, there remains a possibility that other HIV-1 specific HLA-A2 restricted peptides could have contributed to the observed killing. This would be interesting to assay in future studies, although the transduced CTL passed safety concerns in a phase 1 clinical trial NCT0099122 assessing safety and tolerability of 868-TCR transduced CTLs infused into HLA-A2 positive patients with HIV-1 (ClinicalTrials.gov, 2014).

Since HLA-A2 is such a common HLA-type, and the 868-TCR is the strongest natural binding TCR ever recorded, these CTLs could be very useful in enhancing the clearance of latently infected cells post viral reactivation. It may be possible to take patients' own CTLs, transduce them with the 868-TCR pre 'kick' and then adoptively transfer the cells into the patient to improve a natural killing response.

Interestingly, the 868-TCR transduced CTLs were able to target chronically infected cell lines that express p24 below the limit of detection by FACS, even with no 'kick' (Figure 6.1, 6.3, 6.7). This may be because the 868-TCR has an innate ability to recognize very low levels of SLYNTVATL peptide on the cell surface. Also, it could have been an artifact of the constantly dividing latent cell lines, which might have aberrant transcription profiles. The transduction of HLA-A2 also might have led to supraphysiological levels of HLA-A2, thus higher than expected levels of SLYNTVAL could have been expressed for detection. However, the point remains that the kick and kill hypothesis might not be best studied in these cells.

Surprisingly, the latent cell lines also appeared to be less antigenic with a 'kick' as they were cleared less by CTL after drug treatment (Figure 6.8). Understanding this phenomenon is of paramount importance if HDACi inhibitors are to be used to stimulate cells out of latency to be targeted with a CTL based 'kill' mechanism. The data showed that anti-latency drugs down-regulated MHC class I expression (Figure 6.9, 6.10, 6.11), which could contribute to the reduced antigenicity observed. Previous studies demonstrate that modulations in MHC class I expression are most likely drug and cell-type specific. For example, surface MHC I, TAP-1, TAP-2, Tapasin, Calnexin, Calreticulin, ERp57, LMP2 and LMP7 (all proteins

crucial for peptide presentation, whose functions are summarized in Figure 6.19) were upregulated in melanoma cells, human embryonic and pluripotent stem cells on HDACi treatment (Khan et al., 2008, Suarez-Alvarez et al., 2010). HDAC class 1, 2 and 8 downregulated MHC class I expression in cervical cancer cells, meaning their inhibition would lead to an increase in expression (Khan and Tomasi, 2008). However, other studies have shown MHC class I, Tapasin and proteasome catalytic proteins are downregulated on HDACi treatment (Pellicciotta et al., 2008, Song et al., 2011).

Studies have also shown that HDACi drugs may interfere with the peptide presentation pathway, meaning CTLs might kill target cells less efficiently. Indeed, HDAC-6 has a role in regulating peptide stability (Lazaro et al., 2011). Also, decreased tumour antigen hTERT peptide expression was discovered after HDACi treatment of human tumour cells, which lead to impaired killing by hTERT-specific T-cells, despite increased hTERT mRNA after HDACi treatment (Pellicciotta et al., 2008). Analogously, although IFN- γ induced MHC class I upregulation, a decrease in specific tumour peptide expression lead to impaired recognition by antigen-specific CTLs in a mouse model of melanoma (Cho et al., 2011).

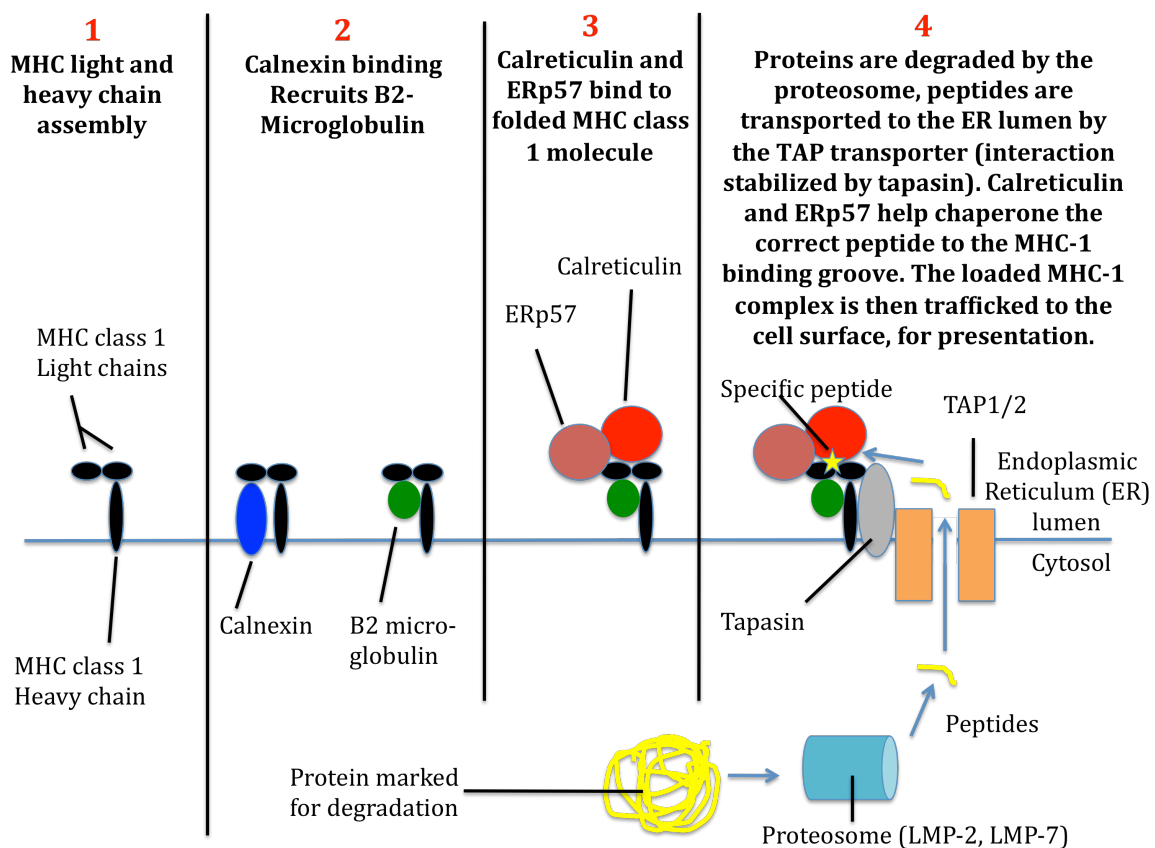


Figure 6.19. Summary of major proteins involved in the MHC Class 1 peptide presentation pathway, whose functions have been shown to be modulated by HDACi treatment.

While the literature potentially supported HDACi inhibiting CTL-mediated clearance of target cells, there was less evidence supporting the results involving PKC agonists. It is possible that these drugs may have caused the target cells to be less antigenic as inhibition of peptide presentation and Gag protein expression might have occurred, since MHC class I expression was increased. However, the decrease in antigenicity of HDACi treated cells could have been solely down to downregulation of MHC class I expression (Figures 6.9, 6.10, 6.11). Therefore, it

would be valuable for future studies aiming to cure HIV by the kick and kill approach, to more thoroughly investigate the relationship between MHC class I expression, antigenicity and CTL mediated clearance after cell stimulation.

However, since latent cell lines are transformed and not HLA-A2 native, the possibility that all of these results were an artefact of the system cannot be ruled out. This is especially likely in light of the differential impact of drug-treatment on native and transduced MHC class I expression. Also, *in vivo* latently infected cells are quiescent unlike cell lines. Aberrant signaling pathways that lead to non-physiological HIV-1 reactivation may be active in cell lines. For example, TNF α does not reactivate latent HIV-1 *in vivo* (Brooks et al., 2003, Tyagi et al., 2010, Yang et al., 2009), but does in ACH2 cells (Duh et al., 1989).

Due to these concerns, it was decided that the effect of 'kick' strategies (particularly HDACi) on CTL clearance was better investigated in a primary model of latency.

Therefore, spinoculation was used in model of latency where resting primary CD4+T cells were directly infected with HIV-1 (Figure 6.15, 6.17). In this model, no reactivation or release of latent virus could be demonstrated conclusively. This goes against what was published originally, where reactivation was reported (Pace et al., 2012) possibly because the infected cells died before analysis, particularly due to the high number of virions used in spinoculation. However, the viral cytopathic effect would perhaps be more limited using a lower MOI, and reactivation was not seen then either. It is also possible that p24 expression is

dynamic and turns on and off over time, meaning that the p24 positive cells post stimulation may not be the same as those detected pre-stimulation.

Despite this, the 868-TCR transduced CTL were able to target GPR cells (Figure 6.18). Additionally, treatment with SAHA may have enhanced the targeting of latently infected cells by 868-TCR transduced CTLs possibly suggesting the 'kick' strategy enhanced antigenicity of the target cells. However, this is an observation that must be verified using other HDACi in this model, 868-TCR transduced CTLs from other donors and in other primary cell models. If this result is supported, a twofold enhancement of p24+ cell antigenicity occurred, with no additional p24 being expressed post 'kick'. A hypothesis for this could be that SAHA but not Prostratin released a block in peptide presentation, but this has not been determined and would be interesting to investigate.

Despite this potential increased killing, similar perturbations in MHC class I expression occurred in CD4+ T cells compared to the chronically infected cell lines (Figure 6.11). However, the impact of HDACi treatment of MHC class I expression was not as pronounced as in the cell line models (Figure 6.9, 6.10). It is unknown whether the MHC class I downregulation seen in human CD4+ T-cells is sufficient to mount a natural killer cell mediated response. This is something that would be interesting to investigate, but was beyond the scope of this chapter.

There has been much recent debate as to which primary latency models are best for truly reflecting HIV-1 latency *in vivo*. A recent collaborative study has attempted to unify a selection of primary latency models in terms of their

reactivation potential (Spina et al., 2013). Reactivation in each model was measured by slightly different methods, but was standardized by normalizing the results to maximal levels (100%) seen in that assay. A disadvantage is it is impossible to assess how small the absolute reactivation levels are. The Greene model (Lassen et al., 2012) closely resembles the O'Doherty model that was assayed in this chapter, the main difference being an NL4-3-Luciferase virus was spinoculated with resting CD4+ T-cells, giving a reading of light units on reactivation from latency. Similar to results presented in this chapter, the Greene model also demonstrated very low reactivation of latent HIV-1 with SAHA, Prostratin or any HDACi tested.

There was much variation in anti-latency drug-induced reactivation across the different models (Spina et al., 2013), which may be because they individually represent the myriad types of latency present *in vivo*. Therefore, multiple models of latency, as discussed in this chapter, will need to be examined in order to understand the potential consequences of anti-latency agents on CTL clearance of the reservoir.

Ideally the targeting of latent cells and antigenicity of the HIV-1 reservoir should be studied in latently infected patient cells, but it is very challenging to isolate enough of them for use in a study. However, with the development of more sensitive methods for detection of reactivated cells and bigger bleeds for getting sufficient latent cells to assay, this may be possible to do in patient samples in the future.

7. Conclusions and Future Directions

The aim of this thesis was to characterise the HIV-1 reservoir and to assess the potential for viral eradication using thermotherapy and TCR transduced CTL. I developed sensitive Q-PCR assays to quantify HIV-1 DNA levels and applied these assays to characterise the HIV-1 reservoir in a cohort of acutely infected patients enrolled in the SPARTAC trial. I investigated a novel nanotechnological approach, which was aimed at enhancing CTL function and that could act as an adjunctive therapy in the eradication of latently infected HIV-1 positive cells. I also explored the potential for TCR-transduced CTLs to target the latent reservoir through the 'kick and kill' approach in a number of cellular models of latency.

In this thesis, patients who had low subtype B HIV-1 DNA levels in untreated infection experienced a delay in time to primary endpoint of the SPARTAC trial. Also, in patients who were treated for 48 weeks, low HIV-1 DNA levels just before treatment interruption were also predictive of a longer time to primary endpoint and time to viral rebound to detectable levels. This characterisation of the HIV-1 reservoir in SPARTAC patients is therefore very relevant to current opinion, whereby low HIV-1 DNA levels are associated with viral control. Indeed, HIV-1 DNA levels at the point of stopping HAART being predictive of the time to viral rebound was shown for the first time in a randomised clinical cohort in this thesis. For future work, it would be interesting to use the multi-clade Q-PCR assays, developed in Chapter 3 to see if subtype C HIV-1 reservoir levels can similarly predict clinical outcome and time to viral rebound in the SPARTAC trial.

Total HIV-1 DNA may thus be a useful biomarker that, by itself or in combination with other markers, could help to inform which patients may be candidates for TI strategies in future HIV eradication clinical trials. For example, a new clinical trial (Eramune-03 (ULTRA-STOP) NCT01876862), investigating factors that predict which patients can safely interrupt therapy, is planned (ClinicalTrials.gov, 2014).

No evidence for sustained Post Treatment Control was observed on analysis of the SPARTAC trial, perhaps because the duration of HAART in primary HIV was too short. However, the 'HEATHER' cohort, a clinical trial as part of the CHERUB collaboration, will address the impact of longer term HAART, also administered in primary HIV-1 infection. The results from this study, which is currently recruiting, would be interesting to compare with the SPARTAC study findings.

Since lower HIV-1 DNA reservoir levels were beneficial in the SPARTAC study, ways to decrease the amount of latently infected cells were then investigated.

Firstly, thermotherapy was employed as a possible technology to increase CTL killing ability of HIV-1-infected cells. Should the technology work in the proof-of-principle study, it could contribute to virological control or be used in concert with other HIV-1 eradication strategies in the 'kick and kill' approach to improve the likelihood of functional cure.

However, the heat generated in CTL was insufficient to cause direct thermal ablation of bound target cells and also was insufficient to enhance the natural killing ability of CTL. However, with techniques to improve SPION uptake, the development of SPION with higher heating capabilities and the generation of

higher alternating magnetic fields, this technology has the potential to show efficacy.

Despite this result, nanotechnology has enormous promise in the future for efficient delivery of HAART to potential HIV-1 sanctuary sites across the blood-brain barrier (Alyautdin et al., 2014). A study in a humanised mouse model showed that the anti-latency agent Bryostratin and the antiretroviral protease inhibitor Nelfinavir could both be conjugated to lipid nanoparticles, thus creating a particle that could simultaneously reactivate latent HIV-1 and inhibit subsequent viral spread (Kovochich et al., 2011). Indeed, the FDA approved PKC agonist Bryostatin was recently shown to induce LTR-specific rather than read-through transcription of latent HIV-1 when HDACi did not, potentially making this drug viable for application to the kick and kill hypothesis (Bullen, Laird et al. 2014).

The final chapter in this thesis attempted to investigate whether the latent viral reservoir could be targeted directly by 868-TCR transduced CTLs. Latent HIV-1 was reactivated using a panel of anti-latency compounds in a variety of latent cell line models. 868-TCR transduced CTL were able to target latent cells pre and post viral reactivation, but the presence of more intracellular p24 protein did not always link with increased antigenicity of cells. This may be in part due to anti-latency drug-induced alterations in MHC-Class 1 expression. It is not known if the MHC class I molecules are degraded or just become intracellular and the mechanism would be interesting to investigate in future studies.

This last point is of special pertinence to the field because if Histone Deacetylase inhibitors decrease the expression of MHC class I molecules, the reservoir may become less antigenic. However, in a primary model of latency, a pilot experiment in this thesis showed that SAHA could potentially have increased antigenicity of the reservoir despite no additional p24 being detected in the cytoplasm. However, this observation needs to be confirmed in other models of latency. Additionally, it remains unknown what peptides are being presented on the surface of latent cells pre-and post stimulation, other than SLYNTVATL. It would be interesting to investigate peptide repertoire expression in latent cells, for example by mass spectrometry. If predominant peptides are presented, it may be possible for a more targeted kick and kill approach to be achieved. It would also be interesting to investigate if MHC class I downregulation by HDACi is sufficient to mount Natural killer cell responses.

Clinical trials are planned as part of the CHERUB initiative to investigate whether anti-latency drug treatment can make the reservoir antigenic. For example, the 'RIVER' study will investigate perturbations in the latent HIV-1 reservoir after CTL vaccination and HDACi treatment. The concerns raised in this thesis may be addressed in studies such as these.

To conclude, the prospect of a generalised HIV-1 cure remains elusive. Only through the development of sensitive assays that measure the HIV-1 reservoir and their application to novel and innovative cure strategies, together with continued support from funding bodies and laboratory cooperation, will HIV-1 ever be functionally cured or eradicated in patients on a large scale.

8. References

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