Immunological and genetic correlates of delayed HIV disease progression in children with perinatally-acquired HIV-1 infection from Harare, Zimbabwe



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Thesis Abstract

Understanding the immunological and genetic mechanisms that contribute to HIV-1 disease progression is important in the development of antiviral therapies and vaccines. HIV-1 has evolved to downregulate the cell surface expression of HLA class I in order to avoid the presentation of viral peptides to cytotoxic CD8+ T cells. HLA class I molecules are loaded with antigenic peptides in the endoplasmic reticulum (ER) by the peptide loading complex (PLC), aided by the molecular chaperone protein tapasin. Tapasin promotes the selection of high affinity peptides on HLA class I for presentation to T cells. HLA class I alleles exhibit different dependencies on tapasin for peptide selection and loading. This thesis aimed to investigate the role of tapasin and HLA class I tapasin-dependency in HIV-1 infection. Chapter three describes an association between tapasin-independent HLA class I alleles and slow disease progression in a cohort of children and adolescents with perinatally-acquired HIV-1 infection from Harare, Zimbabwe. Our study then aimed to use the evidence from our clinical data analyses to inform *in vitro* studies investigating whether HIV-1 interferes with the PLC or tapasin, as has been observed for other viruses. Chapter four describes how HIV-1 downregulates tapasin-dependent HLA class I alleles more efficiently than tapasin-independent alleles. We also show that downregulation of HLA class I by HIV-1 is impeded in PLC knockout cells. Chapter five aimed to assess how HIV-1 affects PLC mRNA and protein expression levels. We show that HIV-1 does not downregulate tapasin, but further experiments are required to interpret the association between HLA class I tapasin-dependency and HIV-1 control. Finally, chapter six describes how tapasin-independent HLA class I alleles are more stably expressed in HIV-1 infection, compared to tapasin-dependent alleles. This thesis provides evidence to show for the first time that HIV-1 may affect tapasin function.

List of Abbreviations

β2-m - β2-microglobulin
β-me – β-mercaptoethanol
ABC – ATP binding cassette

AERD – Aspirin-exacerbated respiratory disease
AF700 – Alexa Fluor 700

AIDS – Acquired immunodeficiency syndrome
AP-1 – Adapter protein complex-1

AP-2 – Adapter protein complex-2
APC – Allophycocyanin
APC-Cy7 - Allophycocyanin-Cyanine7
APOBEC3G – Apolipoprotein B mRNA editing enzyme, catalytic subunit 3G
ARP – AIDS Reagent Programme
ART – Antiretroviral therapy
ATP – Adenosine triphosphate

BFA – Brefeldin A
BiP – Immunoglobulin-binding protein

BLS I – Bare lymphocyte syndrome type I
BMDC – Bone marrow-derived dendritic cell
BRTI – Biomedical Research and Training Institute, Harare, Zimbabwe
BV605 – Brilliant Violet™ 605
BV711 – Brilliant Violet™ 711
CA – Capsid

cART – Combination antiretroviral therapy
cGAS - Cyclic GMP-AMP synthase
CRF – Circulating recombinant forms
CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
CRS - Chronic rhinosinusitis

Ct – Cycle threshold
CTL – Cytotoxic T lymphocyte
CTLA-4 – Cytotoxic T-lymphocyte associated protein 4
CTP – Cytoplasmic transduction peptide

DMEM - Dulbecco's Modified Eagle media
DMSO – Dimethyl sulfoxide

DPBS – Dulbecco's phosphate-buffered saline
DTT – Dithiothreitol
E19 – Adenovirus E3 19 kDa protein
EBV – Epstein-Barr virus
EC – Elite control

EDTA - Ethylenediaminetetraacetic acid
ER – Endoplasmic reticulum
ERAD – Endoplasmic reticulum-associated degradation
EV – Empty Vector
FACS - Fluorescence-activated cell sorting
FBS – Foetal Bovine Serum

FFPE – Formalin-fixed paraffin-embedded
FITC - Fluorescein isothiocyanate

FWB – FACS Wash Buffer
Fwd – Forward
GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
GAS – Interferon gamma activated sequence
gDNA – Genomic DNA

GM-CSF – Granulocyte-macrophage colony-stimulating factor
GWAS – Genome-wide association studies
GzmB – Granzyme B
HBV – Hepatitis B virus
HCMV – Human cytomegalovirus
HCV – Hepatitis C virus
HKG – Housekeeping gene
HLA – Human leukocyte antigen
HPV – Human papillomavirus
HPV-16 – Human papillomavirus type 16
ISRE – Interferon response element
JRA – Juvenile rheumatoid arthritis
ICAM1 – Intracellular adhesion molecule 1
ICAM3 – Intracellular adhesion molecule 3
I-DIRT – Isotopic differentiation of interactions as random or targeted
IFN-α – Interferon alpha
IFN-γ – Interferon gamma
Ig – Immunoglobulin
IHC – Immunohistochemistry
IL-2 – Interleukin-2
IL-4 – Interleukin-4
IMDM – Iscove's Modified Dulbecco's media
INHALE - INvestigation of Heart And Lung disEase among older children in Harare, Zimbabwe
IP - Immunoprecipitation
ISG – Interferon stimulated gene
KIR – Killer immunoglobulin-like receptor
KSHV – Kaposi's sarcoma associated herpesvirus
LCL – Lymphoblastoid cell line
LCMV – Lymphocytic choriomeningitis
LD – Linkage disequilibrium
LN_2 – Liquid nitrogen
LTNP – Long-term non-progression
MCV – Molluscum contagiosum virus
MEM – Minimum Essential Medium
MFI – Median fluorescence intensity
miRNA – microRNA
MOI – Multiplicity of infection
MPN - Myeloprofilerative neoplasms
MS – Mass spectrometry
MTCT – Mother to child transmission
MX1 - Interferon-induced GTP-binding protein Mx1
NaCl – Sodium chloride
NC – Nucleocapsid
NEAA – Non-essential amino acids
NHS – National Health Service
NIH – National Institutes of Health
NIR – Near-infrared
NK cell – Natural killer cell
NSDV – Nairobi sheep disease virus
ORF- Open reading frame
P2 – Position 2 (peptide residue)
PAKC - CRISPR/Cas9 panel of antigen processing machinery knockout cells
PBMC – Peripheral blood mononuclear cell
PBS – phosphate buffered saline
PCR – Polymerase chain reaction
PDI – Protein disulphide isomerase
PDIA3 – Protein disulphide isomerase A3

PE – Phycoerythrin
PEC – Paediatric elite control
PE-Cy7 – Phycoerythrin-Cyanine7
Pen/Strep – Penicillin/Streptomycin

PFA – Paraformaldehyde
PHA – Phytohaemagglutinin
PIC – Pre-integration complex
PLC – Peptide loading complex
PLWH – People living with HIV
PRDM-1 – PR domain zinc finger protein 1
PRF – Perforin
PSP – Paediatric slow progressor
PVC – Paediatric viral controller
PVDF - Polyvinylidene difluoride
PWB – Permeabilisation Wash Buffer

PΩ – C-terminal peptide residue
qPCR – quantitative PCR
Rev – Reverse
RIPA – Radio-immunoprecipitation assay
RPMI – Roswell Park Memorial Institute
RQ – Relative quantification
RSAD2 - Radical S-adenosyl methionine domain-containing protein 2

RT – Room temperature
RT-PCR – Reverse transcription polymerase chain reaction
SAV3 – Salmonid alphavirus 3
SCLC – Small cell lung carcinoma
SDS – Sodium dodecyl sulphate
siRNA – short interfering RNA
SIV – Simian immunodeficiency virus
SNP – Single nucleotide polymorphism
spVL – Set point viral load
STING - Stimulator of IFN gene
SV40 – Simian vacuolating virus 40
T/F – Transmitter/founder

TAP – Transporter associated with antigen processing
TAPBPR – TAP-binding protein related
TAR – Tat activation region
TBS – Tris-Buffered Saline
TCR – T cell receptor
TD – Tapasin-dependency
TNF-α – Tumour necrosis factor alpha

U – Units
UTR – Untranslated region

V – Volts
VHSV – Viral haemorrhagic septicaemia virus
Vif – Viral infectivity factor
VL – Viral load
VLW – Voluntary lay worker
VSV-G – Vesicular stomatitis virus G

WHO – World Health Organisation
WT – Wild type
ZENITH – Zimbabwe study for ENhancing testing and Improving Treatment of HIV in children

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Chapter 1: Introduction

1.1. The HIV Pandemic

The human immunodeficiency virus (HIV) still represents a significant global health concern forty years since the pandemic began. HIV is the causative agent of acquired immunodeficiency syndrome (AIDS), still responsible for approximately 650,000 annual deaths worldwide (UNAIDS estimates, 2022). The most recent estimates predict that over 38 million people globally are living with HIV (UNAIDS estimates, 2022). More than 50% of people infected with HIV are situated in eastern and southern Africa. (UNAIDS estimates, 2022). A small proportion of the number of people living with HIV worldwide are children (approximately 4%), but children make up for 15% of AIDS related deaths (UNAIDS estimates, 2022). With the advent of the availability of combination anti-retroviral therapy (cART) treatment from the mid-1990s, the life expectancy of people living with HIV has drastically improved (Tseng et al., 2015). That being said, reliable access to antiretroviral therapy (ART) is not ubiquitous worldwide and studies have reported on the barriers faced by people living with HIV to gain access to treatment especially in low-income countries (UNAIDS, 2015, Addo-Atuah et al., 2012, Mutevedzi and Newell, 2014, Kouanda et al., 2010, Ankomah et al., 2016). Moreover, the high prevalence of mortality in children is likely due to poor ART adherence and retention in care in this age group (Ferrand et al., 2017). For people living with HIV, the virus does not present an acute often fatal infection as it did at the start of the pandemic. Rather, the infection is a chronic lifelong disease that disproportionately affects vulnerable groups and people from resource-limited settings. As such, further research with the goal to prevent, control and eradicate HIV is vital.

There are two types of HIV: HIV-1 and HIV-2. The origin of HIV-1 can be traced back to the replication of simian immunodeficiency virus (SIV) SIVcpz in chimpanzees (*Pan troglodytes troglodytes*) and gorillas (*Gorilla gorilla gorilla*) in West Africa (Sharp and

Hahn, 2010). Cross-species zoonotic events in south Cameroon from chimpanzees to humans are predicted to be the phylogenetic route of HIV-1 group M viruses that predominate the pandemic (Keele et al., 2006). The origin of less pathogenic HIV-2 which most commonly infects individuals from West Africa is predicted to have originated from SIVsmm in sooty mangabeys (*Cercocebus atys*) (Hirsch et al., 1989).

HIV-1 is classified into four groups: M, N, O and P. As mentioned, group M is the most prevalent and has driven the majority of the global HIV-1 pandemic. Group M is further classified into subtypes or clades denoted as A1, A2, A3, A4, A6, A7, A8, B, C, D, F F1, F2, G, H, J and K (Los Alamos HIV Sequence Database, Los Alamos National Laboratory). These clades are phylogenetically, geographically and epidemiologically linked (Hemelaar et al., 2006). Recombinant HIV-1 isolates of different clades have also been identified, known as circulating recombinant forms (CRFs) (Robertson et al., 1995).

1.2. The Human Immunodeficiency Virus

HIV is a 120 nm enveloped lentivirus retrovirus of the *Lentivirus* genus. The viral genome consists of two identical copies of positive-sense single stranded RNA. The HIV-1 RNA genome encodes for nine viral genes, including the three structural proteins *gag, pol* and *env* as well as multiple accessory/regulatory proteins (Figure 1.1A).

1.2.1. HIV-1 structural proteins

HIV-1 primarily infects CD4+ T cells and macrophages. Viral tropism is governed by the viral envelope; the only surface-expressed viral protein that is made up of trimers of heterodimer complexes of gp120 and gp41. The gp41 subunit spans the viral lipid membrane, which is derived from the host cell membrane. The viral lipid membrane also contains host cell proteins such as integrin α4β7 and intracellular adhesion molecule 1 (ICAM-1) that are selectively, rather than passively, incorporated into the membrane and have been suggested to impact viral infectivity and pathogenesis (Fortin et al., 1997, Guzzo et al., 2017). The gp120 subunit is non-covalently bound to gp41 and expressed completely outside of the viral lipid bilayer (Figure 1.1B). The gp120 subunit of Env is highly variable and heavily glycosylated, impairing the ability to mount a robust humoral response against the surface protein (Leitner et al., 1997, Fenouillet et al., 1994). Conserved regions of gp120 recognise and interact with the CD4 receptor and CCR5/CXCR4 co-receptors on the surface of target cells. Transmitted/founder (T/F) HIV-1 isolates are generally R5-tropic, meaning that they use the CCR5 co-receptor for cell entry to CD4+ T cells. These isolates can also infect macrophages and are commonly found in the central nervous system infecting microglia and contributing to neurodegenerative disorders associated with HIV-1 infection (Gorry et al., 2001). During viral infection, variation in the gp120 glycoprotein can cause HIV-1 to switch co-receptor

usage to CXCR4 (X4-tropic). Co-receptor switching for X4-tropism enables HIV-1 to infect different CD4+ T cell subsets and has been associated with accelerated disease progression and prolonged replication of the virus in an immunosuppressed environment (Schramm et al., 2000).

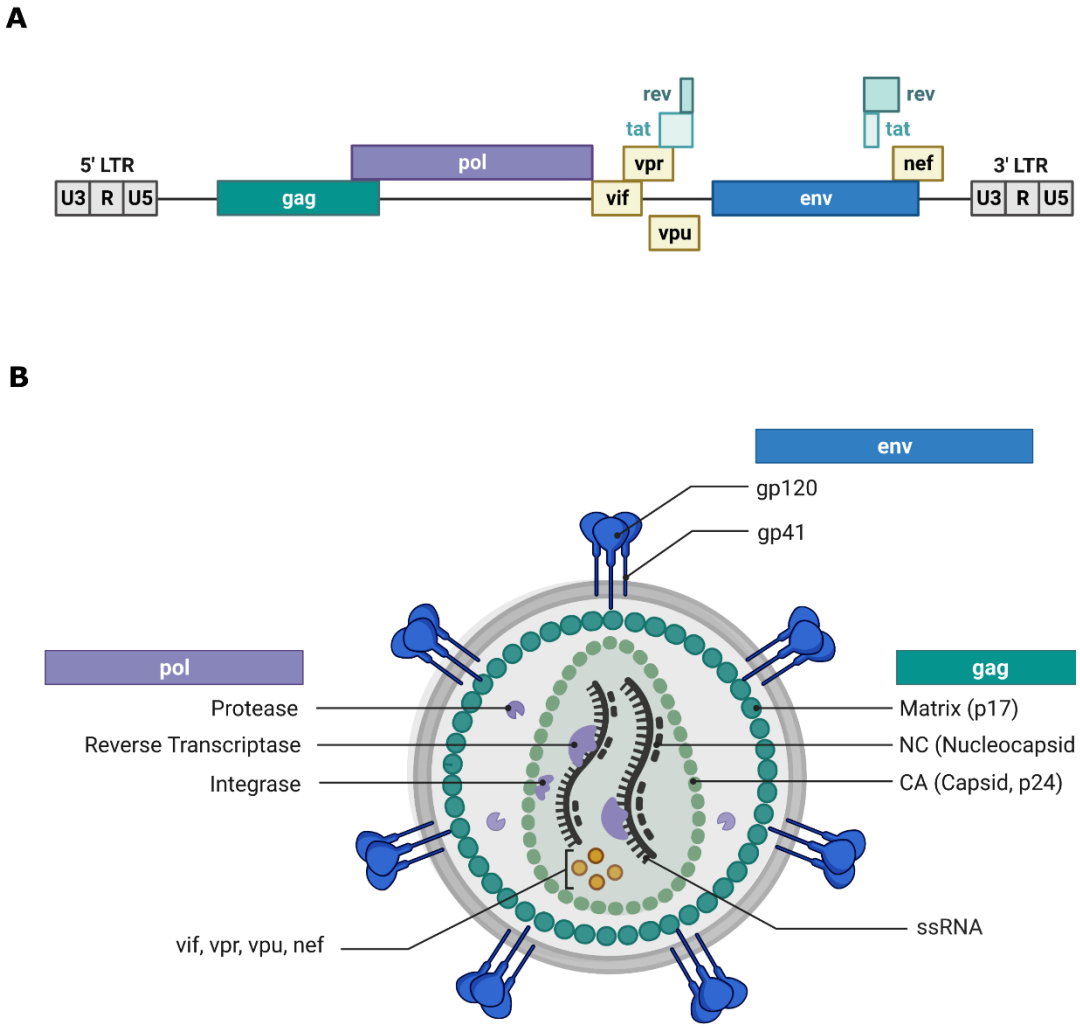


Figure 1.1. The structure of HIV-1. The structural organisation of A) the HIV-1 genome and B) the HIV-1 genome. Illustration created with BioRender.com.

The HIV-1 capsid (p24) and nucleocapsid are encoded by *gag* (Figure 1.1A and 1.1.B). The p24 antigen is essential for the assembly of the core conical capsid that encapsulates the viral RNA, nucleocapsid and viral enzymes (Figure 1.1B). p24 expression is commonly used as an indicator of HIV-1 infection both *in vitro* and *in vivo* (Di Stefano et al., 1995). The nucleocapsid is responsible for chaperoning the viral RNA to ensure efficient reverse transcription of the viral genome by virally-encoded reverse transcriptase (Levin et al., 2010) (Figure 1.2). The viral matrix protein (p17) is anchored to the inside of the viral lipid membrane and maintains viral integrity and viral assembly (Yu et al., 1992, Freed et al., 1994).

Pol encodes the viral enzymes reverse transcriptase, integrase and protease. The virally encoded reverse transcriptase is responsible for the conversion of viral RNA into cDNA. Reverse transcription of HIV-1 RNA lacks intrinsic proof reading capabilities, which leads to the accumulation of mutations in the HIV-1 genome, especially in the *Env* gene. This is advantageous in viral escape from the humoral immune response (Yeo et al., 2020, Sun et al., 2022).

1.2.2. HIV-1 accessory and regulatory proteins

HIV-1 encodes four accessory proteins: *vif, vpr, vpu* and *nef*, and two regulatory proteins *tat* and *rev* that make the virus distinguishable from other primate lentiviruses. HIV-2 contains the same accessory/regulatory proteins but encodes Vpx instead of Vpu (Tristem et al., 1992). Some of the functions for the other accessory proteins also differ between HIV-1 and HIV-2 (reviewed by (Strebel, 2013)).

The accessory and regulatory proteins encoded by HIV-1, Tat, Rev, Vif, Nef Vpu and Vpr, have been shown to interfere with host restriction factors and cellular mechanisms

to enhance HIV-1 replicative capacity. The HIV-1 accessory proteins have also been shown to interfere with host cell defences in order to encourage viral release and spread of progeny virions to neighbouring cells, as well as limit the surveillance of infected cells by the immune system.

Rev is an HIV-1 regulatory protein with a nuclear localisation signal that aids the nuclear exportation of intron-containing unspliced viral RNA transcripts (Fischer et al., 1995). Tat enhances the efficiency of viral transcription from integrated dsDNA (Kao et al., 1987). Tat enables RNA Polymerase II (RNAP II) elongation by Tat binding to Tat activation region (TAR), an RNA stem-loop, in the 5' untranslated region (UTR) of the HIV-1 genome (Dingwall et al., 1989). The HIV-1 accessory protein viral infectivity factor (Vif) has been found to disrupt the enzyme apolipoprotein B mRNA editing enzyme, catalytic subunit 3G (APOBEC3G) by targeting it for ubiquitination and proteasome-mediated degradation (Marin et al., 2003). APOBEC3G causes hyper-mutation of the HIV-1 genome by inducing deoxycytidine to deoxyuridine mutations and also interferes with the processes of HIV-1 reverse transcription and viral DNA integration (Goncalves et al., 1996, Donahue et al., 2008, Mbisa et al., 2007). Vpr has been shown to be required for HIV-1 infection of non-dividing cells such as macrophages, as it is essential for nuclear import of the pre-integration complex (PIC) (Popov et al., 1998). Vpr has also been implied to induce G2 cell cycle arrest and apoptosis in infected cells, contributing to HIV-1-mediated suppression of the immune system (Stewart et al., 2000).

Both the accessory proteins Vpu and Nef encoded by HIV-1 have been shown to downregulate cellular CD4 expression. Nef is an early expressed gene whereas Vpu is late expressed, allowing coordinated and continuous removal of CD4 expression of cells. Downregulation of CD4 in such a way avoids viral superinfection of cells and inhibition

of virus release (Tanaka et al., 2003). Vpu downregulates CD4 by targeting newly synthesised molecules for ubiquitination in the endoplasmic reticulum (ER) for ER-associated degradation (ERAD) by the proteasome, whereas Nef utilises the clathrin adapter protein complex 2 (AP-2)-dependent endocytotic pathway to downregulate CD4 (Lindwasser et al., 2007). Vpu exerts additional effects on HIV-1 infected cells to suppress the antiviral immune response. Vpu also downregulates the expression of the host restriction factor tetherin at the cell surface to antagonise its limitation of HIV-1 virion release upon viral budding (McNatt et al., 2013, Neil et al., 2008). More recent evidence points towards Vpu inhibition of NF-κB-dependent antiviral gene expression and pro-inflammatory cytokine production (Langer et al., 2019). The additional roles of Nef have also been described to promote T cell activation states that encourage viral replication. Nef downregulates cytotoxic T-lymphocyte associated protein 4 (CTLA-4) on activated T cells that signals to antigen-presenting cells to stop interleukin-2 (IL-2) release and stimulation of T cell activation, hereby promoting viral replication (El-Far et al., 2013). Notably, HIV-1 Nef and Vpu also downregulate HLA class I molecules at the cell surface of HIV-1 infected cells, impairing the presentation of viral peptides to T cell receptors (TCRs) on cytotoxic CD8+ T cells (Lubben et al., 2007, Mann et al., 2013).

1.2.3. The HIV-1 replication cycle

The HIV-1 replication cycle is outlined in Figure 1.2. Viral entry begins when interactions between gp120 and CD4 induce conformational changes in the glycoprotein that expose the secondary interacting site on gp120 for the CCR5 co-receptor (Kwong et al., 1998, Rizzuto et al., 1998). The binding of gp120 to CD4 and the CCR5/CXCR4 results in a stable attachment to the target cell (Wyatt and Sodroski, 1998).This is followed by the insertion of the N-terminal fusion peptide from gp41 into the cell membrane which

initiates the fusion of the viral and cell membranes. Following fusion, the viral capsid encapsulating the viral RNA, nucleocapsid and the viral protease and integrase enzymes enters the target cell (Figure 1.1B). Capsid uncoating to release viral RNA and virally encoded enzymes was long hypothesised to occur in the cytoplasm, but more recent evidence has shown that capsid uncoating can occur in the nucleus, probably to avoid detection by intracellular pattern recognition receptors (PRRs) (Muller et al., 2021, Burdick et al., 2020, Rasaiyaah et al., 2013).

Following RT of viral RNA into DNA, the PIC, formed of viral cDNA, viral proteins and host proteins, is translocated to the nucleus. Viral DNA is then integrated as the provirus into the host chromosomal DNA by the viral integrase (reviewed by (Craigie, 2012)). The provirus lies dormant until activation of the cell, which starts the replication of the HIV-1 genome utilising the host cell machinery. During viral replication, the proviral DNA is transcribed back into mRNA, which serves as both genomic RNA for new viral particles as well as being multiply spliced to encode the different viral proteins (reviewed by (Emery and Swanstrom, 2021)). Full length mRNA transcripts encode the structural Gag and Gag-Pol polyproteins whilst single spliced transcripts generate Env, Vif and Vpu. Lastly, fully spliced transcripts are responsible for the expression of the accessory and regulatory proteins Rev, Tat, Vpr and Nef. Viral mRNA copies are transported from the nucleus by Rev where they are translated in the cytoplasm by host cell machinery alongside the mRNA transcripts required for normal cell function (Malim et al., 1989, Feinberg et al., 1986).

After translation of viral proteins, infectious HIV-1 particles are assembled from immature HIV-1 polyproteins. The *Env* gene is translated into the gp160 glycoprotein which is then cleaved by furin into gp120 and gp41. This is anchored to the membrane of the host cell ready for release (Freed et al., 1989). The Gag (p55), Gag-Pol (p160)

polyproteins and newly synthesised viral RNA molecules are also recruited to lie in close proximity to the cell membrane and associate with the new immature budding virus particle. The matrix protein has been suggested to target Gag to the plasma membrane and incorporate Env glycoproteins into the budding virions (Yu et al., 1992, Freed et al., 1994). The new immature virus particle buds from the cell membrane and viral maturation occurs, where the viral protease cleaves the larger Gag and Gag-Pol polyprotein precursors into the HIV-1 Gag structural components (capsid, matrix and nucleocapsid). These then assemble to produce a mature HIV-1 virion capable of viral spread and infecting other cells (Konvalinka et al., 2015).

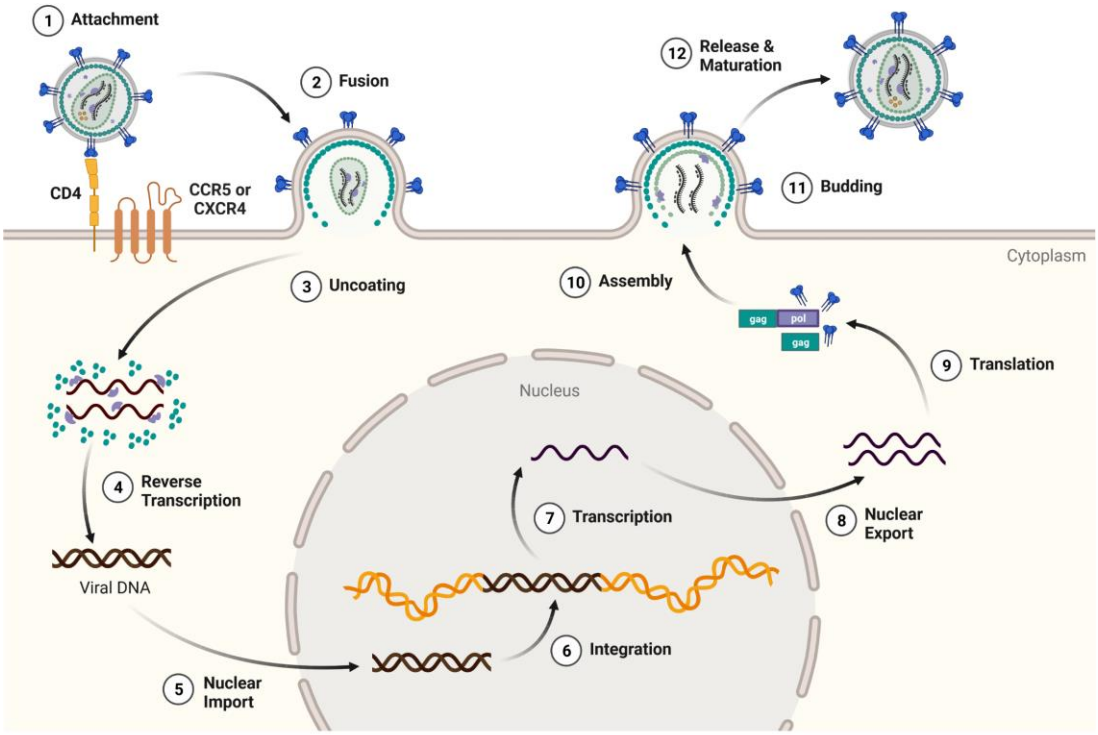


Figure 1.2. **The viral replication cycle of HIV-1 in CD4+ T cells**. HIV-1 Env first interacts with the host CD4 receptor aided by the CCR5 (depicted) or CXCR4 co-receptor. Conformational changes in HIV-1 Env bring the viral and host cell membranes within close proximity to allow fusion to occur and virion contents are injected into the cell, including the HIV-1 RNA genome. After capsid uncoating, viral RNA is reverse transcribed into cDNA by the virally encoded reverse transcriptase for integration of viral DNA into the host genome. The host cell then transcribes and translates viral genetic information alongside host genes. Viral assembly occurs in the cytoplasm and new immature virions are assembled at the cell membrane. After budding from the host cell membrane, the viral particle matures to form newly infectious HIV-1 virions. Illustration created with BioRender.com. Adapted from Engelman and Cherepanov, 2012.

1.3. HIV-1 Disease Progression

The rate of HIV-1 disease progression varies among individuals living with the virus, but the pattern is relatively consistent. After initial transmission of the virus, HIV-1 targets and begins to replicate in CD4-expressing T cells (CD4+ T helper cells). HIV-1 viral load (VL), measured in the number of HIV-1 RNA copies per millilitre of blood (copies / mL), peaks during acute infection only two to four weeks after the initial transmission event. During this acute phase of infection, there is a substantial decrease in CD4+ T cell count as the virus replicates. The person infected with HIV-1 undergoes seroconversion and the initial generation of anti-HIV-1 cellular and humoral immune response is associated with a reduction in HIV-1 VL and a partial recovery of CD4+ T cell counts. As HIV-1 integrates into the host genome and establishes a chronic latent infection, the level of HIV-1 RNA detectable in the blood remains relatively constant. This is referred to as the set point VL (spVL) and has been found to be positively associated with the rate of HIV-1 disease progression (Fraser et al., 2007). The rate of disease progression and the number of years a person remains latently infected varies, but the majority of people living with HIV-1 remain in this latent phase of infection for five to ten years (Pantaleo and Fauci, 1996). The latent phase of untreated HIV-1 infection lasts until CD4+ T cells are depleted below 200 cells per cubic millimetre of blood (cells / mm^3 or cells / µL) signalling the onset of AIDS. At this stage, immune dysregulation secondary to HIV-1 replication and the destruction of CD4+ T cells results in rapid HIV-1 viral replication, the inability to combat opportunistic infections and cancers and ultimately, death.

HIV-1 infected individuals who display extreme disease phenotypes such as spontaneous control of infection, long-term non-progression (LTNP) of disease or rapid disease progression are more commonly described for HIV-1 infected adults compared to their paediatric counterparts. The terminology used to describe adult non-progressors is varied

and extensive, but not consistent in the literature. Adult LTNPs have been described as ART-naïve patients who maintain CD4+ T cell counts above 500 cells / µL for up to ten years (Okulicz et al., 2009, Mandalia et al., 2012). Elite controllers (EC) of HIV-1 are prevalent in less than 1% of HIV-1 infected adults and generally defined as patients with an undetectable HIV-1 plasma VL for at least one year without ART intervention (Okulicz et al., 2009, Deeks and Walker, 2007, Yang et al., 2012). Viraemic controllers are described similarly to ECs, but maintain detectable levels of HIV-1 RNA in the blood lower than 2,000 copies / mL (Okulicz et al., 2009, Walker, 2007, Emu et al., 2008).

Studying the immunological and genetic markers in patients who display extreme phenotypes of HIV-1 disease provides an opportunity to try and understand the immune response against HIV-1 and the host-pathogen interactions that lead to slow or rapid progression of HIV-1 infection and disease.

1.4. HLA Class I Peptide Presentation to CD8+ T cells Influences HIV-1 Disease Progression

Cohorts of people living with HIV-1 (PLWH) who exhibit different rates of disease progression are invaluable to study in an attempt to identify the genetic and immunological correlates of delayed or rapid HIV-1 disease. The human leukocyte antigen (HLA) class I locus on chromosome 6 was the first genetic factor identified as being associated with the rate of HIV-1 disease progression. HLA class I molecules present endogenous antigen on the surface of infected cells to CD8+ cytotoxic T lymphocytes (CTLs) (Figure 1.3).

The TCR on CD8+ T cells is recognised by peptide-bound HLA class I molecules expressed at the cell surface. T cells develop tolerance for self-antigens presented on HLA class I molecules in the thymus. T cells with sufficient affinity for self-HLA molecules are positively selected for in the thymus, but negatively selected if they recognise self-antigen, to avoid the generation of autoreactive CD8+ T cells (reviewed by (Klein et al., 2014)). HLA class I is ubiquitously expressed throughout the body and across cell types. The TCRα TCRβ complex on the surface of CD8+ T cells is in complex with the CD3 heterodimers CD3δε, CD3γε and the CD3 homodimer CD3ζζ (Figure 1.3) (Ashwell and Klusner, 1990). Upon TCR recognition of antigenic HIV-1 peptides presented by HLA class I, CD8+ CTLs release perforin and granzymes: cytotoxic granules that permeate the cell membrane and induce apoptosis of the infected target cell. CD8+ CTLs also release inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) to promote an anti-viral immune state.

Robust CD8+ T cell responses in HIV-1 infection have been associated with a reduction in HIV-1 viraemia in acute infection and protection against HIV-1 infection in

persistently exposed but uninfected sex workers in Nairobi and The Gambia (Rowland-Jones et al., 1998a, Rowland-Jones et al., 1995, Rowland-Jones et al., 1998b, Ndhlovu et al., 2015, Koup et al., 1994, Goonetilleke et al., 2009). CTL activity has also been associated with spontaneous immune control of HIV-1 viraemia in untreated patients and is associated with control of SIV infection in non-human primates (Oxenius et al., 2004, Hersperger et al., 2011, Migueles et al., 2002, Migueles et al., 2008, Monel et al., 2019, Matano et al., 1998, Schmitz et al., 1999, Jin et al., 1999).

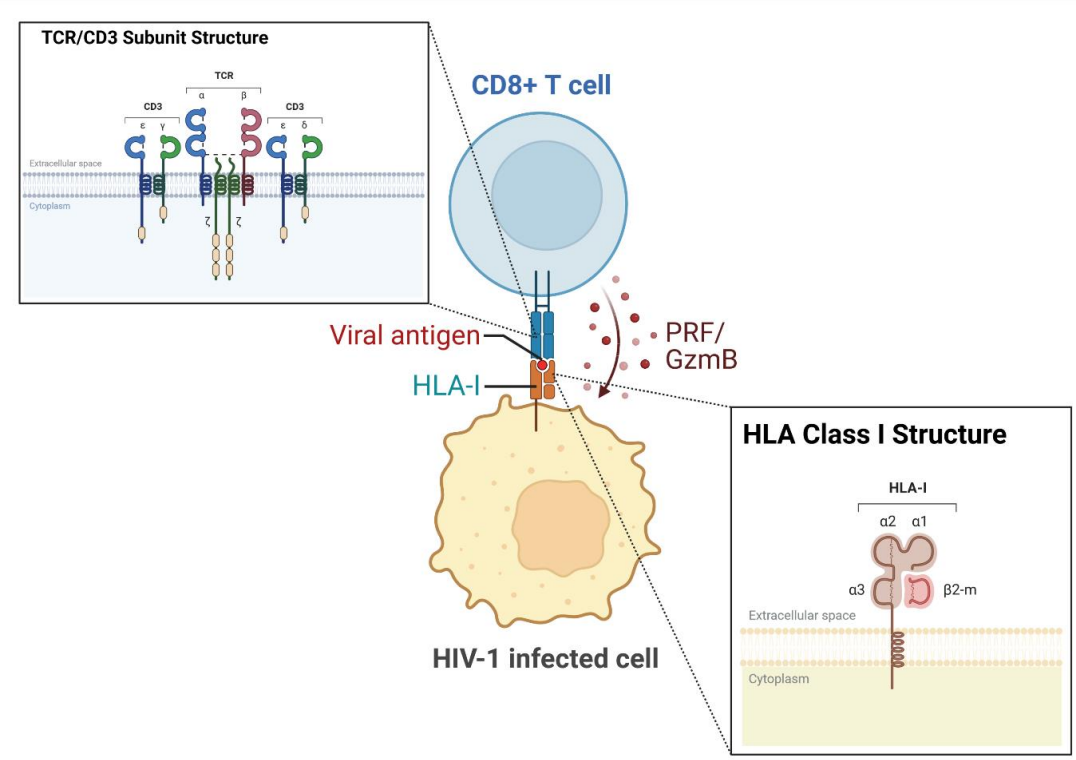


Figure 1.3. CD8+ T cell killing of HIV-1 infected cells. Simplified illustration showing the interaction between the cytotoxic CD8+ T cell and HIV-1 infected CD4+ cell expressing viral antigen on HLA class I molecules. TCR signalling after recognition of foreign antigen signals the release of perforin (PRF) and granzymes (GzmB) to induce apoptosis of the virally infected cell. The TCR/CD3 subunit structure on CD8+ T cells is shown in blue (left) and the structure of the HLA class I molecules complexed with β2-microglobulin (β2-m) is shown in yellow (right).

Common immunogenetic association of the HLA class I locus with control of HIV-1 disease in genome-wide association studies (GWAS) supports the role of the CTL response in the control of HIV-1 infection (Fellay et al., 2007, Pereyra et al., 2010, McLaren et al., 2015). HLA-B*57:01 has been consistently associated with protection from HIV-1 pathogenesis and delayed disease progression (Kaslow et al., 1996, Migueles et al., 2000, Fellay et al., 2007, McLaren et al., 2015, Arora et al., 2019). HLA-B*57 positive patients with long term suppression of HIV-1 VL in plasma were shown to be capable of generating *de novo* T cells responses against B*57 Gag epitope escape mutants, eliciting superior responses against HIV-1 evolution *in vivo* (Bailey et al., 2006). HLA class I allele association with HIV-1 resistance or susceptibility is ethnically distinct (Rowland-Jones et al., 1995, Rowland-Jones et al., 1998b, Fabio et al., 1992, Cao et al., 2001). The protective HLA-B*57:01 allele is virtually absent in African populations, whereas another member of the same B58 supertype, HLA-B*57:03, is strongly associated with HIV-1 control and slow disease progression in African and African-American HIV-1 infected individuals (Pelak et al., 2010, Lazaryan et al., 2006, Shrestha et al., 2009, Tang et al., 2002). Lesser but still significant contributions of HLA-B*39:10, HLA-A*30-Cw*03 and HLA-B*15:17 (also of the HLA B58 supertype) have also been associated with HIV-1 control in African populations (Pelak et al., 2010, Tang et al., 2002).

Shepherd and colleagues (2015) showed HLA-C*08:02 and HLA-C*08:04 as being significantly enriched in adolescent long-term survivors of perinatally-acquired HIV-1 infection from the Teenaids project in Harare, Zimbabwe compared to age and ethnically matched HIV-1 uninfected adolescents. The major limitation of this study was the absence of a vertically-infected group with rapid progression to HIV-1 disease. Rapidly progressing children and adolescents are hard to identify and study, but in the absence of

this group ethnicity-matched HIV-1 uninfected population controls are useful to model the population-wide distribution of HLA class I alleles. Using these HIV-1 uninfected control patients, the relative enrichment of HLA class I alleles in adolescent long-term survivors that may have likely contributed to the control of HIV-1 infection can be inferred. This study identified a significant enrichment of HLA–A*02:01, HLA-A*34:02 and HLA-B*58:02 in the HIV-1 uninfected control group. HLA-B*58:02 has previously been reported to be associated with increased HIV-1 spVL and rapid HIV-1 disease progression in Zambian and Rwandan children, respectively, and the HLA-A*02-Cw*16 haplotype was most significantly associated with higher subtype-C HIV-1 viral load in Zambian adults (Tang et al., 2002, Lazaryan et al., 2006). Although inferential, these findings imply that uninfected age and ethnicity-matched controls are the best-case comparison group in the absence of genetic data from rapid progressors.

Protective HLA class I alleles present epitopes from conserved HIV-1 viral proteins which are mutationally-constrained and functionally costly for the virus to mutate to escape HLA restriction (reviewed by (Kloverpris et al., 2015)). For example, the epitope regions of the HIV-1 genome that are restricted by protective HLA class I alleles HLA-B*57 and B*27 stimulate immunodominant CTL responses against conserved Gag epitopes essential for capsid assembly (Altfeld et al., 2003, Schneidewind et al., 2007, Leslie et al., 2004, Goulder et al., 1996, Gillespie et al., 2002, Kelleher et al., 2001).

HLA class I protection is also associated with the ability to present a broader repertoire of the HIV-1 peptidome. HLA heterozygous individuals are able to present a quantitatively and qualitatively broader array of HIV-1 epitopes to CD8+ T cells which is correlated with lower HIV-1 spVLs (Carrington et al., 1999, Arora et al., 2019). HLA allele heterozygosity can be measured as a binary factor (0: homozygous or 1: heterozygous) or on a continuous scale by measuring the total difference in amino acid

residues between each allele of a heterozygous pair. There is a negative association between heterozygosity at the HLA-B locus and HIV-1 spVL (Arora et al., 2019). HIV-1 disease progression is associated with many properties of HLA class I and warrants even further investigation to help guide anti-HIV vaccine and therapeutic strategies.

1.5. Peptide Loading of HLA Class I by the PLC

Loading of peptides onto HLA class I molecules is mediated by the peptide loading complex (PLC) in the endoplasmic reticulum (ER) (Figure 1.4). The HLA class I complex is made up of a transmembrane-spanning heavy chain non-covalently bound to β2-microglobulin (β2-m). The nascent HLA class I heavy chain is synthesised directly into the ER, where it is initially stabilised by the ER chaperone proteins immunoglobulin-binding protein (BiP) and calnexin (Koch and Tampe, 2006, Nossner and Parham, 1995). BiP is then released from the HLA class I heavy chain and β2-m binds to form the HLA class I heterodimer complex. The ER chaperone calreticulin replaces calnexin and the HLA class I molecule is incorporated into the PLC. Calreticulin and calnexin are lectin-like chaperone proteins that bind to conformational intermediates during protein folding in order to promote efficient assembly of proteins and discourage ER export of misfolded proteins (Jackson et al., 1994, Ou et al., 1993, Peterson et al., 1995).

The proteasome is largely responsible for degrading endogenous proteins in the cytosol. (Koopmann et al., 1996). The transporter associated with antigen processing (TAP) translocates degraded protein peptides to the lumen of the ER. The functional TAP heterodimer is an ATP binding cassette (ABC) composed of the TAP1 and TAP2 subunits. Each subunit of the TAP heterodimer has a C-terminal cytosolic nucleotide-binding domain responsible for ATP binding, a core transmembrane spanning six helix domain and a further N-terminal transmembrane domain with four transmembrane helices (Lehnert and Tampe, 2017). Peptides bind to TAP in an ATP-dependent manner and the isomerisation of the TAP complex triggers the translocation of peptides across the ER membrane into the lumen (Gorbulev et al., 2001, Oancea et al., 2009). TAP-deficient cells have critically low concentrations of peptide in the ER which correlates with inefficient peptide loading of the HLA class I complex and instability of the class I

molecule at the cell surface (Paulsson et al., 2001, Aladin et al., 2007). Nevertheless, TAP-independent peptide presentation on HLA class I is possible, albeit inefficient and associated with diseases such as recurrent respiratory infections, chronic granulomatous disease and bare lymphocyte syndrome type I (BLS I) (Del Val et al., 2020, Shrestha et al., 2012, Law-Ping-Man et al., 2018).

The TAP transporter is bridged to HLA class I molecules by the ER-resident chaperone protein tapasin to form a functional PLC. Each TAP heterodimer is associated up to with four tapasin:HLA class I complexes (Ortmann et al., 1997). By bridging TAP to HLA class I molecules, tapasin ensures that HLA class I can access the pool of TAP-translocated peptides. HLA class I molecules expressed in the tapasin-deficient lymphoblastoid cell line (LCL) 721.220 do not associate with the TAP transporter, egress faster from the ER and are expressed at the cell surface at lower levels compared to when tapasin is exogenously expressed in the same cell line (Grandea et al., 1995, Grandea et al., 1997). This association of TAP with multiple HLA class I molecules is suggested to increase the likelihood that ER translocated peptides encounter a suitable HLA class I molecule to which they can bind.

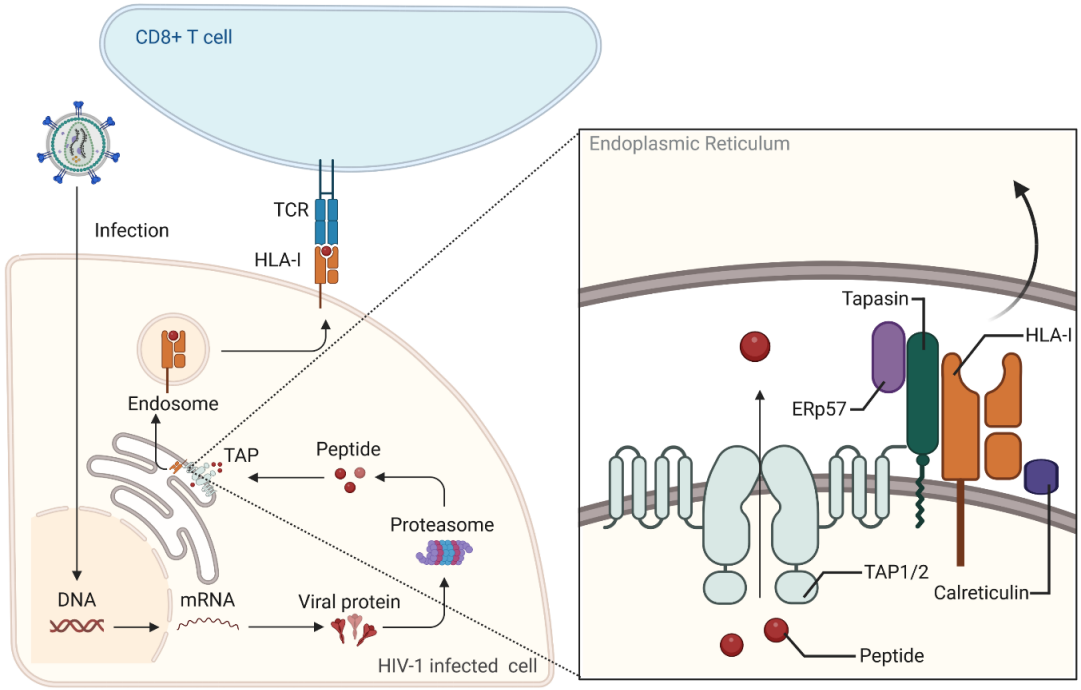


Figure 1.4. **Peptide loading on HLA class I molecules by the peptide loading complex (PLC) in the endoplasmic reticulum (ER).** HIV-1 infection is shown simplified from the stages outlined in Figure 2. The left hand panel shows a simplified schematic of HIV-1 protein degradation by the proteasome in the cytosol into peptides which are shuttled into the ER by the TAP1/TAP2 heterodimer (light blue). The right hand panel shows a more detailed schematic of the PLC in the ER. HLA class I (orange) molecules are shuttled to the PLC by calnexin (not shown) and calreticulin (dark purple), TAP1/2 is bridged to HLA class I by tapasin (green), aided by ERp57 (light purple). Tapasin holds open the peptide binding groove of HLA class I in a peptide receptive state and when peptide of sufficient affinity has bound, HLA class I molecules egress to the cell surface for presentation to CD8+ T cells. Illustration created withBioRender.com.

1.5.1. Quality control of peptide loading on HLA class I molecules by tapasin

Tapasin is a 48 kDa protein encoded by the *TAPBP* gene on chromosome 6 (Sadasivan et al., 1996, Jackson et al., 1990, Herberg et al., 1998). Tapasin is a type I transmembrane protein encoded across eight exons (Herberg et al., 1998). The N-terminal domain of tapasin is located in the ER lumen whilst the protein has a single transmembrane spanning domain and a cytosolic C-terminal domain (Sadasivan et al., 1996, Dong et al., 2009). The N-terminal domain of tapasin is a seven-stranded β-barrel (β-strands S1-S7) with an immunoglobulin (Ig)-like fold (β-strands S8-S14) that interacts with residues 128 to 136 of the α2-1 helix of the HLA class I peptide binding groove (Dong et al., 2009, Lewis et al., 1996, Yu et al., 1999). A second interaction point between tapasin and HLA class I is predicted between the C-terminal Ig-like domain transmembrane/cytosolic domain of tapasin and residues 222 to 229 of the HLA class I α3 domain (Kulig et al., 1998, Suh et al., 1999). This secondary interaction of tapasin with HLA class I was shown to be critical for tapasin facilitating assembly of the mouse HLA class I homologue H-2Kd and H-2Ld molecules (Simone et al., 2012a, Turnquist et al., 2004b). The C-terminal cytosolic domain has a di-lysine ER retention motif that ensures its compartmentalisation to the ER (Paulsson et al., 2006). The C-terminal domain of tapasin interacts with the N-terminal accessory domain of the TAP1 and TAP2 subunits (Dong et al., 2009, Raghuraman et al., 2002, Procko et al., 2005, Leonhardt et al., 2014). Tapasin does not change TAP's functionality in its selectivity for peptides or the rate of peptide translocation into the ER (Howarth et al., 2004, Bangia et al., 1999). Tapasin instead maximises the time that HLA class I is exposed to peptides in the ER by increasing the steady-state levels of TAP and enhancing HLA class I retention (Garbi et al., 2003).

Tapasin is an interferon stimulated gene (ISG) (Abarca-Heidemann et al., 2002). The 5' UTR promoter region of *TAPBP* contains IFN-γ activated sequence (GAS) motifs and an

IFN-response element (ISRE) and is upregulated by type-I and type-II interferon (IFN) stimulation (Abarca-Heidemann et al., 2002, Herrmann et al., 2003). Exogenous IFN-γ stimulation of murine cells has been shown to significantly improve antigen presentation on HLA class I, which correlated with a significant upregulation in the expression of tapasin, TAP1, TAP2 and HLA class I mRNA levels (Boulanger et al., 2022). Tapasin transcript and protein levels have been shown to be affected by β_2-m and calreticulin deficiency in the cell, indicating that tapasin stability is dependent on other protein members of the PLC (Del Cid et al., 2010, Simone et al., 2012b).

Tapasin does not contact the peptide directly. The N-terminus of tapasin has been shown to interact with the β-sheet forming the groove's floor and cradles the alpha helices of the HLA class I peptide-binding groove (Fisette et al., 2016a). Tapasin cradles α2-1 helices of the HLA class I binding groove and pulls on the β7-8 strands supporting the α2 helix (Fleischmann et al., 2015). This pulls the α1 and α2 helices outward and widens the binding groove by up to 2Å (Fleischmann et al., 2015, Fisette et al., 2016a). Through these interactions tapasin stabilises the HLA class I binding groove into a wide peptide-receptive open conformation state (Fisette et al., 2016a, Dong et al., 2009, Kanaseki et al., 2013).

Tapasin exerts a qualitative control over the peptide repertoire presented by HLA class I molecules. Tapasin catalyses the dissociation of peptides with low affinities and fast off-rates from HLA class I and promotes the exchange for a higher affinity, immunodominant peptides (Chen and Bouvier, 2007, Elliott and van Hateren, 2018, Fisette et al., 2016a, Praveen et al., 2010, Wearsch and Cresswell, 2007). Tapasin and incoming peptides compete and engage in a "molecular tug-of-war" over the opening and closing of the peptide-binding groove, competing with peptide for binding (Fisette et al., 2016a, Schneeweiss et al., 2009). Positions 2 and 9 of the peptide are essential anchors for stable

binding to the B and F pockets of the binding groove, respectively (reviewed by (Nguyen et al., 2021)). The F-pocket accommodates the C-terminal residue (PΩ) of the incoming presented peptide whereas the B-pocket is occupied by the anchor residue at position 2 (P2) of the peptide. Contact between the C-terminus of a high-affinity peptide and the F-pocket of HLA class I exerts an opposing force on the α2-1 helix to close the binding groove and allow egress of HLA class I to the cell surface (Fisette et al., 2016a). Tapasin functions to weaken these binding attractions between the C-terminus of an incoming peptide and the F-pocket of the binding groove, accelerating the release of lower affinity peptides (Fisette et al., 2017). Therefore a peptide with sufficiently high binding affinity is needed to surpass the forces exerted by tapasin on the F-pocket and close the binding groove to initiate peptide-bound HLA class I trafficking from the ER to the cell surface for presentation to CD8+ T cells (Fleischmann et al., 2015, Fisette et al., 2016a).

Tapasin increases the stability and promotes the presentation of peptides with a slower rate of decay on HLA class I molecules for both tapasin-dependent and tapasin–independent class I alleles (Barnden et al., 2000, Williams et al., 2000, Zarling et al., 2003, Boulanger et al., 2022). Increased stability of HLA class I molecules at the cell surface is associated with increased binding affinity and decreased off-rate of the presented peptide (Fahnestock et al., 1992). Studies *in vitro* have shown that HLA class I favourably presents high affinity peptides when loaded in complex with tapasin and TAP (Williams et al., 2002, Tan et al., 2002). Increased stability of the peptide bound HLA class I complex presented at the cell surface has been associated with governing the immunodominance hierarchy of the CD8+ T cell response (Galea et al., 2012, Howarth et al., 2004).

As tapasin expression increases, HLA class I molecules bound to incoming peptide experience greater flux through the PLC loading pathway (Bailey et al., 2015). Increasing

levels of tapasin expressed in the cell influences the degree of filtering of the peptide repertoire that is to be presented. As such, with greater tapasin expression a much less diverse and selectively optimised peptide cargo is presented by HLA class I. Recently, Boulanger and colleagues showed *in vitro* that peptides presented on HLA class I molecules have different optimal levels of tapasin expression, depending on peptide abundance and peptide stability (Boulanger et al., 2022). Immunodominant CD8+ T cell responses in acute HIV-1 infection were associated with the stable presentation of highly abundant Gag peptides on HLA class I molecules (Kaseke et al., 2021). Increased tapasin expression preferentially selects for the presentation of a narrower repertoire of high affinity peptides at the expense of a more diverse range of peptide ligands, including those of low affinity (Boulanger et al., 2022). In viral infection or disease scenarios where tapasin is targeted for downregulation or degradation, favouring the presentation of abundant but low affinity peptides at optimal tapasin expression levels may be a valid strategy for anti-viral and anti-cancer vaccine design.

It has been observed that tapasin-deficient mice elicit CD8+ T cells responses favouring subdominant peptides with fast off-rates and decreased stability (Boulanger et al., 2010, Garbi et al., 2000, Grandea et al., 2000). Tapasin-deficient mice also show reduced thymic selection of CD8+ T cells and an 80% reduction in total peripheral CD8+ T cells (Garbi et al., 2000, Grandea et al., 2000). Reduced tapasin expression was also associated with impaired cross-presentation of peptides by dendritic cells in mice (Garbi et al., 2000).

Tapasin is covalently bound at its N-terminus to the protein disulphide isomerase (PDI) A3 (PDIA3), also known as ERp57, in the ER (Dick et al., 2002, Dong et al., 2009, Peaper et al., 2005). ERp57 and tapasin are linked via a disulphide bond between the cysteine residues 57 on ERp57 and 95 on tapasin (Dick et al., 2002). The disulphide bond between ERp57 and tapasin is suggested to have a role in stabilising the PLC (Dick et al., 2002,

Dong et al., 2009). Removal of the interaction between ERp57 and tapasin has been shown to result in the downregulation of HLA-B*44 alleles, but no further evidence for this observation in other HLA class I allotypes has been described (Dick et al., 2002).

The TAP-binding protein related (TAPBPR) is a more recently identified additional member of the peptide loading quality control machinery. TAPBPR has 22% sequence homology with tapasin, but is not physically associated with tapasin, ERp57 or calreticulin in the ER (Teng et al., 2002, Boyle et al., 2013). TAPBPR has been shown to associate with HLA class I and β_2-m and promote retention of HLA class I in the ER for prolonged association with the PLC (Boyle et al., 2013, Hermann et al., 2015a, Hermann et al., 2015b). TAPBPR binds to a loop below the $\alpha_{2\text{-}1}$ helix and a β-strand of the α3 domain of HLA class I, similar to tapasin (Jiang et al., 2017, Thomas and Tampe, 2017). TAPBPR is suggested to have a similar function as tapasin in the ER to promote high affinity peptide binding to HLA class I (Hermann et al., 2015b, Jiang et al., 2017, Morozov et al., 2016, Thomas and Tampe, 2017). Therefore, it is important to consider TAPBPR effects in experiments studying the effect of tapasin-deficiency on HLA class I cell surface expression and peptide loading, especially as evidence suggests that TAPBPR association with HLA class I is increased *in vitro* when tapasin is knocked out (Hermann et al., 2013).

1.6. HLA Class I Tapasin-Dependency

Of the proteins expressed in the ER as the PLC, HLA class I expression is most closely linked to tapasin (Thuring et al., 2015, Belicha-Villanueva et al., 2008). An interesting cellular phenomenon is the differential dependency of HLA class I allotypes on tapasin for their ability to load peptide and be expressed at the cell surface (Barber et al., 2001, Barnden et al., 2000, Beissbarth et al., 2000). HLA class I molecules such as HLA-A*1, HLA-B*8, HLA-A*30:01 and HLA-B*44:02 are retained in the ER rather than egressing to the cell surface in the absence of tapasin and are deemed "tapasin-dependent" (Greenwood et al., 1994, Park et al., 2003). In contrast, tapasin-independent class I molecules such as the HLA-A*3 and HLA-B*7 families, HLA-A*02:01, HLA-B*44:05 and HLA-B*2705 do not require tapasin for their normal cell surface expression (Greenwood et al., 1994, Barber et al., 2001, Barnden et al., 2000, Grandea et al., 1997, Lewis et al., 1998).

Tapasin-independent HLA class I alleles have an increased intrinsic stability of the peptide binding groove compared to tapasin-dependent alleles (Chen and Bouvier, 2007, Elliott and van Hateren, 2018). Tapasin-independent alleles have a faster conversion rate between the open and closed conformational states of the peptide-binding groove, allowing them to filter the peptide repertoire more efficiently without the help of tapasin. Computational analyses of the biochemical properties and spatial arrangement of amino acids in the peptide binding groove of tapasin-independent HLA class I alleles show that they hold the groove open in a more naturally peptide-receptive state and do not require tapasin to allow peptides to efficiently bind (Ozbek, 2016).

Polymorphisms in the α1 and α2 domains of the HLA class I heavy chain making up the peptide binding groove have been suggested to be associated with the phenomenon of HLA class I allele dependency on tapasin (Blanco-Gelaz et al., 2009, Beissbarth et al.,

2000). In particular, residue 116 of the F-pocket in the peptide-binding groove is one of the four residues (alongside 77, 80 and 81) that are considered key for PΩ specificity and has been associated with changes in HLA class I dependence on tapasin (Blanco-Gelaz et al., 2009, Neisig et al., 1996). HLA-B*44:02 and HLA-B*44:05 differ only at residue 116, expressing aspartic acid and tyrosine respectively. Despite this solitary polymorphism, HLA-B*44:02 and HLA-B*44:05 display vastly different phenotypic dependencies on tapasin for their normal cell surface expression (Bashirova et al., 2020, Park et al., 2003). The hydrophobic tyrosine residue expressed at position 116 in HLA-B*44:05 is associated with reduced energy fluctuation of the F-pocket of the peptide-binding groove and stabilise the C-terminus of an incoming peptide (Fisette et al., 2017, Ozbek, 2016, Fisette et al., 2016a). This has been suggested to allow HLA-B*44:05 to adopt a more intrinsically stable and peptide-receptive conformation state (Sieker et al., 2007, Ozbek, 2016).

The evidence to suggest that residue 116 is associated with differences in HLA class I tapasin-dependency is not definitively or solely explanatory. HLA-B*44:02, B*27:05 and A*03:01 all display phenotypic differences in their dependency on tapasin, but encode aspartic acid at position 116 of the HLA class I heavy chain (Park et al., 2003). However, it has been noted that tapasin-independent HLA class I alleles HLA-B*27:05 and HLA-B*27:02 both encode histidine at position 114, whilst the tapasin-dependent HLA-B*44:02 encodes aspartic acid (Park et al., 2003, Peh et al., 1998). Increased acidity of the amino acid residue encoded at position 114 of the D-pocket of the peptide-binding groove has also been associated with higher tapasin-dependency of the HLA class I allele (Park et al., 2003). The D-pocket of the peptide binding groove binds secondary anchor residues at positions 3, 5 or 6 of the peptide (Nguyen et al., 2021). Acidic amino acids with negatively charged side chains have been suggested to cause a steric hindrance to

incoming peptide binding and therefore require tapasin to open the binding groove (Blanco-Gelaz et al., 2009).

Despite the capacity of tapasin-independent HLA class I to be expressed at more normal levels at the cell surface in the absence of tapasin, tapasin is not redundant in its function promoting the loading of high affinity peptides. Tapasin-independent HLA class I alleles that are expressed at the cell surface in the absence of tapasin egress have been found to be bound to low affinity peptides or no peptide at all, limiting the immune response that can be mounted against foreign antigens (Lewis et al., 1998)(Lewis et al., 1998). HLA-A*02:01 and HLA-B*27:05 are both expressed at similar levels at the surface of cells both in the presence and the absence of tapasin. However, in tapasin-deficient cells both alleles were found to present lower affinity peptides and recycle from the cell surface faster compared to when tapasin is expressed (Barber et al., 2001, Purcell et al., 2001). This evidence suggests that the expression of tapasin exerts a qualitative effect over the peptide repertoire presented, even when quantitative changes in cell surface expression are not observed for tapasin-independent HLA class I alleles. Purcell and colleagues (2001) also showed that CD8+ T cell responses could discriminate between the peptide repertoire presented by HLA-B*27:05 alleles in cell systems that were tapasin-proficient or deficient, respectively. These findings further indicate the influence of tapasin on T cell-specific responses aside from its impact on HLA class I expression alone.

1.7. The PLC is an Intracellular Target for Dysfunction in Disease

1.7.1. Tapasin and PLC downregulation in cancer

Tapasin-independent HLA class I alleles may have evolved in response to selective pressure from pathogens and tumorigenesis. Tapasin and other components of the PLC have been found to be downregulated or disrupted in multiple different tumour types and in viral infections. Inadequate presentation of endogenous foreign peptides to CD8+ T cells can have a significant impact on disease progression and outcome.

Tapasin is more commonly found to be downregulated in tumour cells over other members of the PLC. Firstly, tapasin protein expression measured *ex vivo* has been found to be downregulated in colorectal tumours, squamous cell carcinoma, melanoma, lung carcinoma, renal cell carcinoma, adenocarcinoma, neuroblastoma and bladder cancer (Cabrera et al., 2005, Ogino et al., 2003, Seliger et al., 2003, Lou et al., 2005, Atkins et al., 2004, Raffaghello et al., 2005). Decreased tapasin expression has been associated with increased neoplasticity, faster disease progression and poor prognoses (Dissemond et al., 2003, Ogino et al., 2003, Bandoh et al., 2010). Decreased levels of TAP and the LMP2/LMP7 subunits of the immunoproteasome have also been found in cancer cells studied *ex vivo* (Romero et al., 2005).

Studies of PLC expression from patient tumour cells examined *ex vivo* is limited by the post-hoc nature of the analyses performed on archived formalin-fixed paraffin-embedded (FFPE) tissue. This can result in small case sample sizes that lack sufficient statistical power to detect changes compared to healthy tissue. These studies are also limited in that they often fail to report - or do not know - the age, sex or ethnicity of tested samples. Furthermore, studies of the expression of tapasin in tumour cells *ex vivo* are often conducted using immunohistochemistry (IHC) staining protocols for proteins in the PLC.

These experiments are limited by largely qualitative data analysis techniques. Applying more robust quantitative molecular methods to study changes in tapasin expression in different disease scenarios would provide greater analytical power to decipher the role that tapasin and the PLC plays in disease progression and outcome.

In addition to the differences in PLC expression observed in clinical tumour samples, coordinated downregulation of components of the PLC is also evident in tumour-derived immortalised cell lines. A number of immortalised cell lines derived from small cell lung carcinoma (SCLC), pancreatic carcinoma, colon carcinoma or murine melanoma show decreased expression of TAP1, TAP2, tapasin or the LMP2/LMP7/LMP10 subunits of the immunoproteasome, as well as simultaneous downregulation of multiple components in many of these tested cell lines (Delp et al., 2000, Seliger et al., 2001b). These findings may be due to impaired antigen processing machinery in cancer cells, or may be due to the method of cell line immortalisation employed, as transformation by Epstein-Barr virus (EBV) may aberrantly reduce PLC expression levels, as EBV protein BNLF2a prevents peptide and adenosine triphosphate (ATP) binding to TAP (Hislop et al., 2007, Horst et al., 2009). A small study of 36 Japanese patients with nasopharyngeal carcinoma showed tapasin to be significantly downregulated in cancerous lesions that were also infected with EBV (Ogino et al., 2007). Whether the downregulation of tapasin in these cells was due to dysfunction during tumorigenesis or EBV infection was not established. The functional mechanism behind reduced PLC expression in tumour cells has been examined in a number of studies. Little evidence suggests that functional defects in the tapasin protein itself might be associated with decreased expression in cancer cells.

Human tapasin has limited polymorphism (Williams et al., 2000). However a substitution of tyrosine for aspartic acid at position 59 of exon 2 and a G to A substitution in intron 2 of tapasin was associated with increased susceptibility to human papillomavirus (HPV)

type 16-associated cervical cancer (Deshpande et al., 2008). No functional consequences from the polymorphism in exon 2 have been reported, but it is feasible that this nonsynonymous mutation change in the N-terminal ER luminal domain affects HLA class I binding and interrupts tapasin's function to optimise the antigen presentation of tumour epitopes. Tapasin expression in tumour cells can be restored by interferon alpha (IFN-α), IFN-γ, TNF-α and interleukin-4 (IL-4) stimulation *in vitro* and *ex vivo* (Seliger et al., 2001a, Seliger et al., 2001b, Corrias et al., 2001, Delp et al., 2000). Tapasin was undetectable in Panc02 pancreatic tumour cells and this was not rectified even after IFN-γ stimulation, suggesting a transcriptional defect in this cell line (Turnquist et al., 2004a). An investigation of murine tumour cells overexpressing the cancer-associated protein HER2/neu revealed that downregulation of the tapasin gene was partially attributable to the binding of the transcriptional repressor ER2F-1 (Bukur et al., 2010). The evidence available therefore suggests that tapasin downregulation in disease may result from transcriptional rather than structural defects.

1.7.2. Tapasin downregulation and polymorphisms is associated with other non-tumorigenic diseases

Tapasin dysfunction and downregulation has also been described in other disease models aside from cancer. Tapasin was found to be downregulated in patients with poor outcomes in a cohort with chronic myeloproliferative neoplasms (MPN) (Skov et al., 2013). Bare lymphocyte syndrome type I (BLS I) is characterised by the lack of HLA class I expression at the cell surface and manifests as an inability to mount an immune response against invading pathogens. BLS I is usually associated with TAP-deficiency, but a single patient was identified by Yabe *et al.* in 2002 who instead had a four exon deletion of exons 4 to 7 of *TAPBP* (Yabe et al., 2002). This association between BLS I and deletions

in *TAPBP* have not been investigated in other cases of the disease, but might indicate that significant structural deformities in tapasin could be associated with diseases characterised by changes in the cell surface expression of HLA class I molecules. Polymorphisms in *TAPBP* have also been found to be associated with aspirin-exacerbated respiratory disease (AERD) in an Asian population (Cho et al., 2013). This study took a biased approach and targeted five single nucleotide polymorphisms (SNPs) in the TAPBP gene to find a significant association between a C to G polymorphism in exon 4 coding for a variation of threonine to arginine at position 260 of tapasin. This variation at position 260 of exon 4 has been studied for its association with juvenile rheumatoid arthritis (JRA) and schizophrenia (Bukulmez et al., 2005, Fellerhoff and Wank, 2009). Expression of arginine at position 260 was both enriched in patients presenting with schizophrenia compared to controls and was associated with inheritance of JRA in a cohort of Caucasian children from the United States. Position 260 of tapasin is located in an Ig-like domain of tapasin, sterically close to an interaction site for ERp57 (Dong et al., 2009). The studies that observed an association of the variant at residue 260 of tapasin with AERD, JRA and schizophrenia failed to identify or explain a functional difference between the arginine and threonine alleles. There is no evidence in Caucasians that the variant at position 260 of exon 4 of tapasin is in linkage disequilibrium (LD) with any HLA-A, -B or -C or HLA class II allele (Williams et al., 2000), This indicates that the disease associations observed with this variant of tapasin were unlikely to be inadvertently identified due to linkage with protective/susceptible HLA class I genotypes.

The *TAPBP* intronic SNP rs2282851 was significantly associated with an increased risk for chronic rhinosinusitis (CRS) (Alromaih et al., 2013). Homozygotes for the rs2282851 T allele were associated with increased risk of eczema and higher levels of circulating IgE, in CRS, which the authors suggested might be associated with reduced clearance of

Staphylococcus aureus in these patients. It is unknown whether rs2282851 is associated with changes in tapasin transcript or protein expression.

SNPs in a AP-2α transcription factor (rs111686073) and microRNA (rs59097151) binding site of *TAPBP* were associated with increased tapasin mRNA expression and decreased prevalence of *Plasmodium falciparum* in Ugandan individuals (Walker-Sperling et al., 2022). These tapasin variants were associated with lower incidence of clinical malaria in the same Ugandan cohorts, especially in patients who expressed tapasin-dependent HLA class I alleles. These data suggest that intrinsically higher expression levels of tapasin may benefit antigen presentation and contribute to protection against malaria.

These data indicate that tapasin dysfunction is not solely the target for pathogenic infection or cellular tumorigenesis to avoid CD8+ T cell recognition of infected or cancerous cells. Changes in tapasin expression are indicated in a whole range of clinical scenarios and thus proves an interesting target to focus on in terms of its expression and function in a variety of different disease types.

1.7.3. Viral interference with the peptide loading complex

Viral pathogen interaction with and disruption of the PLC is well documented in the literature. Viruses have evolved host immune evasion strategies to exploit the PLC in order to avoid the presentation of viral antigens to CD8+ T cells. Studies have discovered a number of viral virulence factors that interrupt the function of tapasin and other ER chaperone proteins within the PLC. In tapasin-deficient mouse models, defects in viral antigen presentation have been shown to result in poor CD8+ T cell responses against

pathogens such as influenza, vaccinia virus, lymphocytic choriomeningitis virus (LCMV) and listeria monocytogenes (Grandea et al., 2000).

Different viral pathogens have evolved to interfere with TAP function and expression in order to avoid translocation of viral peptides to the ER for loading on HLA class I. Herpes simplex virus 1 (HSV1), EBV and human cytomegalovirus (HCMV) all encode proteins that work to reduce the available pool of peptides in the ER for HLA class I presentation to CD8+ T cells. As previously mentioned above, EBV protein BNLF2a prevents peptide and ATP binding to TAP, resulting in TAP being unable to access the energy requirements needed to translocate peptides into the ER for presentation on HLA class I molecules (Hislop et al., 2007, Horst et al., 2009). Similarly, HSV1 encodes protein ICP47 that has been shown to bind to and destabilise TAP *in vitro*, preventing peptide binding to TAP (Lacaille and Androlewicz, 1998, Hill et al., 1995, Fruh et al., 1995, Hill et al., 1994, Tomazin et al., 1996). HCMV also encodes protein US6 that binds TAP and arrests it in a structural state that cannot bind ATP, blocking peptide translocation similar to the function exerted by protein BNLF2a in EBV infection (Hewitt et al., 2001, Hengel et al., 1996, Lehner et al., 1997).

Viruses have also evolved to interact with and inhibit tapasin function in the ER to interfere with viral antigen presentation by HLA class I. HLA class I expression on adenovirus-infected cells is significantly reduced compared to uninfected cells (Burgert and Kvist, 1985). The virally encoded adenovirus type 2 E3 19 kDa protein (E19) possesses a di-lysine ER-retention motif in its cytoplasmic domain (Jackson et al., 1990). E19 binds the alpha helices of the HLA class I binding groove and disrupts tapasin bridging TAP to HLA class I, preventing class I molecules from correctly folding with peptides from the cytosol and trafficking to the cell surface (Flomenberg et al., 1992, Sester and Burgert, 1994, Sester et al., 2013, Bennett et al., 1999).

Like adenovirus protein E19, the glycoprotein US3 encoded by HCMV also contains an ER-retention motif in its luminal domain (Lee et al., 2003). HCMV US3 has been shown to inhibit tapasin expression at a pre-transcriptional level, blocking *de novo* synthesis of tapasin transcripts *in vitro* (Halenius et al., 2011). HCMV US3 preferentially downregulates tapasin-dependent HLA class I alleles and promotes the binding of low affinity peptides with fast off-rates from HLA class I at the cell surface (Park et al., 2004a, Zernich et al., 2004). Human poxvirus molluscum contagiosum virus (MCV) protein MC80 and γ2-Herpesvirus 68 protein mK3 have also evolved mechanisms to interfere with tapasin that result in the specific downregulation of the cell surface expression of tapasin-dependent HLA class I alleles (Harvey et al., 2019, Lybarger et al., 2003). These proteins have been found to exert their immunomodulatory effect on HLA class I by sequestering tapasin for degradation via the ubiquitin/proteasome pathway. The evidence in the literature indicates that undiscovered or understudied mechanisms of viral inhibition of tapasin and the PLC might be due to virally encoded proteins physically interacting with the host protein itself and blocking its function, or alternatively due to the mobilisation or interruption of host cellular transcriptional machinery. For example, viral proteins might possibly recruit natural transcriptional repressors of *TAPBP* such as PR domain zinc finger protein 1 (PRDM-1) (Doody et al., 2007).

Changes in tapasin expression and gene function have been described in viral infection. The expression of an alternate splice form of tapasin lacking residues 50 to 137 of exon 3 (tapasin- ΔEx3) was shown to be upregulated during the course of HCMV infection, in the place of WT tapasin transcripts (Halenius et al., 2011, Beutler et al., 2013). The tapasin-ΔEx3 was also found to be expressed in human melanoma cells (Belicha-Villanueva et al., 2010). Tapasin-ΔEx3 fails to interact with ERp57 and does not rescue tapasin-dependent HLA-B*44:02 cell surface expression in 721.220 cells (Beutler et al.,

2013). In addition, tapasin-ΔEx3 could not stabilise the expression of the tapasin-independent allele HLA-B*44:05, suggesting that significant structural alterations to tapasin have the potential to impact the peptide repertoire. As such, viral immune evasion mechanisms may have evolved the potential to avoid antigen presentation via the induction of less stable, dysfunctional isoforms of proteins making up the PLC.

PBMCs from patients with chronic hepatitis B virus (HBV) infection showed reduced tapasin mRNA expression compared to both healthy controls and patients with acute HBV infection (Tang et al., 2016). The CD8+ T cells from these chronically infected HBV patients showed signs of dysfunction and exhaustion with a high apoptotic ratio and decreased IFN-γ production. The authors did not clarify whether decreased tapasin expression might be a causative factor of the dysfunctional CD8+-mediated anti-HBV response observed in the study, or whether reduced tapasin expression might have been measured due to the loss of IFN-γ production from exhausted CD8+ T cells in chronic HBV infection.

Resolution of chronic hepatitis C virus (HCV) infection and stronger CD8+ T cell IFN-γ responses were observed in European Caucasians who expressed the aforementioned arginine mutation at position 260 of exon 4 of tapasin (Ashraf et al., 2013, Hydes et al., 2015). However, the associations were not repeatable in other population groups (Ashraf et al., 2013, Huang et al., 2015). The association of the variation in tapasin at residue 260 with clearance of HCV infection indicates that even though a functional role could not be identified between this variant and its association with AERD, JRA or schizophrenia, this *TAPBP* variant may serve a functional role in efficient or optimal peptide loading of HLA class I molecules for presentation to CD8+ T cells (Bukulmez et al., 2005, Fellerhoff and Wank, 2009, Cho et al., 2013).

Viral pathogens have been noted to encode multiple virulence factors that cooperatively work to evade host immune recognition. HCMV is a prime example of the sophisticated mechanisms viruses, particularly persistent viruses, have evolved to evade the multifaceted adaptive immune response. In addition to proteins US3 and US6 described for their interference with tapasin and TAP respectively above, HCMV also encodes US2 and US11 which directly target HLA class I molecules for degradation by the proteasome (Barel et al., 2006). Downregulation of HLA class I expression at the cell surface leaves the infected cell susceptible to natural killer (NK) cell lysis through a lack of HLA class I binding to inhibitory killer immunoglobulin-like receptor (KIR) ligands. KIR ligands regulate NK cell activity. Their major ligands are HLA class I molecules and when this ligand signal is missing, NK cells initiate killing of infected, “non-self” cell (Caligiuri, 2008, Smyth et al., 2005). HCMV has evolved a multifaceted approach to avoid this mechanism. HCMV encodes UL18, a homologue of HLA class I which is presented at the cell surface to replace the ”missing self” signal and prevent NK cell lysis (Kim et al., 2008). Interestingly, UL18 relies on functioning TAP for its expression, but is resistant to HCMV UL6-mediated TAP dysfunction.

The evidence shows viral pathogens have evolved to target the PLC, in particular tapasin, using sophisticated and varied methods in order to evade the cellular immune response. We next considered the published evidence to suggest that HIV-1 may employ similar methods targeting the PLC in order to downregulate HLA class I expression and antigen presentation to CD8+ T cells, similar to other chronic viral infection such as HCMV or EBV.

1.7.4. HIV-1 and tapasin

Evidence has shown since the start of the HIV-1 pandemic that the virus downregulates HLA class I at the cell surface (Scheppler et al., 1989, Kerkau et al., 1989). It was also identified that the HIV-1 Nef protein was responsible for the downregulation of HLA class I and that a reduction in CD8+ T cell killing of HIV-1 infected cells could be observed when Nef was expressed (Schwartz et al., 1996, Collins et al., 1998). HIV-1 Nef has been shown to downregulate the cell surface expression of HLA class I molecules by binding to the HLA class I cytoplasmic tail, recruiting the adapter protein complex-1 (AP-1) and redirecting HLA class I molecules to the endolysosome for degradation (Schwartz et al., 1996, Lubben et al., 2007, Roeth et al., 2004). HIV-1 Nef has been shown to specifically downregulate HLA-A and HLA-B molecules rather than HLA-C (Cohen et al., 1999). As previously mentioned, viral immune evasion of CD8+ T cell recognition via the downregulation of HLA class I molecules comes at the price of exposing infected cells to NK cell lysis. This is due to "missing self" signals and lack of HLA class I binding to inhibitory KIR ligands. It was long thought that HIV-1 had evolved an elegant mechanism to simultaneously reduce CD8+ T cell killing yet avoid NK cell lysis by leaving HLA-C ligands intact at the cell surface for recognition by inhibitory KIRs (Cohen et al., 1999, Specht et al., 2010). However, Apps and colleagues showed that this was only true for the lab-adapted HIV-1 strain NL4-3 and HLA-C was able to be downregulated by primary HIV-1 isolates (Apps et al., 2016). HLA-C downregulation by primary HIV-1 isolates was found to be mediated by Vpu rather than Nef. These findings highlight the importance of using primary viral isolates and consideration of the potential effects overlooked by solely using highly lab-adapted strains such as NL4-3.

HCMV is a prime example of a viral pathogen that has evolved multiple proteins that work cooperatively to downregulate HLA class I and different components of the PLC,

as described above. With a genome approximately 25 times larger than HIV-1, HCMV can afford to expend much more genomic space across its 173 open reading frames (ORFs) to the effort of exerting multiple immune evasion mechanisms in the cell. However, given the evidence that HIV-1 Nef and Vpu may work together to downregulate HLA class I, it is worth investigating whether HIV-1 may also employ a multifaceted approach to avoid CD8+ T cell recognition of viral antigens by also targeting the PLC.

Viral interruption of the PLC components can be initially identified by the co-precipitation of viral proteins with the PLC protein components. The function of viral inhibition of the PLC is often elucidated later. Kasper *et al.* (2005) found that Nef interacts with HLA class I early in the secretory pathway and used fluorescent microscopy to show that Nef also colocalised with tapasin in the ER. This study went on to show that HIV-1 Nef co-precipitated with tapasin via immunoprecipitation (IP) pulldown experiments (Kasper et al., 2005). However, it was deemed that the observation of Nef co-precipitating with tapasin may have been due to an indirect interaction via both protein's shared association with HLA class I. Tapasin interacts with HLA class I heavy chains after they are complexed with β2-m and chaperoned to the PLC by calnexin and calreticulin (Jackson et al., 1994, Ou et al., 1993, Peterson et al., 1995). When β2-m was knocked down in CEM T cells, the co-IP observed between Nef and tapasin was reduced but not abolished (Kasper et al., 2005). This study did not further investigate the interaction between Nef and tapasin, nor did they study any other immunoprecipitates of other PLC proteins potentially interacting with Nef. The Nef interactome and whether this might include tapasin or the PLC is still to be concluded.

A 2016 study of the HIV-1 interactome with host proteins revealed tapasin to be the ninth top hit for HIV-1 Env (Luo et al., 2016). The study used the mass spectrometry (MS) technique isotopic differentiation of interactions as random or targeted (I-DIRT) to

identify specific potential interacting proteins of both Env and Vif with an unbiased approach. The resulting analyses gave tapasin a high I-DIRT specificity average score, but the study failed to perform any confirmatory IP experiments nor investigate the interactome of any other HIV-1 proteins.

HIV-1 envelope peptides have been presented on HLA class I independent of TAP1/2 processing, indicating that HIV-1 may have evolved mechanisms to avoid the PLC (Hammond et al., 1993). However, it has not been investigated whether epitopes from other HIV-1 proteins might be restricted by tapasin or TAP-independent pathways. Evidence also indicate a potential role of the PLC in HIV-1 infection as variant polymorphisms in TAP2 were associated with the rate of HIV-1 disease progression (Kaslow et al., 1996).

Matsui et al. (2001) showed that HepG2 cells overexpressing tapasin were still unable to present an HIV-1 envelope WI9 peptide to HLA-A*24-restricted CD8+ T cells (Matsui et al., 2001). HLA-A*24-restricted CD8+ T cells were however able to recognise WI9 when the cells were pulsed with the peptide, suggesting that failure to present WI9 is due to dysfunction of the cellular peptide processing machinery. The study reported by Matsui and colleagues in 2001 was limited to a single HIV-1 envelope peptide restricted by HLA-A*24, but it revealed preliminary evidence that HIV-1 peptides may escape the conventional antigen processing pathway via the PLC and fail to be presented to CD8+ T cells.

Perhaps the most convincing evidence that the tapasin-dependency spectrum and the PLC might play a role in HIV-1 disease progression were the recent findings reported by Bashirova *et al.* in 2020. The study examined the tapasin-dependency spectrum of HLA class I alleles in a cohort of nearly one thousand HIV-1 positive treatment naïve patients

and found that the expression of highly tapasin-dependent HLA class I alleles was associated with faster progression to AIDS (Bashirova et al., 2020). This study also showed that higher mean longitudinal HIV-1 VL was associated with the expression of HLA class I alleles with increased dependency on tapasin. The authors investigated a functional role behind this observation and showed that tapasin-dependent HLA class I alleles presented a much narrower repertoire of the HIV-1 peptidome, imposing little selective pressure on the virus and making it easier for HIV-1 to adapt to these HLA class I types.

Biochemical analyses of HLA class I alleles have also revealed how tapasin-dependency may be associated with the progression of HIV-1 disease. The less common alleles of the HLA-B*35 subtype, HLA-B*35:02 and HLA-B*35:03 are associated with much faster progression to AIDS compared to the most common HLA-B*35 allele, HLA-B*35:01 (Gao et al., 2001). The hypothesis that HLA class I tapasin-independent alleles are associated with HIV control due to the presentation of an increased breadth of the HIV peptidome is further supported by the differences between these HLA-B*35 alleles. The F-pocket of HLA-B*35:01 is large and accommodates a wider range of large and small C-terminal anchor peptide residues, compared to the small F-pocket of B*35:02 and B*35:03 (Nguyen et al., 2021). HLA-B*35:01 encodes a tyrosine at position 116, similar to the highly tapasin-independent HLA-B*44:05 allele mentioned above (Gao et al., 2001). HLA-B*35 subtype alleles that do not encode tyrosine at position 116 (HLA-B*35-Px alleles) are associated with faster progression to AIDS in both Caucasian and African cohorts (Gao et al., 2001). These biochemical analyses further indicate that limiting the incoming peptide repertoire that can be accommodated with the expressed HLA class I allele may be associated with HIV-1 disease progression. HLA-B*35-Px alleles are largely tapasin-independent for their cell surface expression (Bashirova et al.,

2020). It is evident that the spectrum of control of HIV-1 infection may be attributed to a number of mechanisms at play. Further investigation into the key role that tapasin plays in HIV peptide repertoire editing is warranted to elucidate its association with the control of HIV infection.

1.8. Rationale and Aims

It has been well documented in the literature how viral infections and neoplastic cells have evolved to exploit the individual protein components of the PLC. In doing so, cells are able to interfere with HLA class I antigen presentation of endogenous viral or cancer peptides. HLA class I alleles have been shown to display different dependencies on tapasin for their normal cell surface expression. Recent evidence has suggested that increased tapasin-dependency is associated with faster HIV-1 disease progression and increased viral loads. Furthermore, two independent studies using different methodologies have identified tapasin as a potential interacting host protein with the HIV-1 viral proteins Nef and Env. If the interaction between HIV-1 proteins and tapasin confers some functional defect or re-directs tapasin for degradation within the cell, then it is plausible that the expression of tapasin-independent HLA class I alleles confers some evolutionary advantage for people infected with HIV-1. In a scenario where HIV-1 downregulates or interferes with tapasin function in the cells, the expression of tapasin-independent HLA class I alleles would circumvent this effect and allow HIV-1 infected patients to express HLA class I at more normal levels on the cell surface (Figure 1.5).

This thesis aims to provide further evidence that tapasin-independent HLA class I alleles are involved in the control of HIV-1 disease. We further aim to elucidate the potential cellular mechanisms that may be behind this phenomenon.

We aim as follows:

1) To investigate whether the relationship between tapasin-independent HLA class I alleles and the control of HIV-1 infection is repeatable in other treatment-naïve patient populations. We aim to correlate HIV-1 VL with the tapasin-dependency spectrum of HLA class I alleles expressed by a cohort of ART-naïve children and adolescents with perinatally-acquired HIV-1 *ex vivo*.
2) To assess whether HIV-1 downregulation of HLA class I molecules is dependent on tapasin, TAP1, TAP2, ERp57 or calreticulin expression *in vitro*.
3) To assess whether tapasin-dependent or tapasin-independent HLA class I alleles are more efficiently downregulated by HIV-1 *in vitro*.
4) To measure whether HIV-1 infection affects the levels of tapasin, TAP1, TAP2, ERp57 and calreticulin expression *in vitro*.
5) To measure whether HIV-1 Nef expression *in vitro* affects the levels of tapasin, TAP1, TAP2, ERp57 and calreticulin expression.
6) To investigate whether the stability of HLA class I molecules expressed on the surface of HIV-1 infected cells is reliant on tapasin expression *in vitro*.
7) To investigate whether the stability of tapasin-dependent or tapasin-independent HLA class I alleles differs in HIV-1 infection.

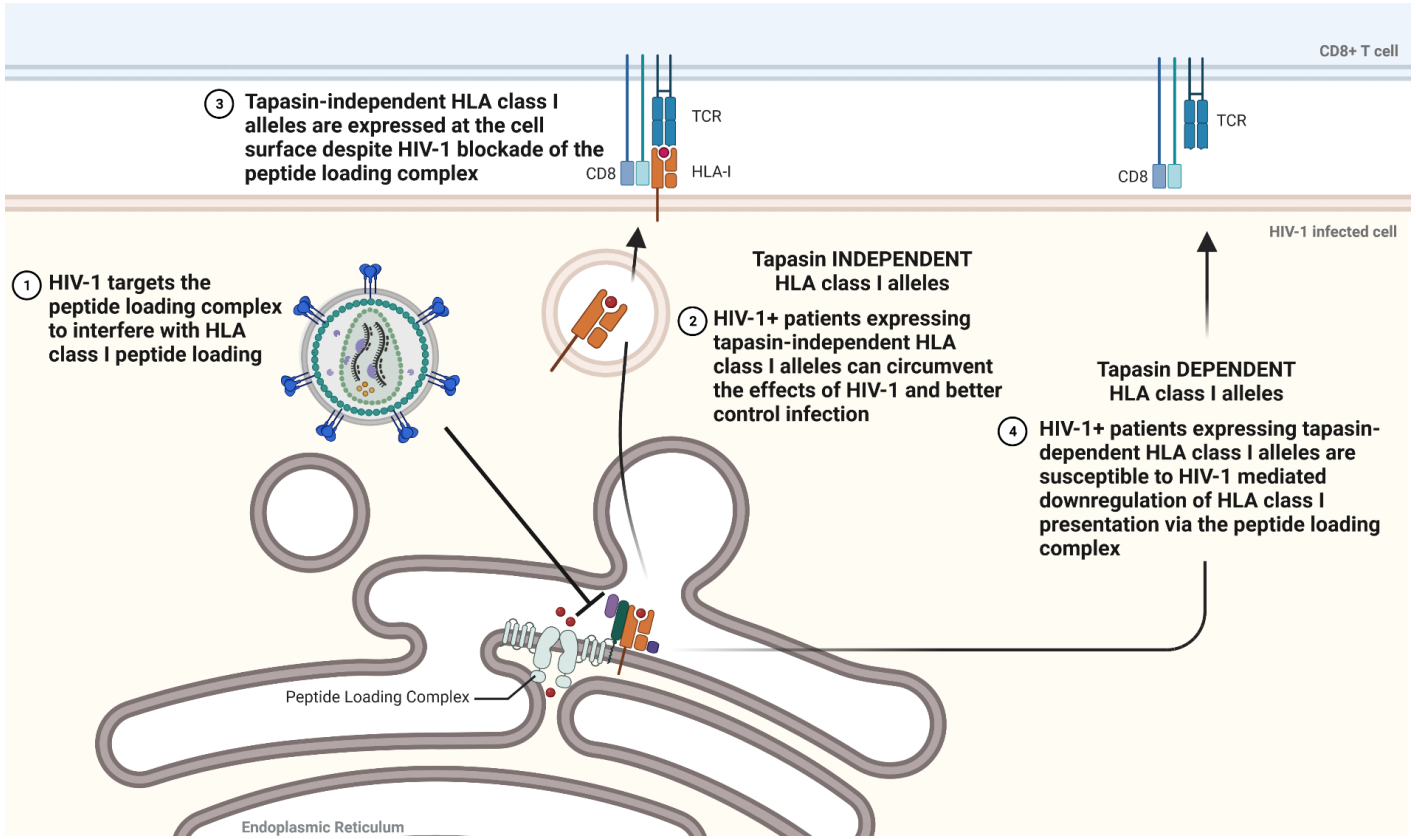


Figure 1.5. **Thesis hypothesis.** Proposed mechanistic hypothesis that describes HIV-1 targeting the peptide loading complex (PLC) in order to downregulate HLA class I expression at the cell surface and evade recognition of HIV-1 infected cells by CD8+ T cells. Tapasin-independent HLA class I alleles have been shown to be associated with delayed HIV-1 disease progression (Bashirova et al., 2020), which would be beneficial to be expressed in patients infected with HIV-1 if tapasin function or expression was affected by HIV-1 itself. Illustration created with BioRender.com.

Chapter 2: Methods

2.1. Methods for Chapter 3

2.1.1. HIV-1 infected patients with long-term non-progression of disease

Children and adolescents with slow progressing HIV-1 infection were recruited as part of the Zimbabwe study for ENhancing testing and Improving Treatment of HIV in children (ZENITH) trial (Ferrand et al., 2017). Participants of the ZENITH trial were recruited from primary health care clinics (PHC) in Harare, Zimbabwe between 2008 and 2015 with their first diagnosis of HIV-1 infection. The trial participants were recruited aged between 6 and 16 years of age and were assessed by healthcare workers at the recruiting clinic to have contracted HIV-1 perinatally. All patients were ART-naïve upon recruitment to the trial.

Patients were randomly assigned to either the control arm or intervention arm of the original ZENITH trial. The control arm consisted of optimised primary HIV care services alone and the intervention arm had primary care services plus support provided by voluntary lay workers (VLWs) to provide additional psychosocial counselling and advice. The primary outcomes of the ZENITH trial aimed to assess the impact of VLW supplemented care on the proportion of patients who died, had virological failure 12 months after ART initiation or were not retained in care 18 months post enrolment. The arms of the ZENITH trial are not biologically relevant in any further stratification of patients from the cohort in this study.

During the trial, blood samples were taken at baseline and at 6 monthly intervals (3 monthly post ART initiation) for plasma separation, peripheral blood mononuclear cell (PBMC) isolation and total circulating CD4+ T cell count measurements.

2.1.2. HIV-1 uninfected patients

Age, sex and ethnicity matched HIV-1 uninfected control patients were included in this study from patients recruited as part of the INvestigation of Heart And Lung disEase among older children in Harare, Zimbabwe (INHALE) trial (Rylance et al., 2016).

2.1.3. Blood sample processing

Whole blood collected from ZENITH participants was processed at the Biomedical Research and Training Institute (BRTI), Harare, Zimbabwe. Blood samples were centrifuged at 400 x g and the upper plasma layer collected and frozen at -80°C. PBMCs were then separated by layering of the plasma-free fraction on Ficoll-Paque (GE Healthcare) and centrifugation at 400 x g for 20 minutes with the acceleration and brake turned off. The buffy coat was recovered, washed in RPMI-1640 and frozen at -80°C in FBS with 10% dimethyl sulfoxide (DMSO). The pelleted red blood cells containing polymorphonuclear granulocytes and erythrocytes were stored at -80°C for genomic DNA extraction.

2.1.4. HIV-1 plasma viral load measurements

Circulating viral RNA was extracted from patient plasma using the QIAamp Viral RNA Mini Kit (Qiagen), following the manufacturer's protocol. Plasma HIV-1 VLs were measured from extracted viral RNA using the RealStar HIV reverse transcription-polymerase chain reaction (RT-PCR) kit v.1.0 (Altona Diagnostics) at the BRTI, Harare, Zimbabwe by Dr Louis-Marie Yindom and Dr Dan Hameiri Bowen of the Professor Sarah Rowland-Jones group. HIV-1 VLs are reported in International Units (IU) / mL plasma, as per the manufacturer's specifications. VL measurement data reported in IU / mL was converted to copies / mL as appropriate and specified herein using a conversion factor of 1.74 IU per copy according to the 1st World Health

Organisation (WHO) International Standard for HIV-1 RNA (NIBSC code: 97/656), against which the RealStar HIV RT-PCR kit v.1.0 (Altona Diagnostics) was validated (Altona Diagnostics, personal communication).

2.1.5. Sub-definitions of long-term viral controllers and slow progressors from the ZENITH cohort

Patients from the ZENITH cohort were further defined as long-term viral controllers or slow progressors. Slow progressors patients (PSP) were defined as having WHO stage I or II disease (asymptomatic or mildly symptomatic) at baseline and an absolute CD4+ T cell count above 350 cells / μL for at least two time points off ART, based upon the definition of paediatric slow progressors (PSPs) as described by Leitman *et al.* (2017) (Leitman et al., 2017). During the time that the ZENITH study was conducted, the WHO guidelines for ART initiation in children over two years of age recommended that ART could be deferred until CD4+ T cell counts fell below 350 cells / μL and/or the patient reached WHO disease stage criteria of III/IV (moderately and severely symptomatic), as such guiding the stratification of patients from the study.

Paediatric viral controller patients (PVC) were defined as trial participants who had at least two undetectable HIV-1 plasma VL measurements off ART regardless of disease stage or CD4+ T cell count - similar to the definition of paediatric EC (PEC) as described by (Vieira et al., 2019).

2.1.6. Genomic DNA extraction

2.1.6.1. Salting-out method

Human genomic DNA (gDNA) was extracted from stored granulocyte/erythrocyte pellets at the BRTI, Harare, Zimbabwe by Dr Louis Marie Yindom and Justin Mayini using a modified salting-out protocol (Miller et al., 1988). Red blood cells were first lysed by repeatedly washing the cell pellet in 40 mL Tris 20 mM-Ethylenediaminetetraacetic acid (EDTA) 5 mM solution (pH 8.0) until the pellet appeared white. The cell pellet was homogenised by thorough vortexing for at least two minutes, followed by the addition of 2 mL Tris 20 mM-EDTA 5 mM solution (pH 8.0) and 100 µL 20% sodium dodecyl sulphate (SDS) to lyse the white blood cells. The samples were mixed by inversion and 200 µg proteinase K was added to facilitate protein digestion. Samples were incubated at 42°C in a water bath overnight. Subsequently, 2.5 mL of 7.5 M ammonium acetate followed by 10 mL of ice-cold ethanol was added to samples to precipitate a DNA-ammonium salt complex. The samples were centrifuged at 700 x g for 30 minutes and the supernatant removed. The DNA pellet was dissolved in 4 mL Tris 20 mM-EDTA 5mM-Sodium chloride (NaCl) 0.2 M solution (pH 8.0) at 42°C in a water bath. The DNA was then re-precipitated in 8 mL ice-cold ethanol and centrifuged at 700 x g for 30 minutes. The supernatant was removed and the final DNA pellet allowed to dry at room temperature (RT). The DNA pellet was resuspended in 150 µL Tris 20mM-EDTA 1 mM and re-dissolved at 58°C overnight in a water bath.

2.1.6.2. Silica-membrane-based DNA purification

gDNA was extracted from isolated PBMCs at the University of Oxford using the QIAamp DNA Blood Mini Kit (Qiagen). DNA extraction was carried out from 200 µL PBMCs according to the manufacturer's spin protocol and eluted in 200 µL dH_2O. The eluted DNA

from either extraction method was checked for quantity and quality using the NanoDrop One spectrophotometer (ThermoFisher).

2.1.7. Polymerase-chain reaction (PCR)

Each of the three classical HLA class I genes (HLA-A, HLA-B and HLA-C) were amplified from gDNA using the PrimeSTAR GXL DNA Polymerase (Takara Bio, Japan) with 1X PrimeSTAR GXL Buffer, 0.3 μM each of the forward and reverse primers (Table 2.1), dNTPs (200 μM each), 1.25 U DNA Polymerase and 50-200 ng DNA in a 50 μL reaction volume. The reaction was incubated at 98°C for 1 minute followed by 30 cycles of PCR (95°C for 10 seconds followed by 60°C for 4 minutes). The amplified HLA class I gene PCR products were then assessed by gel electrophoresis. PCR clean-up for excess primers and nucleotides was performed using the ExoSAP PCR Product Clean-up Reagent (ThermoFisher) according to the manufacturer's protocol.

Gene	Primer Sequence (5' to 3')
HLA-A-Fwd	AGA GGG GTC AGG GCG AAG
HLA-A-Rev	CAT CAA CCT CTC ATG GCA AGA ATT T
HLA-B-Fwd	AGA GTT TAA TTG TAA TGC TGT TTT GAC ACA
HLA-B-Rev	AGG TGA ATG GCT CTG AAA ATT TGT CTC
HLA-C-Fwd	CCA TGG TGA GTT TCC CTG TAC AAG AG
HLA-C-Rev	GGC CGC CTG TAC TTT TCT CAG CAG

Table 2.1. HLA class I PCR primer sequences for full gene amplification. Fwd: Forward primer, Rev: Reverse primer.

2.1.8. HLA class I genotyping

The clean HLA class I gene PCR products were genotyped at the HLA Typing Laboratory, Weatherall Institute of Molecular Medicine, University of Oxford by Timothy Rostron. HLA class I genotyping was performed to 4-digit resolution by sequence-specific primer technology using the BigDye Terminator reagent and sequence-specific primers targeting exons 2 and 3 of each HLA class I locus.

2.1.9. Tapasin-dependency assignment of HLA class I alleles

HLA class I tapasin-dependency (TD) scores were extrapolated from the data published and provided as supplementary information by Bashirova *et al.* (2020) (Bashirova et al., 2020). The original study calculated TD scores for 97 common HLA class I allotypes by transducing lentiviral FLAG-tagged HLA allele expression constructs individually into 721.220 cells. The human B cell line 721.220 does not express tapasin, HLA-A or HLA-B but may express insignificantly low levels of HLA-C. The same HLA expression constructs were also transduced into 721.220 cells reconstituted with tapasin (721.220-tpn cells). The cell surface expression levels of each HLA class I allele was quantified in 721.220 (tapasin-deficient) and 721.220-tpn (tapasin-expressing) cells by flow cytometry using an anti-FLAG monoclonal conjugated to allophycocyanin (APC). TD scores were then calculated from the ratio of HLA class I expression in tapasin-expressing (721.220-tpn cells) compared to tapasin-deficient (721.220 cells) (Bashirova et al., 2020). Higher TD scores indicate that a specific HLA class I allele exhibits higher dependency on tapasin for its cell surface expression.

The TD scores for each HLA locus (HLA-A, HLA-B or HLA-C) for each patient was calculated by summing the TD values on each of the two alleles expressed at each locus (TD(HLA-A) = TD(A1) + TD(A2); TD(HLA-B) = TD(B1) + TD(B2); TD(HLA-C) = TD(C1) + TD(C2), where A1/A2, B1/B2, and C1/C2 represent the two allele's expressed by a patient

at each locus, respectively. The global tapasin dependency score for each patient was calculated by summing the TD scores of all three classical HLA class I genes so that global TD = TD(HLA-A) + TD(HLA-B) + TD(HLA-C).

2.1.10. Statistics for Chapter 3

The Fisher's exact test was used to identify HLA class I allele enrichment in either the slow progressor or viral controller groups compared to HIV-1 uninfected age and sex matched controls. Significant associations were set at $p < 0.05$, or using the Bonferroni correction for multiple comparisons. HLA class I alleles expressed at an overall frequency of >3% in the study population are represented in the analysis. HLA allele frequencies were calculated taking into account homozygosity at each locus and as such, alleles were recorded for their absence/presence in patients (i.e. a homozygous patient for HLA-A*02:01 would only contribute a count of 1 for that allele to the analysis). The Kruskal-Wallis non-parametric test was used to investigate HLA class I TD association with HIV-1 sero-status. Pairwise comparisons between patient groups were conducted using the Wilcoxon rank sum test. The pairwise Wilcoxon rank sum test was also used to look at the association of TD with HIV-1 viral suppression (< 50 copies / mL, undetectable) at baseline. For HIV-1 VL associations with TD, regression analysis were performed in R (v 3.6.1) using the lmer, rstatix and ggplot2 packages. Random effects due to individual patients, time post enrolment and individual HLA-A, HLA-B and HLA-C alleles were included in these models. All graphs and statistical analyses were carried out in R (v 3.6.1).

2.2. Common Methods for Chapters 4, 5 and 6

2.2.1. Cells and cell culture

HEK293FT cells were a kind gift from the group of Professor Persephone Borrow, University of Oxford. HEK293FT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 10% foetal bovine serum (FBS), 1% L-Glutamine and 1% Penicillin/Streptomycin (Pen/Strep). GHOST X4/R5 cells were obtained from the National Institutes of Health (NIH) AIDS Reagent Programme (ARP) and cultured in DMEM supplemented with 10% FBS, 1% L-Glutamine, 1% Pen/Strep, 1% sodium pyruvate and 1% non-essential amino acids (NEAA). HAP1 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FBS and 1% Pen/Strep. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 panel of APM knockout cell (PAKC) lines were a kind gift from Dr Robbert Spaapen (Department of Immunopathology, Sanquin Research, Amsterdam, The Netherlands and the Landsteiner Laboratory, University of Amsterdam, Amsterdam, The Netherlands) (de Waard et al., 2021). The PAKC cell lines are all derived from HAP1 cells and were cultured in the same way as the parental HAP1 cell line. 721.221-CD4-HLA-B*44:02 and 721.221-CD4-HLA-B*44:05 cells were a kind gift from Professor Persephone Borrow, University of Oxford. These cells were generated by transduction of single HLA class I allotypes into the HLA class I-deficient LCL 721.221. 721.221-CD4- HLA-B*44:02/05 cells were cultured Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% FBS, 1% Pen/Strep and 1% L-Glutamine. All a cell lines were cultured at 37°C, 5% CO_2 atmosphere and adherent cells were passaged using the TrypLE Express cell dissociation reagent (Gibco).

PBMCs were processed from healthy donor blood samples obtained from the National Health Service (NHS) Blood and Transplant service as residual blood leukocyte cones. Blood was

recovered from the leukocyte cone and diluted 1:3 in Dulbecco's phosphate-buffered saline (DPBS) (Gibco). The diluted blood was layered onto Ficoll-Paque solution and the PBMC and plasma layers separated by density centrifugation at 400 x g for 20 minutes with no acceleration or brake on the centrifuge. The central PBMC layer was carefully aspirated with a Pasteur pipette and washed three times in DPBS. PBMCs were either used directly in HIV-1 infection assays after isolation or frozen for long-term storage. Cells were frozen by resuspension of the cell pellet after centrifugation in FBS with 10% DMSO at a density of $5x10^7$ cells / mL. PBMCs were frozen in 1 mL aliquots for 24 hours at -80°C in a Mr Frosty freezing container, before being transferred to liquid nitrogen (LN_2) storage.

2.2.2. Plasmid DNA

The full-length HIV-1 infectious molecular clone (IMC) of the CXCR4-tropic subtype B pNL4-3 was kindly donated by the group of Professor Persephone Borrow, University of Oxford and originally obtained through the NIH ARP (HIV-1 strain NL4-3 IMC pNL4-3, ARP-2852, contributed by Dr Martin Martin). The full length IMC of the dual CCR5/CXCR4 tropic T/F subtype B pCH077 was obtained through the NIH ARP (HIV-1 strain CH077 IMC pCH077.t/2627, ARP-11742, contributed by Dr John Kappes and Dr Christina Ochsenbauer). The full length CXCR4-tropic pNL4-3 derivative clone defective in *nef* (pNL4-3 ΔNef) was obtained through the NIH ARP (HIV-1 strain NL4-3 ΔNef IMC pNL4-3ΔNef ARP-12755, contributed by Dr. Olivier Schwartz). The pMD2.G plasmid from the Trono lab encoding the Vesicular stomatitis virus G protein (VSV-G) envelope was kindly donated by the group of Professor Persephone Borrow, University of Oxford.

2.2.3. Virus production and titration

Vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV-1 viral particles were produced in HEK293FT cells in 6 well plates. Briefly, 8 µL of TransIT-293 (Mirus Biosciences) per well was diluted in 200 µL serum-free Opti-Minimum Essential Medium (MEM)™ and incubated at RT for 20 minutes. A total of 0.4 µg pDM2G and 2.6 µg full-length HIV DNA per well was added to the mixture and incubated for 30 minutes at RT. The plasmid DNA mixture was added drop-wise to $0.9x10^6$ HEK293FT cells per well plated 12-18 hours prior to transfection. The cell culture media was replaced after overnight incubation with the transfection mix and the supernatant containing virus particles was harvested 48 hours post transfection, filtered at 0.2 µM, aliquoted and frozen at -80°C. Viral titres were determined by titration on GHOST X4/R5 cells, as described by Manel *et al*, 2010. GHOST X4/R5 cells express GFP under the HIV-1/2 promoter and possess both co-receptors for HIV infection (Manel et al., 2010). The virus supernatant was first diluted 1:2, then serially diluted 1:5 until a final dilution volume of $1.28x10^{-5}$ µL virus supernatant in 200 µL media. Each virus dilution was incubated with $8x10^3$ GHOST X4/R5 cells per well in a flat-bottom 96 well plate for 48 hours at 37°C, 5% CO_2. Cells were harvested using the TrypLE Express reagent and fixed in 4% paraformaldehyde (PFA). The cells were then analysed by flow cytometry on the BD LSRFortessa™ X-20 Cell Analyzer (BD Biosciences) or Attune NxT Flow Cytometer with the CytKick Max plate autosampler (ThermoFisher) under excitation of the 488nm laser and analysed in FlowJo v10.8.1 (BD Biosciences). The slope between the number of GFP positive GHOST X4/R5 cells and volume of initial virus inoculation was modelled on a Poisson distribution to determine the HIV viral titre in IU / mL as described by (Manel et al., 2010). HIV virus titres were calculated from the mean slope of duplicate titration curves.

2.2.4. HIV-1 infection assays in cell lines

HEK293FT, HAP1/PAKC and 721.221-CD4-HLA-B*44:02/05 cells were seeded at a density of $1.5x10^5$ cells per well in a 24 well plate in the appropriate cell culture growth media and allowed to adhere overnight at 37°C, 5% CO_2. The cell culture media was substituted for fresh media immediately prior to infection and cells were harvested immediately prior to infection as time 0 of the assay.

Cell lines were infected with VSV-G pseudotyped NL4-3, CH077 or NL4-3-ΔNef at a multiplicity of infection (MOI) of 1.0 unless otherwise specified. Cell lines were mock-infected with supernatant collected from cultured HEK293FT cells that were transfected with just pMD2.G during virus production as a control. The cell culture medium was replaced six hours post infection and the infected cells were left to incubate with virus at 37°C, 5% CO_2 for up to 48 hours post infection, as specified in each experiment.

2.2.5. Flow cytometry of cell lines

Cells were harvested by pipetting after dissociation from an adherent surface using the TrypLE Express reagent. Cells were washed once in fluorescence-activated cell sorting (FACS) Wash Buffer (FWB; 1% FBS, 2mM EDTA in DPBS) and once in DPBS before staining in 50 µL with a 1:100 dilution of antibody W6/32 (anti-HLA class I) conjugated to Phycoerythrin (PE) (Biolegend) in DPBS. Cells were incubated with anti-HLA class I for 15 minutes at RT. Unstained cells and controls were incubated with DPBS only. The cells were then washed three times with 100 µL FWB and fixed in 4% PFA solution for 15 minutes at RT. To stain intracellular antigens, the cells were spun down after fixation and resuspended in 100 µL Permeabilisation Wash Buffer (PWB, 1% FBS, 2mM EDTA, 1% saponin in DPBS). Cells were incubated in PWB for 30 minutes at RT. Following permeabilisation, the cells were spun down

and resuspended in PWB with the Fluorescein isothiocyanate (FITC)-conjugated anti-HIV gag p24 antibody clone KC57 (1:1,000) (Beckman Coulter) for 15 minutes at RT. Unstained cells and single stain controls were incubated with PWB alone. The cells were washed three times with PWB and stored in 4% PFA solution at 4°C until resuspension in 200 µL DPBS for processing by flow cytometry. The samples were analysed on the BD LSRFortessa™ X-20 Cell Analyzer (BD Biosciences) or the Attune NxT Flow Cytometer with the CytKick Max plate autosampler (ThermoFisher) and analysed in FlowJo v 10.8.1 (BD Biosciences).

2.2.6. Statistical analyses for Chapters 4 and 5

Pairwise comparisons between groups were made using the student's t-test with Benjamini-Hochberg correction for multiple comparisons unless otherwise stated. The Wilcoxon rank sum test was used for non-parametric data. Comparisons between three or more groups were made using the non-parametric Kruskal-Wallis test. The alpha level was set to 0.05 (5%) to determine significant associations. All graphs and statistical analyses were performed in R (v 3.6.1) using the rstatix and ggplot2 packages.

2.3. Methods for Chapter 5

2.3.1. HIV-1 infection assays in primary human PBMCs

Prior to infection, PBMCs from healthy donors were activated in RPMI 1640 with 10% FBS, 1% L-Glutamine, 1% Pen/Strep (R10 media) supplemented with 5 µg / mL phytohemagglutinin (PHA) plus 50 units (U) / mL IL-2 and incubated at 37°C, 5% CO_2 for 48 hours. The cell culture media was replaced with fresh R10 media supplemented with 50 U / mL IL-2 after activation and allowed to rest for a further 48 hours at 37°C and 5% CO_2. Activated PBMCs were cultured at a density of $1x10^6$ cells / mL for the assay and infected with VSV-G pseudotyped NL4-3, CH077 or NL4-3-ΔNef at an MOI of 1.0 unless otherwise specified. Activated PBMCs were mock-infected with supernatant collected from cultured HEK293FT cells that were transfected with just pMD2.G during virus production as a control.

2.3.2. Flow cytometry of primary human PBMCs

Primary human PBMCs were processed by flow cytometry as described above in the methods outlined for Chapters 4, 5 and 6. PBMCs were stained with a surface antigen cocktail (Table 2.2) in a total staining volume of 50 µL DPBS per sample for 15 minutes at RT. Washing procedures in FWB, fixation in 4% PFA and permeabilisation of cells in PWB was carried out according to the procedure outlined in the common methods. Intracellular staining of HIV-1 gag with the FITC-conjugated antibody clone KC57 was carried out at a 1:100 dilution for PBMCs and incubated with cells for 15 minutes at RT. All subsequent wash procedures in PWB and analysis on the Attune NxT (ThermoFisher) were carried out according to the common protocol outlined for Chapters 4, 5 and 6.

Surface Marker	Fluorophore	Clone	Dilution
CD3	Phycoerythrin-Cyanine7 (PE-Cy7)	UCHT1	1:200
CD8	Alexa Fluor 700 (AF700)	TPA-T8	1:50
CD4	Allophycocyanin (APC)	TPA-T8	1:100
CD19	Allophycocyanin-Cyanine7 (APC-Cy7)	SJ25C1	1:50
CD14	Brilliant Violet™ 711 (BV711)	63D3	1:100
CD16	Brilliant Violet™ 605 (BV605)	3G8	1:50
HLA class I	Phycoerythrin (PE)	W6/32	1:100
Live/Dead™	Fixable Violet	NA	1:1,000

Table 2.2. **Surface marker antibody panel optimised for the immunophenotyping of peripheral blood mononuclear cells (PBMCs) by flow cytometry.** The surface marker targeted, conjugated fluorophore, antibody clone name and dilution factor used for PBMC staining in this study are detailed.

2.3.3. Plasmid DNA

The HIV-1 HXB2 Nef His Expression vector (pNef-His) produces an HIV-1 HXB2 Nef protein with a C-terminal 6X His-tag under control of the CMV promoter. The Nef-His plasmid clone was obtained through the NIH ARP (HIV-1 HXB2 Nef His Expression Vector, ARP-13145, contributed by NIAID, DAIDS). The Gateway™ pcDNA™-DEST47 vector was purchased from ThermoFisher Scientific (ThermoFisher).

2.3.4. DNA transfection

DNA transfection of HEK293FT, HAP1 and HAP1 CRISPR/Cas9 PAKC cells was carried out in 24 well plates. Per well, 1.6 µL TransIT-293 (HEK293FT cells) or TransIT-LT1 (HAP1 and PAKC cells) was incubated with 40 µL Opti-MEM™ for 20 minutes at RT. A total of 0.6 µg plasmid DNA (pNef-His or empty vector (EV) pcDNA™-DEST47) per well was incubated with the transfection reagent mix at RT for 30 minutes before being added dropwise to $1.5x10^5$ cells per well of a 24 well plate. The transfection mixture only, without exogenous DNA, was added to cells as a mock transfection control. The cell culture media was replaced after overnight incubation with the transfection mix and cells were collected as described at the appropriate time point for the assay. Transfection reactions were scaled up 20% for 6 well plates for Western Blot analyses.

2.3.5. Flow cytometry of HIV-1 Nef-His transfected cell lines

Cell lines transfected with HIV-1 Nef-His were processed by flow cytometry as described above in the methods outlined for Chapters 4, 5 and 6. Nef-His transfected cells were first stained with the anti-HLA class I clone W6/32 conjugated to PE. After wash steps in FWB, fixation in 4% PFA and permeabilisation in PWB: Nef-His transfected cells were intracellularly stained with a 1:100 dilution of the FITC-conjugated anti-6X His Tag antibody (clone AD1.1.10, Invitrogen). All subsequent wash and analysis steps on the Attune NxT (ThermoFisher) were carried out according to the protocol described above for the Chapters 4, 5 and 6.

2.3.6. RNA extraction

RNA was extracted from cells using the RNeasy Mini Kit (Qiagen). Cells were first harvested by dissociation from an adherent surface using the TrypLE Express reagent and washed twice in DPBS. The cells were pelleted by centrifugation at 400 x g for 5 minutes and the cell pellet were lysed in 350µL Buffer RLT (Qiagen) supplemented with 1% β-mercaptoethanol (β-me) with agitation by vortexing for 1 minute. The cell lysates were frozen at -80°C until extraction. Frozen lysates were subsequently thawed at 37°C in a water bath, mixed 1:1 with 70% ethanol and applied to the RNeasy extraction column. The column was washed once with buffer RW1 and on-column DNaseI digestion was performed with the RNase-Free DNase kit (Qiagen) for 15 minutes at RT to remove potential gDNA contamination. Subsequent wash procedures were performed with buffers RW1 and RPE as recommended by the manufacturer. The RNA was finally eluted in 60 µL nuclease-free H_2O: the RNA was eluted twice in 30 µL nuclease-free H_2O and the eluates were subsequently pooled, in order to maximise RNA yield. Nuclease-free H_2O and the first eluate used to elute again from the same column. RNA was frozen at -80°C before quantification on the NanoDrop One (ThermoFisher).

2.3.7. Reverse transcription reaction and quantitative PCR (qPCR)

Reverse transcription of RNA into cDNA was carried out using the qPCRBIO cDNA Synthesis Kit (PCR Biosystems) using 1X cDNA Synthesis Mix, 1X RTase and 0.1 µg RNA per 20 µL reaction volume. No reverse transcription controls were carried out in the same reaction mixture with 0.1 µg RNA but without 1X RTase. The reverse transcribed and control reactions were incubated at 42°C for 30 minutes followed by 85°C for 10 minutes to denature the RTase using the PCRmax Alpha Cycler 4 Thermal Cycler (Cole Parmer).

qPCR reactions were carried out using the qPCRBIO SyGreen Lo-ROX Master Mix (PCR Biosystems) with 1X Sygreen Mix, 0.4 µM each of the forward and reverse primers (Table 2.3) and 2 µL cDNA in a 20 µL reaction volume. qPCR reactions were carried out in duplicate and run on the 7500 Fast System (Applied Biosystems), incubating at 95°C for 2 minutes followed by 40 cycles of PCR at 95°C for 5 seconds followed by 60°C for 30 seconds.

Raw cycle threshold (Ct) values were analysed according to the ΔΔCt method. The Ct values across technical qPCR duplicates were averaged and then normalised to the average Ct value of GAPDH (housekeeping gene, HKG) for each sample (ΔCt). The ΔCt values for each experimental group were then normalised to either the non-infected control or the non-infected control at time 0 of the assay, per biological (experimental) replicate, as appropriate for each experiment (ΔΔCt). The relative quantification (RQ) signal for each sample was calculated as $2^{-\Delta\Delta Ct}$.

Gene	Primer Sequence (5' to 3')
TAPBP-Fwd	GAG CCT GTT CTC ATC ACC ATG G
TAPBP-Rev	GTA GGC AAA GCT CAA GTC CAG C
ERp57-Fwd	GTC AGC CAC TTG AAG AAG CAG G
ERp57-Rev	TAG GAA CTC GGA GTG AGC CTC A
TAP1-Fwd	GCA GTC AAC TCC TGG ACC ACT A
TAP1-Rev	CAA GGT TCC CAC TGC TTA CAG C
TAP2-Fwd	ATG CCC TTC ACA ATA GCA GCG G
TAP2-Rev	CCA AAA CTG CGA ACG GTC TGC A
Calreticulin-Fwd	TCA AGG AGC AGT TTC TGG ACG G
Calreticulin-Rev	GCA TCC TGG CTT GTC TGC AAA C
MX1 -Fwd	GGC TGT TTA CCA GAC TCC GAC A
MX1-Rev	CAC AAA GCC TGG CAG CTC TCT A
RSAD2-Fwd	CCA GTG CAA CTA CAA ATG CGG C
RSAD2-Rev	CGG TCT TGA AGA AAT GGC TCT CC
GAPDH-Fwd	GTC TCC TCT GAC TTC AAC AGC G
GAPDH-Rev	ACC ACC CTG TTG CTG TAG CCA A

Table 2.3. **Peptide loading complex (PLC), interferon stimulated gene (ISG) and housekeeping gene (HKG) primer sequences for qPCR reactions.** The housekeeping gene used to normalise target gene Ct values in this study was Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). TAPBP: Tapasin, MX1: Interferon-induced GTP-binding protein Mx1, RSAD2: Radical S-adenosyl methionine domain-containing protein 2 (also known as Viperin). Fwd: Forward primer, Rev: Reverse primer.

2.3.8. Immunoblotting

Cells were harvested after dissociation from an adherent surface using the TrypLE Express reagent, washed twice in ice-cold DPBS and lysed in 100 µL / $1x10^6$ cells ice-cold radio-immunoprecipitation assay (RIPA) buffer (50mM Tris-HCl, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150mM NaCl). Cells were lysed for 45 minutes at 4°C with constant agitation before being spun down at 14,000 x g for 10 minutes to remove cellular debris. The supernatant containing cellular proteins was collected and stored at -80°C.

Cell lysates were thawed on ice and combined with 1X Laemmli buffer (Bio-Rad) and 10 mM dithiothreitol (DTT) to reduce and denature proteins in a total volume of 40 µL. The samples were boiled at 95°C for 5 minutes on a heat block and placed on ice for 2 minutes prior to loading on a 12% Novex™ WedgeWell™ Tris-Glycine mini protein gel (ThemoFisher Scientific) with 5 µL of the PageRuler™ Prestained near-infrared (NIR) Protein Ladder (ThermoFisher Scientific) in adjacent wells as a protein marker. Gels were run in 1X running buffer (2.5 mM Tris, 19.2 mM Glycine and 0.1% SDS) for 10 minutes at a constant 100 volts (V) followed by 1 hour and 20 minutes at a constant 150 V. The gel was then transferred semi-dry onto a 0.45 µm polyvinylidene difluoride (PVDF) membrane for 1 hour at 15 V using the Trans-Blot SD system (Bio-Rad) with ice-cold 1X transfer buffer (2.5 mM Tris, 19.2 mM Glycine and 0.01% SDS, pH 8.3) containing 10% methanol. Following transfer, the membrane was dried at 37°C for 10 minutes and reactivated in methanol for 30 seconds before blocking in 1X Tris-Buffered Saline (TBS) (20 mM Tris and 150 mM NaCl, pH 7.6) with 5% non-fat dried milk for 1 hour at RT. The membrane was then incubated with the primary antibody cocktail of 1:200 dilution rat anti-tapasin clone 7F6 (Sigma Aldrich), 1:1000 dilution of mouse anti-ERp57 clone MaP.Erp57 (Abcam) and 1:5000 dilution mouse anti-GAPDH clone GA1R (Invitrogen) in 1X TBS-T (1X TBS with 0.1% Tween-20) with 5% non-fat dried milk overnight at 4°C with gentle agitation. The membrane was washed three times with TBS-T for 5 minutes

each at RT on a rocker before incubation with a 1:15,000 dilution of IRDye 800CW Goat anti-Rat IgG (LI-COR) and/or 1:15,00 dilution of IRDye 800CW Goat Anti-Mouse IgG (LI-COR) in 1X TBS-T with 5% non-fat dried milk for 1 hour at RT. Following secondary antibody incubation, the membrane was washed three times with 1X TBS-T for 5 minutes each time at RT on a rocker and finally kept in phosphate buffered saline (PBS). Membranes were imaged in the 800 channel at 169 µm resolution, High quality with Automatic intensity correction on the Odyssey CLx Infrared Imaging System (LI-COR).

The densitometry data obtained from each western blot image were analysed using Image Studio Lite (LI-COR) using an average local background correction. For each membrane, the target protein (tapasin or ERp57) signal was normalised to the GAPDH signal for each sample. The corrected signal was then normalised to the non-infected control, per biological replicate, as appropriate for each experiment.

2.4. Methods for Chapter 6

2.4.1. Brefeldin A decay assay

HAP1, PAKC Tapasin KO HAP1 and 721.221-CD4-HLA-B*44:02/05 cells were seeded at a density of $0.35x10^6$ cells / mL in a flat-bottomed 96 well plate. 721.221-CD4-HLA-B*44:02/05 cells were seeded at a density of $0.25x10^6$ cells / mL in a flat-bottomed 96 well plate. Cells were infected at an MOI of 1.0 with VSV-G pseudotyped NL4-3, CH077 or NL4-3ΔNef or mock infection media only as a control. The cell culture medium was replaced six hours post infection and the infected cells were left to incubate with virus at 37°C, 5% CO_2 for 48 hours post infection.

After 48 hours, the cell media was replaced with IMDM supplemented with 10% FBS, 1% Pen/Strep (HAP1 and PAKC Tapasin KO HAP1 cells) or RPMI 1640 supplemented with 10% FBS, 1% Pen/Strep (721.221-CD4 cells) plus 10 µg / mL Brefeldin A (BFA) (GolgiPlug™, BD Biosciences) to block *de novo* transport of HLA class I alleles to the cell surface. The cells were harvested immediately after BFA addition at time 0 and also incubated at 37°C, 5% CO_2 for 0.5, 1, 2 and 4 hours in order to follow the decay of HLA class I from the cell surface. Cells were harvested at time 0, 0.5, 1, 2 and 4 hours, washed once in FWB (1% FBS, 2mM EDTA in DPBS) and once in DPBS before staining with a 1:100 dilution of antibody W6/32 (anti-HLA class I) conjugated to PE (Biolegend) in DPBS at RT for 15 minutes. Following antibody staining, the cells were washed three times in FWB then fixed in 4% PFA for 15 minutes at RT. Following fixation, cells were permeabilised in PWB (1% FBS, 2mM EDTA, 1% saponin in DPBS) for 30 minutes at RT and then stained with a 1:1,000 dilution of antibody KC57 (anti-HIV gag p24) conjugated to FITC (Beckman Coulter) in PWB for 15 minutes at RT. The cells were washed three times in PWB and resuspended in 200 µL DPBS to be analysed by flow cytometry on the Attune NxT with the CytKick Max plate autosampler (ThermoFisher). Data

analysis was performed in FlowJo v10.8.1 (BD Biosciences). Median fluorescence intensity (MFI) values were corrected for background by subtracting the MFI value of the unstained control for each cell line and stain from each sample.

2.4.2. Statistical analysis for Chapter 6

Peptide-HLA class I complex half-lives were determined by fitting exponential trend lines in R (v 3.6.1) using the lmer, rstatix and ggplot2 packages and calculated using Formula 2.1. Pairwise comparisons between half-lives were made using the student's t-test with Benjamini-Hochberg correction for multiple comparisons unless otherwise stated. The alpha level was set to 0.05 (5%) to determine significant associations. All graphs and statistical analyses were performed in R (v 3.6.1) using the rstatix and ggplot2 packages.

Calculation of peptide-HLA class I complex half-life.

$$N_t = N_0 \left(\frac{1}{2}\right)^{\frac{t}{t_{\frac{1}{2}}}}$$

where:

$\mathbf{N_0}$ is the initial quantity;

$\mathbf{N_t}$ is the remaining quantity after time, **t**;

$\mathbf{t_{½}}$ is the half-life.

Formula 2.1. **Calculation of peptide-HLA class I complex half-life.** HLA class I half-lives were calculated from exponential trend lines fitted to the data.

Chapter 3: HLA class I allele expression and tapasin-dependency in older children and adolescents with slow progressing HIV-1 disease from Harare, Zimbabwe

3.1. Chapter 3 Introduction

Of the estimated 38.4 million people living with HIV-1 worldwide, sub-Saharan Africa is burdened with approximately 67% of cases and 58% of new daily infections (UNAIDS estimates, 2022). Focused studies on people living with HIV-1 from these regions are vital to provide data on the pandemic from the most affected populations who stand to reap the greatest benefit from viral prophylaxis and vaccines.

Zimbabwe is a land-locked country in southern Africa with a population of approximately 16 million (2022), bordered by South Africa, Zambia, Namibia, Botswana and Mozambique. One point three million people are estimated to be infected with HIV-1 in Zimbabwe, with almost 6% of these cases being children aged between 0 and 14 years of age (UNAIDS estimates, 2022).

Childhood acquisition of HIV-1 infection is mostly caused by perinatal infection from mother to child across the placenta, during birth or via breastfeeding (Simonon et al., 1994, Bertolli et al., 1996, Mock et al., 1999, De Cock et al., 2000). Early mortality is prevalent in untreated HIV-1 infected African infants without ART intervention, 50% of whom are not expected to survive past two years of age (Newell et al., 2004, Marinda et al., 2007). A study on the rate of infant mortality by two years of age in perinatally HIV-1 infected infants infected *in utero* or *intra partum* in Zimbabwe in 2007 predicted death in up to two thirds of infected children; which is striking when compared to the 3% mortality rate of HIV-1 uninfected infants born to HIV-1 negative mothers in Zimbabwe in the same year (Marinda et al., 2007).

Despite children progressing to AIDS much faster on average compared to adults without ART intervention, a proportion of HIV-1 infected infants are expected to have slow progressing disease: at least one third of perinatally-infected children have slow-progressing disease with

a median survival of at least 16 years and expected to survive into childhood (Ferrand et al., 2009, Stover et al., 2006, Ferrand et al., 2010, Marston et al., 2005).

During the early 2000s, increasing numbers of perinatally-infected children with slow progressing disease started presenting to health services as the epidemic matured and these children reached their older years (Devendra et al., 2013, Desai et al., 2018). A number of children and adolescents living with untreated paediatric HIV-1 infection were observed presenting to healthcare facilities with chronic comorbidities associated with HIV infection (Ferrand et al., 2009). This epidemic of long-term survivors and slow progressors of paediatric HIV-1 disease was likely caused by the high prevalence of adult HIV-1 infected patients in the 1990s who lacked early access to therapy for prevention of mother to child-transmission (MTCT), the use of which wasn't introduced in Zimbabwe until 2001 (Musarandega et al., 2020, Ferrand et al., 2007, Eaton et al., 2013). Children with slow progression of HIV-1 disease are an interesting cohort to study immunologically and genetically to determine the factors that underlie slow progression of HIV-1 disease.

The immunological factors contributing to spontaneous control and slow progression of HIV-1 disease in adults are discussed in Chapter 1, but are thought likely to involve HLA class I allele distribution, KIR gene interaction and the magnitude and kinetics of the HIV-specific CD8+ T cell response. That being said, immature infant immune systems are proposed to control HIV-1 infection less efficiently compared to adults (Garcia-Knight et al., 2016).

Paediatric non-progression of HIV-1 disease shares more common immunological features with natural non-pathogenic SIV infection in sooty mangabeys and African green monkeys than adults (Muenchhoff et al., 2016). Protective HLA class I alleles such as HLA-B*27, HLA-B*57, HLA-B*58:01 and HLA-B*81:01 are not consistently associated with non-progression of HIV disease in children as these alleles are for adults (Adland et al., 2015b). Instead, delayed

disease progression is associated with low immune activation (CD38/HLA-DR expression on CD4+ and CD8+ T cells) and low CCR5 expression and infection in long-lived central memory CD4+ T cell subsets, despite these patients having a high HIV-1 viraemia (Adland et al., 2015b, Muenchhoff et al., 2016, Ssewanyana et al., 2007, Paul et al., 2005, Klatt et al., 2014).

Paediatric LTNPs are more common compared to their adult counterparts, but not as extensively studied (Paul et al., 2005, Blanche et al., 1997, Mphatswe et al., 2007). The few studies on the immunological factors contributing to the control of HIV-1 infection and non-progression of disease in paediatric patients are often limited by unstructured *post hoc* analyses and the identification of controllers on an *ad hoc* basis from different cohorts. The latter issue is compounded when cohorts from different geographical locations and of different ethnicities are pooled and studied together. This makes it more difficult to elucidate individual immunological traits and genetic signatures that contribute to spontaneous control of viral infections and slow progression of disease.

There are also differences in the criteria and terminology used to define these patients with control or slow progression of disease from wider cohorts in the first place. Firstly, the definition of paediatric LTNP of HIV-1 disease differs from adults. Adult LTNP of HIV-1 disease is commonly defined for patients who maintain high CD4+ and CD8+ T cell counts despite being ART-naïve for a period ranging between 8 and 10 years (Zeller et al., 1996, Easterbrook, 1999). Children express and maintain higher CD4+ T cell counts and thus CD4+ T cell percentages are thought to be a better biomarker of infection control in paediatric disease (Dunn and Group, 2003). The definition for paediatric LTNPs generally describes children with perinatal HIV-1 infection who have normal-for-age CD4+ T cell percentages or counts and no severe symptoms of HIV-1-related disease or evidence of immunosuppression, despite not having received ART treatment for a period of over ten years (Warszawski et al., 2007, Mphatswe et al., 2007, Muenchhoff et al., 2016, Ananworanich et al., 2010, Hofer et al., 2009).

A lack of centralised international guidelines on the use of clinical parameters such as CD4+ T cell counts and HIV-1 plasma VLs as well as the time period over which control is measured results in heterogeneity and inconsistencies between the phenotypes of control described in the literature. A 2014 systematic review of the published terminology used to describe the extremes of HIV-1 disease phenotypes found that the level of redundancy between studies reached 600 definitions used to describe in reality only 26 of the same patient subgroups (Gurdasani et al., 2014). This makes it difficult to compare the findings from various patient subgroups and infer the biological mechanisms underlying HIV-1 control.

Vieira *et al.* (2019) described 11 ART-naïve children perinatally infected with HIV-1 from ten clinics across South Africa, Brazil, Spain, Sweden, Thailand and the United Kingdom. These patients met the criteria for EC previously defined for adults: three or more consecutive undetectable HIV-1 plasma VL measurements over a period of at least one year (Olson et al., 2014, Yang et al., 2017). This definition was made independent of CD4+ T cell count and estimated PEC prevalence in their study population to be less than 0.1%, which is five to ten fold less prevalent than their adult counterparts (Vieira et al., 2019). Due to the rarity of EC in paediatric HIV-1 infected patients, the main drawback of this study was the necessity to pool patients from very ethnically diverse cohorts in order to identify this subgroup of ECs.

As discussed above, paediatric patients with non-progressor phenotypes of disease are found to maintain normal CD4+ T cell counts despite high HIV-1 viraemia. Leitman *et al.* (2017) described the CD8+ T cell responses for 11 slow progressors from the Paediatric Early highly active RT (HAART) and Strategic Treatment Interruption Study (Mphatswe et al., 2007, Thobakgale et al., 2009, Prendergast et al., 2008). The study defined 11 slow progressor patients who failed to meet the clinical criteria for commencing ART (WHO stage III/IV disease or absolute CD4+ T cell counts < 350 cells / μL) by 5 years of age and found that the breadth and magnitude of the CD8+ T cell response was positively associated with HIV-1

viraemia in these slow progressor patients, further indicating that lower levels of CTL immune activation is associated with non-progression of disease (Leitman et al., 2017, Muenchhoff et al., 2016).

We aimed in this study to utilise previously defined criteria for viral controllers/ECs based on that described by Vieira *et al.* (2019) and slow progressors based on that described by Leitman *et al.* (2017) in order to define patient subgroups with increased consistency with the published literature (Gurdasani et al., 2014). Patients from the ZENITH cohort were sub-defined in order to examine host genetic associations with delayed disease progression in an immunologically defined and focused subgroup of clinically interesting patients.

The ZENITH trial poses a unique opportunity to study the genetic factors contributing to LTNP of HIV-1 disease to answer these questions. ZENITH was a randomised controlled trial conducted in Harare, Zimbabwe between 2012 and 2016 (Ferrand et al., 2017). The trial recruited children and adolescents aged between 6 and 15 years of age with a new diagnosis of HIV-1 infection. Children and adolescents have a higher risk of poor adherence to ART and failure of retention in care (Fetzer et al., 2011, Campbell et al., 2012, Rehman et al., 2022). The primary aim was to investigate whether a package of VLW supplemented services at the primary healthcare level could improve ART adherence and retention into HIV care for children and adolescents. The ZENITH trial found that community level HIV-1 VLs were significantly decreased in the patients randomised to the intervention arm (VLW supplemented care) compared to the control arm (primary health care clinic-based care only).

Outside of the original findings from ZENITH, the patients recruited to the trial are an interesting and unique cohort to study the immunological mechanisms underlying slow progression of HIV disease in children. As the patients were infected perinatally, the duration

of HIV-1 infection survived without ART intervention can be extrapolated from the patient's age at recruitment to the trial.

The expression of protective HLA class I alleles is associated with slower disease progression and lower viral replicative capacity in adults, but has not been reported in children (Goulder and Walker, 2012, Adland et al., 2015b). A recent study also provided evidence that the level of HLA class I dependency on tapasin (discussed in detail in Chapter 1) was associated with slower progression to AIDS and lower HIV-1 VLs in adults, even when controlling for the expression of protective HLA class I alleles (Bashirova et al., 2020). The highly protective allele HLA-B*57:01 is highly tapasin-dependent, suggesting an uncoupling of the relationship between tapasin-dependency and HLA class I allotypes being protective perhaps solely due to their individual peptide repertoire. These findings indicate that the relationship between HLA class I and HIV-1 disease progression may extend beyond what has already been studied in HIV-1 cohorts and be influenced by the tapasin dependence.

No previous study has addressed if tapasin-independence is associated with viral control or slow progression of HIV-1 disease in children and adolescents. The objective of the study described in this chapter was to address the potential contribution of not only HLA class I allele expression, but also tapasin-dependency in a unique population of perinatally-HIV-1 infected paediatric patients from Harare, Zimbabwe with distinct phenotypes of disease control.

3.2. Chapter 3 Results

3.2.1. Paediatric slow progressors and viral controllers from the ZENITH cohort

Children and adolescents were recruited to the ZENITH trial from primary healthcare clinics in Harare, Zimbabwe with new diagnoses of HIV-1 infection, having survived for 6 to 15 years without ART intervention. Subgroups of participants with particular phenotypes of long-term non progression of HIV-1 disease were defined from the wider ZENITH cohort (n=384) (Figure 3.1). Firstly, thirty-three patients were defined as PSPs with a WHO stage disease I/II at recruitment to the trial in addition to CD4+ T cell counts above 350 cells / μL for two or more time points throughout the trial follow up, despite being ART-naïve (Figures 3.2 and 3.3). Two patients from this subgroup were determined by healthcare professionals at the recruiting primary healthcare clinics to have contracted HIV-1 infection through horizontal transmission and were excluded from further analysis, leaving 31 patients deemed to have perinatally-acquired slow progressing HIV-1 infection.

The PVC patient subgroup was defined as those patients with an undetectable HIV-1 plasma VL measured at two instances during follow up of the trial (Figures 3.4 and 3.5). Fourteen patients were originally defined as PVC but one patient was deemed by healthcare professionals at recruitment to have contracted HIV-1 infection through parenteral transmission and was excluded from the study. PVC and PSP patients were treated as mutually exclusive groups in our analyses. Six PVC patients also met the criteria as PSPs but were henceforth only included in the analysis as PVC, unless otherwise stated. Two PVC patients had an undetectable HIV-1 plasma VL over a period of more than one year off ART. As such these patients met the criteria generally accepted for paediatric EC (PEC) (Vieira et al., 2019). One PEC patient's VL was persistently undetectable for four measurements taken over a period of 17 months (patient Z1170, Figure 3.4) and the other PEC patient was also persistently

undetectable over a period of 18 months, but with VL only measured at two consecutive clinic visits over this time (patient Z1168, Figure 3.4). The latter therefore only loosely met the criteria set out for PEC as described by Viera *et al* (2019).

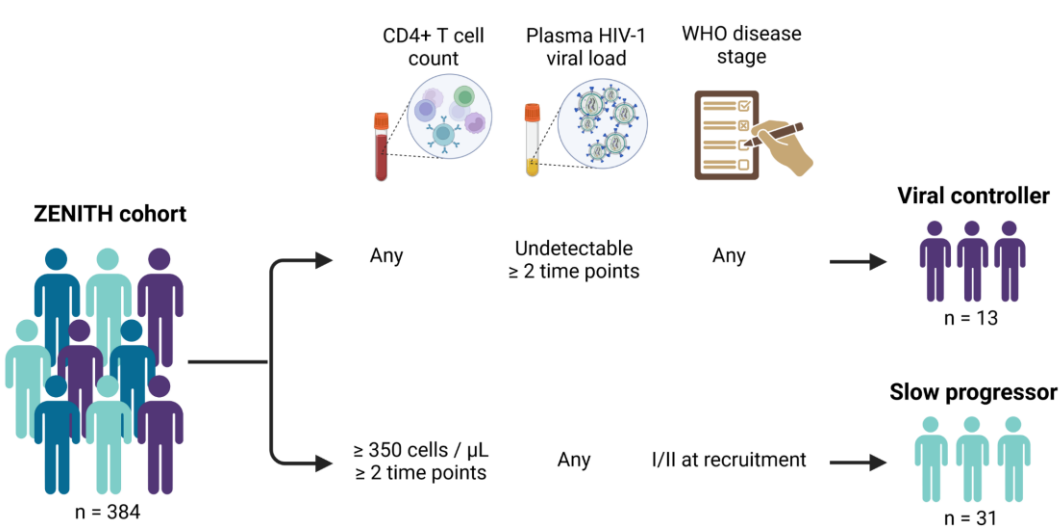


Figure 3.1. **Characterisation of viral controllers and slow progressors from the ZENITH cohort.** Schematic showing decision tree for defining viral controllers and slow progressors from the ZENITH cohort. Viral controller patients were defined as those with undetectable HIV-1 VL for at least two time points off ART. Slow progressor patients were defined as those who had normal CD4+ T cell counts (above 350 cells / µL) for at least two time points off ART as well as having a WHO disease stage of I/II at recruitment to the ZENITH trial. Only patient data collected prior to ART initiation was considered.

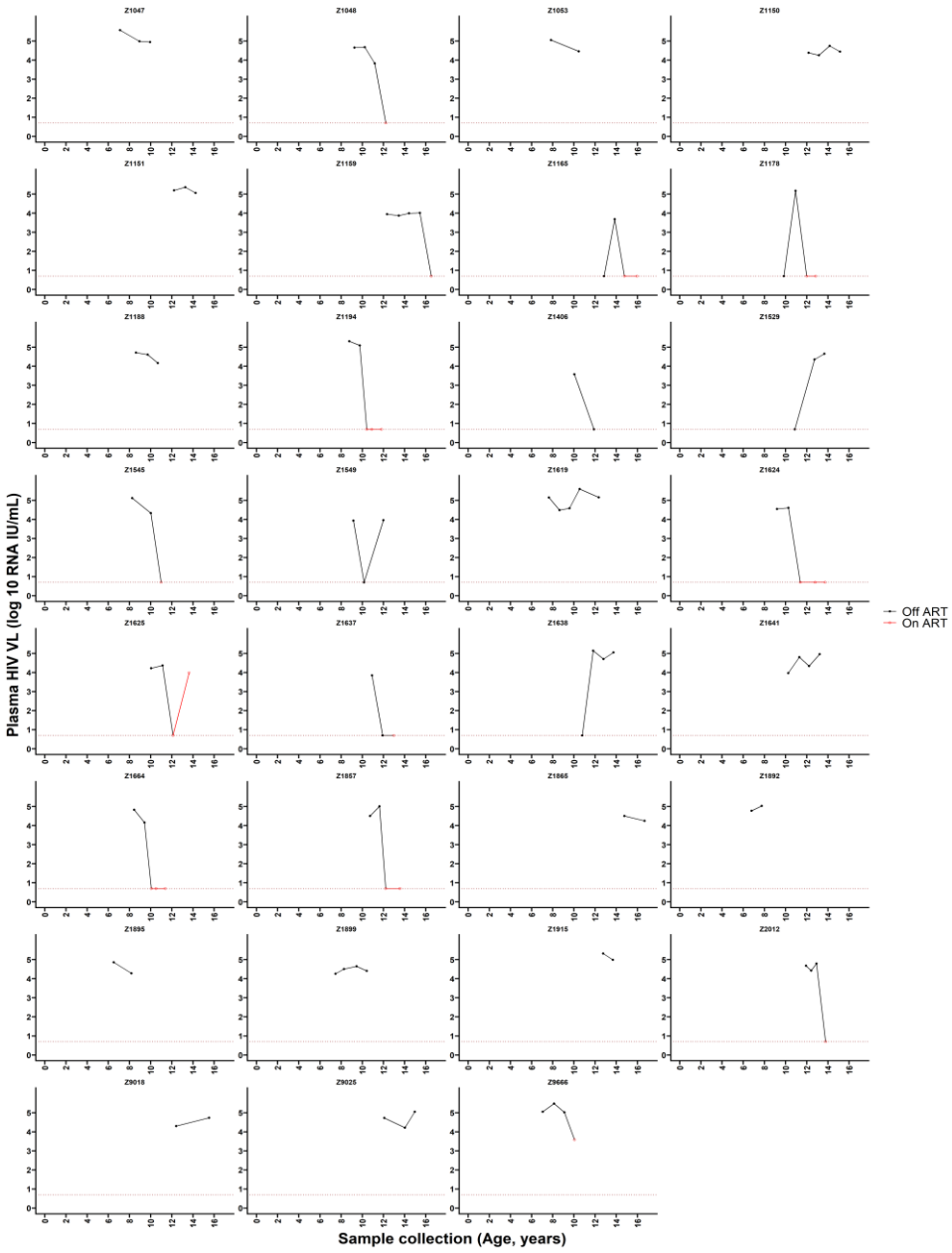


Figure 3.2. **HIV-1 viral loads in PSP patients.** Plasma HIV-1 VL measurements (measured as HIV-1 RNA IU / mL blood) from ZENITH PSP patients (n = 31) over time, from the age of recruitment. Both the HIV-1 plasma VL measurements taken from samples taken pre-ART initiation (black) and after ART-initiation (red) are shown. The assay cut-off for accurate detection of HIV-1 VL is set at 5 IU / mL (red dashed line), below which is considered an "undetectable" viral load.

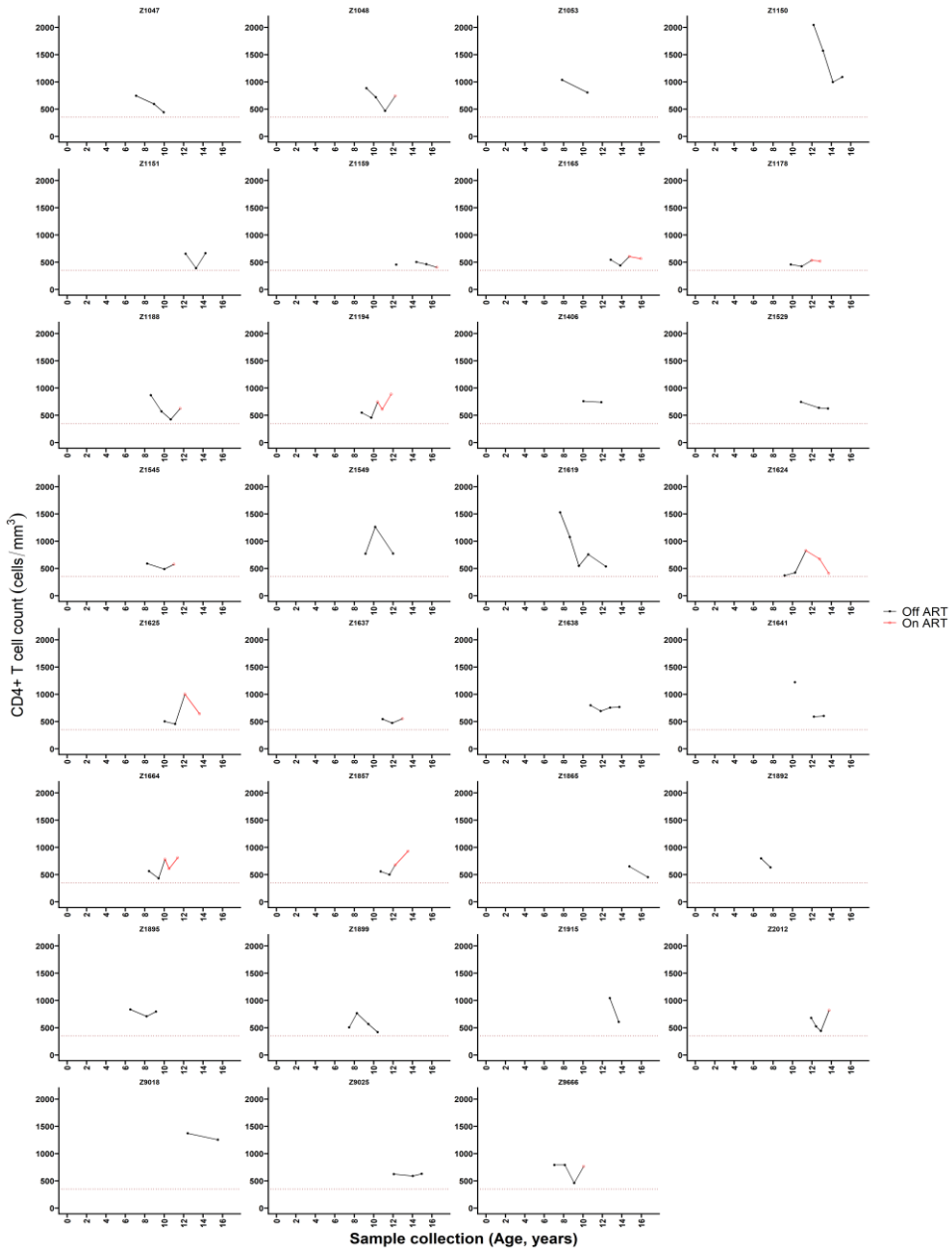


Figure 3.3. **CD4+ T cell counts in PSP patients.** Total CD4+ T cell count per µL of blood from ZENITH PSP patients (n = 31) over time, from the age of recruitment. Samples are shown at time points taken from patients when they were ART-naïve (black) and following when patients were initiated on ART (red), as appropriate. Both the total CD4+ T cell counts / µL measurements taken from samples taken pre-ART initiation (black) and after ART-initiation (red) are shown. A total CD4+ T cell count of 350 cells / µL is shown (dashed red line), below which children and adolescents were eligible to initiate ART (according to national policy at the time of the ZENITH trial).

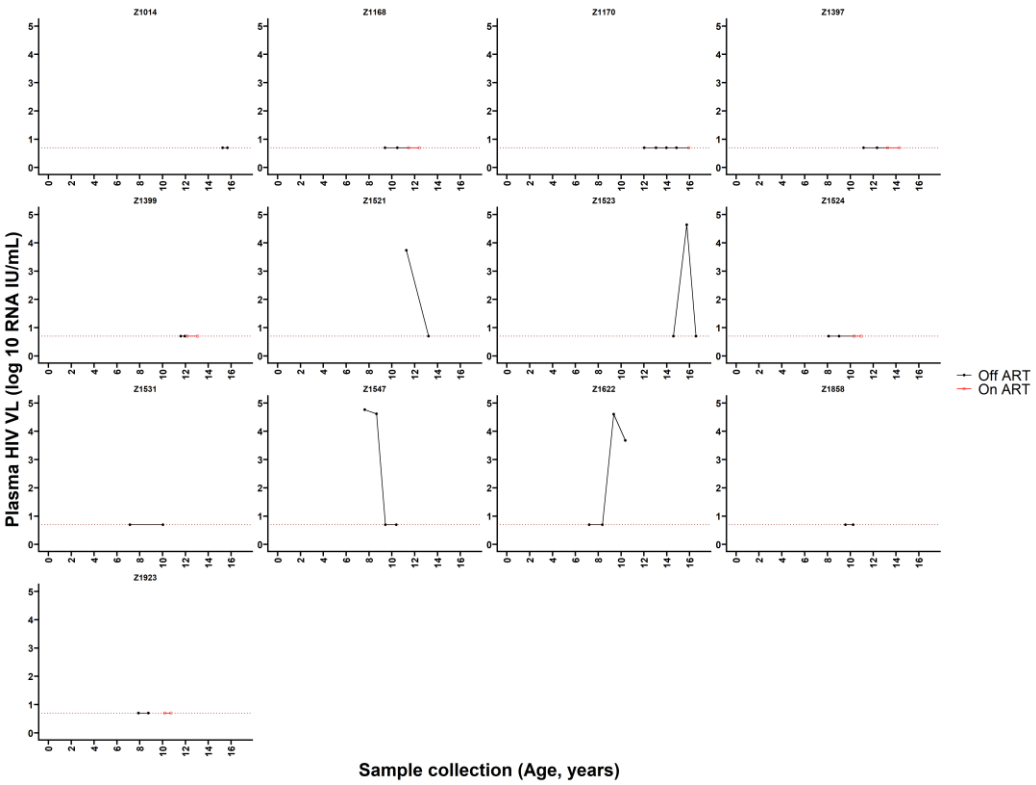


Figure 3.4. **HIV-1 viral loads in PVC patients.** Plasma HIV-1 VL measurements (measured as HIV-1 RNA IU / mL blood) from ZENITH PVC patients (n = 13) over time, from the age of recruitment. Both the HIV-1 plasma VL measurements taken from samples taken pre-ART initiation (black) and after ART-initiation (red) are shown. The assay cut-off for accurate detection of HIV-1 VL is set at 5 IU / mL (red dashed line), below which is considered an "undetectable" viral load.

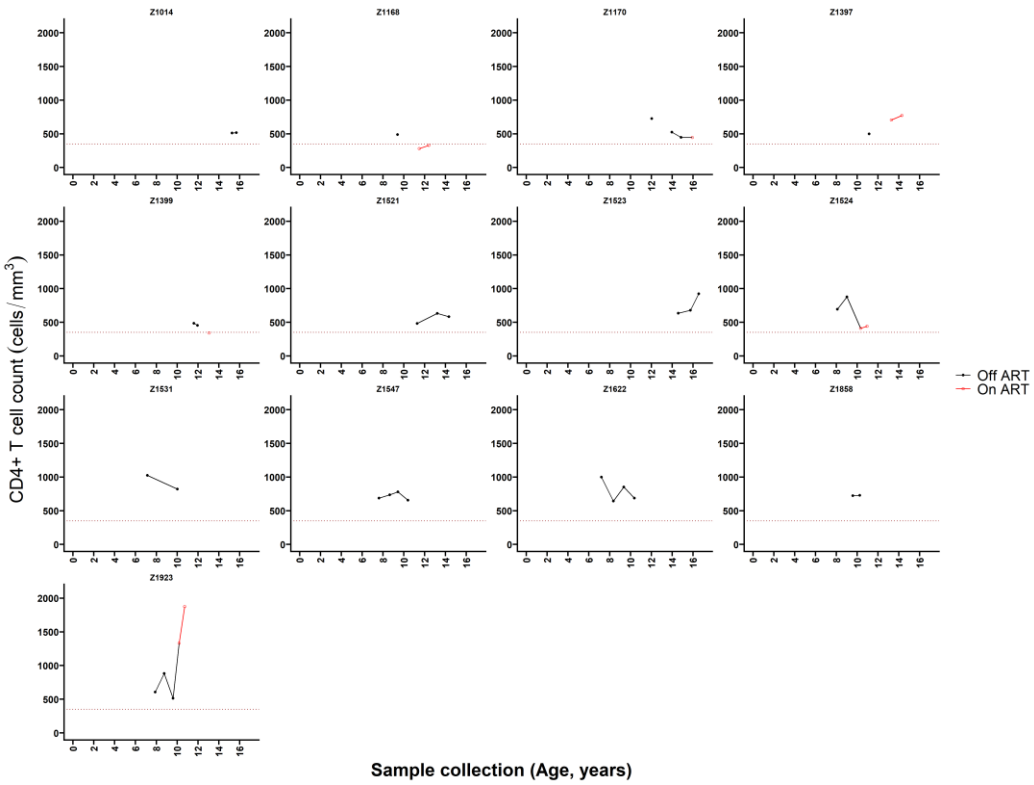


Figure 3.5. CD4+ T cell counts in PVC patients. Total CD4+ T cell count per µL of blood from ZENITH PVC patients (n = 13) over time, from the age of recruitment. Samples are shown at time points taken from patients when they were ART-naïve (black) and following when patients were initiated on ART (red), as appropriate. Both the total CD4+ T cell counts / µL measurements taken from samples taken pre-ART initiation (black) and after ART-initiation (red) are shown. A total CD4+ T cell count of 350 cells / µL is shown (dashed red line), below which children and adolescents were eligible to initiate ART (according to national policy at the time of the ZENITH trial).

3.2.2. Enrichment of specific HLA class I alleles in PSP and PVC patients

The PSP and PVC subgroups of patients from the ZENITH cohort were HLA class I genotyped to determine the genetic correlates at the HLA class I locus associated with paediatric HIV-1 control. Extracted genomic DNA from patient cells was HLA class I-typed by sequence-specific primer sequencing to 4-digit resolution at the HLA-A, HLA-B and HLA-C loci. HIV-1 uninfected age, sex and ethnicity matched controls were also HLA class I typed to measure the HLA class I allele frequencies in the background population of children and adolescents in Harare, Zimbabwe. Twenty-five of the 31 PSP patients were successfully HLA class I typed for all three HLA class I genes, one patient was only typed at the HLA-B and HLA-C loci and one patient was only typed for HLA-C (Table 3.1). Four PSP patients were unable to be HLA class I typed at all due to poor quality genomic DNA. Ten of the 13 PVC patients were successfully HLA class I typed for all three HLA class I genes and one patient was only typed at the HLA-B and HLA-C loci (Table 1). Two PVC patients were unable to be HLA class I typed due to poor quality DNA. We identified a total of 20 unique HLA-A alleles, 28 unique HLA-B and 16 unique HLA-C alleles across the 27 individual PSP and 11 individual PVC patients whom were able to be HLA class I typed across any or all of the HLA class I gene loci in this study (Table 3.2).

HLA class I locus	HIV-1 Infected		HIV-1 Uninfected
	PSP n = 31	PVC n = 13	
HLA-A	26	11	55
HLA-B	26	11	55
HLA-C	26	10	55

Table 3.1. **Collection of HLA class I genotyping data from HIV-1 infected and uninfected patients.** Total number of patients from whom HLA class I gene typing data was able to be collected from the PSP and PVC sub groups of the ZENITH cohort as well as age, sex and ethnicity matched HIV-1 uninfected controls from the INHALE cohort.

HLA class I locus	Uninfected n = 55	PSP n = 31	PVC n = 13
A * 01:01	0	1	0
A * 02:01	9	3	1
A * 02:02	3	0	0
A * 02:05	2	2	0
A * 03:01	7	2	0
A * 23:01	10	6	3
A * 23:17	2	0	0
A * 26:01	3	0	0
A * 29:02	11	2	2
A * 30:01	10	2	0
A * 30:02	19	5	6
A * 30:04	0	2	1
A * 30:09	0	1	0
A * 32:01	1	1	0
A * 33:01	2	0	1
A * 33:03	1	1	1
A * 34:02	3	3	2
A * 36:01	7	2	1
A * 66:01	1	4	0
A * 68:01	0	1	0
A * 68:02	7	1	1
A * 74:01	8	8	1
A * 80:01	1	0	0
B * 07:02	9	1	0
B * 07:05	0	0	1

B * 08:01	5	2	0
B * 13:02	0	0	2
B * 13:03	0	1	0
B * 14:01	0	2	0
B * 14:02	6	3	1
B * 15:03	13	6	2
B * 15:10	9	3	1
B * 15:16	1	1	0
B * 15:17	1	0	0
B * 18:01	8	0	0
B * 35:01	0	2	1
B * 39:10	1	1	0
B * 40:16	0	1	0
B * 40:80	0	0	1
B * 41:02	0	1	0
B * 42:01	11	4	2
B * 42:02	0	1	0
B * 44:03	6	3	0
B * 44:15	0	1	0
B * 45:01	7	1	1
B * 49:01	1	0	1
B * 53:01	9	3	2
B * 57:02	0	1	0
B * 57:03	5	2	4
B * 58:01	4	3	2
B * 58:02	5	7	1
B * 81:01	4	1	0

C * 02:02	1	1	1
C * 02:10	13	5	2
C * 03:02	2	2	1
C * 03:04	7	2	0
C * 04:01	16	6	3
C * 04:07	0	2	0
C * 05:01	1	0	0
C * 06:02	5	6	3
C * 07:01	6	7	2
C * 07:02	7	0	0
C * 07:04	7	0	0
C * 07:18	5	0	0
C * 08:02	5	6	1
C * 08:04	3	0	1
C * 12:03	1	1	0
C * 14:02	0	1	0
C * 15:05	1	1	0
C * 16:01	9	1	2
C * 17:01	11	6	2
C * 18:01	0	5	4
C * 18:02	4	0	0

Table 3.2. **HLA class I allele frequencies in HIV-1 infected and uninfected patients**. HLA class I allele frequencies as sequenced from HIV-1 uninfected (n=55), HIV-1 infected PSP (n=31) or HIV-1 infected PVC (n=13) participants.

None of the PVC patients in this study were homozygous any of the three HLA class I genes. Two of the PSP patients were homozygous for HLA-A (both homozygous for HLA-A*23:01) and one was homozygous for HLA-C (HLA-C*18:01). One patient was homozygous for both HLA-B (HLA-B*57:03) and HLA-C (HLA-C*18:01), but was unable to be typed at the HLA-A locus. The proportion of patients in either the PSP or PVC sub group that were homozygous at any of the three HLA class I gene loci did not significantly differ to the proportion of HIV-1 uninfected age and sex matched control patients who were homozygous for any of the HLA class I genes ($p > 0.05$, Fishers exact test). However, this finding is limited given the limited number of patients in each subgroup.

We tested for enrichment of any of the individual HLA class I alleles identified in this study in the PSP and PVC patients, compared to HIV-uninfected controls. Both HLA-A*66:01 ($p = 0.0383$) and HLA-C*18:01 ($p = 0.00296$) were enriched in the PSP subgroup compared to the HIV-1 uninfected controls (Figure 3.6A and 3.6C, respectively), whereas a significantly lower proportion of PSP patients possessed the HLA-B*18:01 allele compared to control patients ($p = 0.0479$) (Figure 3.6B). HLA-B*13:02 ($p = 0.0256$), HLA-B*57:03 ($p = 0.0356$) and HLA-C*18:01 ($p = 0.000458$) were all enriched in PVC patients compared to HIV-1 uninfected controls (Figure 3.6D-F). Only the difference in HLA-C*18:01 expression between HIV-uninfected controls and PVC patients remained significant after Benjamini-Hochberg correction for multiple comparisons (Figure 3.6F).

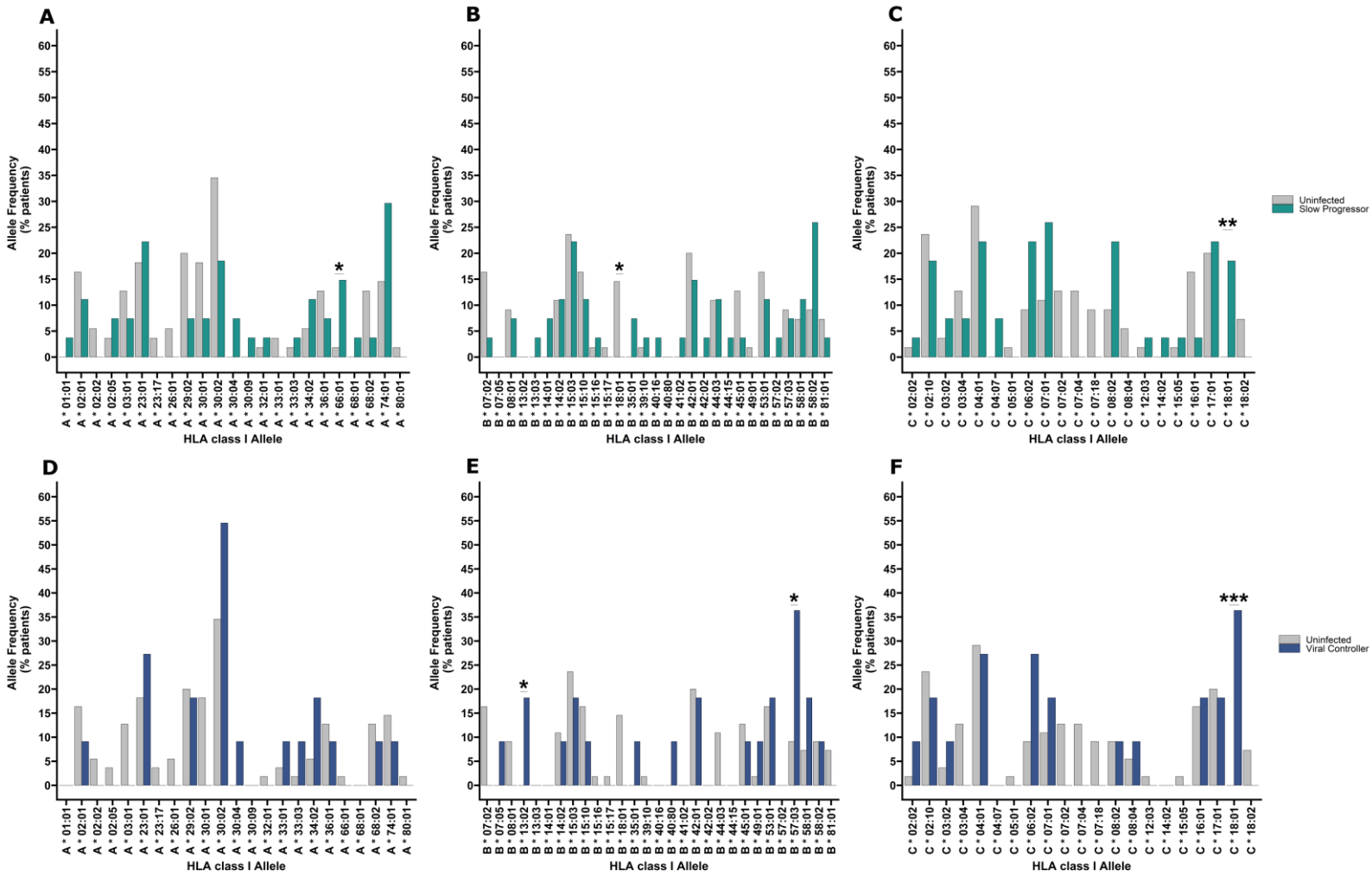


Figure 3.6. HLA class I allele frequencies in PSP and PVC patients.

Figure 3.6. **HLA class I allele frequencies in PSP and PVC patients.** Distribution of HLA class I allele frequencies in HIV-1 uninfected participants (grey bars) compared to HIV-1 infected PSP participants (green bars) and HIV-1 infected PVC participants (purple bars). P-values were estimated using Fisher's exact test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. A) SP HLA-A, B) SP HLA-B, C) SP HLA-C, D) VC HLA-A, E) VC HLA-B, C) VC HLA-C. The difference in HLA-C*18:01 expression between PVC participants and HIV-1 uninfected controls remained significant after Benjamini-Hochberg correction for multiple comparisons.

3.2.3. HLA class I tapasin-dependency is not associated with HIV-1 status or PVC/PSP patient status

TD scores were assigned to HLA class I alleles expressed by HIV-1 uninfected age and sex matched controls as well as PVC and PSP participant subgroups of long-term non-progression of disease from the ZENITH cohort. TD scores were extrapolated from the data described for 97 4-digit HLA class I allotypes published by Bashirova *et al.* (2020). The original study calculated HLA class I TD scores from the ratio of HLA class I expression of each individual allele in tapasin knockout cells compared to tapasin-expressing cells. Higher scores indicate an allele's increased dependency on tapasin for their normal cell surface expression. Global TD scores were calculated as the summation of TD scores from all six HLA alleles expressed by a participant (where the data was available) (n = 6: 3 loci, diploid) and HLA-A, HLA-B and HLA-C TD scores were each calculated from the sum of alleles at each locus for each participant (n=2: diploid).

The aim was to assess the association of HLA class I allele tapasin dependency with odds of HIV-1 status or belonging to the PSP and PVC subgroup of disease.

The global or individual HLA-A/B/C locus TD spectra of the HLA class I alleles expressed by PSP and PVC participant subgroups individually (Figure 3.7A-D) or PVC/PSP patients pooled together (Figure 3.7E-H) did not significantly differ from the TD spectra of HLA class I alleles expressed by HIV-1 uninfected age and sex matched controls ($p > 0.05$, Wilcoxon signed rank test), indicating that HLA class I TD does not predict participant's HIV-1 status in this cohort nor does it predict the specific phenotype of long-term non-progression in the HIV-1 infected participants described in this study.

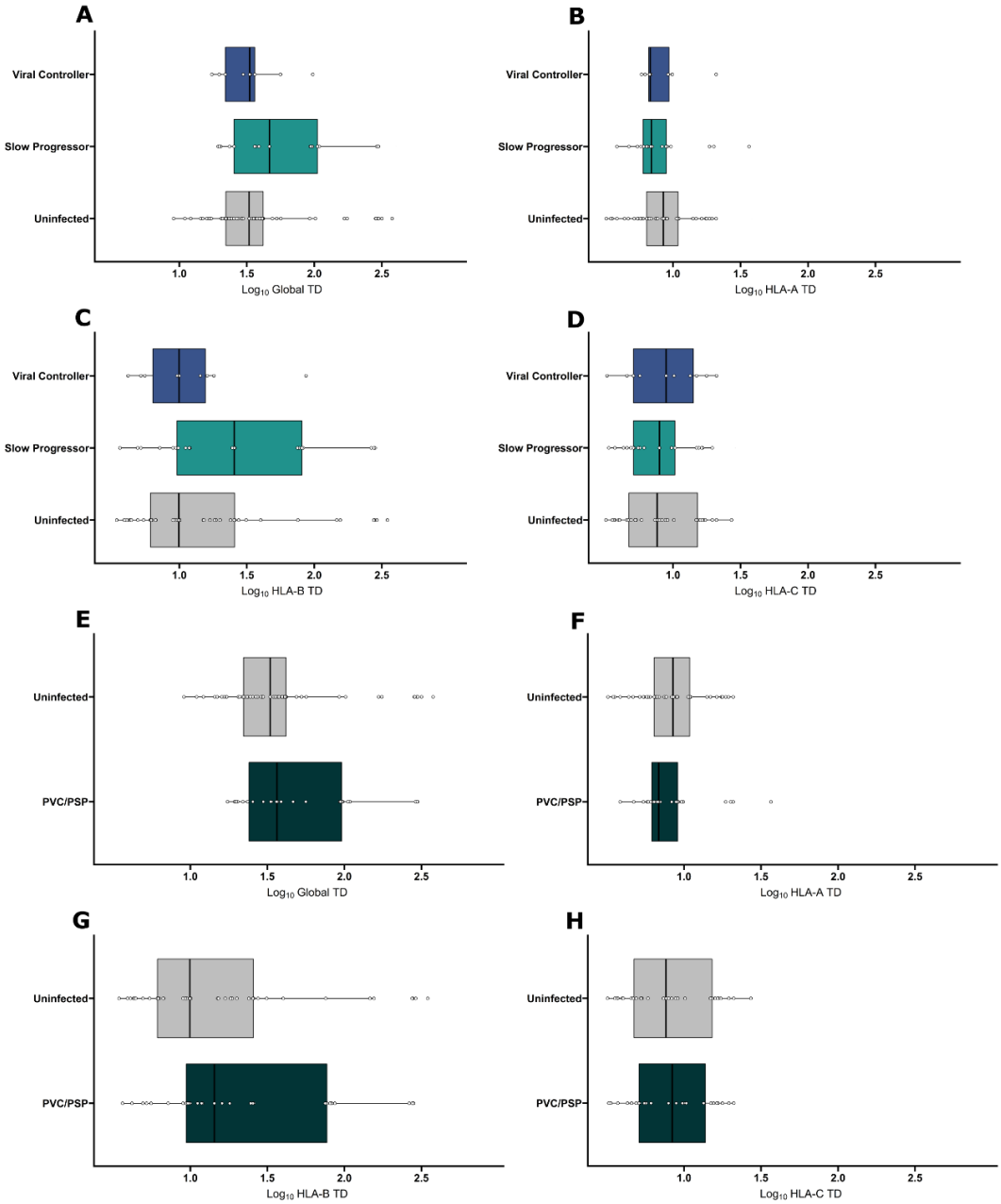


Figure 3.7. HLA class I tapasin-dependency spectra in PSP and PVC patients. Comparisons of the global HLA class I (HLA-A + HLA-B + HLA-C) and individual HLA-A, HLA-B and HLA-C loci tapasin dependency spectrums A-D) between each patient subgroup included in this study: HIV-1 uninfected controls (grey), PVC (purple) and PSP (teal) and E–H) between HIV-1 uninfected controls (grey) and PVC/PSP patients pooled as one subgroup (dark green). All comparisons are non-significant in a paired Wilcoxon signed-rank test $p > 0.05$.

3.2.4. HLA class I tapasin-dependency is associated with increased HIV-1 viral loads in PSP and PVC patients

We next aimed to assess whether HLA class I TD was associated with clinical parameters of HIV-1 disease within the long-term non-progressor PVC and PSP patients described here. All measurements were analysed from patient samples taken pre-ART initiation.

Consistent with the findings of Bashirova *et al.* (2020), higher HIV-1 plasma VLs measured at baseline (on recruitment to the ZENITH trial) in PSP/PVC patients was correlated with the expression of HLA-I alleles with greater dependency on tapasin ($R^2_{adj} = 0.1997$, $p = 0.01828$) (Figure 3.8A). This positive trend was also observed in the PSP and PVC participant subgroups independently, but these comparisons were not significant ($p > 0.05$) (Figure 3.8B-C). The association between HLA class I TD and HIV-1 plasma VL at baseline was not observed when tested against the TD spectra of each individual HLA class I locus (HLA-A, B or C, $p > 0.05$) (data not shown). Global HLA class I TD did not predict any change in PSP/PVC patient's odds of having been recruited to the ZENITH trial with an undetectable HIV-1 plasma VL (< 87 IU / mL), when accounting for participant age at recruitment to the trial and the random contribution of individual HLA class I alleles expressed by each patient (log odds estimate = -0.05357, $p > 0.05$) (Figure 3.8D). Neither was global HLA class I TD associated with absolute CD4+ T cell count (cells / µL) at baseline for PSP/PVC patients ($p > 0.05$) (Figure 3.8E), nor in either the PSP (Figure 3.8F) or PVC (Figure 3.8G) patient subgroups independently.

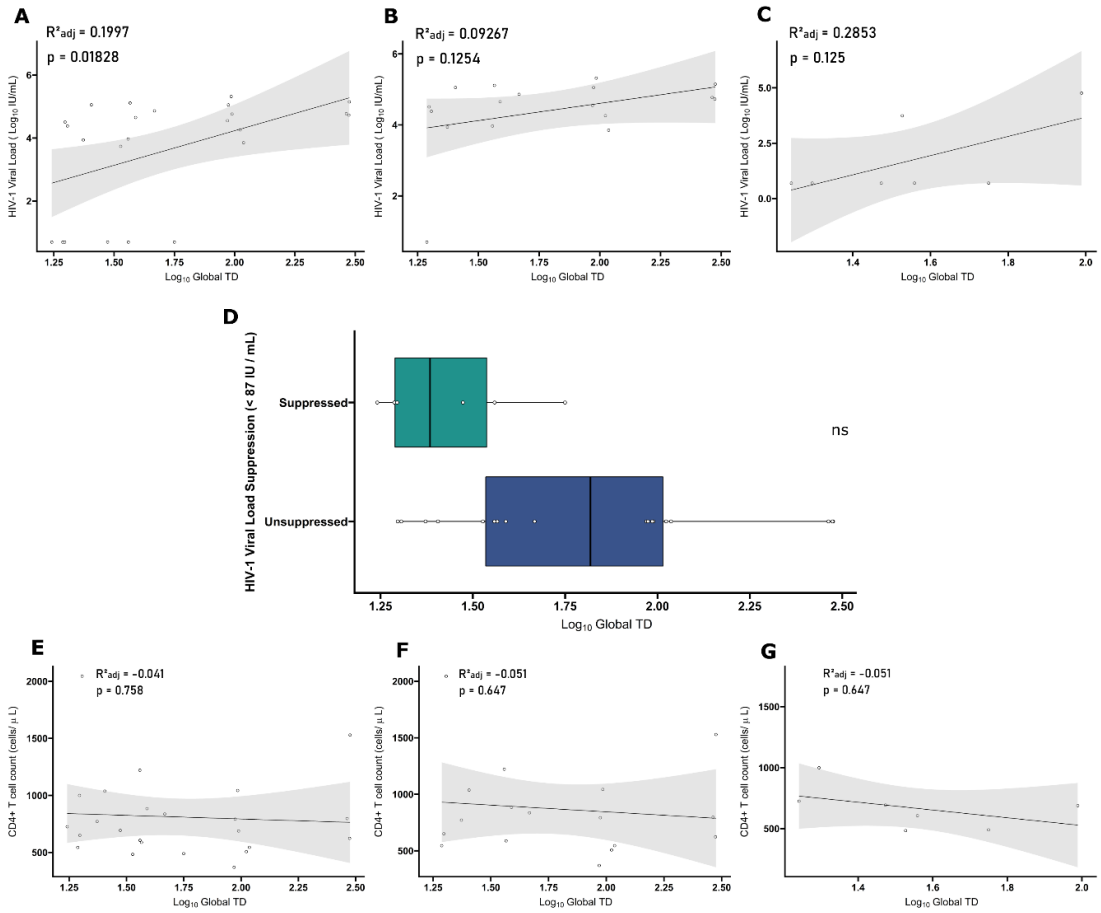


Figure 3.8. **The association of HLA class I tapasin-dependency with HIV-1 viral load and CD4+ T cell count at baseline.** Analysis of the association of HLA class I TD with HIV-1 viral load and CD4+ T cell counts at recruitment to the ZENITH trial (baseline) in PVC and PSP patients with long-term non-progression of HIV-1 disease. Global HLA class I TD association with baseline HIV-1 plasma VL in A) PVC/PSP patients, B) PSP patients and C) PVC patients. D) The association of HLA class I TD with the odds of being virally suppressed (undetectable HIV-1 viral load: < 87 IU / mL) at recruitment to the ZENITH trial. The odds estimation of viral suppression was calculated using a binomial generalised linear regression model with correction for participant age at time of enrolment to the trial as well as individual HLA-I alleles. E-G) Linear regression model of HLA-TD association with CD4+ T cell counts in E) PSP/PVC patients, F) PSP patients and G) PVC patients.

Next, we tested the correlation between HLA class I TD and longitudinal HIV-1 VL measurements over the course of follow-up in the ZENITH trial before ART initiation. Given the finding that lower HIV-1 plasma VLs at baseline were associated with the expression of HLA class I alleles with a significantly increased tapasin-independent phenotype (Figure 3.8A), the aim was to examine whether HLA class I TD is associated with HIV-1 viral control over time. The expression of HLA class I alleles with greater dependency on tapasin was significantly associated with increased mean HIV class I plasma VL in PSP/PVC patients (R^2_{adj} = 0.1849, p = 0.0283) (Figure 3.9A). Independently, the TD spectrum of HLA-B alleles was significantly positively associated with mean HIV-1 plasma VLs in PSP/PVC patients (R^2_{adj} = 0.14, p = 0.0176) (Figure 3.9B) and in the PSP patient subgroup alone (R^2_{adj} = 0.16, p = 0.0335) (Figure 3.9D). Despite HLA-B TD being positively correlated with mean HIV-1 plasma VL in PSP patients, the same trend was not observed for global HLA class I TD scores (p > 0.05) (Figure 3.9C). There was no association between mean HIV-1 plasma VL in PVC patients and HLA class I TD (p > 0.05) (Figure 3.9E), nor was HLA-A or HLA-C TD associated with mean HIV-1 VL in PSP/PVC patients or either patient subgroup independently (p > 0.05, data not shown). Furthermore, increased global HLA class I (estimate = 2.2753, p = 0.00629) and HLA-B (estimate = 1.1965, p = 0.00986) TD was associated with increased mean HIV-1 plasma VLs in PSP/PVC patients when controlling for time post-enrolment to the trial, age, sex and the random contribution of individual HLA class I alleles expressed by each patient (Table 3.3). The association of HLA-B TD with HIV-1 plasma VL in the PSP group did not remain significant in the same linear mixed effects model controlling for the same variables (p > 0.05) (Table 3.3).

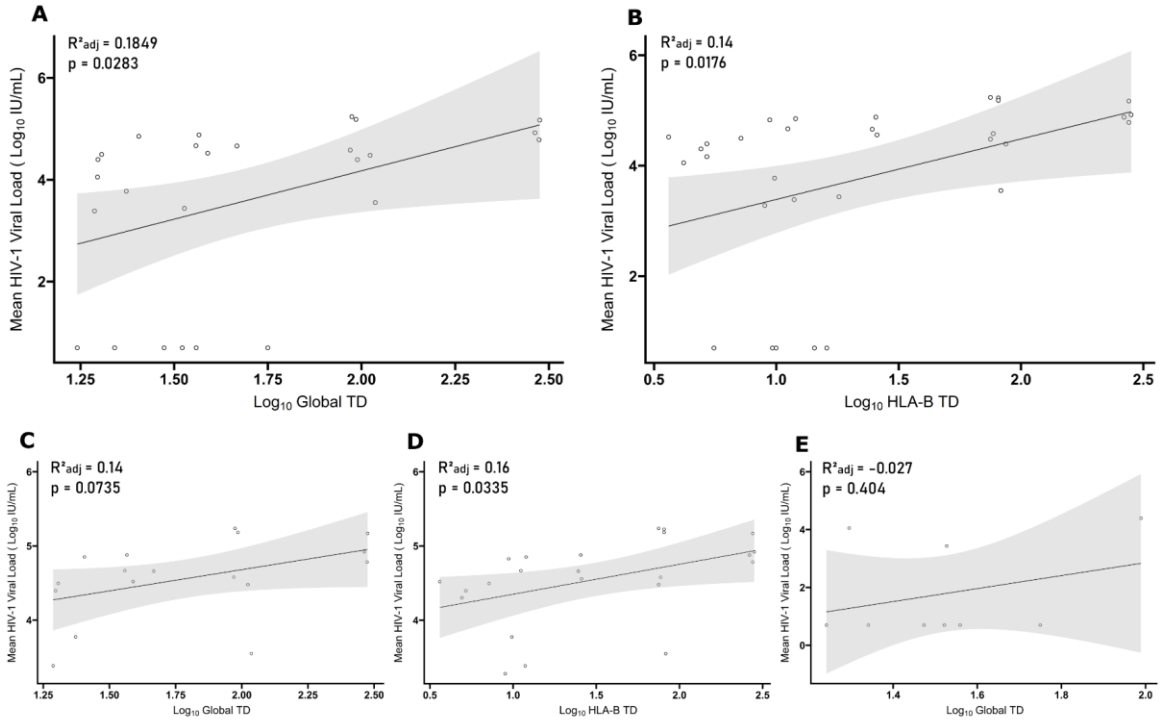


Figure 3.9. **The association of HLA class I tapasin-dependency with mean HIV-1 viral load**. Linear regression models describing the relationship between HLA class I TD and mean HIV-1 plasma VL in A) PSP/PVC patients and global HLA class I TD score, B) PSP/PVC patients and HLA-B TD score, C) PSP patients and global HLA class I TD score, D) PSP patients and HLA-B TD score and E) PVC patients and global HLA class I TD score. Adjusted R^2 values and p-values for the estimation coefficient from each model are shown.

	Patient group					
	PSP/PVC		**PSP**		**PVC**	
HLA class I locus	**VL est. per log_{10} TD increase**	**p-value**	**VL est. per log_{10} TD increase**	**p-value**	**VL est. per log_{10} TD increase**	**p-value**
Global (HLA-A/B/C)	**2.2753**	**0.00629**	0.8305	0.1105	1.3616	0.420
HLA-A	-0.4168	0.73080	-0.8225	0.32	0.2826	0.845
HLA-B	**1.1965**	**0.00986**	0.4055	0.137	0.6011	0.5
HLA-C	-1.352	0.30311	0.3582	0.618	-1.110	0.4195

Table 3.3. **Linear mixed effects model of the relationship between longitudinal HIV-1 plasma VL and global HLA class I/HLA-A/HLA-B/HLA-C tapasin-dependency.** The models describe the relationship between HIV-1 VL and TD in PSP/PVC, PSP and PVC patient subgroups. Models were adjusted for random effects due to each study participant, individual HLA-A, HLA-B and HLA-C alleles, age and time post-enrolment to the trial. Estimate p-values where $p < 0.05$ are highlighted in bold.

3.3. Chapter 3 Discussion

The contribution of HLA class I alleles to protection against disease progression in paediatric cohorts is disputed. Recent data found that HLA class I tapasin-dependency was also associated with slower time to AIDS and lower HIV-1 VLs in a pooled cohort of HIV-1 infected adults, independent of the actual HLA class I alleles expressed and their level of protectiveness (Bashirova et al., 2020). Individual HLA class I alleles that have been found to be associated with slow progression of HIV-1 disease are not necessarily tapasin-independent. For example the highly protective allele HLA-B*57:01 is highly tapasin dependent whereas the disease-susceptible allele HLA-B*35:01 is highly tapasin-independent. Instead, the findings of Bashirova *et al.* (2020) suggest an another mechanism of protection against HIV-1 disease, independent of previous data assigning "protective" or "susceptible" status to individual HLA class I alleles. It is important to consider that given the uncoupling of HLA class I protectiveness from its association with tapasin-dependence and disease progression, whether the findings in adults might be corroborated in HIV-1 infected paediatric patients.

In this chapter we studied the expression of HLA class I alleles and their spectra of tapasin-dependency in a cohort of Zimbabwean perinatally-infected paediatric LTNP patients. These patients were defined as PSPs and PVCs from the ZENITH cohort based on CD4+ T cell count, WHO disease staging and HIV-1 plasma VL described and utilised in the studies published by Leitman *et al.* (2017), Olson *et al.* (2014), Yang *et al.* (2017) and Vieira *et al.* (2019). Our analyses performed here were aligned with these definitions in an attempt to contribute to the harmonisation of data from slow progressor and viral controller patients between studies.

A higher proportion of the PSP and PVC patients identified in this study were female, compared to remaining wider ZENITH cohort (61.7% versus 50.93%, respectively). Although not significant, this apparent enrichment of females is not unexpected, as females have been found to be represented at comparatively higher proportions in both paediatric and adult slow

progressor and EC cohorts (Yang et al., 2017, Anastos et al., 2000, Collazos et al., 2007, Napravnik et al., 2002). Females have also been found to have a slower decline of CD4+ T cell counts, lower viral set points and lower HIV-1 VLs compared to males (European Collaborative Study, 2002, Ruel et al., 2011, Mori et al., 2015, Vieira et al., 2019).

The demographics of the PSP and PVC patients identified from the ZENITH cohort are not surprising and consistent with the literature. Contrary to other published findings from paediatric cohorts however, we did identify protective HLA class I alleles enriched in the slow progressor and viral controller patient groups compared to patients representing an HIV-1 uninfected background population in Harare, Zimbabwe. Different HLA class I alleles have long been known to be associated with HIV-1 disease control or susceptibility in adults, but this association has not previously been seen in paediatric control of HIV-1 disease. The HLA class I alleles significantly enriched in the PSP and PVC subgroups in this study are biologically relevant and consistent with alleles identified in adults. HLA-A*66:01 and HLA-C*18:01 were enriched in PSPs compared to the background population; HLA-B*13:02, HLA-B*57:03 and HLA-C*18:01 were enriched in PVC patients and HLA-B*18:01 was found to be enriched in the HIV-uninfected control group compared to PSPs.

HLA-A*66:01 has been associated with lower HIV-1 VLs and protection against TB infection in exposed South African adults (Seedat et al., 2021, Leslie et al., 2010). HLA-A*66:01 is in LD with HLA-B*58:02 (Matthews et al., 2011): an allele associated with rapid progression to AIDS and poor viral control of HIV-1 (Kiepiela et al., 2004, Ngumbela et al., 2008). However in patients not expressing the disease-susceptible HLA-B*58:02 allele, HLA-A*66:01 has been shown to be independently and significantly associated with control of HIV-1 viraemia (Leslie et al., 2010). Indeed, 50% (2/4) of PSPs in this study also expressed HLA-B*58:02, indicating that HLA-A*66:01 may provide an independent protective effect against drastic CD4+ T cell

loss and development of severe disease despite loss of viral control, as is the phenotype often seen in paediatric slow progressors.

HLA-B*13:02 was significantly enriched in PVCs. HLA-B*13:02 is commonly associated with slow HIV-1 disease progression and low HIV-1 VLs (Honeyborne et al., 2007, Hempel et al., 2009). Similarly, HLA-B*57:03 was also enriched in PVCs compared to HIV-negative controls. HLA-B*57:03 and HLA-B*57:01 are consistently associated with slower disease progression in African and Caucasian patients, respectively (Kaslow et al., 1996, Kiepiela et al., 2004, Migueles et al., 2000, Altfeld et al., 2006, Altfeld et al., 2003). It is striking that even within the low number of PVC patients identified and studied here, significant enrichment of common protective HLA class I alleles can be identified. These findings highlight the potential protective effect of HLA-B*13:02 and HLA-B*57:03 in a paediatric cohort with natural control of HIV-1 viral replication.

HLA-C*18:01 was found to be significantly enriched in both the PSP and PVC groups. Mhandire *et al.* (2018) showed that HLA-C*18:01 was associated with lower HIV-1 VLs and higher CD4+ T cell counts in ART-naïve HIV-1 infected Zimbabwean adults compared to HIV-1 infected patients whom did not carry the allele (Mhandire et al., 2018). We provide evidence here that HLA-C*18:01 may also contribute to HIV-1 viral control and slower disease progression in Zimbabwean children and adolescents. HLA-C*18:01 is restricted to expression in African populations and is in strong LD with the highly protective alleles HLA-B*57 and HLA-B*81 (Mhandire et al., 2018, Kiepiela et al., 2004). Previous studies have suggested that HLA-C*18:01 may be independently protective despite being in strong LD with highly protective alleles (Lazaryan et al., 2006, Tang et al., 2008). That being said, seven out of the nine HLA-C*18:01 positive PSP/PVC patients identified in this study also expressed either HLA-B*57 or HLA-B*81, with one patient being homozygous at both the HLA-B locus for HLA-B*57:03 and at the C-locus for HLA-C*18:01. This brings into question whether the

enrichment of HLA-C*18:01 in both PSPs and PVCs is an indication of its protectiveness in paediatric HIV-1 disease or whether it is due to it being in LD with other protective alleles such as HLA-B*57:03, also found to be enriched in this cohort.

HLA-B*18:01 was enriched in the HIV-uninfected age and sex matched control group of children and adolescents when compared to PSP patients. HLA-B*18 has been associated with a significantly lower risk of HIV-1 MTCT intra-partum or via breastfeeding in Kenyan infants (Farquhar et al., 2004). HLA-B*18:01 is additionally associated with a reduced risk of HIV-1 seroconversion in highly exposed but seronegative cohorts of both Kenyan and Thai sex workers (MacDonald et al., 2000, Beyrer et al., 1999). It is plausible that a proportion of the HIV-1 uninfected controls included in this study may have been born to HIV-1 infected mothers. Enrichment of HLA-B*18:01 in this group may have protected these children from vertical transmission and HIV-1 seroconversion. HLA class I discordance between HIV-1 infected mothers and their children is also associated with a reduced risk of perinatal transmission (MacDonald et al., 1998, Mackelprang et al., 2008). If the HLA-B*18:01 allele was not inherited and therefore shared with the mother this would also confer some protection against perinatal HIV-1 infection in the control group. Nevertheless, neither the HIV-1 status nor the HLA class I types of the mothers of the age and sex matched HIV-1 uninfected control children used in this study are not known. This interpretation that HLA-B*18 enrichment in these children has arisen from protection against HIV-1 MTCT is purely hypothetical. However, it highlights the importance of proper characterisation and consideration of comparative control patients included in studies from populations with high prevalence of HIV-1 infection.

These findings challenge the idea that protective HLA class I alleles do not play a role in HIV-1 control in children and adolescent disease course. We then asked whether tapasin independent HLA class I alleles might be associated with HIV-1 control in paediatric patients, similar to

that seen in adults (Bashirova et al., 2020). The PVC and PSP cohorts were analysed independently and also pooled as a PSP/PVC group to assess the spectrum of tapasin-dependency of the alleles expressed by these patients. There was no association between the levels of tapasin-dependency of the HLA class I alleles expressed by PSP, PVC or PSP/PVC patients versus background population controls. This indicates that the identification of protective alleles in these groups was probably not a by-product of tapasin-dependency correlating with protection, but likely due to the expression of the alleles themselves.

Increased tapasin-dependency of HLA class I alleles in the HIV-1 infected PSP/PVC group was associated with higher baseline and mean HIV-1 VLs. This association was true for both global HLA class I (HLA-A+HLA-B+HLA-C) and HLA-B when controlling for age, sex, time post enrolment to the trial and the expression of individual HLA class I alleles to account for the different contribution of some protective HLA class I alleles to control. This relationship between HLA-B tapasin-dependency and mean HIV-1 VLs was significant in the PSP group when analysed independently, but the association was not true when corrected for individual HLA class I alleles, sex, age and time post enrolment to the trial. Tapasin dependency did not correlate with CD4+ T cell counts, indicating that in this cohort the presence of more tapasin-independent alleles could confer some protection against viral replication and promote viral clearance rather than preservation of normal immune cell counts. Bashirova *et al.* (2020) also showed that increased HLA class I tapasin-dependency was associated with higher mean HIV-1 VLs and faster progression to AIDS in adult patients from six US and one European cohort (Bashirova et al., 2020). Progression to AIDS was not measurable in the ZENITH cohort due to the number of patients in this study, the commencement of ART by many of the patients during the trial and the relatively short follow up period of the ZENITH trial. However, we have independently validated in Zimbabwean paediatric HIV-1 infected patients the association between tapasin-dependency and HIV-1 VL.

The study by Bashirova *et al.* (2020) went further to interrogate why tapasin-independent HLA class I alleles might contribute to control of HIV-1 infection and slower progression of disease. Tapasin-independent HLA class I alleles were found to present a greater breadth of the HIV-1 peptide repertoire compared to alleles that were more dependent on tapasin (Bashirova et al., 2020). Presentation of a greater breadth of the HIV-1 peptide repertoire leads to a broader cytotoxic CD8+ T cell response. The breadth of the HIV-1 peptide repertoire presented to CD8+ T cells is associated with disease control due to the greater exertion of selection pressure at more sites across the viral genome, making it difficult to escape immune recognition (Carlson et al., 2012, Muenchhoff et al., 2016, Kiepiela et al., 2007). Presentation of a broader HIV-1 peptide repertoire is also associated with heterozygosity of the HLA class I genes (Arora et al., 2020). HLA class I homozygosity is associated with more rapid progression to AIDS and also poor prognosis for non-small cell lung cancers, compared to heterozygous patients (Abed et al., 2020, Lee et al., 2021, Carrington et al., 1999). We did not observe a significant enrichment in heterozygosity at any of the HLA class I gene loci in PSP or PVC patients compared to HIV-1 uninfected control patients, but one major limitation of this study is the lack of a comparative paediatric rapid progressor group. This group would be very difficult to identify given the high likelihood of death among infants with perinatally-acquired HIV-1 before 2 years of age (Marinda et al., 2007). Nevertheless, it would be interesting to assess whether highly tapasin-dependent HLA class I alleles or higher levels of homozygosity might be associated with rapid disease progression or higher VLs and loss of CD4+ T cells in paediatric patients. Nevertheless, greater promiscuity in HIV-1 peptide presentation explains why tapasin-independent HLA class I alleles are associated with delayed disease progression and lower HIV-1 VLs in paediatric and adult HIV-1 infected patients. It would be interesting to study HIV-1 peptide repertoire breadth in other paediatric cohorts of different ethnicities and

rates of disease progression to further elucidate the role and mechanisms behind HLA class I tapasin dependency and HIV-1 disease.

Rizvi *et al.* (2014) originally presented data that suggested that tapasin-independent alleles were associated with a greater risk of death following HIV-1 infection (Rizvi et al., 2014). Bashirova and colleagues highlighted in their most recent study that their findings directly contrast with the 2014 study, which was limited by smaller samples sizes and lack of validation of the relationship for HLA class I allotypes aside from HLA-B. The data we have described here from ZENITH PSPs and PVCs support the renewed analysis by Bashirova *et al.* (2020) of tapasin-dependent alleles and their association with faster HIV-1 disease progression.

Tapasin-independent HLA class I allele expression and greater breadth of the peptide repertoire presented to CD8+ T cells has been associated with the control of other viral diseases. Tapasin-independent HLA class I alleles also present a broader range of the Dengue virus peptide repertoire and greater peptide repertoire breadth was found to be associated with protection against Marek's disease in chickens (Bashirova et al., 2020, Chappell et al., 2015). Challenge of rhesus macaques with the RhCMV/SIVgag vaccine resulted in the control and clearance of highly pathogenic SIV in over half of the challenged animals (Hansen et al., 2016). The success of RhCMV/SIVgag was attributed to the generation of protective CD8+ T cell responses against a broad range of low affinity Mamu-E (non-classical class I HLA-E orthologue in macaques) restricted peptides. These findings provide further evidence to suggest that the presentation of a broader range of even low affinity peptides may be protective against the development of severe viral disease. This highlights the potential benefit for targeting responses against a wider range of peptide antigens in HIV-1 vaccine design.

As discussed in Chapter 1, different viral pathogens are known to interfere with and downregulate tapasin to evade HLA class I mediated immune responses. As we and others have shown, the expression of tapasin-independent HLA class I alleles is associated with control of HIV-1 infections and better disease outcomes. The relationship between tapasin-dependency and HIV-1 disease progression has been interrogated to show that tapasin-dependency influences the breadth of the peptide repertoire presented to CD8+ T cells. We further speculate that, similar to other viral pathogens, HIV-1 may have evolved some mechanism to downregulate or impair tapasin function. This would result in an evolutionary benefit for HIV-1 infected patients expressing tapasin-independent HLA class I alleles: they would be able to circumvent this hypothesised viral immune evasion mechanism and be less reliant on tapasin for their cell surface presentation to CD8+ T cells. We therefore sought to address this question in the following chapters to try and elucidate the mechanism behind the relationship between HLA class I tapasin-dependency and HIV-1 disease progression.

Chapter 4: The role of the peptide loading complex in HLA class I downregulation by HIV-1

4.1. Chapter 4 Introduction

Tapasin-independent HLA class I alleles are associated with slower progression of HIV-1 disease and lower HIV-1 VLs in both children and adults ((Bashirova et al., 2020), Chapter 3). Tapasin-independent HLA class I alleles present a broader viral peptide repertoire, making it harder for HIV-1 to escape immune recognition from CD8+ T cells (Bashirova et al., 2020, Di Pucchio and Sekaly, 2020). However, there are alternative viral immune evasion mechanisms that should be considered as they may explain the association of tapasin-independent HLA class I alleles with control of HIV-1 disease. Other chronic viral pathogens such as HCMV and EBV are known to exploit the PLC in order to retain HLA class I molecules in the ER and downregulate their expression at the cell surface. HIV-1 downregulates HLA class I (Kerkau et al., 1989). We have presented an mechanistic cellular hypothetical mechanism by which HIV-1 may downregulate or impair PLC function in order to downregulate HLA class I. This hypothesis would explain the correlation we and others see between tapasin-independent HLA class I molecules and slow progression of HIV-1 disease ((Bashirova et al., 2020) and Chapter 3). Individuals who express tapasin-independent HLA class I alleles may have an evolutionary advantage in HIV-1 infection, as they would be able to circumvent the potential downregulation or interference of tapasin/the PLC by HIV-1 and express HLA class I at less reduced levels at the cell surface. This is seen in HCMV infection. The HCMV protein US3 binds to and downregulates tapasin to selectively inhibit peptide presentation on tapasin-dependent HLA class I alleles, such as HLA-B*44:02, but not tapasin-independent HLA class I alleles like HLA-B*27:05 (Park et al., 2004b, Halenius et al., 2011).

In Chapter 1, we outlined the current understanding surrounding HIV-1 downregulation of HLA class I molecules. However, there is little evidence thus far regarding HIV-1's interaction with the PLC and how this might reduce HLA class I presentation in HIV-1 infection. Kasper *et al.* (2005) showed that HIV-1 Nef co-precipitated with tapasin, but this interaction was

significantly diminished when β2-m was knocked out in CEM T cells (Kasper et al., 2005). The authors suggested that this showed that potential tapasin-Nef interactions might occur due to a by-product of Nef and tapasin each independently interacting with HLA class I. Nevertheless, in this same study co-precipitation immunoblots showed that the interaction between HIV-1 Nef and tapasin was not completely abrogated in β_2-m knockout cells. MCV protein MC80 downregulates HLA class I expression by degrading tapasin via the ubiquitination pathway (Harvey et al., 2019). MC80 was shown to be stabilised by and dependent on β2-m expression (Harvey et al., 2019). It begs the question whether a similar mechanism regarding β2-m expression may have been occurring in the interaction seen between HIV-1 Nef and tapasin in the original 2005 study. It is possible that this interaction was destabilised when β2-m was knocked out, rather than being a by-product of independent HLA class I interactions by Nef and tapasin. Therefore, it should be considered that potential interactions between HIV-1 Nef and tapasin have not been fully explored in the correct cellular models *in vitro.*

HIV-1 Nef is not the only viral protein that has been suggested to be interacting with the PLC. A study of the HIV-1-host interactome of the viral Env and Vif proteins revealed both tapasin and TAP2 as candidate proteins in the interactome of Env gp160 (Luo et al., 2016). Env gp160 has also been found to complex with the ER chaperones calnexin and calreticulin (Otteken and Moss, 1996, Papandreou et al., 2010). No further experiments were described in this study or others to investigate potential Env/PLC interactions in the ER and what impact this may have on HLA class I expression.

Given the limited knowledge that exists to date regarding HIV-1 interaction with the PLC and how this might contribute to HLA class I expression and progression of disease, we considered this an important avenue of immunological research to explore. The correlation between tapasin-dependency as a distinct immunological phenotype associated with viral disease has

not been extensively explored beyond the recent study by Bashirova *et al.* (2020) and the results outlined in Chapter 3 of this thesis. Bashirova *et al.* (2020) correlated their findings with the presentation of a broader repertoire of the HIV-1 peptidome to CD8+ T cells, but a potential direct mechanism of viral interactions with the PLC in HIV-1 infection has not been explored.

We sought to address whether HIV-1 exploits tapasin or other components of the PLC in order to potentiate the downregulation of HLA class I and evade the immune response. In the following chapter, we firstly aimed to assess whether we could measure HLA class I downregulation *in vitro* in a VSV-G pseudotyped model of HIV-1 infection in HEK293FT and HAP1 cells. VSV-G pseudotyped HIV-1 isolates were used in our experiments in order to maximise infection efficiency and infect cell lines that did not express the CD4 or CCR5 co-receptors. Next we evaluated whether the expression of tapasin or the wider PLC in the ER was essential for HIV-1-mediated reduction of HLA class I expression. We lastly aimed to evaluate the susceptibility of tapasin-dependent and tapasin-independent HLA class I alleles to downregulation by HIV-1. This chapter aims to elucidate other potential mechanisms behind HLA class I tapasin-dependency and its association with HIV-1 disease progression.

4.2. Chapter 4 Results

4.2.1. VSV-G pseudotyped HIV-1 downregulates HLA class I in HEK293FT and HAP1 cells

We first aimed to establish an *in vitro* infection model of HIV-1 in our lab in order to study the effects on HLA class I and PLC expression. HEK293FT cells were used due to their transfection efficiency and reproducibility (Ferreira et al., 2020). HAP1 cells were also used in our experiments as, due to their haploid nature, they were able to be efficiently edited by CRISPR/Cas9 technology by the lab of Dr Robbert Spaapen (Sanquin Research and University of Amsterdam, The Netherlands).

HEK293FT cells were infected with VSV-G pseudotyped NL4-3 and CH077 strains (hereby referred to as NL4-3-G and CH077-G) of HIV-1 at an MOI of 1.0 for 48 hours. NL4-3 is a highly lab-adapted strain of clade B HIV-1 commonly used in *in vitro* studies. It has been noted that HIV-1 NL4-3 does not downregulate HLA-C to the same degree as primary HIV-1 isolates (Apps et al., 2016). CH077 is a primary T/F virus strain that has been shown to downregulate both HLA-C and HLA-E expression in CD4+ T cells (Hopfensperger et al., 2020, van Stigt Thans et al., 2019). Given these previous findings, both the NL4-3 and CH077 strains of HIV-1 were studied to maximise the potential of capturing any effect on HLA class I and PLC expression after infection.

Viral infection efficiency (percentage of HIV-1 Gag positive cells) and cell surface HLA class I expression (MFI) in HIV-1 infected HEK293FT cells were measured by flow cytometry after 48 hours post infection (Figure 4.1A). NL4-3-G and CH077-G-infected cells were detectable by flow cytometry by staining with the anti-HIV-1 gag p24 antibody clone KC57 (Figure 4.1A and 4.1C). HLA class I expression was measured with the pan-HLA class I antibody clone W6/32. HLA class I was significantly downregulated in infected Gag-positive (Gag+) cells

compared to bystander (Gag-) and uninfected cells (Figure 4.1A and 4.1D). Both NL4-3-G (Figure 4.1D and 4.1E) and CH077-G (Figure 4.1D and 4.1F) downregulated HLA class I by similar levels in HEK293FT cells in our *in vitro* infection model.

We then measured HLA class I expression and HIV-1 infection efficiency in the human HAP1 cell line to test the reproducibility of HLA class I downregulation in the haplotype cell line used for PLC KO. HAP1 cells were infected with both NL4-3-G and CH077-G at an MOI of 1.0 for 48 hours followed by analysis by flow cytometry. HAP1 cells were able to be infected with NL4-3-G and CH077-G, as detected by Gag+ cells (Figure 4.1B and 4.1G). HIV-1 infection with both the NL4-3-G and CH077-G strains significantly downregulated HLA class I levels after 48 hours of infection (Figure 4.1H-J).

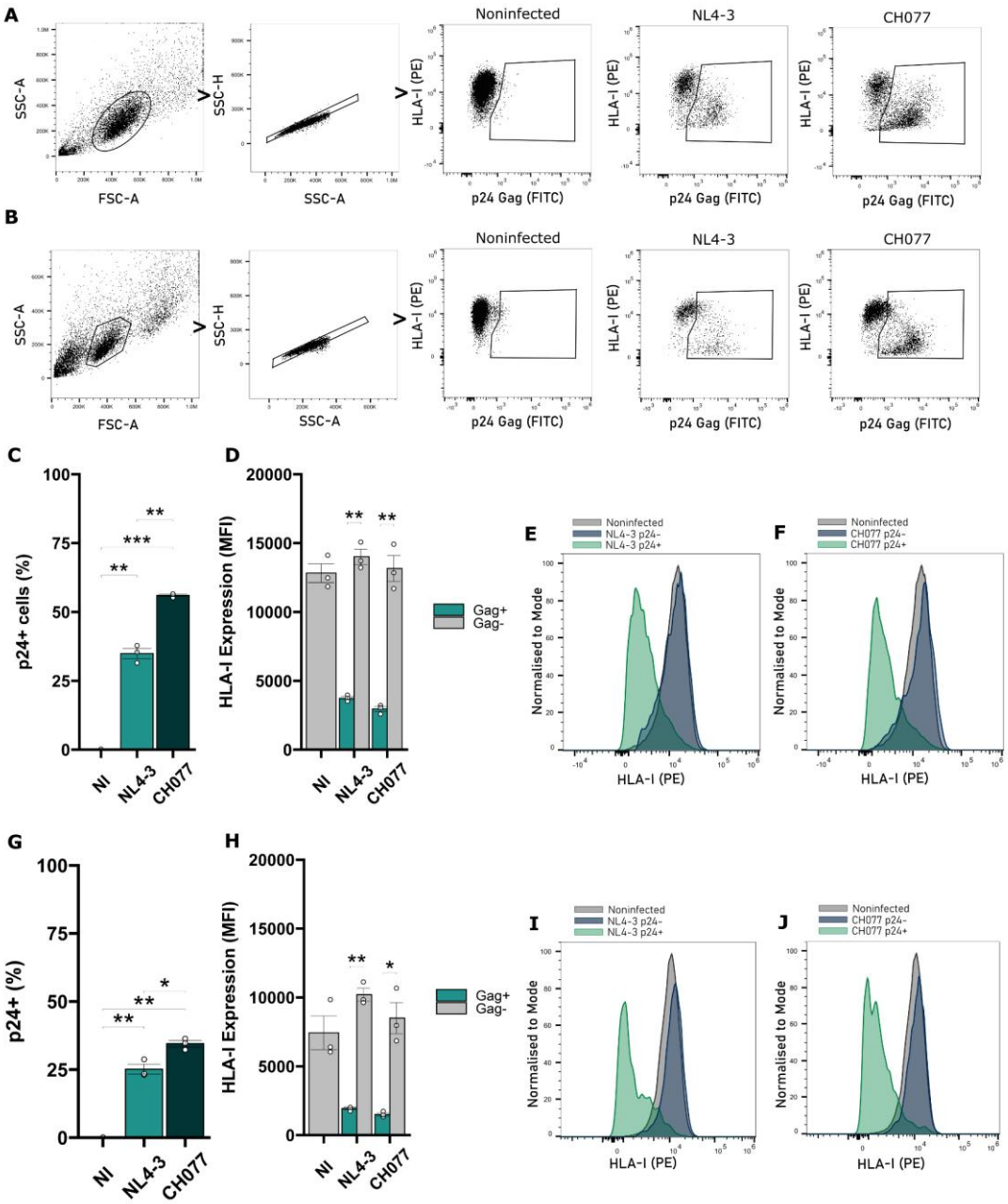


Figure 4.1. HLA class I downregulation measured by flow cytometry in HEK293FT and HAP1 cell lines after HIV-1 infection.

Figure 4.1. **HLA class I downregulation measured by flow cytometry in HEK293FT and HAP1 cell lines after HIV-1 infection.** A) Gating strategy for HEK293FT cells to identify and compare HLA class I MFIs in HIV-1 Gag+ cells 48 hours post-infection. B) Gating strategy for HEK293FT cells to identify and compare HLA class I MFIs in HIV-1 Gag+ cells 48 hours post-infection. C) HIV-1 Gag+ positive HEK293FT cells detected by flow cytometry with FITC-conjugated anti-HIV gag p24 antibody clone KC57 after infection with HIV-1. D-F) Cell surface HLA class I expression (MFI) in Gag+ and Gag- (bystander) HEK293FT cells infected with NL4-3 (D, E) or CH077 (D, F). G) HIV-1 Gag+ positive HAP1 cells detected by flow cytometry with FITC-conjugated antibody clone KC57 after infection with HIV-1. H-J) Cell surface HLA class I expression (MFI) in Gag+ and Gag- (bystander) HAP1 cells infected with NL4-3 (H, I) or CH077 (H, J). Individual points correspond to independent experiments (n=3). P-values were estimated using student's t-test with Benjamini-Hochberg correction for multiple comparisons where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NI: Noninfected.

4.2.2. HIV-1 downregulates HLA class I in PLC knockout cells

HLA class I was downregulated *in vitro* by both the lab-adapted NL4-3-G and T/F CH077-G strains of HIV-1 in both HEK293FT and HAP1 cell lines. Given the evidence that our infection model was working as expected and consistent with the published literature, we next asked whether knockout (KO) of various components of the PLC might abrogate HIV-1's ability to downregulate HLA class I in HAP1 cells.

PLC KO HAP1 cells were generated by CRISPR/Cas9 editing of the HAP1 genome in the laboratory of Dr Robbert Spaapen, Sanquin Research and the University of Amsterdam, Amsterdam, The Netherlands (de Waard et al., 2021). Tapasin, TAP1, TAP2, calreticulin and ERp57 KO and unedited WT HAP1 cells were selected for analysis in this study. Unfortunately, the ERp57 KO cell line was not viable upon thawing from LN_2 storage due to an unknown contamination.

Prior to HIV-1 infection, it was evident that knockout of various components of the PLC resulted in significantly reduced levels of HLA class I expression at the cell surface compared to WT HAP1 cells (Figure 4.2). These findings are consistent with the published literature on HLA class I expression in cells with reduced levels of components of the PLC.

HAP1 PAKC KO and WT cells were infected with both NL4-3-G and CH077-G at an MOI of 1.0 for 48 hours followed by analysis by flow cytometry. NL4-3-G and CH077-G infected WT and all PLC KO cells at similar levels (Figure 4.3A-B). Results showed that NL4-3-G and CH077-G infection resulted in significantly reduced levels of HLA class I in HAP1 WT, tapasin, TAP1, TAP2 and calreticulin PLC KO cells (Figure 4.3C). This data indicates that tapasin, TAP1, TAP2 or calreticulin are not essential components of the PLC required for HLA class I downregulation to occur in HIV-1 infection.

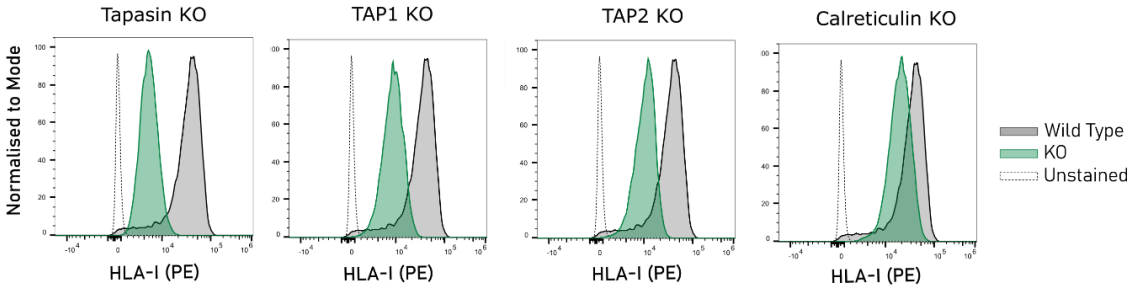


Figure 4.2. **HLA class I expression in HAP1 WT compared to HAP1 PAKC cells.** Expression of HLA-A*02:01, HLA-B*40:01 and HLA-C*03:04 in HAP1 WT cells (grey) compared to HAP1 PAKC cells (green). Background PE MFI in unstained controls shown as dashed line.

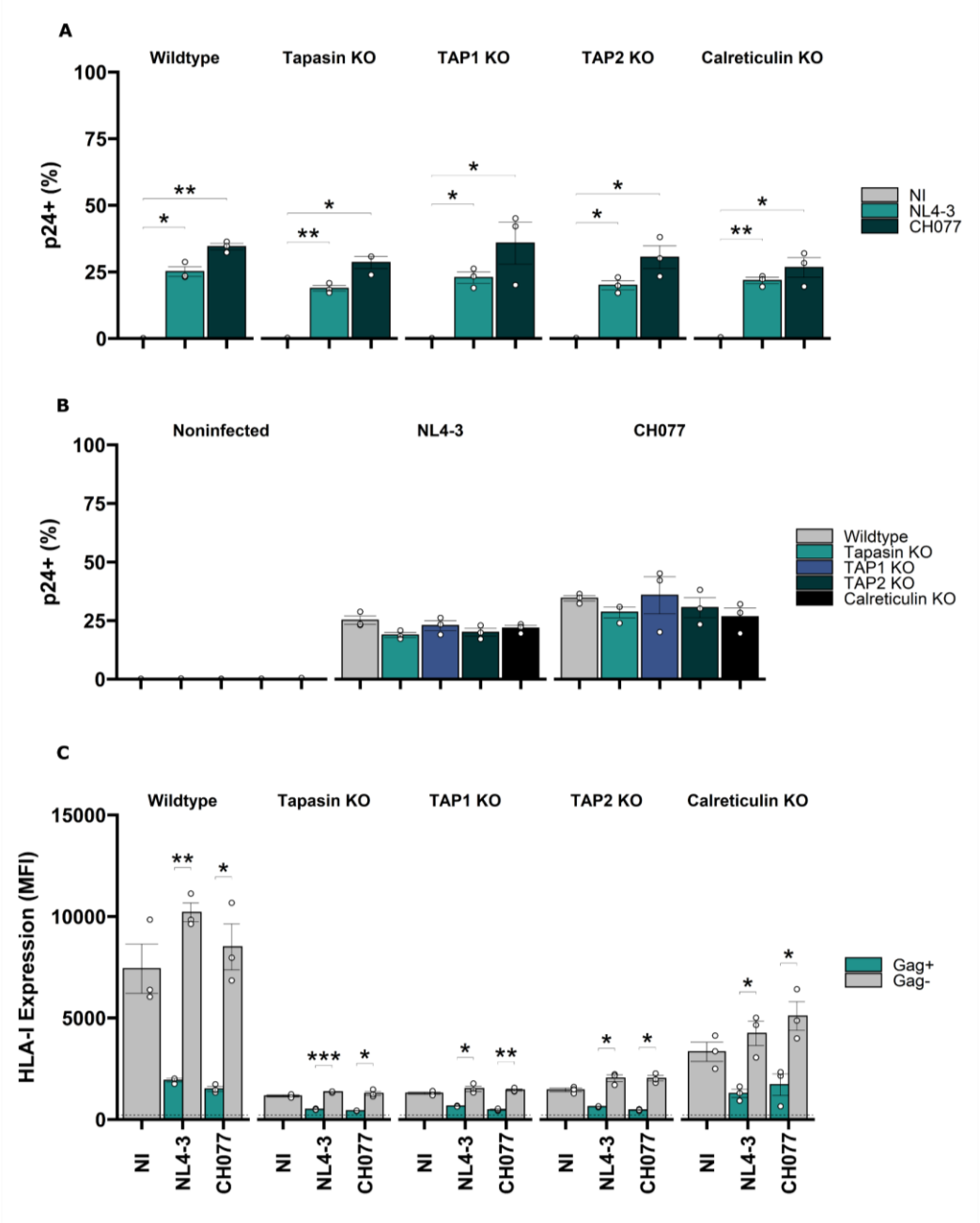


Figure 4.3. **HLA class I downregulation in HAP1 PAKC cells after HIV-1 infection.** A) Percentage of HIV-1 infected cells in each HAP1 PAKC cell line as detected by flow cytometry with anti-HIV gag p24 antibody KC57. B) Comparison of HIV-1 infection levels for NL4-3 and CH077 in each PAKC cell line. C) Cell surface HLA class I expression (MFI) in Gag+ and Gag- (bystander) HAP1 PAKC cells after infection with NL4-3 or CH077 for 48 hours. Individual points correspond to the mean value of technical duplicates from independent experiments (n=3). P-values were estimated using student's t-test with Benjamini-Hochberg correction for multiple comparisons where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NI: Noninfected.

4.2.3. Downregulation of HLA class I by HIV-1 is less efficient in tapasin, TAP1 or TAP2 KO HAP1 cells compared to WT HAP1 cells

We next sought to ask the question whether the expression of PLC components can increase the downregulation of HLA class I by HIV-1 in HAP1 cells. To do so, we evaluated whether the level of downregulation of HLA class I by HIV-1 was the same between WT cells and PLC KO cells. We have shown that HIV-1 NL4-3-G and CH077-G are both capable of downregulating HLA class I in HAP1 WT and tapasin, TAP1 and TAP2 KO cells (Figure 4.3C). It is also evident that HAP1 WT and PLC KO cells display significantly different levels of HLA class I at the cell surface prior to HIV-1 infection (Figure 4.2, Figure 4.3C). To understand if the efficiency of HLA class I downregulation might differ between WT cells and PLC KO cells, we normalised each value to the initial level of HLA class I expression before infection for each cell line respectively.

We calculated the fold change in HLA class I expression measured between HIV-1 Gag+ (infected) and HIV-1 Gag- (bystander) cells as an indication of the level of HLA class I downregulation being achieved in each cell line. A fold change of 1.00 would indicate no difference in HLA class I expression between Gag+ and Gag- cells and a complete abrogation in HIV-1's ability to downregulate HLA. The fold change in HLA class I expression measured on tapasin, TAP1 and TAP2 KO cells was significantly higher compared to WT cells when infected with both NL4-3-G and CH077-G (Figure 4.4). There was no evidence that the fold change in HLA class I expression between WT and CRT KO cells significantly differed (Figure 4.4). These findings indicate that although HIV-1 can still downregulate HLA class I in PLC KO cells; tapasin, TAP1 and TAP2 may be required components of the PLC for HIV-1 to achieve optimal downregulation of HLA class I at the cell surface.

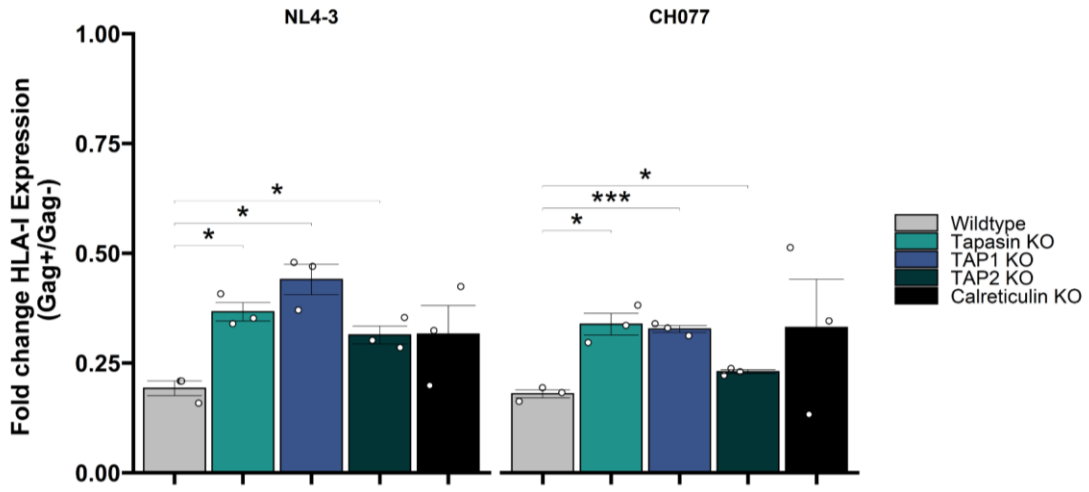


Figure 4.4. **Effect of PLC components on the efficiency of HLA class I downregulation by HIV-1.** Fold change in HLA class I expression (gated on Gag+/Gag- bystander cells) in HAP1 PAKC cells after infection with VSV-G pseudotyped NL4-3 or CH077 for 48 hours. A fold change closer to 1.00 indicates less downregulation of HLA class I in Gag+ compared to the expression of HLA class I in the Gag- bystander cells. Individual points correspond to the mean value of technical duplictaes from independent experiments (n=3). P-values were estimated using student's t-test with Benjamini-Hochberg correction for multiple comparisons where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.2.4. Tapasin-independent HLA class I alleles are more resistant to downregulation by HIV-1 compared to tapasin-dependent alleles

The evidence from the infection of HAP1 WT and PLC KO cells indicated that tapasin, TAP1 and TAP2 are not required for the downregulation of HLA class I by HIV-1 (Figure 4.3C). In our HAP1 WT and PLC KO cell infection model, our data suggests that optimal downregulation of HLA class I by HIV-1 may not be achieved in tapasin, TAP1 or TAP2 KO cells (Figure 4.4). These findings raise the question whether components of the PLC, in particular tapasin, might be exploited by HIV-1 in order to downregulate HLA class I. This might explain the loss of HLA class I downregulation seen in tapasin KO cells compared to WT cells (Figure 4.4).

In order to test this hypothesis, we used the intrinsic tapasin-dependent/independent nature of HLA class I allotypes to see whether HIV-1-mediated downregulation might differ between these two phenotypes. HLA-B*44:02 and HLA-B*44:05 are two HLA class I alleles that differ by only a single amino acid (position 116), but lie on opposite ends of the tapasin dependency spectrum (Williams et al., 2002, Bashirova et al., 2020). HLA-B*44:02 is highly tapasin-dependent whereas HLA-B*44:05 is tapasin-independent. The HLA class I negative LCL 721.221 was transduced with single HLA class I molecules to produce single HLA-B*44:02 and HLA-B*44:05 expression cell lines in the lab of Professor Persephone Borrow, University of Oxford. 721.221-HLA-B*44:02/05 cells express CD4 (Figure 4.5A-B and 4.5F-G) as well as each of their single allele HLA class I molecules (Figure 4.5A-B and 4.5C-E) at the cell surface. This makes them useful biological tools to study peptidomics and cellular responses governed by single HLA class I allotypes.

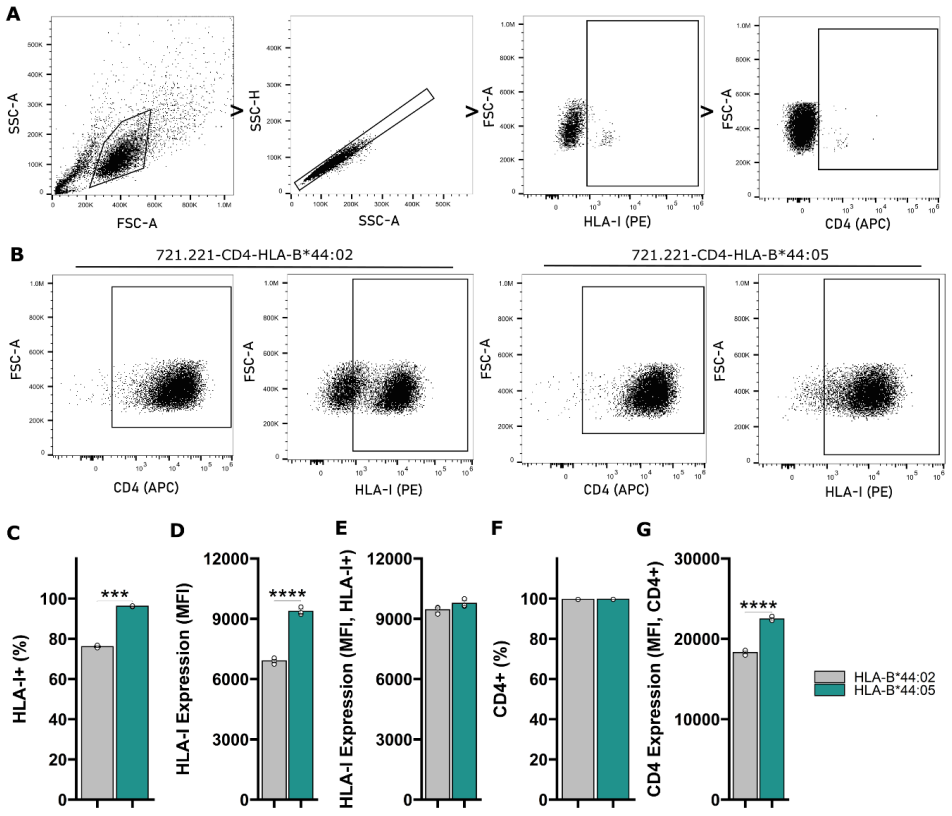


Figure 4.5. **Steady state HLA class I and CD4 surface expression markers in 721.221 tapasin-dependent and independent cell lines.** A) Gating strategy for 721.221 cells to identify and compare HLA class I (third panel) and CD4 (fourth panel) positive populations and median fluorescent intensities (MFI). Data representative of 721.221-CD4-HLA-B*44:02 unstained cells showing lack of HLA class I and CD4 expression when gating on single cells with lymphocyte-like morphology. B) CD4+ (first and third panel) and HLA class I+ (second and fourth panel) populations identified after staining with PE conjugated antibody W6/32 (anti-HLA class I) and APC conjugated antibody RPA-T4 (anti-CD4), respectively. Staining shown for both 721.221-CD4-HLA-B*44:02 and 721.221-CD4-HLA-B*44:05 cells. C-G) Comparison of the proportion of HLA class I+ (C) and CD4+ (F) cells identified in each cell line as well as a comparison of the expression levels (MFI) for each positive population respectively (E and G) or for ungated single cells (D). Individual points correspond to the mean value of technical duplicates from independent experiments (n=3). P-values were estimated using student's t-test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Tapasin-dependent 721.221-CD4-HLA-B*44:02 and tapasin-independent 721.221-CD4-HLA-B*44:05 cells were infected with NL4-3-G and CH077-G and HLA class I expression was measured by flow cytometry 48 hours post infection. Flow cytometry data from HIV-1 infected 721.221-CD4-HLA-B*44:02/05 cells were gated on singlets with lymphocyte-like morphology that were HLA class I and HIV-1 Gag-positive (Figure 6A-B). The level of HLA class I expression in infected (Gag+) cells compared to uninfected, bystander (Gag-) cells was measured to assess the level of HLA class I downregulation achieved in both cell lines. Both NL4-3-G and CH077-G strains of HIV-1 were able to significantly downregulate HLA-B*44:02 and HLA-B*44:05 in 721.221-CD4 cells (Figure 4.6C). There was no significant difference in the level of HLA class I expression measured between the noninfected cell controls and Gag- bystander cell populations (Figure 4.6C). We did observe some variation in the difference between the expression levels of HLA-B*44:02 and HLA-B*44:05 in 721.221-CD4 cells in the noninfected cell population from experiment to experiment, however (compare Figure 4.5D-E and 4.6C NI grey bars).

The tapasin-dependency phenotype of the HLA-B*44 alleles studied here did not impair the ability of HIV-1 to downregulate HLA class I (Figure 4.6C). We next wanted to consider whether HIV-1 was able to more efficiently downregulate HLA-B*44:02 (tapasin-dependent allele) compared to HLA-B*44:05 (tapasin-independent allele). Similar to the analysis performed for HAP1 PAKC KO cells (Figure 4.4), we looked at the fold change in HLA class I expression measured between HIV-1 Gag+ (Figure 4.6C, green bars) and HIV-1 Gag- bystander (Figure 4.6C, grey bars) cells, as an indication of the level of HLA class I downregulation being achieved in each cell line. A fold change of 1.00 would indicate no difference in HLA class I expression between Gag+ and Gag- cells. We found that HLA-B*44:02 was significantly more efficiently downregulated compared to HLA-B*44:05 by both NL4-3-G and CH077-G (Figure 4.7). These findings indicate that tapasin-independent HLA

class I alleles such as HLA-B*44:05 may be more resistant to downregulation compared to tapasin-dependent alleles such as HLA-B*44:02. These results collectively imply that tapasin likely plays a role in HIV-1 downregulation of HLA class I.

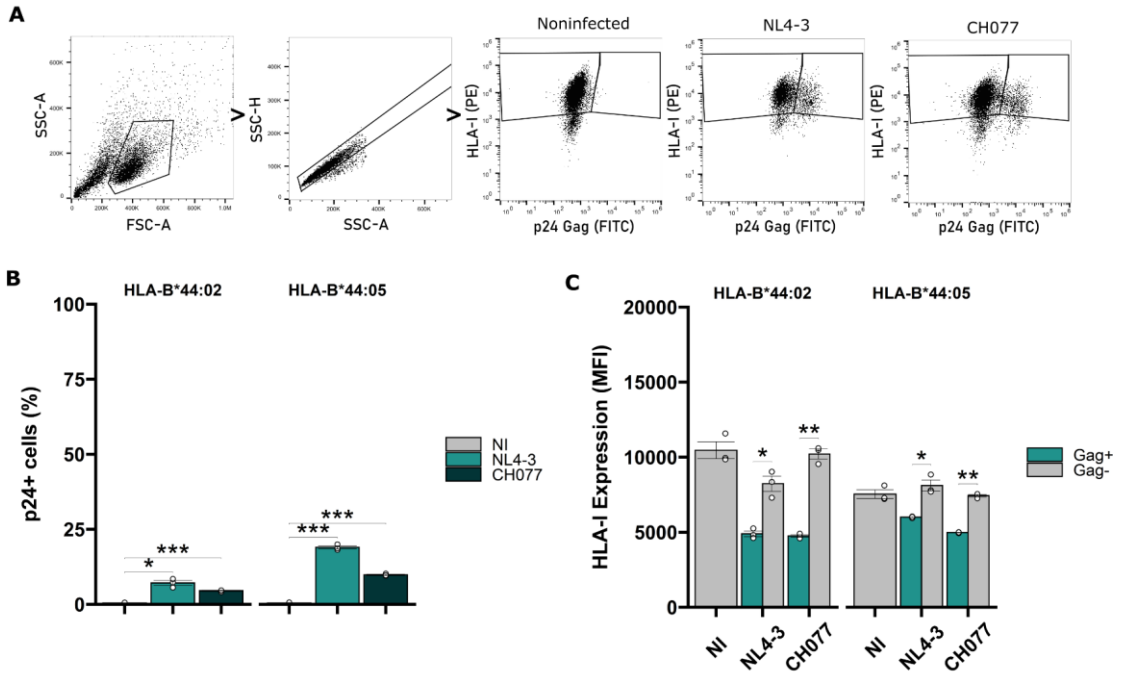


Figure 4.6. **HLA class I surface expression after HIV-1 infection in 721.221 tapasin-dependent and independent cell lines.** A) Gating strategy for 721.221-CD4-HLA-B*44:02/05 cells to identify and compare HLA class I MFIs in HIV-1 Gag+ cells. 721.221-CD4-HLA-B*44:05 cells shown as the representative gating strategy used for both cell lines. B) HLA class I expression (MFI) in the tapasin-dependent 721.221-CD4-HLA-B*44:02 cell line (left panel) compared to the tapasin-independent 721.221-CD4-HLA-B*44:05 cell line (right panel). C) Fold change in HLA class I expression (gated on Gag+/Gag- bystander cells) in 721.221-CD4-HLA-B*44:02 and 721.221-CD4-HLA-B*44:05 cells after infection with VSV-G pseudotyped NL4-3 or CH077. A fold change closer to 1.00 indicates less downregulation of HLA class I in Gag+ compared to the expression of HLA class I in the Gag- bystander cells. Individual points correspond to the mean value of technical duplicates from independent experiments (n=3). P-values were estimated using student's t-test with Benjamini-Hochberg correction for multiple comparisons where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

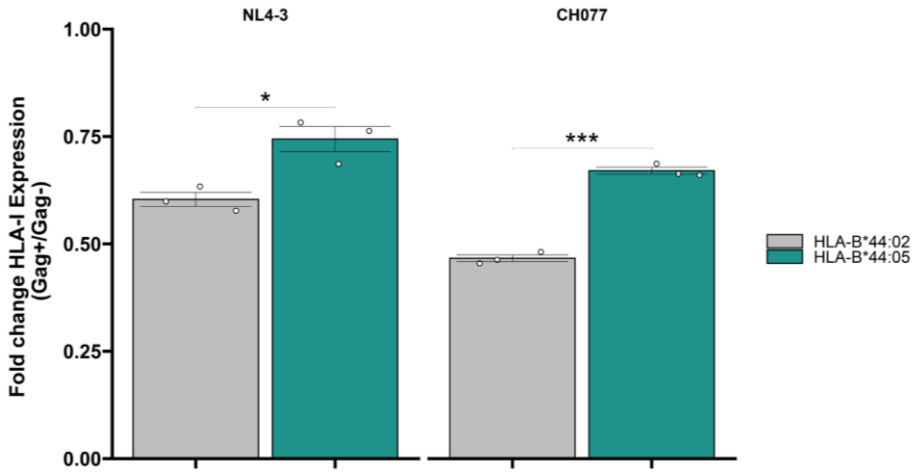


Figure 4.7. **Efficiency of HLA class I downregulation by HIV-1 in 721.221 tapasin-dependent and independent cell lines.** Fold change in HLA class I expression (gated on Gag+/Gag- bystander cells) in 721.221-CD4-HLA-B*44:02 and 721.221-CD4-HLA-B*44:05 cells after infection with VSV-G pseudotyped NL4-3 or CH077 for 48 hours. A fold change closer to 1.00 indicates less downregulation of HLA class I in Gag+ compared to the expression of HLA class I in the Gag- bystander cells. Individual points correspond to the mean value of technical duplicates from independent experiments (n=3). P-values were estimated using student's t-test with Benjamini-Hochberg correction for multiple comparisons where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4.3. Chapter 4 Discussion

In Chapter 3, we provided evidence from a cohort of paediatric HIV-1 infected patients that tapasin-independent HLA class I alleles were associated with control of HIV-1 infection. Bashirova *et al.* (2020) originally observed this phenomenon in adults and provided evidence to show that tapasin-independent HLA class I alleles present a broader repertoire of the HIV-1 peptidome to CD8+ T cells. This makes it harder for HIV-1 to generate sufficient escape mutations that maintain viral replicative capacity whilst evading CD8+ T cell recognition (Carlson et al., 2012, Kiepiela et al., 2007, Muenchhoff et al., 2016). In this thesis, we have presented an mechanistic hypothesis to describe a direct cellular mechanism behind the association between HLA class I tapasin-dependency and HIV-1 disease progression and implicate tapasin-independent HLA class I alleles in being able to avoid viral immune-evasion mechanisms.

Our hypothesis is that the expression of tapasin-independent HLA class I alleles would be beneficial to HIV-1 infected individuals if tapasin or the PLC function was affected by immune-evasion mechanisms of HIV-1 itself. This method of immune evasion has been described in other viral infections that downregulate or impair the PLC in order to reduce HLA class I presentation of virally-derived peptides. Firstly, we were able to produce a reproducible model of VSV-G pseudotyped HIV-1 infection with both the NL4-3 lab adapted strain and the primary T/F virus CH077 *in vitro* in HEK293FT and HAP1 cells (Figure 4.1). This infection model can be used to explore the impact of HIV-1 infection on HLA class I and PLC expression in different cellular scenarios.

We showed that knockout of tapasin, TAP1, TAP2 or calreticulin in HAP1 cells resulted in reduced levels of cell surface HLA class I presentation (Figure 4.2). Although this is not a novel finding, significant HLA class I downregulation in PLC knockouts highlights the potential impact on HLA class I expression that would occur if HIV-1 were to hijack or target

the components of the PLC. In particular tapasin, as tapasin knockout cells showed the most significant decrease in HLA class I expression. This hypothetical targeting of the PLC/tapasin by HIV-1 would be less effective in downregulating HLA class I in patients or cells expressing tapasin-independent HLA class I alleles. Indeed, this phenotype was seen when we infected an LCL that singly expressed closely-related tapasin-dependent and tapasin-independent HLA class I alleles. Downregulation of the tapasin-independent HLA-B*44:05 allele was significantly less efficient compared to tapasin-dependent HLA-B*44:02, supporting this hypothesis (Figure 7). These findings implicate a role of tapasin and/or the PLC in helping to maximise HIV-1-mediated downregulation of HLA class I. Similarly, it has been observed that the immediate early expressed HCMV protein US3 selectively prevents the expression of tapasin-dependent HLA class I alleles over tapasin-independent alleles (Park et al., 2004b). The mechanism behind this has been investigated and HCMV US3 has been shown to bind to tapasin and TAP and reduce the level of interaction between components of the PLC (Park et al., 2004b). HCMV US3 has also been found to block *de novo* synthesis of tapasin mRNA transcripts, which contributes to the decreased levels of tapasin-dependent HLA class I allotypes (Halenius et al., 2011).

We found evidence that HLA class I downregulation by HIV-1 was less efficient in cells not expressing tapasin, TAP1 or TAP2 (Figure 4.4). This indicates that targeting of these components of the PLC by HIV-1 may be contributing to diminished HLA class I presentation. HIV-1 Nef downregulates HLA-A and HLA-B by recruiting the clathrin-associated AP-1 and directing HLA class I molecules for lysosomal degradation (Roeth et al., 2004, Lubben et al., 2007). Nef was shown to reduce the level of the LMP7 (β5i) subunit of the immunoproteasome in HeLa and Jurkat cells (Yang et al., 2020). Nef is still capable of downregulating HLA class I in cells treated with short interfering RNA (siRNA) targeting LMP7, but the level of HLA class I downregulation is significantly greater when LMP7 is expressed. This indicates that Nef

interaction with the LMP7 subunit of the immunoproteasome is not necessary for HLA class I downregulation, but the effect is amplified when Nef is expressed with LMP7 and able to inhibit of the antigen processing pathway and the immunoproteasome. These findings are similar to that observed in the tapasin, TAP1 and TAP2 knockout cells infected with HIV-1 in this study: HLA class I is still downregulated in the absence of these proteins, but the level of HLA class I downregulation is dampened in their absence compared to cells that express all of the components of the PLC (Figure 4.4). This suggests that HIV-1 is using the PLC to potentiate the reduction in HLA class I presentation.

There is limited published data thus far that indicates HIV-1 targets the PLC for HLA class I downregulation. There is evidence, however, to suggest that HIV-1 viral proteins may interact with tapasin. As previously mentioned, Nef was co-precipitated with both HLA class I and tapasin in β2-m-expressing cells (Kasper et al., 2005). Other HIV-1 proteins have also been implicated in associating with HLA class I and potentially the PLC. HIV-1 Vpu downregulates HLA-C molecules (Apps et al., 2016) and HIV-1 Env non-covalently associates with HLA-C molecules on HLA-C-positive virions (Beretta et al., 1987, Zipeto and Beretta, 2012, Serena et al., 2017). More interestingly in the findings for HIV-1 Env in the context of this study, tapasin came out as the ninth top hit as a potential interactor for Env in a study of the HIV-1-host interactome by MS analysis (Luo et al., 2016). No further functional experiments such as IP or two-hybrid system analyses were performed to clarify this potential interaction. This finding is of interest as other host proteins including intracellular adhesion molecule 3 (ICAM3) and β2-integrin that have been described in the literature for their involvement in HIV-1 infection were also identified in this study and were shown to have similar MS association scores as tapasin (Sipsas and Sfikakis, 2004, Pelchen-Matthews et al., 2012).

It is possible that HIV-1 viral proteins may not work alone in targeting the PLC for the downregulation of HLA class I. The downregulation of a member of the ABC transporter

family (to which the TAP heterodimer belongs), ABCA1, is implemented in HIV-1-associated atherosclerosis (Mukhamedova et al., 2016). Proper ABCA1 folding in the ER is dependent on calnexin, but the interaction between calnexin and ABCA1 in the ER is diminished by Nef (Hunegnaw et al., 2016, Jennelle et al., 2014). Nef instead redirects calnexin to preferentially bind to Env gp160 over ABCA1. Env and Nef working cooperatively together to impair calnexin's chaperone function in the ER for ABCA1 folding might be considered as a potential mechanism relating to the PLC and HLA class I retention in the ER. For example, HCMV US3 has been shown to function synergistically with other HCMV proteins US2 and US11 in order to retain HLA class I molecules in the ER (Noriega et al., 2012). This may explain both Nef and Env's independent identification as potential interactors with tapasin.

It is well known that components of the PLC are targets for downregulation in viral infections (Roder et al., 2008). Given this new evidence to suggest that HIV-1 may utilise the PLC to downregulate HLA class I and selectively target tapasin-dependent HLA class I allotypes, we considered this an interesting scientific avenue to explore in the following chapters of this thesis.

Chapter 5: The effect of HIV-1 infection on tapasin and peptide loading complex expression *in vitro*

5.1. Chapter 5 Introduction

The data presented in Chapter 3 independently described the association between tapasin-independent HLA class I alleles, slow HIV-1 disease progression and low plasma VLs. The association between tapasin-independent HLA class I alleles and HIV-1 disease control was found to be associated with the presentation of a greater breadth of the HIV-1 peptide repertoire by Bashirova and colleagues in 2020. In Chapter 4, we investigated an mechanism that might describe this association. We hypothesised that HIV-1 may interact with tapasin or other components of the PLC as a means of retaining sub-optimally loaded HLA class I molecules in the ER and thereby reducing viral antigen presentation to CD8+ T cells at the cell surface. Downregulation of HLA class I by HIV-1 was impaired when any one of tapasin, TAP1 or TAP2 was knocked out *in vitro*, implying that HIV-1 may use these components of the PLC to enhance the reduction in HLA class I expression at the cell surface. We also showed that HIV-1 downregulated the tapasin-dependent HLA-B*44:02 allele more efficiently compared to the tapasin-independent HLA-B*44:05 allele. This further supported our findings to suggest that HIV-1 may interfere with the PLC, particularly tapasin, in order to evade the cellular immune response.

Viral interference with the PLC has been well described in the literature. Viral proteins have been shown to interact with components of the PLC and interfere with their function or the interactions with one another that enable the formation of functioning antigen processing machinery in the ER. HSV1 protein ICP47 destabilises the TAP heterodimer by directly competing with peptides for binding to TAP and blocking conformational changes in TAP that allow for peptide translocation across the ER membrane (Ahn et al., 1996, Tomazin et al., 1996, Matschulla et al., 2017). ICP47 significantly limits the peptide pool in the ER available for loading and thus retains sub-optimally loaded HLA class I molecules in the ER (Fruh et al., 1995). HCMV protein US6 and EBV BNLF2a similarly block TAP's function as a peptide

shuttle across the ER membrane. HCMV US6 and EBV BNLF2a block ATP binding to TAP and shut off the energy requirements needed to reorganise the TAP1/TAP2 protein components and translocate peptide to the ER (Hewitt et al., 2001, Kyritsis et al., 2001, Horst et al., 2009, Wycisk et al., 2011, Hislop et al., 2007). Bovine herpesvirus type 1 UL49.5 also inhibits TAP function by preventing conformational changes occurring in the complex that enable peptide translocation (Koppers-Lalic et al., 2005, Verweij et al., 2011b, Verweij et al., 2011a). The adenovirus E19 protein inhibits TAP and HLA class I interactions in the ER by binding to TAP, inhibiting tapasin's function but not mimicking it (Bennett et al., 1999). In such a way, E3-19 destroys the tapasin bridge between TAP and HLA class I, therefore indirectly interfering with tapasin's function in the ER. Adenovirus E19 has also been shown to directly bind to HLA class I molecules and block their transport from the ER to the cell surface (Sester et al., 2013, Jefferies and Burgert, 1990). This highlights the potential polyfunctionality of viral proteins to inhibit different components of the PLC.

In addition to directly interfering with PLC component function, viral pathogens have also been shown to downregulate or degrade PLC expression as a means of reducing HLA class I presentation at the cell surface. Murine gamma-2 herpesvirus 68 mK3 targets HLA class I molecules for ubiquitination and proteasomal degradation only when HLA class I is complexed with TAP and tapasin in the PLC (Boname et al., 2004, Boname et al., 2005, Yu et al., 2002). Viral protein homologues may also target the same host protein by different mechanisms. For example, the murine gamma-2 herpesvirus 68 mK3 homologues kK3 and kK5 of Kaposi's sarcoma associated herpesvirus (KSHV) downregulate HLA class I expression by inducing endocytosis of HLA class I from the cell surface (Stevenson et al., 2000, Coscoy and Ganem, 2000, Ishido et al., 2000). HCMV US2 and US11 divert HLA class I molecules from the ER to the cytosol where they are then degraded by the proteasome (Barel et al., 2006, Tirosh et al., 2005). HIV-1 Nef directs HLA class I molecules for lysosomal degradation, exerting its effect

by recruiting the clathrin-associated AP-1 to HLA class I molecules and diverting them to endocytic compartments within the cell (Roeth et al., 2004, Lubben et al., 2007, Schaefer et al., 2008). Viral proteins have also been shown to target PLC components aside from HLA class I itself in order to indirectly downregulate HLA class I expression. MCV protein MC80 targets tapasin for ubiquitination and ERAD (Harvey et al., 2019). HCMV inhibits the synthesis of tapasin transcripts *in vitro* via its US3 protein (Halenius et al., 2011). The mechanism behind HCMV-mediated impairment of tapasin transcription was not fully elucidated in this study, but the authors bioinformatically identified an HCMV-encoded microRNA (miRNA) mi-RUS25.2 that was predicted to target the 3' UTR of the tapasin gene. However this miRNA was not shown to have any biological effect on tapasin expression *in vitro.* Nevertheless, miRNA disruption of PLC gene expression via direct inhibition of mRNA translation or targeting transcripts for degradation has been described in cancer (reviewed in (Dhatchinamoorthy et al., 2021).

Plentiful evidence gathered from other viral pathogens indicates that targeting the PLC in order to downregulate the cell surface expression of HLA class I is a common immune evasion mechanism. The findings presented in Chapter 4 indicate that HLA class I downregulation by HIV-1 is more efficient when tapasin, TAP1 or TAP2 are expressed or when HLA class I alleles are dependent on tapasin, indicating that HIV-1 may interfere with the PLC to downregulate HLA class I expression. We therefore asked whether there was any evidence that HIV-1 downregulated the expression of components of the PLC. We first aimed to investigate whether infection with HIV-1 is associated with the differential expression of PLC components (tapasin, ERp57, TAP1, TAP2 and calreticulin) compared to noninfected cells. We then aimed to try and elucidate whether HIV-1 Nef might have an independent effect on PLC component expression. This hypothesis was based on the evidence in the literature describing Nef's role in the downregulation of HLA class I, as well as the findings reported in 2005 by Kasper and

colleagues who first co-IP HIV-1 Nef with tapasin. We hypothesised that HIV-1 infection may lead to significantly reduced levels of individual protein components of the PLC in a Nef-dependent manner.

5.2. Chapter 5 Results

5.2.1. PLC expression after HIV-1 infection in HEK293FT cells

5.2.1.1. There is no significant difference in PLC expression after HIV-1 infection in HEK293FT cells

HEK293FT cells were infected with VSV-G pseudotyped NL4-3 or CH077 at an MOI of 1.0 for up to 48 hours. Infected cells or noninfected cell controls were collected at 0, 6, 12, 24 and 48 hours post infection. At each time point, cell surface HLA class I expression was measured by flow cytometry and RNA was extracted to measure PLC transcript expression by RT-qPCR. The proportion of HIV-1 infected cells at each time point was measured by flow cytometry using the anti-HIV gag p24 antibody (clone KC57), which showed that productive infection with either NL4-3-G or CH077-G was achieved 48 hours post infection (Figure 5.1A). HIV-1 Gag+ cells showed significantly reduced levels of HLA class I expression compared to Gag- bystander cells 48 hours post infection with both NL4-3-G and CH077-G (Figure 5.1B). There was no significant change in HLA class I expression between noninfected cells and HIV-1 infected Gag- bystander cells at any time point, nor did HLA class I expression significantly change over time in any condition (Figure 5.1B).

The kinetics of PLC mRNA transcript levels were measured by RT-qPCR from RNA extracted from HEK293FT cells 6, 12, 24 and 48 hours post infection with NL4-3-G and CH077-G and from noninfected controls at the same time points. The RQ of transcripts encoding tapasin, ERp57, TAP1, TAP2 and calreticulin were calculated by normalising Ct values to the housekeeping gene GAPDH (ΔCt) and normalisation of ΔCt values to the noninfected control at each time point (ΔΔCt). RQ values were calculated as $2^{-\Delta\Delta Ct}$, where the RQ of noninfected controls equals 1.00. We saw no significant difference in tapasin, ERp57, TAP1, TAP2 or

calreticulin transcript levels in HEK293FT cells at any point up to 48 hours post infection with either NL4-3-G or CH077-G compared to noninfected controls (Figure 5.1C – G).

We next looked into whether HIV-1 infection might influence PLC protein expression in HEK293FT cells compared to noninfected controls over time. Murine gamma-herpesvirus-68 MK3 protein rapidly degrades TAP1, TAP2 and tapasin protein levels without impacting the transcription or translation processes of these proteins directly (Boname et al., 2004). Therefore, even though we see no influence of HIV-1 infection on PLC transcripts, it is plausible that protein levels may be differently impacted. HEK293FT cells infected with NL4-3-G or CH077-G or noninfected controls were lysed in RIPA buffer at 0, 6, 12, 24 and 48 hours post infection and analysed by semi-quantitative Western Blot. Unfortunately, of the antibodies available to us in this study that target components of the PLC, only anti-tapasin monoclonal antibody 7F6 (Sigma-Aldrich) and anti-ERp57 monoclonal antibody MaP.Erp57 (Abcam) were shown to detect proteins by Western Blot at the expected weights of 48 kDa and 68 kDa, respectively (Figure 1H). We were not able to optimise Western Blot conditions that produced specific binding of monoclonal antibody mAb 148.3 to TAP1 (87 kDa, Merck), polyclonal antibody PA5-37414 (Thermo Fisher Scientific) or monoclonal antibody 429.4 (Merck) to TAP2 (72 kDa) or monoclonal antibody 16B11.1 to calreticulin (46 kDa, Merck) (data not shown). We also attempted to measure intracellular tapasin expression by flow cytometry either by indirect staining with monoclonal antibody 7F6 (rat anti-tapasin primary antibody) with an APC conjugated goat anti-rat secondary antibody (Thermo Fisher Scientific) or by conjugating the fluorophore XFD488 (structurally identical to Alexa Fluor™ 488) to clone 7F6 using the ReadliLink™ xtra Rapid Labelling Kit from Stratech Scientific (data not shown). Neither of these approaches of detecting tapasin by flow cytometry were successful. Indirect staining of tapasin with fluorophore-conjugated secondary antibodies resulted in the detection of a false-positive signal in tapasin KO HEK293FT and tapasin KO HAP1 cells (data not shown). Both

of these cell lines were confirmed to be tapasin negative by Western Blot (data not shown). We suspect that a large primary antibody/secondary antibody complex may give rise to high levels of background signal that hides any true detection of tapasin when staining for intracellular targets. This background signal could not be resolved with increased wash steps after staining. Quantification of protein expression levels by semi-quantitative Western Blot does not allow direct measurement of absolute protein quantity, but provides data on the relative protein levels in the sample. Tapasin and ERp57 protein levels were calculated as the RQ of protein measured normalised to the housekeeping gene GAPDH and the noninfected sample at each time point (Figure 5.1H). In correlation with the mRNA data, we observed no significant difference in the protein expression of tapasin (Figure 5.1I) or ERp57 (Figure 5.1J) measured in NL4-3-G or CH077-G infected HEK293FT cells compared to noninfected controls, although levels of ERp57 did appear to be upregulated in HEK293FT cells after infection with CH077.G for 48 hours compared to noninfected controls, although these findings were not significant and inconsistent at other time points measured in this study (Figure 5.1J).

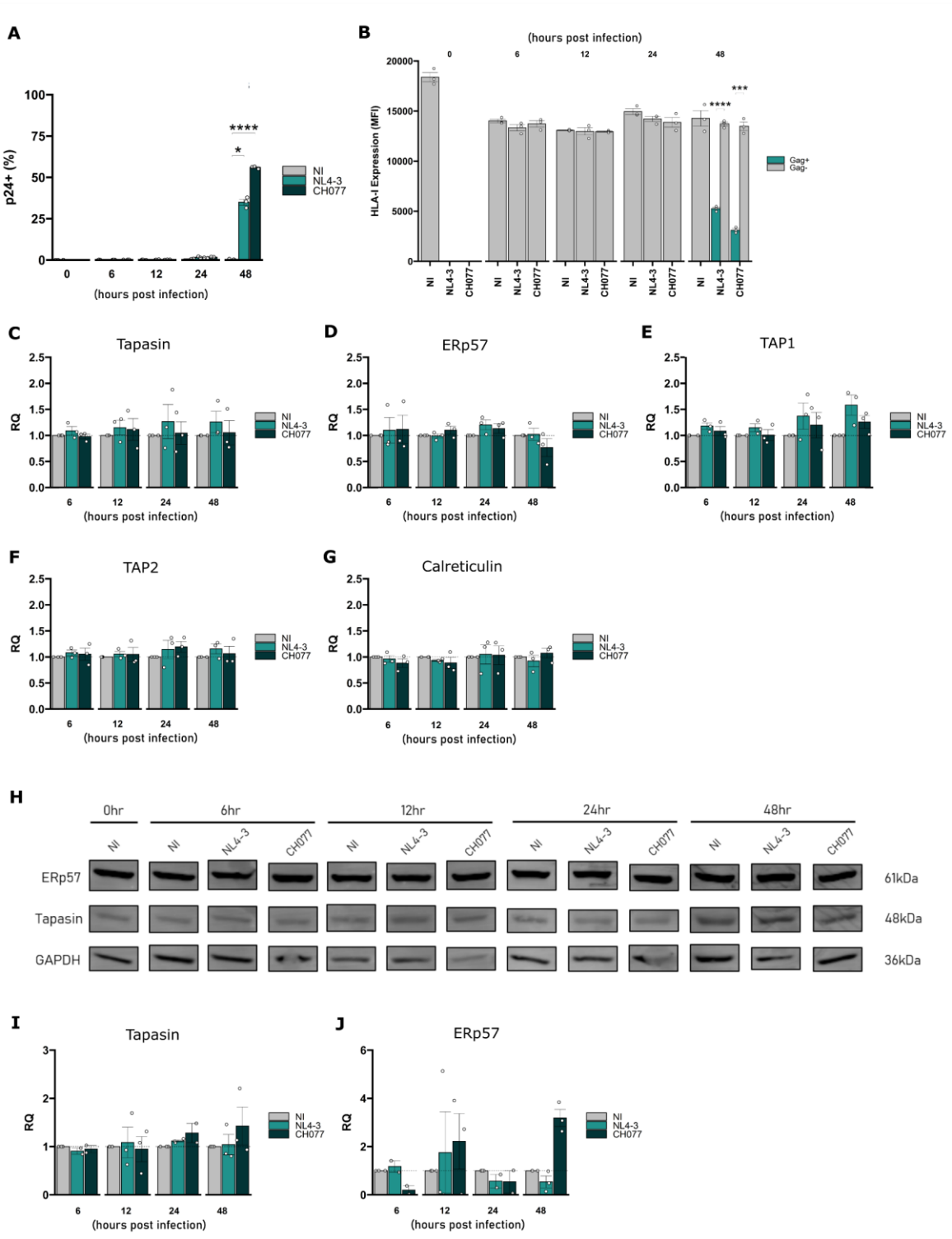


Figure 5.1. **PLC expression kinetics in HEK293FT cells infected with HIV-1.**

Figure 5.1. PLC expression kinetics in HEK293FT cells infected with HIV-1. A) The proportion of Gag+ HEK293FT cells was measured by flow cytometry pre-infection (0 hours) and at 6, 12, 24 and 48 hours post infection with NL4-3-G or CH077-G. Cells were stained with FITC conjugated anti-HIV gag p24 antibody clone KC57. B) HLA class I expression in Gag- (grey) and Gag+ (green) HEK293FT cells infected with NL4-3-G or CH077-G compared to noninfected (NI). HLA class I expression was analysed by flow cytometry staining with the PE-conjugated anti-HLA antibody clone W6/32. C – G) Relative Quantification (RQ) of C) tapasin, D) ERp57, E) TAP1, F) TAP2 and G) Calreticulin gene expression measured in bulk HEK293FT cells after infection with NL4-3-G or CH077-G at an MOI of 1.0 over time, compared to noninfected (NI) controls. RQ of PLC gene expression was measured by qPCR. RQ values were calculated according to the ΔΔCt method where $RQ = 2^{-\Delta\Delta Ct}$. H) Western Blot analysis of tapasin and ERp57 protein expression in HEK293FT cells infected over time with NL4-3-G or CH077-G, compared to noninfected (NI) controls. Loaded samples were normalised for protein content by bicinchoninic acid (BCA) protein assay I – J) Semi-quantitative analysis of relative protein expression measured by Western Blot (H) for I) tapasin and J) ERp57. Semi –quantitative RQ values calculated by normalisation of tapasin and ERp57 band intensities to GAPDH and further normalising to the NI control at each time point. Individual points correspond to the mean value of technical duplicates from independent experiments (n=3). P-values were estimated using student's t-test with Benjamini-Hochberg correction for multiple comparisons where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.2.1.2. HEK293FT cells infected with HIV-1 do not represent a good model for our system, as they do not appropriately upregulate ISGs

All of the expression experiments carried out thus far in vitro and described in Figure 5.1 were carried out in HEK293FT cells. We considered that the use of immortalised cell lines to study transcription of IFN-stimulated PLC genes may be masking some potential effect of HIV-1 infection on gene and protein expression (Abarca-Heidemann et al., 2002). To investigate this, we examined the expression of two ISGs: IFN-induced GTP-binding protein Mx1 (MX1) and viperin (radical S-adenosyl methionine domain-containing protein 2, RSAD2), which have previously been shown to be significantly upregulated in primary macrophages infected with HIV-1 (Decalf et al., 2017). We found that compared to PLC genes and the housekeeping gene GAPDH, the Ct values of MX1 and Viperin were very high (Ct > 35) and did not change over time or differ between noninfected and HIV-1 infected HEK293FT cells (Figure 5.2A – D). This led us to believe that HEK293FT cells did not mount an effective ISG response after HIV-1 infection comparable to primary cell types.

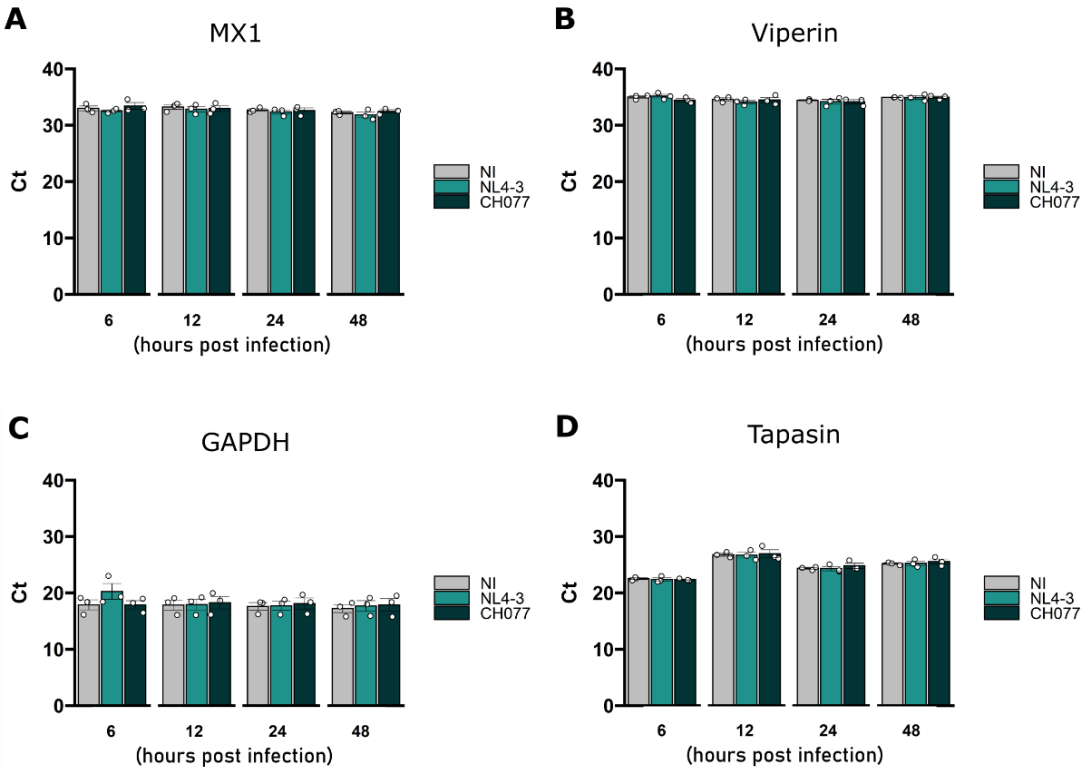


Figure 5.2. **Detection of ISG mRNA expression in HEK293FT cells.** Raw IFN stimulated gene (ISG) MX1 and Viperin (ISGs), GAPDH housekeeping gene (HKG) and tapasin (PLC) Ct values in noninfected (NI) HEK293FT cells or HEK293FT cells infected with VSV-G pseudotyped NL4-3 or CH077 over time. A) MX1, B) Viperin (RSAD2) and C) GAPDH and D) Tapasin. Individual points correspond to the mean value of technical duplicates from independent experiments (n=3).

5.2.2. PLC expression after HIV-1 infection in PBMCs

5.2.2.1. HIV-1 predominately infects the CD3+CD4+ cell subset in activated PBMCs and downregulates HLA class I expression

To evaluate whether HIV-1 infection impacts PLC expression via promotion or interference with IFN signalling, we infected bulk primary human PBMCs with VSV-G pseudotyped HIV-1. PBMCs were first activated with PHA and IL-2 in R10 for 48 hours to promote expansion of T lymphocytes. Following activation, PBMCs were rested for a further 48 hours with R10 containing 50 U / mL IL-2 before infection with HIV-1 NL4-3-G or CH077-G at an MOI 1.0 for up to 48 hours. Activated PBMCs were cultured with media only as a control. HIV-1 infected and noninfected PBMC controls were collected prior to PHA activation, prior to HIV-1 infection, then 12, 24 or 48 hours post infection for analysis of cellular CD3+, CD4+, CD8+, CD19+, CD14+, CD16+ composition and HLA class I expression by flow cytometry. Cellular RNA was also extracted after activation but before HIV-1 infection (0 hour post infection) and 6, 12, 24 and 48 hours post infection with HIV-1 to analyse how PLC transcript levels change over time with HIV-1 challenge.

Flow cytometry data gained from noninfected and HIV-1 infected PBMCs were gated according to the strategy employed by Ende *et al.* (2018) (Figure 5.3A). We also stained PBMCs at every time point with the monocytic markers CD14+(BV711) and CD16+(BV605) although the cellular subsets expressing these markers were underrepresented after activation with PHA and were not found to be infected with HIV-1 under these assay conditions (data not shown). Live lymphocytes were gated as CD3+CD8-CD4+Gag- or CD3+CD8-CD4-Gag+ (Figure 3A). Neither infection with NL4-3-G nor CH077-G influenced the percentages of CD3+, CD19+, CD4+ or CD8+ cells (Figure 5.3B). We also first gated PBMCs as Gag+/ Gag- in order to distinguish the phenotype of cells that were productively infected with HIV-1 under

these conditions (Figure 5.3C). The efficiency of pseudotyped HIV-1 infection in primary PBMCs was quite low compared to the infection of both NL4-3-G and CH077-G in immortalised cells lines (Figure 5.3D). We suspect this observation was due to the presence of activated cytotoxic CD8+ T cells in the same culture that were able to induce apoptosis of HIV-1 infected cells and clear infection.

We also saw a very low level of infection of CD3+CD8+ cells (3% and 6% of Gag+ for NL4-3-G and CH077-G infection, respectively). CD8+ T cells can be infected under our assay conditions due to the use of VSV-G pseudotyped HIV-1 isolates that overcome the entry step of HIV-1 replication (Figure 5.3E). We speculate that the reason for significantly lower infection levels of CD8+ cells compared to CD4+ cells, even with VSV-G pseudotyped HIV-1, may be due to secondary infections after integration and production of replication competent HIV-1 virions lacking the VSV glycoproteins that can only use CD4 molecules for viral entry.

The cell surface expression of HLA class I molecules on Gag+ cells productively infected with NL4-3-G and CH077-G was significantly reduced compared to Gag- bystander cells and noninfected cells (Figure 5.3F and 5.3G). Although the efficiency of HIV-1 infection in bulk PBMCs was low, we found that HLA class I was downregulated as expected in Gag+ cells.

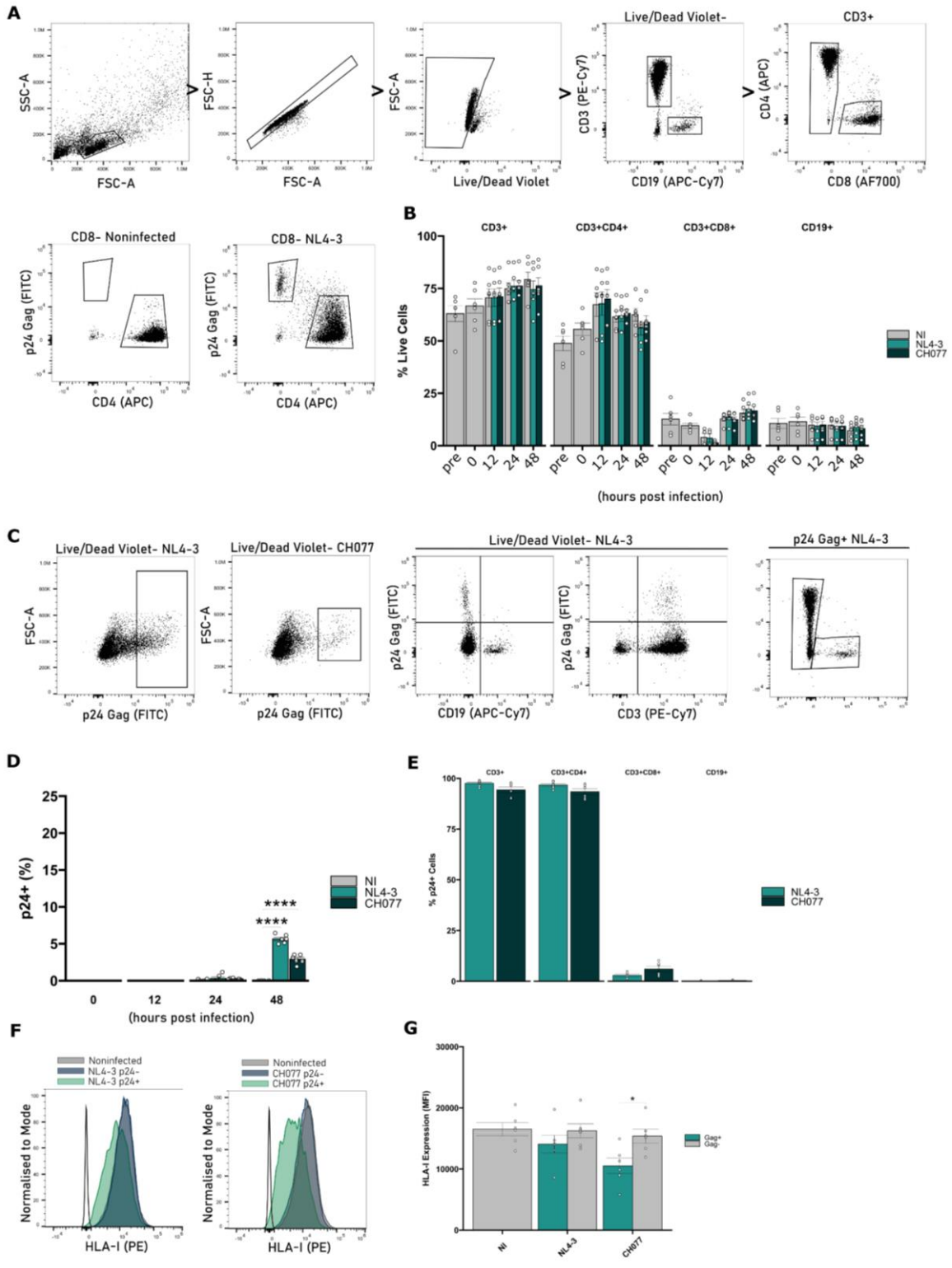


Figure 5.3. HLA class I downregulation measured by flow cytometry in activated PBMCs after infection with HIV-1.

Figure 5.3. HLA class I downregulation measured by flow cytometry in activated PBMCs after infection with HIV-1. A) Gating strategy for activated PBMCs to identify CD3+CD4+, CD3+CD8+ and CD19+ populations infected with NL4-3 or CH077 48 hours post infection. Top panel shows representative data from HIV-1 uninfected PBMCs from a single healthy blood donor. Gag+ single lymphocytes were gated as Live+CD3+CD8-. Productively HIV-1 infected cells were gated as CD4-Gag+ whilst bystander cells were gated as CD4+Gag- (bottom panel, NL4-3 shown as representative sample for HIV-1 infection of PBMCs). B) The proportion of cells expressing the lymphocyte markers CD3, CD4, CD8 or CD19 in PBMCs derived from healthy donor blood. Population percentages as a proportion of live cells (Live/Dead-) are shown prior to activation with PHA and IL-2 (pre-activation) then 0, 12, 24 and 48 hours post infection with NL4-3-G or CH077-G to show any changes in population make-up after HIV-1 challenge. C) Gating strategy to identify the cellular subsets in PBMCs that are productively infected with NL4-3-G or CH077-G. Gag+ cells were gated to identify double positive cells expressing CD3, CD19, CD4 or CD8 markers at the cell surface (HIV-1 infected). D) Levels of VSV-G pseudotyped NL4-3 and CH077 infection in PBMCs measured by flow cytometry using the anti-HIV gag p24 antibody clone KC57. E) Population characteristics of cellular markers expressed on Gag+ NL4-3-G or CH077-G infected PBMCs. The proportion of Gag+ cells expressing CD3, CD4, CD8 or CD19 are shown in the gated population of Gag+ productively infected cells. F) HLA class I expression (MFI) in Gag+ HIV-1 infected, Gag- (bystander) and noninfected PBMCs infected with NL4-3 or CH077. Representative data from a single healthy blood donor is shown in these histograms. G) HLA class I expression (MFI) in Gag+ and bystander Gag- healthy donor PBMCs uninfected or infected with NL4-3-G or CH077-G. Individual points correspond to the mean value of technical duplicates from independent experiments (n=3). P-values were estimated using student's t-test with Benjamini-Hochberg correction for multiple comparisons where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NI: Noninfected. pre: pre-activation.

5.2.2.2. HIV-1 infection upregulates ERp57, TAP1 and TAP2 mRNA expression in activated PBMCs

We found that VSV-G pseudotyped NL4-3 and CH077 isolates infected bulk PBMCs and downregulated HLA class I under our assay conditions. We next asked how HIV-1 infection affected PLC mRNA transcript levels *in vitro*. We first addressed whether we could detect an ISG response in primary human PBMCs infected with HIV-1, as this was not the case for HEK293FT cells (Figure 5.2A and 5.2B). In PBMCs, the highest levels of productive infection achieved by NL4-3-G and CH077-G 48 hours post infection (Figure 5.3D) correlated with a significant upregulation of the mRNA expression of the ISGs MX1 and Viperin (Figure 5.4A and 5.4B). These results indicated that ISG induction in primary human cells was functional, as has been previously described (Decalf et al., 2017, Deng et al., 2021, Sidharthan et al., 2014). The induction of MX1 and Viperin mRNA expression was only seen in cells infected with NL4-3-G compared to noninfected controls. We could not detect ISG induction after CH077-G infection, which may have be due to the lower levels of infection achieved with this virus (Figure 5.3D).

We found that the mRNA expression levels of ERp57, TAP1 and TAP2 were upregulated in NL4-3-G infected cells compared to noninfected controls and in CH077-G infected cells 48 hours post infection (Figure 5.4D – F). We did not observe any changes in the expression levels of tapasin and calreticulin mRNA between HIV-1 infection and noninfected controls at any time point post infection (Figure 5.4C and 5.4G). The expression of TAP2 mRNA was also upregulated in NL4-3-G infected PBMCs 24 hours post infection, prior to detection of significant productive infection in PBMCs (Figure 5.3D). We could not detect upregulation of any PLC gene in CH077-G infected PBMCs compared to noninfected controls (Figure 5.4A and 5.4B). This is consistent with the pattern of ISG mRNA expression seen in CH077-G

infection and is suspected to be due to the low levels of infection seen with CH077-G (Figure 5.3D).

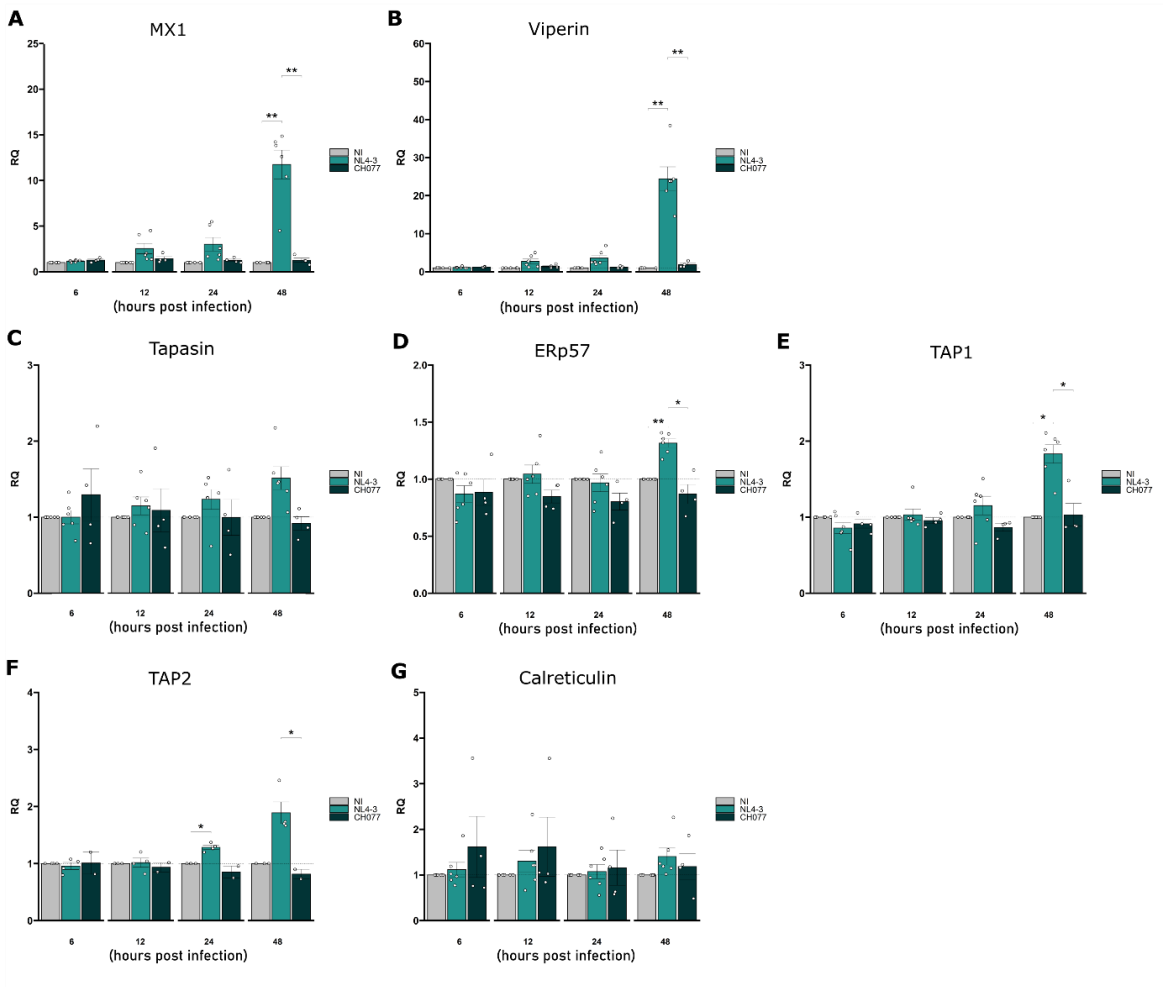


Figure 5.4. **PLC mRNA expression kinetics in activated PBMCs infected with HIV-1.** Relative Quantification (RQ) of interferon stimulated genes (ISGs) A) MX1 and B) Viperin (RSAD2) and peptide loading complex genes C) tapasin, D) ERp57, E) TAP1, F) TAP2 and G) Calreticulin gene expression measured in PBMCs after infection with NL4-3-G or CH077-G at an MOI of 1.0 over time, compared to noninfected (NI) controls. RQ of PLC gene expression was measured by qPCR. RQ values were calculated according to the ΔΔCt method where RQ = $2^{-\Delta\Delta Ct}$. Individual points correspond to the mean value of technical duplicates from independent experiments (n=3). P-values were estimated using student's t-test with Benjamini-Hochberg correction for multiple comparisons where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.2.2.3. Nef-deleted HIV-1 fails to downregulate HLA class I in activated PBMCs

The data suggest that infection with NL4-3-G and CH077-G in primary human PBMCs induces the expression of PLC transcripts likely via the IFN signalling pathway (Figure 5.4). This change in PLC mRNA expression was not observed in HEK293FT cells (Figure 5.1). We next asked what HIV-1 viral protein might be driving the upregulation of PLC gene transcription in PBMCs.

HIV-1 Nef downregulates HLA class I by directing intracellular HLA class I molecules for lysosomal degradation (Dirk et al., 2016). Consistent with previous published findings, we showed that infection of HEK293FT cells with NL4-3ΔNef-G abrogated the virus' ability to downregulate HLA class I (Figure 5.5A). HIV-1 Env and Vpu are also viral proteins of potential interest in the context of this current study due to their identification as interacting with tapasin and ability to downregulate HLA-C molecules, respectively (Luo et al., 2016, Apps et al., 2016). However, infection of HEK293FT cells with NL4-3ΔEnv-G or NL4-3ΔVpu-G downregulated HLA class I to a similar degree to WT NL4-3-G (Figure 5.5A). Therefore in the setting of studying HIV-1 evasion of the cellular immune response via the downregulation of HLA class I and the potential mechanisms underlying this that may involve the PLC, we decided to focus this study on HIV-1 Nef.

Primary human PBMCs were similarly infected with NL4-3-G (WT virus) and NL4-3ΔNef-G at an MOI of 1.0 for 48 hours after activation of the cells with PHA and IL-2. HLA class I and cell surface lineage marker (CD3, CD4, CD8, CD19, CD14 and CD16) expression was analysed prior to activation with PHA (pre-activation), prior to HIV-1 infection (0 hour) and 12, 24 and 48 hours post infection by flow cytometry. Cellular mRNA was also extracted at 0, 6, 12, 24 and 48 hours in noninfected controls and post infection with NL4-3-G or NL4-3ΔNef-G for analysis by RT-qPCR to quantify PLC transcript levels.

The gating strategy described in Figure 5.3A was used for the analysis of flow cytometry data from PBMCs infected with NL4-3-G and NL4-3ΔNef-G. HIV-1 Nef downregulates CD4 molecules, which are endocytosed from the cell surface and degraded by lysosomes (Aiken et al., 1994, Rhee and Marsh, 1994). Therefore, the gating strategy for CD3+CD8- PBMCs (Figure 5.3A) was modified to gate all Gag+ cells (Gag+CD3+CD4high/low) in NL4-3ΔNef-G infected PBMCs (Figure 5.5B). The same gate was also applied to NL4-3-G infected and noninfected cell control data in this experiment (Figure 5.5B). We saw that, as expected, infection of PBMCs in our VSV-G pseudotyped infection model of NL4-3ΔNef resulted in higher CD4 expression compared to infection with Nef-expressing NL4-3-G (Figure 5.5B, histograms). We did however observe low infection rates with NL4-3ΔNef-G, comparable to that seen for NL4-3-G and CH077-G infection (Figure 5.5C). We did not see any preference for different cellular subsets after infection with NL4-3-G or NL4-3ΔNef-G (Figure 5.5D). NL4-3-G and NL4-3ΔNef-G primarily infected CD3+CD4+ cells, with a very small percentage (3% and 5% of Gag+ for NL4-3-G and NL4-3ΔNef-G infection, respectively) infecting CD3+CD8+ cells (Figure 5.5E). Gag+ cells infected with WT virus showed reduced HLA class I expression compared to Gag- bystander and noninfected cells, whereas the HLA class I expression in Gag+ cells infected with NL4-3ΔNef-G virus was significantly upregulated compared to Gag- bystanders (Figure 5.5F and 5.5G). Therefore, even with the low infection efficiencies achieved with NL4-3-G and NL4-3ΔNef-G in PBMCs, we consistently saw the expected phenotypes of HLA class I downregulation with each virus.

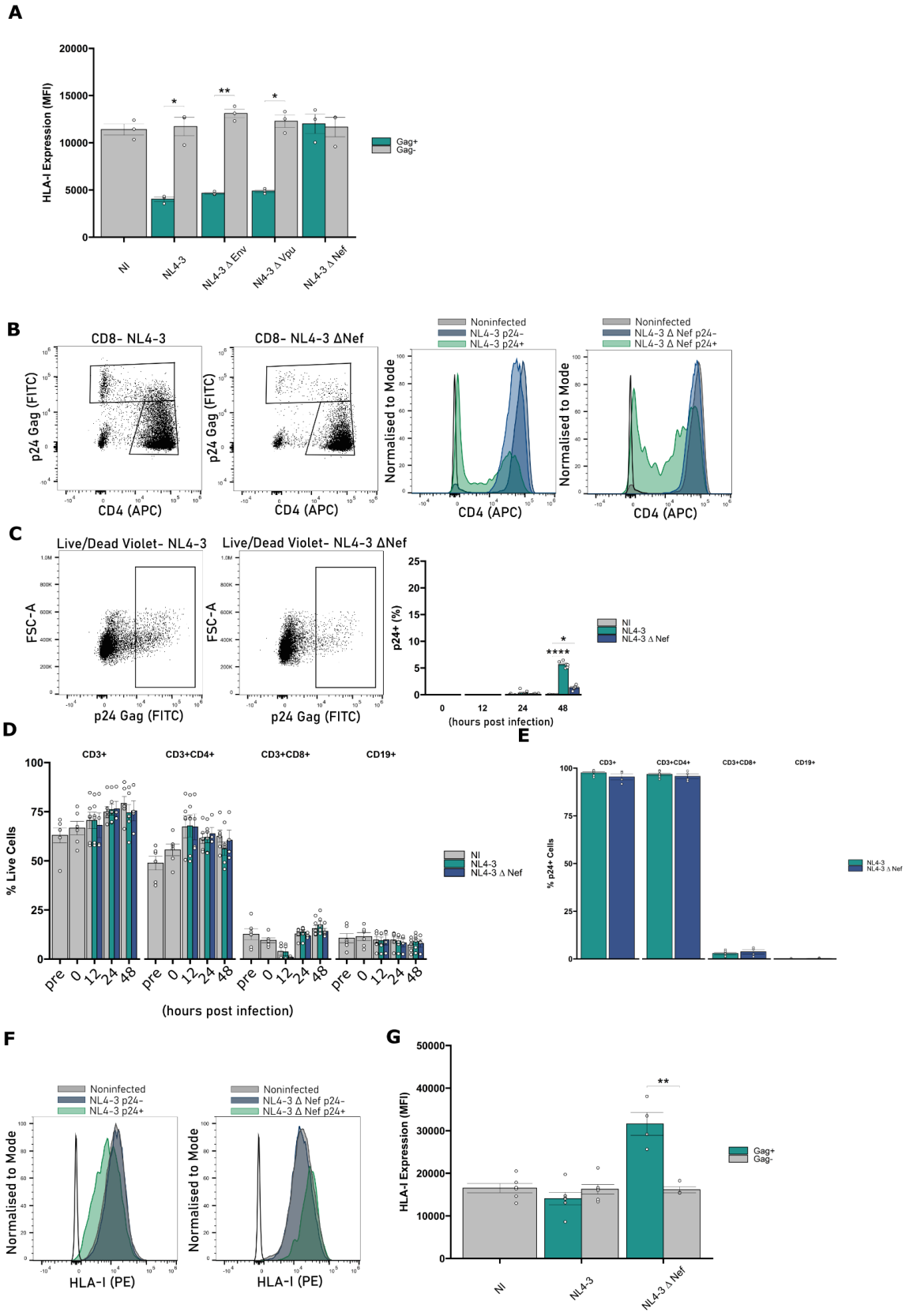


Figure 5.5. HLA class I downregulation measured by flow cytometry in activated PBMCs after infection with WT and ΔNef HIV-1.

Figure 5.5. HLA class I downregulation measured by flow cytometry in activated PBMCs after infection with WT and ΔNef HIV-1. A) Downregulation of HLA class I in HEK293FT cells after infection with VSV-G pseudotyped NL4-3, NL4-3ΔEnv, NL4-3ΔVpu or NL4-3ΔNef in Gag+ (productively infected) versus Gag- (bystander) cells. B) Gating strategy on activated CD3+CD8- PBMCs to identify CD3+CD4+Gag- and CD3+CD4-Gag+ cells infected with NL4-3-G or NL4-3ΔNef-G 48 hours post infection. Histograms show level of CD4 expression 48 hours post infection with NL4-3-G (left) or NL4-3ΔNef-G (right) in Gag+ PBMCs (green) compared to bystander (blue) and noninfected PBMCs (grey). C) Level of NL4-3-G and NL4-3ΔNef-G infection in PBMCs measured by flow cytometry using the anti-HIV gag p24 antibody clone KC57. D) The proportion of cells expressing the lymphocyte markers CD3, CD4, CD8 or CD19 in PBMCs derived from healthy donor blood. Population percentages as a proportion of live cells (Live/Dead-) are shown prior to activation with PHA and IL-2 (pre-activation) then 0, 12, 24 and 48 hours post infection with NL4-3-G or NL4-3ΔNef-G to show any changes in population composition after HIV-1 challenge. E) Population characteristics of cellular markers expressed on Gag+ NL4-3-G or NL4-3ΔNef-G infected PBMCs. The proportion of Gag+ cells expressing CD3, CD4, CD8 or CD19 are shown in the gated population of Gag+ productively infected cells. F) HLA class I expression (MFI) in Gag+ HIV-1 infected, Gag- (bystander) and noninfected PBMCs infected with NL4-3-G or NL4-3ΔNef-G. Representative data from a single healthy blood donor is shown in these histograms. G) HLA class I expression (MFI) in Gag+ and bystander Gag- healthy donor PBMCs uninfected or infected with NL4-3-G or NL4-3ΔNef-G. Individual points correspond to the mean value of technical duplicates from independent experiments (n=3). P-values were estimated using student's t-test with Benjamini-Hochberg correction for multiple comparisons where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NI: Noninfected. pre: pre-activation.

5.2.2.4. Nef-deleted HIV-1 fails to upregulate ERp57, TAP1 or TAP2 mRNA expression in activated PBMCs

We next examined the role of Nef in the mRNA expression of the PLC components by using the Nef-deleted NL4-3-G virus. The cellular mRNA from NL4-3-G or NL4-3ΔNef-G infected PBMCs was extracted at 6, 12, 24 and 48 hours post infection and analysed by RT-qPCR. The results showed that infection of PBMCs with NL4-3ΔNef-G did not upregulate the ISGs MX1 or Viperin compared to infection with NL4-3-G (Figure 5.6A and 5.6B). Moreover, infection with NL4-3ΔNef-G did not upregulate ERp57, TAP or TAP2 compared to NL4-3-G (Figure 5.6D – 5.6F). We observed a significant reduction in TAP1 and TAP2 mRNA expression 24 hours post infection with NL4-3ΔNef-G compared to noninfected cells and NL4-3-G infection, respectively (Figure 5.6E and 5.6F). TAP2 mRNA expression was also significantly lower in NL4-3ΔNef-G infected cells compared to infection with NL4-3-G 48 hours post infection (Figure 5.6F). These findings suggest a role for Nef in triggering the ISG response in primary human cells, including the response of elements of the PLC. However, as mentioned previously, the infection efficiency of NL4-3-G and NL4-3ΔNef-G was very low. These results provide preliminary evidence to suggest that HIV-1 Nef triggers the expression of TAP1, TAP2 and ERp57, but not the expression of tapasin, calreticulin or other known ISGs, in primary human cells. Further experiments should be conducted to provide additional evidence to support this observation.

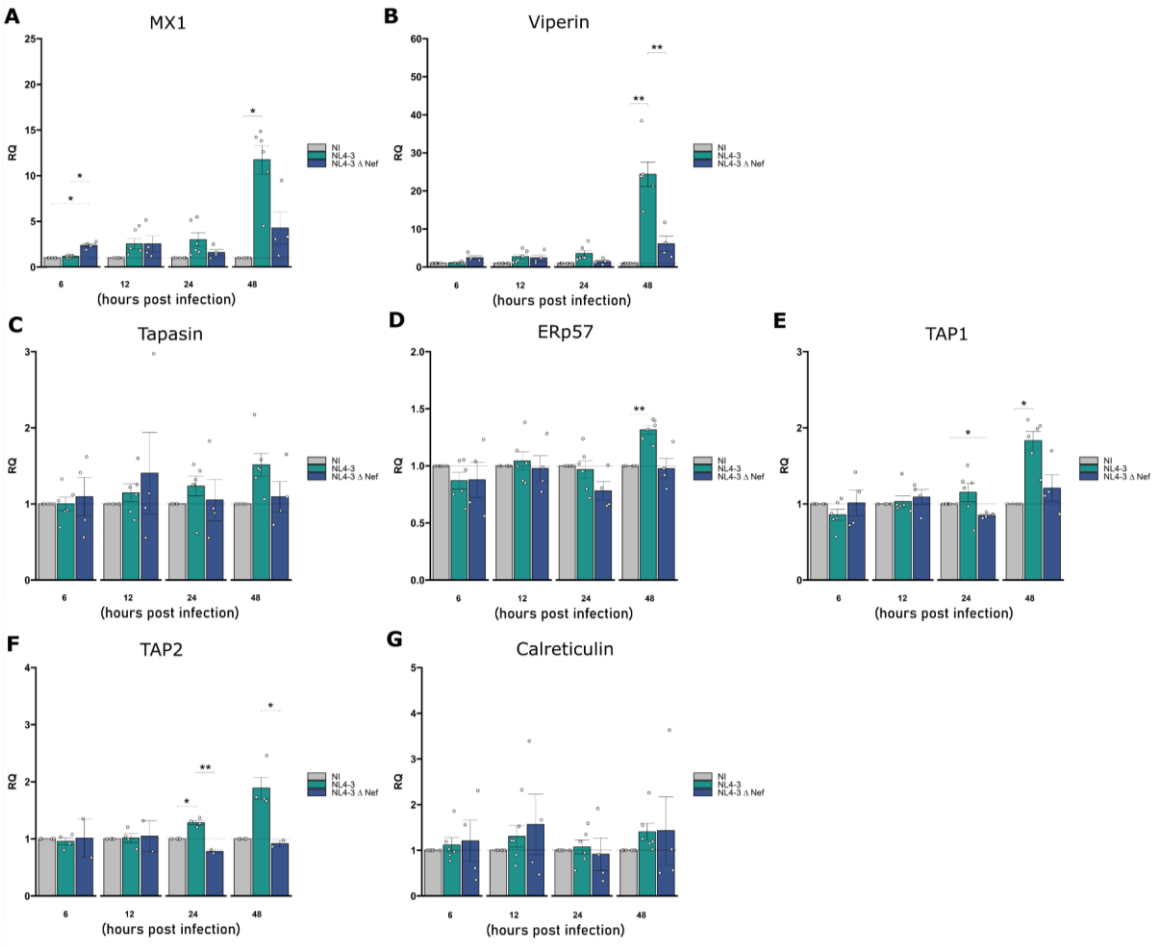


Figure 5.6. **PLC mRNA expression kinetics in activated PBMCs infected with WT and ΔNef HIV-1.** Relative Quantification (RQ) of IFN stimulated genes (ISGs) A) MX1 and B) Viperin (RSAD2) and peptide loading complex genes C) tapasin, D) ERp57, E) TAP1, F) TAP2 and G) Calreticulin gene expression measured in PBMCs after infection with NL4-3-G or NL4-3ΔNef-G at an MOI of 1.0 over time, compared to noninfected (NI) controls. RQ of PLC gene expression was measured by qPCR. RQ values were calculated according to the ΔΔCt method where RQ = $2^{-\Delta\Delta Ct}$. Individual points correspond to the mean value of technical duplicates from independent experiments (n=3). P-values were estimated using student's t-test with Benjamini-Hochberg correction for multiple comparisons where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.2.3. The effect of HIV-1 Nef on PLC expression in HEK293FT cells

5.2.3.1. There is no significant difference in PLC expression after infection with WT or Nef-deleted HIV-1 in HEK293FT cells

As expected from our previous findings, these results were not corroborated in HEK293FT cells infected with NL4-3-G or NL4-3ΔNef-G for up to 48 hours (Figure 5.7). Cellular mRNA was extracted 6, 12, 24 and 48 hours post infection and PLC component expression was analysed by RT-qPCR, consistent with previous experiments. Again, we found no evidence that the expression of the ISGs MX1 or Viperin were induced in response to infection of HEK293FT cells with NL4-3-G or NL4-3ΔNef-G (Figure 5.7F and 5.7G). Nor did we find any evidence that tapasin, ERp57, TAP1, TAP2 or calreticulin mRNA expression was different between noninfected and NL4-3-G or NL4-3ΔNef-G infected HEK293FT cells (Figure 5.7A – G).

Tapasin and ERp57 protein expression from cell lysates collected 6, 12, 24 and 48 hours post infection with NL4-3-G and NL4-3ΔNef-G and in noninfected cells was also analysed by Western Blot. Both tapasin and ERp57 protein expression appeared to be upregulated in HEK293FT cells infected with NL4-3ΔNef-G 48 hours post infection, but the relationship was not statistically significant.

The results of the experiments conducted in HEK293FT cells suggest that changes in PLC expression is likely due to induction of the IFN signalling pathway, which is intact and responsive in bulk activated PBMCs infected with VSV-G pseudotyped HIV-1 but not in HEK293FT cells which fail to induce type I IFN responses.

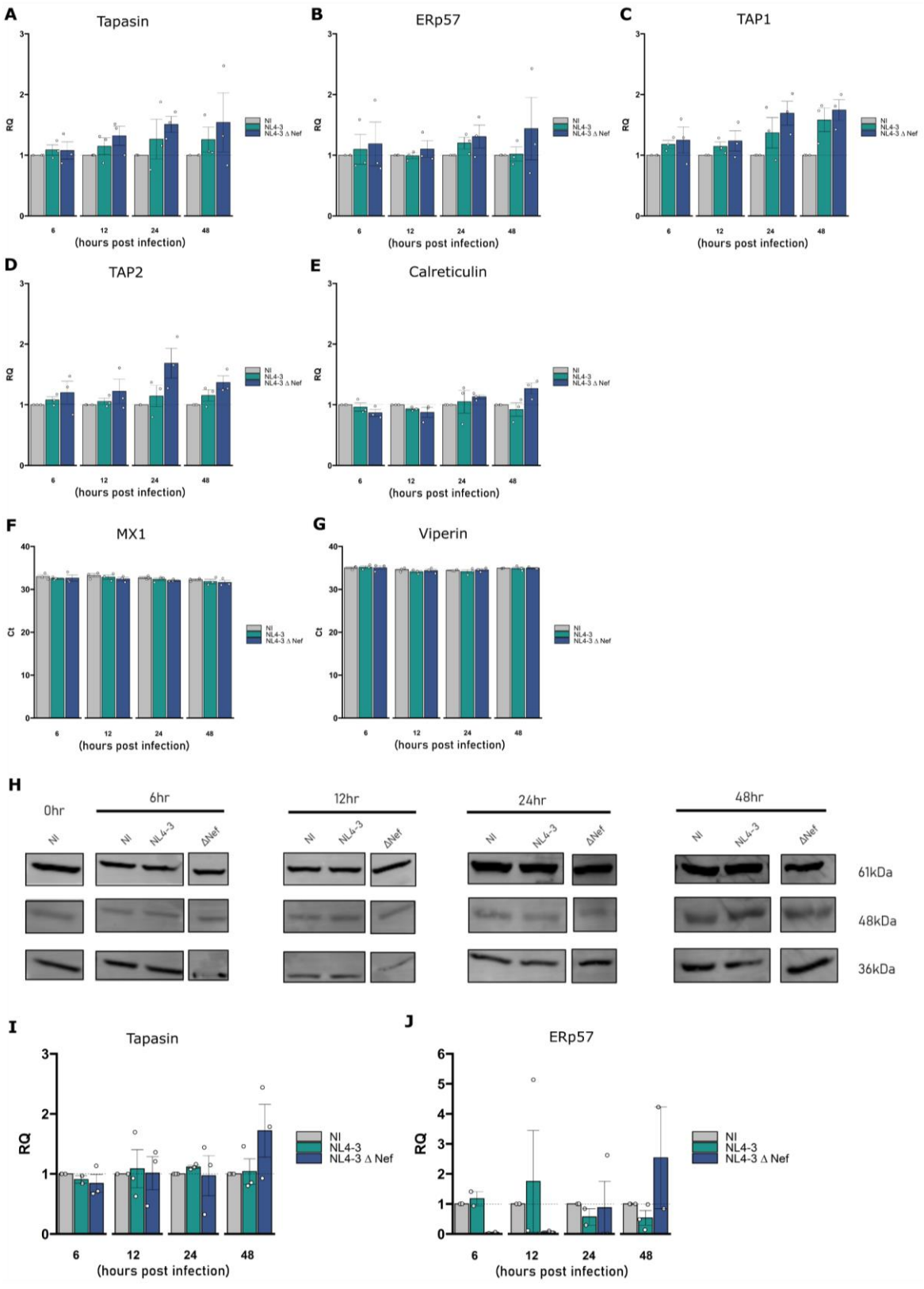


Figure 5.7. PLC expression kinetics in HEK293FT cells infected with WT and ΔNef HIV-1.

Figure 5.7. PLC expression kinetics in HEK293FT cells infected with WT and ΔNef HIV-1. Relative Quantification (RQ) of peptide loading complex genes A) tapasin, B) ERp57, C) TAP1, D) TAP2 and E) Calreticulin gene expression measured in HEK293FT cells after infection with NL4-3-G or NL4-3ΔNef-G at an MOI of 1.0 over time, compared to noninfected (NI) controls. RQ of PLC gene expression was measured by qPCR. RQ values were calculated according to the ΔΔCt method where $RQ = 2^{-\Delta\Delta Ct}$. F – G) Raw interferon stimulated gene (ISG) F) MX1 and G) Viperin (RSAD2) Ct values in noninfected (NI) HEK293FT cells or HEK293FT cells infected with NL4-3-G or NL4-3ΔNef-G over time. H) Western Blot analysis of tapasin and ERp57 protein expression in HEK293FT cells infected over time with NL4-3-G or NL4-3ΔNef-G, compared to noninfected (NI) controls. Loaded samples were normalised for protein content by BCA protein assay. I – J) Semi-quantitative analysis of relative protein expression measured by Western Blot (H) for I) tapasin and J) ERp57. Semi –quantitative RQ values calculated by normalisation of tapasin and ERp57 band intensities to GAPDH and further normalising to the NI control at each time point. Individual points correspond to the mean value of technical duplicates from independent experiments (n=3). P-values were estimated using student's t-test with Benjamini-Hochberg correction for multiple comparisons where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.2.3.2. Transient expression of Nef in HEK293FT cells results in the specific upregulation of ERp57 mRNA expression

We next wanted to examine whether Nef expression alone was sufficient to upregulate ERp57, TAP1 or TAP2 expression as shown in the above results with HIV-1 infection. Due to constraints on the experimental conditions that could be employed to investigate this question, HEK293FT cells were used to investigate the role of Nef expression in PLC expression.

The pcDNA3.4 TOPO expression vector encoding the HIV-1 strain HXB2 Nef protein with a C-terminal 6X His-tag under the control of the HCMV promoter was transfected into HEK293FT cells. The amino acid sequence of HXB2 Nef has three nonsynonymous mutations that differ it from NL4-3 Nef at positions 15 (threonine to alanine), 29 (arginine to glycine) and 51 (threonine to asparagine) (Figure 5.8). These residues are not involved in HLA class I (methionine at position 20) or CD4 (asparagine at position 35 and glutamic acid at position 59) downregulation (Mann et al., 2015).

Cells were also either mock transfected with DMEM and TransIT-293 transfection reagent only (DMEM controls) or with pcDNA™-DEST47 vector only as an EV DNA only control. Transfected cells were collected 48 hours post transfection and cell surface HLA class I expression and intracellular 6X His-tag expression was analysed by flow cytometry. Transfected cells were also lysed for cellular RNA and total protein to analyse PLC expression by RT-qPCR and Western Blot.

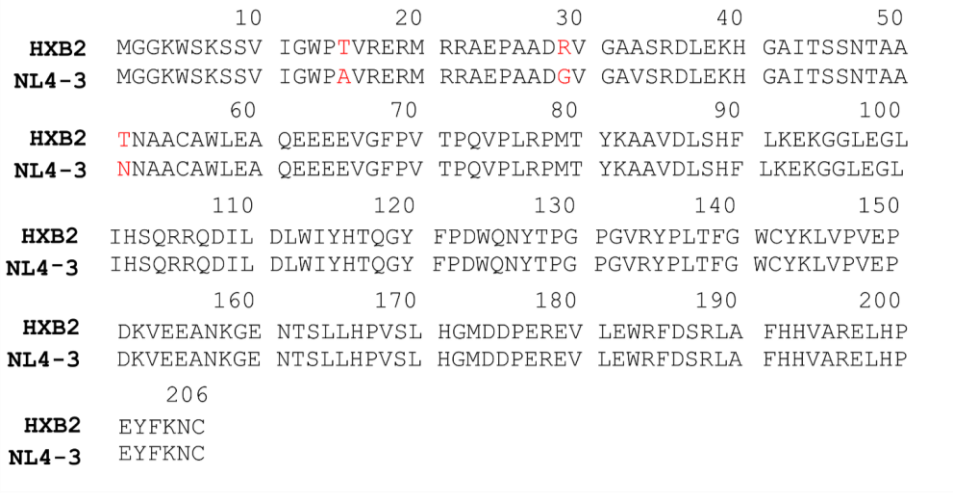


Figure 5.8. **HIV-1 HXB2 and NL4-3 Nef sequences.** Comparison between subtype B HXB2 and NL4-3 HIV-1 Nef amino acid sequences. Residues shown in red denote nonsynonymous mutations between the two sequences.

Flow cytometry data collected from Nef-His, EV and DMEM only transfected HEK293FT cells were gated as single cell populations that were positive or negative for 6X His-tag expression (Figure 5.9A and 5.9B). We then examined HLA class I expression in His-tag+ (Nef-His transfected) versus His-tag- (transfection bystander or non-transfected cells). Transfection of EV DNA non-specifically downregulated HLA class I expression in HEK293FT cells compared to mock transfection (Figure 5.9C and 5.9E). We also saw that the expression of HLA class I was lower in His-tag negative bystander HEK293FT cells after transfection with the Nef-His construct (Figure 5.9D and 5.9E). His-tag positive HEK293FT cells transfected with Nef-His had significantly reduced levels of HLA class I compared to DMEM mock transfected cells, His-tag- bystander cells and EV DNA transfected cells (Figure 5.9D and 5.9E). These results indicated that Nef-His expression in HEK293FT specifically downregulated HLA class I in the transfection model utilised here.

As our transfection model of Nef-His expression in HEK293FT cells gave the expected phenotype of HLA class I downregulation, we next asked whether Nef expression in this cell line impacted PLC expression. Cellular mRNA was extracted 48 hours post infection in HEK293FT cells transfected with Nef-His, EV DNA or mock transfected with DMEM only. We found no evidence that tapasin, TAP1, TAP2 or calreticulin mRNA expression levels were significantly different in Nef-His transfected HEK293FT cells compared to mock transfected or EV DNA transfected cells (Figure 5.9F, 5.9H, 5.9I and 5.9J). However we did find that ERp57 expression levels were significantly upregulated in Nef-His transfected cells compared to EV DNA or mock transfected cells (Figure 5.9G). Although not significant, the level of ERp57 protein expression also appeared to be greater in cell lysates from HEK293FT cells transfected with Nef-His compared to EV DNA and mock transfection after analysis by Western Blot (Figure 5.9K and 5.9M). This was less evident and not significant when tapasin

protein expression levels were analysed by Western Blot in the same cellular lysates (Figure 5.9K and 5.9L).

The upregulation of ERp57 by Nef-His in HEK293FT cells appeared to be independent of Nef's impact on ERp57 transcription via IFN. As was also evident in HEK292FT cells infected with VSV-G pseudotyped HIV-1 (Figure 5.2), the expression of exogenous EV or Nef-His DNA, much like HIV-1 infection, did not induce ISG mRNA expression in this cell line (Figure 5.9N). Further experiments to elucidate HIV-1 Nef influence on ERp57 expression would be interesting to interpret the potential biological role of this chaperone protein in HIV-1 infection.

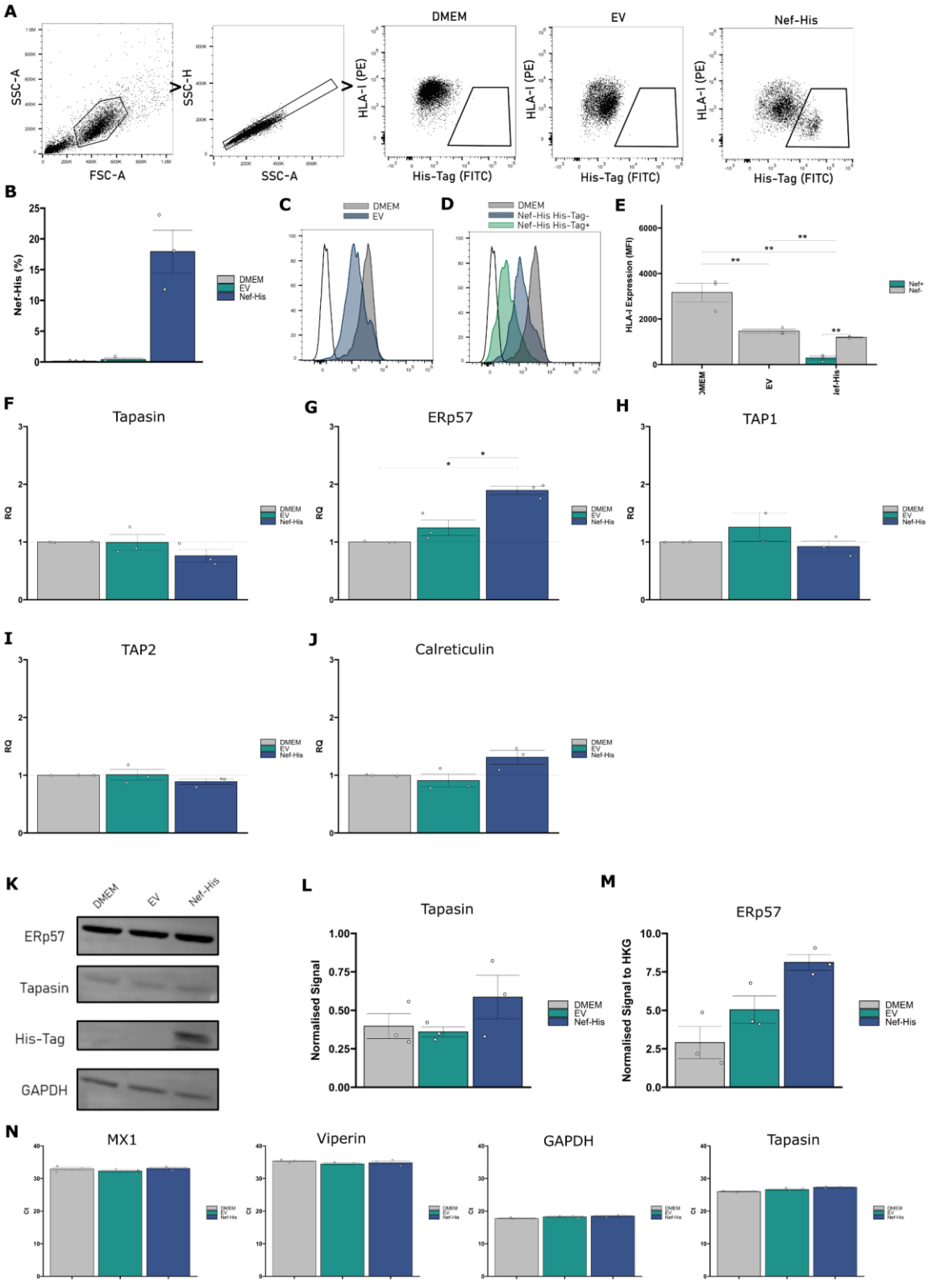


Figure 5.9. PLC expression kinetics in HEK293FT cells transfected with HIV-1 Nef.

Figure 5.9. PLC expression kinetics in HEK293FT cells transfected with HIV-1 Nef. HLA class I downregulation and PLC gene and protein expression kinetics measured by flow cytometry, qPCR and Western Blot analyses in HEK293FT cells after transfection with DMEM only (control), empty vector pcDNA™-DEST47 (EV) DNA or C-terminally 6xhistidine tagged HIV-1 HXB2 Nef tagged HIV-1 HXB2 Nef (Nef-His). A) Nef-His positive cells were measured by flow cytometry using the anti-His-Tag antibody clone AD1.1.10. HLA class I expression was measured in His-Tag negative cells or His-Tag positive (Nef-His transfected) cells by flow cytometry by staining with the anti-HLA class I antibody clone W6/32. B) Proportion of HEK293FT cells expressing Nef-His (His-Tag+) after 48 hours post-transfection. C – E) HLA class I expression in His-Tag- cells (blue) compared to nontransfected DMEM (grey) and His-Tag+ cells (green) in HEK293FT cells transfected with C) EV DNA or D) Nef-His encoding plasmid. E) Quantification of HLA class I expression in Nef-His+ (green) or Nef-His- (grey) cells 48 hours post transfection. F - J) Relative Quantification (RQ) of peptide loading complex genes F) tapasin, G) ERp57, H) TAP1, I) TAP2 and J) Calreticulin gene expression measured in HEK293FT cells after transfection with DMEM, EV DNA or Nef-His for 48 hours. RQ of PLC gene expression was measured by qPCR. RQ values were calculated according to the ΔΔCt method where $RQ = 2^{-\Delta\Delta Ct}$. K) Western Blot analysis of tapasin and ERp57 protein expression in HEK293FT cells transfected with EV DNA or Nef-His for 48 hours, compared to DMEM controls. L – M) Semi-quantitative analysis of relative protein expression measured by Western Blot (H) for L) tapasin and M) ERp57. Semi –quantitative RQ values calculated by normalisation of tapasin and ERp57 band intensities to GAPDH and further normalising to the DMEM control at each time point. N) Raw interferon stimulated gene (ISG) MX1 and Viperin (RSAD2), GAPDH housekeeping gene (HKG) and tapasin (PLC) Ct values in DMEM control HEK293FT cells or HEK293FT cells transfected with EV DNA or Nef-His for 48 hours. Individual points correspond to the mean value of technical duplicates from independent experiments (n=3). P-values were estimated using student's t-test with Benjamini-Hochberg correction for multiple comparisons where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3. Chapter 5 Discussion

In this chapter we aimed to examine the possible cellular mechanisms to explain the data presented in Chapter 4. In Chapter 4 we showed that HLA class I downregulation by HIV-1 was impeded when tapasin, TAP1 or TAP2 were knocked out *in vitro* (Figure 4.4). We also showed that HIV-1 more efficiently downregulated tapasin-dependent HLA-B*44:02 molecules compared to the tapasin-independent HLA-B*44:05 (Figure 4.7). These data imply that HIV-1 may interfere with or downregulate the PLC itself in order to exert an effect on HLA class I expression. In their 2020 study, Bashirova and colleagues showed that HIV-1 infected adult patients who express tapasin-independent HLA class I alleles experienced slower disease progression and lower HIV-1 VLs. We found further evidence to support these findings in Chapter 3 of this thesis. We showed in a paediatric cohort of patients perinatally-infected with HIV-1 that lower plasma HIV-1 VLs were associated with the expression of more tapasin-independent HLA class I alleles. Bashirova *et al.* (2020) explained that these phenotypes of disease and their association with tapasin-dependency may be explained by tapasin-independent alleles presenting a broader repertoire of the HIV-1 peptidome to CD8+ T cells. Our data presented in Chapter 4 supported the hypothetical mechanism we proposed in that the association of tapasin-independent HLA class I alleles with control of HIV-1 infection may have evolved in response to viral immune evasion mechanisms that target the PLC, as has been observed for other viral infections (reviewed by (Roder et al., 2008) and (Hansen and Bouvier, 2009)). The data presented in this chapter looked at how HIV-1 might affect PLC gene and protein expression. Our hypothesis was that HIV-1 infection may result in reduced expression of tapasin or other components of the PLC, and patients or cells that express tapasin-independent HLA class I alleles might be able to circumvent this interference.

HEK293FT cells and primary human PBMCs from healthy blood donors were infected with VSV-G pseudotyped NL4-3, CH077 and NL4-3ΔNef to try and determine the impact of HIV-

1 infection on PLC mRNA and protein expression. We showed in Chapter 4 that infection of HEK293FT cells with VSV-G pseudotyped HIV-1 resulted in efficient infection levels of HIV-1 measured by intracellular Gag protein and significant downregulation of HLA class I molecules at the cell surface. However, we found no evidence that HIV-1 infection with Nef-expressing NL4-3-G or CH077-G or Nef-deficient NL4-3ΔNef-G led to differential expression of any of the PLC genes when analysed by RT-qPCR (Figure 5.1). Furthermore, we found no evidence that expression of a Nef construct alone in HEK293FT affected PLC gene expression (Figure 5.9). We also found no significant upregulation of tapasin or ERp57 protein expression in HEK293FT cells in response to either HIV-1 infection or Nef expression. These data do not suggest that infection with HIV-1 blocks the transcription of PLC genes, nor downregulates or degrades PLC mRNA post-transcription or pre-translation. Although the quantitation of tapasin and ERp57 protein levels by semi-quantitative Western Blot is limited due to crude analysis of densitometry data from raw blots (Figure 5.1H – J), we also do not find any evidence in HEK293FT cells that HIV-1 infection degrades or promotes the expression of tapasin or ERp57 translated proteins.

We considered whether the absence of any changes in PLC expression might be due to a dysfunctional IFN response in HEK293FT cells. HEK293 cells have been shown to be deficient in endogenous cyclic GMP-AMP synthase (cGAS) stimulator of IFN gene (STING) and IFN-β expression (Sun et al., 2013, Zhang et al., 2014). The expression of components of the PLC are upregulated in response to pathogens via the IFN signalling pathway (van den Elsen et al., 2004, Kelly and Trowsdale, 2019, Abarca-Heidemann et al., 2002, Kuchtey et al., 2005, Schiffer et al., 2002). IFN release from cells that sense viral infection triggers ISG upregulation via the JAK/STAT signalling pathway and the production of proteins that guide the antiviral immune response (reviewed in (Walter, 2020)). The tapasin gene promoter region contains an ISRE and GAS that makes the gene responsive to IFN signalling (Herberg et al., 1998). TAP1

expression is upregulated by IFN-α and IFN-γ in primary cells (Heise et al., 2016, Brucet et al., 2004, Chatterjee-Kishore et al., 1998). TAP1, TAP2 and tapasin are also shown to be significantly upregulated in dendritic cells differentiated from monocytes in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN-α, which have highly efficient cross-priming/cross-presentation capabilities (Lattanzi et al., 2011). Targeting PLC gene expression via exploitation of the IFN signalling pathway would enable HIV-1 evasion of antigen presentation to CD8+ T cells by stopping the upregulation of key peptide processing machinery components.

To interrogate this possibility, we followed up the expression profiling studies of the PLC genes in bulk primary human PBMCs infected with VSV-G pseudotyped HIV-1 strains. We found that the expression of the ISGs MX1 and Viperin were significantly upregulated in NL4-3-G infection. The expression of ERp57, TAP1 and TAP2 mRNA was also upregulated in NL4-3-G infected PBMCs. These findings imply that in bulk PBMCs ERp57, TAP1 and TAP2 transcription is upregulated in HIV-1 infection, potentially via IFN induction of these genes. The expression of neither ISG nor PLC mRNA was significantly increased in CH077-G or NL4-3ΔNef-G infected PBMCs (Figure 5.4 and Figure 5.6). These findings are most likely due to the significantly reduced levels of infection achieved by CH077-G and NL4-3ΔNef-G in primary PBMCs (Figure 5.3D and 5.5C). RT-qPCR analyses were performed on bulk PBMCs infected with HIV-1. With infection efficiencies of less than 10% (Figure 5.3D and 5.5C), differential PLC gene expression with small effect sizes may have been masked by the disproportionately high number of uninfected cells in the infected sample. The use of bulk PBMCs in the experiments with primary human cells may have contributed to the low infection efficiencies of all three HIV-1 isolates tested in this study. First isolating CD4+ T cells via immunomagnetic negative selection followed by infection or spinoculation with pseudotyped HIV-1 may avoid CD8+ T cell recognition and killing of infected CD4+ T cells in the bulk

infected sample and achieve higher infection rates in primary cells. Alternatively, it would be interesting to study and compare PLC gene expression specifically in productively HIV-1 infected by single-cell RNA sequencing or gene expression profiling by RT-qPCR following FACS sorting of Gag+ cells.

Despite the low infection rates achieved by NL4-3ΔNef-G infection in PBMCs, we did observe that TAP1 mRNA expression was significantly downregulated in PBMCs infected with NL4-3ΔNef-G compared to noninfected controls (Figure 5.6E). Productive infection with any of the pseudotyped HIV-1 isolates in this study were not measurable until at least 48 hours post infection. Thus, it appears that Nef-expressing HIV-1 may impact TAP1 transcription prior to detection of intracellular viral proteins. This was also seen in the expression levels of TAP2 (Figure 5.6F) mRNA 24 hours post infection with NL4-3-G. Taken together, these data imply a role of Nef in HIV-1 infection for the upregulation of PLC genes. These findings require further validation in experiments that achieve higher infection efficiencies and in different cell types.

The question remains why no differential expression in tapasin mRNA expression was observed in NL4-3-G infected PBMCs, as was seen for ERp57, TAP1 and TAP2. Tapasin has been shown to be upregulated in response to viral infection with viral haemorrhagic septicaemia virus (VHSV) and salmonid alphavirus 3 (SAV3) in trout and salmon macrophages, respectively, and is upregulated in hepatocytes that clear HBV infection (Wieland et al., 2003, Wieland et al., 2004, Xu et al., 2019, Landis et al., 2006). Tapasin has also been shown to be upregulated in some cancer microenvironments (Seliger et al., 2001a). On the other hand, as previously mentioned in the introduction to this chapter, HCMV US3 has been shown to inhibit the synthesis of tapasin transcripts (Halenius et al., 2011). This was shown to contribute to the specific downregulation of tapasin-dependent HLA class I alleles in HCMV infection. Tapasin expression has also conversely been shown to be downregulated in

a number of different cancers (Dissemond et al., 2003, Thuring et al., 2014, Shionoya et al., 2017, Jiang et al., 2010). The data in this chapter does not provide any evidence that tapasin mRNA expression levels significantly differ between infected and noninfected cells. It would be interesting to investigate whether this is as a result of flaws in the data, or due to an inhibition of the upregulation of tapasin gene expression, as opposed to targeting tapasin transcripts for degradation or blocking transcription altogether.

The data indicated that ERp57, TAP1 and TAP2 mRNA expression was upregulated in NL4-3-G but not NL4-3ΔNef-G infected cells. We found that Nef expression in HEK293FT cells significantly upregulated ERp57 mRNA expression levels, but in contrast to the data from PBMC infection, not any of the other PLC components. As previously described, it appeared from our data on MX1 and Viperin expression that the IFN response was not intact in HEK293FT cells. Therefore, the mechanism behind the upregulation of ERp57 during infection with HIV-1 strains with intact Nef or Nef DNA expression in immortalised cell lines should be further investigated. HIV-1 proteins Vpu, Vif and Nef have been shown to antagonise the IFN response and block the induction of ISGs (Sanchez et al., 2015, Nguyen et al., 2018, Gargan et al., 2018). Further experiments to explore the effect of infection with NL4-3ΔVpu, NL4-3ΔVif and dual NL4-3ΔORF viruses on PLC gene expression would be interesting to understand the impact of these viral proteins on antigen processing machinery expression.

ERp57 has been implicated in a number of different diseases including cancer, neurodegenerative disease and heart failure (Tohda et al., 2012, Vitello et al., 2012, Hettinghouse et al., 2018). ERp57 is found to be upregulated in a number of different cancers and increased expression is also associated with the expression of the protein HERG A561V in patients with the heart arrhythmia disorder long QT syndrome (Chen et al., 2007, Park et al., 2018, Li et al., 2019, Wu et al., 2021, Liu et al., 2019). ERp57 has also been implicated in viral and bacterial infection. ERp57 is primarily located in the ER, but has also been found in other

cellular compartments such as the nucleus and mitochondria as well as at the cell surface (reviewed in (Chichiarelli et al., 2022)). Infection with Nairobi sheep disease virus (NSDV) (similar to the human Crimean-Congo haemorrhagic virus) promotes translocation of ERp57 from the ER to the cell surface (Lasecka and Baron, 2014). At the cell surface, ERp57, as a member of the PDI family, catalyses thiol-disulphide exchange reactions to reductively cleave protein disulphide bond (Frickel et al., 2004). This disulphide reductase activity has been described as aiding membrane fusogenic reactions and contributing to cellular entry after infection with simian vacuolating virus 40 (SV40), *Burkholderia cenocepacia*, murine polymavirus, rotavirus, HBV, influenza A and B and HIV-1 (Schelhaas et al., 2007, Walczak and Tsai, 2011, Santana et al., 2013, Pacello et al., 2016, Perez-Vargas et al., 2021, Roberson et al., 2012, Kim and Chang, 2018, Liu et al., 2017). PDI has been shown to have a critical role in HIV-1 cellular entry. PDI expressed at the cell surface bound to CD4 and reduces disulphide bridges in gp120 that trigger adjacent gp41 conformation changes that enable viral cell entry (Ryser and Fluckiger, 2005). The upregulation of ERp57 identified in this study after infection of primary human PBMCs with NL4-3-G or expression of Nef in HEK293FT cells may indicate a Nef driven upregulation of disulphide isomerase activity to mediate disulphide bond reduction in gp120 and promote HIV-1 cellular entry and replication. Further experiments with VSV-G pseudotyped NL4-3ΔEnv and un-pseudotyped viruses should be conducted to establish a direct correlation between viral proteins that contribute to the upregulation of ERp57 in primary human cells and HEK293FT cells after infection with HIV-1. Agents that block thiol-disulphide exchange activity or downregulate the expression of PDI and ERp57 can inhibit HIV-1 cell entry and replication (Ryser et al., 1994, Khan et al., 2011, Barbouche et al., 2003, Fenouillet et al., 2001, Gallina et al., 2002, Markovic et al., 2004, Ou and Silver, 2006). Thus the findings described in this chapter contribute to the knowledge that highlights ERp57 as a potential therapeutic target to combat HIV-1 infection.

Aside from ERp57, TAP1 and TAP2 were also upregulated in primary human PBMCs infected with NL4-3-G (Figure 5.4E and 5.4F). Increased expression of TAP1 and TAP2 mRNA was not seen in HEK293FT cells transfected with HIV-1 Nef. As mentioned, we found no ISG response in HEK293FT cells (Figure 5.2) and hypothesised that TAP1/2 upregulation of expression may be via an IFN response. HCV infection has been shown to upregulate TAP1 expression in order to increase HLA class I expression and subvert lysis by natural killer (NK) cells (Herzer et al., 2003). By downregulating HLA class I molecules, HIV-1 leaves infected cells open to NK cell lysis via "missing–self" recognition (reviewed in (Raulet, 2006). HIV-1 can avoid this by upregulating HLA-E expression, which interacts with the CD94/NKG2A C-lectin receptor on NK cells and provides inhibitory signals to reduce NK cytolytic activity (Braud and McMichael, 1999, Nattermann et al., 2005). Upregulation of HLA-E has been highlighted as an important immune evasion strategy for pathogens that downregulate HLA class I molecules. HCMV encodes gpUL40, a HLA class I signal sequence homologue that binds to HLA-E for presentation to inhibitory receptors on NK cells (Ulbrecht et al., 2000, Tomasec et al., 2000). HLA-E presents peptides in a TAP-dependent manner, but Natterman *et al.* (2005) reported that the HLA-E upregulation observed in their model of HIV-1 infection was not due to the upregulation of TAP1 or TAP2 mRNA, like in HCV infection. The data provided in this chapter have highlighted that HIV-1 infection of primary human cells is capable of inducing TAP1/2 transcription, which might indicate that HLA-E can be upregulated in HIV-1 infected cells via an effect on TAP1/2 expression. Specific measurement of HLA-E expression in HIV-1 infected primary PBMCs with anti-HLA-E antibody 3D12 would be interesting to provide data to contribute to this hypothesis and elucidate further whether HLA-E is upregulated or downregulated in response to HIV-1 infection (Nattermann et al., 2005, van Stigt Thans et al., 2019). This data would be important to contribute to the ongoing work involving HLA-E restricted T cell antigens for HIV-1 vaccination strategies (Yang et al., 2021).

The data presented previously in Chapters 3 and 4 described a relationship between tapasin-independent HLA class I alleles and HIV-1 disease control or relative resistance to downregulation by HIV-1. In conclusion, from the data presented in this chapter, we cannot find any evidence to show that these associations were due to HIV-1 infection downregulating the expression of tapasin mRNA or protein levels. Instead, we found that the mRNA expression of ERp57, TAP1 and TAP2 were upregulated in response to HIV-1 infection in primary PBMCs. ERp57 mRNA upregulation was independently driven by Nef expression in HEK293FT cells. The data presented in this chapter may point toward HIV-1 upregulating antigen processing genes to enhance viral cell entry or avoid NK cell lysis. The hypotheses that this data have generated present interesting avenues for exploration in future HIV-1 research. Further work into the impact of HIV-1 infection on PLC expression and function are necessary to try and fully understand the functional mechanisms behind the association between tapasin-independent HLA class I alleles and resistance to downregulation by HIV-1.

Chapter 6: Peptide-HLA class I complex stability in HIV-1 infected cells *in vitro*

6.1. Chapter 6 Introduction

The function of tapasin and its role in promoting the presentation of high affinity epitopes on HLA class I alleles is described in detail in Chapter 1. Tapasin catalyses the fluctuation between the open and closed conformational states of HLA class I to filter and sample the peptide repertoire (Fisette et al., 2016b, van Hateren and Elliott, 2021). Some HLA class I alleles are intrinsically more stable in the open conformational state and are less reliant on tapasin for their peptide sampling abilities and subsequent presentation at the cell surface. These tapasin-independent HLA class I alleles are well presented at the cell surface in tapasin-deficient cells, albeit with a sub-optimal peptide cargo in this scenario of limited tapasin expression (Bailey et al., 2015, van Hateren et al., 2015). In tapasin-deficient cells the expression of HLA class I molecules is reduced, but the half-life of HLA class I expression is also significantly diminished, indicating that tapasin plays a role in the half-life of HLA class I expression (Boulanger et al., 2022, Howarth et al., 2004, Zarling et al., 2003). Tapasin expression optimises the peptide cargo of both tapasin-dependent and tapasin-independent HLA class I alleles (Williams et al., 2002). Peptide epitopes have been shown to have a "tapasin bonus" of editing provided by tapasin, which is correlated with the abundancy and off-rate of the peptide (Boulanger et al., 2022). Interestingly, the relationship between tapasin expression and HLA class I expression is not linear, nor does it plateau at higher tapasin concentrations (Boulanger et al., 2022). Rather, beyond optimal expression levels the benefit of tapasin for HLA class I expression actually starts to decrease. Optimal HLA class I expression and stability of the HLA class I complex at the cell surface is a key driver in immunogenic CD8+ T cell responses, central to viral clearance of pathogens such as HIV-1 (van der Burg et al., 1996). However, the recent study published in 2022 by Boulanger *et al.* only looked at the tapasin-dependent mouse HLA homologue H2-Db. It would be interesting to see if these findings are repeatable in human HLA class I alleles, particularly those with different tapasin-dependency phenotypes.

HIV-1 peptide epitopes derived from highly abundant and structurally constrained viral proteins are associated slower off rates of HLA class I from the cell surface (Kaseke et al., 2021, Bassani-Sternberg et al., 2015). HLA class I stability has been shown to be associated with HIV-1 epitope immunodominance and immunogenicity (Harndahl et al., 2012, Rasmussen et al., 2016, Kaseke et al., 2021). As mentioned, the stable and immunogenic HLA class I presentation of peptides is essential for optimal CD8+ T cell responses.

Tapasin has a role in promoting the stability of HLA class I expression at the cell surface and HLA class I stability is associated with the anti-HIV-1 immune response. We therefore aimed to examine the impact of HIV-1 infection on tapasin-dependent and tapasin-independent HLA class I allele stability and expression dynamics. We have shown in previous chapters that the expression of tapasin-independent HLA class I alleles is associated with HIV-1 control (Chapter 3). We found further evidence to suggest that HIV-1 may utilise or target tapasin in order to downregulate HLA class I and avoid the cellular immune response. In Chapter 4, we showed that HIV-1 is less efficient in downregulating HLA class I both in tapasin deficient (KO) cells as well as in cells that express tapasin-independent HLA class I alleles. We have also provided preliminary evidence to suggest that HIV-1 Nef dampens the upregulation of tapasin expression after HIV-1 infection, although this data requires further validation (Chapter 5). These data together suggest that HIV-1 may have evolved immune evasion mechanisms to target tapasin. The aim for this chapter was to examine whether HIV-1 infection affects the dynamics of HLA class I stability differently in tapasin-deficient or tapasin-independent cells. These aims are in contrast to the objectives outlined and studied in Chapter 4, which focused on HLA class I expression in tapasin-independent and dependent cell lines and PLC KO cells after 48 hours infection with HIV-1. Instead, we aim to examine how HIV-1 infection affects HLA class I off-rate from the cell surface, as a surrogate measurement indicating the stability of the peptide cargo presented at the cell surface.

If HIV-1 targets tapasin in the ER, this may suggest that HIV-1 may affect tapasin's peptide filtering function. We theorise that this would have a greater impact on the stability and off-rate of tapasin-dependent HLA class I alleles. Our hypotheses are that 1) the expression dynamics and stability of HLA class I molecules expressed on HIV-1 infected cells would differ between cells that expressed tapasin or were tapasin-deficient and 2) that the stability of the expression of highly tapasin-dependent HLA class I alleles would be different to the stability of expression of tapasin-independent HLA class I alleles. Understanding factors that contribute to the stabilisation of HLA class I molecules presented on HIV-1 infected cells is important to further elucidate cellular features that could be exploited in T-based-vaccine design to target immunodominant viral epitopes.

6.2. Chapter 6 Results

6.2.1. HLA class I alleles recycle at the same rate in tapasin KO compared to WT HAP1 cells

The level of HLA class I expression at the cell surface of tapasin KO HAP1 cells is significantly reduced when compared to WT HAP1 cells (Figure 4.2 and Figure 6.1A). WT and tapasin KO HAP1 cells were treated with BFA to block the *de novo* transport of HLA class I alleles from the ER to the cell surface. BFA blocks protein transport from the ER to the Golgi complex. The stability of HLA class I alleles expressed at the cell surface prior to BFA addition can then be determined by measuring HLA class I expression over time after *de novo* transportation of HLA class I was blocked. Using these measurements, the internalisation rate of HLA class I alleles can be calculated and correlated with their stability. The level of HLA class I expression 0, 0.5, 1, 2 and 4 hours post BFA addition was analysed by flow cytometry to measure the decay of HLA class I from the cell surface. The half-life of HLA class I alleles can then be calculated after fitting exponential regression models to the data.

HLA class I expression gradually diminished over time for both WT and tapasin KO cells (Figure 6.1A). The level of HLA class I expression over 4 hours post BFA treatment was then expressed as a percentage of the maximum level of expression measured at time 0 (pre-BFA treatment), to calculate the speed of decay of HLA class I from the cell surface. Even though the level of HLA class I expression was significantly reduced in tapasin KO cells, we found no evidence that this impacted the half-life of HLA class I presenting peptide at the cell surface (Figure 6.1B). The half-life of HLA class I from the surface of HAP1 WT HAP1 cells (2.91 ± 0.36 hours) and tapasin KO HAP1 cells (2.71 ± 0.37 hours) did not significantly differ ($p > 0.05$). These data indicate that the cell surface stability of HLA class I on HAP1 cells is not reliant on tapasin expression in the absence of viral infection *in vitro*.

Although we do not see a difference in the rate of decay between HLA-A*02:01, HLA-B*40:01 and HLA-C*03:04 alleles in WT and tapasin KO cells, we presented data in Chapter 4 that indicates that tapasin expression is beneficial for HIV-1 in its ability to downregulate HLA class I (Figure 4.4). The downregulation of HLA class I is less efficient in tapasin KO cells compared to WT. Our next step was to evaluate the dynamics of HLA class I surface expression during HIV-1 infection.

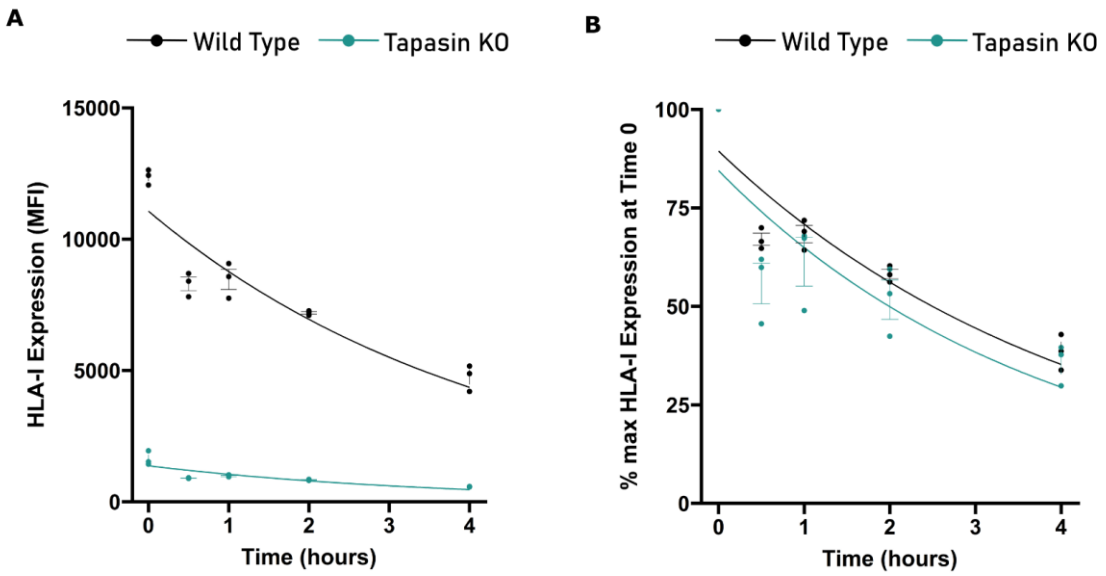


Figure 6.1. **HLA class I decay from the cell surface in WT and tapasin KO PAKC cells.** A) Total HLA class I surface expression (MFI) measured over time after addition of Brefeldin A (BFA) to cells at time 0. MFI was measured by flow cytometry after staining with anti-HLA class I antibody W6/32 in WT (black) or tapasin KO (green) cells. B) Percentage of HLA class I surface expression over time in relation to HLA class I expression levels measures at time 0 after addition of BFA. WT cells (black) or tapasin KO cells (green). HLA class I expression was measured by flow cytometry after staining with anti-HLA class I antibody W6/32. Individual points correspond to the mean value of technical duplicates from independent experiments (n=3).

6.2.2. HIV-1 infection of WT HAP1 cells stabilises the presentation of HLA class I

HAP1 WT and tapasin KO HAP1 PAKC cells were infected with VSV-G pseudotyped NL4-3-G, CH077-G or NL4-3ΔNef-G at an MOI of 1.0. Cells were cultured with IMDM only as an uninfected control. After 48 hours of infection with HIV-1, the cells were treated with BFA for 0, 0.5, 1, 2 and 4 hours. At each time point, the cells were assayed for their surface HLA class I and intracellular Gag expression by flow cytometry to follow the decay of HLA class I from the cell surface.

Uninfected tapasin KO HAP1 cells present significantly lower levels of HLA class I at the cell surface compared to WT cells at all of the time points measured post BFA addition (Figure 6.1A and Figure 6.2A, green lines). Consistent with the data presented in Chapter 4 and 5 of this thesis, NL4-3-G and CH077-G (but not NL4-3ΔNef) were able to downregulate HLA class I in both WT and tapasin KO cells (Figure 6.2A, dashed lines).

HLA class I molecules expressed on infected Gag+ WT HAP1 cells after infection with CH077-G were significantly more stable at the plasma membrane compared to the Gag- bystander cells ($p = 0.00816$, Figure 6.2B and Table 6.1). Intriguingly, this association was not observed in WT HAP1 cells infected with NL4-3-G, indicating that this effect might be virus-specific (Figure 6.2B and Table 6.1). The stability of HLA class I molecules presented on HIV-1 infected tapasin KO cells could not be analysed due to undetectable levels of expression (Figure 6.2B).

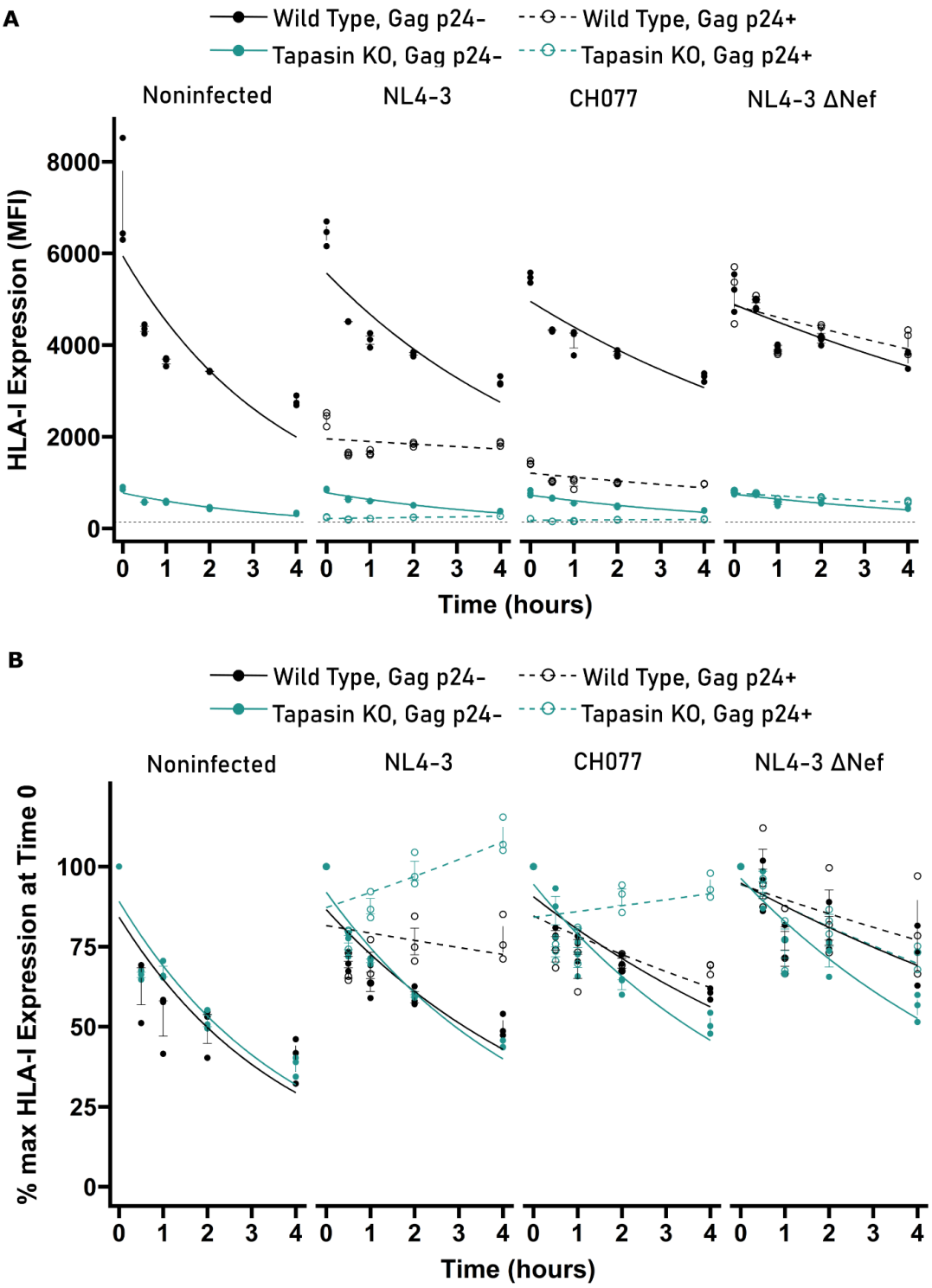


Figure 6.2. HLA class I decay from the cell surface of WT and tapasin KO PAKC cells after infection with HIV-1.

Figure 6.2. **HLA class I decay from the cell surface of WT and tapasin KO PAKC cells after infection with HIV-1.** A) The level of HLA class I surface expression (MFI) measured over time after infection with VSV-G pseudotyped NL4-3, CH077 or NL4-3ΔNef and addition of Brefeldin A (BFA) over 4 hours 48 hours post infection. HLA class I expression was measured by flow cytometry after staining with anti-HLA class I antibody W6/32. HLA class I expression was measured after gating HIV-1 positive cells as Gag+ (dashed lines) or HIV-1 negative bystander cells as Gag- (solid lines) on WT (black) or tapasin KO (green) cells. B) The level of HLA class I surface expression was measured over time after addition of BFA to cells by staining with anti-HLA class I antibody W6/32. Measurements plotted at the percentage of the level of HLA class I measured at time 0 on WT cells (black) or tapasin KO cells (green). HLA class I expression was measured after gating HIV-1 positive cells as Gag+ (dashed lines) or HIV-1 negative bystander cells as Gag- (solid lines). Individual points correspond to the mean value of technical duplicates from independent experiments (n=3).

Sample	WT HAP1				Tapasin KO HAP1			
	Gag+		Gag-		Gag+		Gag-	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Noninfected	*NA*	*NA*	3.03	1.05	*NA*	*NA*	2.85	0.80
NL4-3	10.74	1.05	4.00	0.96	*NA*	*NA*	3.40	0.63
CH077	7.26	0.68	5.48	0.85	*NA*	*NA*	4.09	0.81
NL4-3ΔNef	12.89	1.48	8.66	0.85	8.46	1.17	4.78	0.88

Table 6.1. Half-life of HLA class I molecules expressed on WT and tapasin KO HAP1 cells infected with HIV-1. The half-lives of HLA class I molecules expressed on Gag+ and Gag- (bystander) WT and tapasin KO HAP1 cells after mock infection (noninfected) and infection for 48 hours with VSV-G pseudotyped NL4-3, CH077 or NL4-3ΔNef are shown. *NA* denotes non-applicable data for Gag+ cells in noninfected group and inability to calculate half-lives from Gag+ tapasin KO HAP1 cells infected with NL4-3 or CH077, due to complete loss of HLA class I expression from the cell surface in these cell subsets. Data expressed as the mean and standard deviation (SD) of independent experiments (n=3).

6.2.3. Nef-deleted HIV-1 stabilises the presentation of HLA class I in tapasin KO HAP1 cells

HLA class I molecules decay marginally faster in tapasin KO cells compared to WT HAP1 cells (Figure 6.1B). When tapasin KO cells were infected with NL4-3ΔNef.-G, the internalisation rate of Gag+ tapasin KO cells was returned to the level of Gag- WT bystander cells (Figure 6.2B and Table 6.1). The internalisation rate of HLA class I molecules on Gag+ tapasin KO cells was significantly slower compared to Gag- tapasin KO cells ($p = 0.0224$, Figure 6.2B and Table 6.1). These findings preliminarily indicate that tapasin is not required for the stable presentation of HIV-1 peptides other than those derived from Nef.

6.2.4. HLA class I molecules expressed on HIV-1 uninfected bystander cells are more stable compared to uninfected controls

As also shown in Figure 6.1B, there was no significant difference in the decay of HLA class I molecules from the cell surface between noninfected WT HAP1 cells and noninfected tapasin KO HAP1 cells (Figure 6.2B panel 1 and Table 6.1). However, we found that the HLA class I molecules in the bystander (Gag-) cell population were significantly more stable in CH077-G infected WT HAP1 cells compared to CH077-G infected tapasin KO HAP1 cells ($p = 0.0105$, Figure 6.2B panel 3, solid lines and Table 6.1). HLA class I molecules expressed on CH077-G Gag- bystander WT HAP1 cells were also significantly more stable compared to noninfected WT HAP1 cells ($p = 0.021$). We did not observe a similar increase in HLA class I stability in other bystander WT HAP1 cell populations after infection with NL4-3-G or NL4-3ΔNef-G.

HLA class I molecules expressed on the bystander cells of NL4-3-G, CH077-G or NL4-3ΔNef-G infected tapasin KO HAP1 samples were all significantly more stable compared to the

uninfected tapasin KO HAP1 control cell population (Figure 6.2B, green solid lines and Table 6.1; $p = 0.047$).

This indicates that there may be a bystander effect of productive HIV-1 infection on the uninfected neighbouring cells that may influence HLA class I stability, perhaps due to IFN release from infected cells into the cell supernatant. However for reasons elaborated on below, it was impossible to draw comparisons involving the stability of HLA class I molecules presented on Gag+ HIV-1 infected tapasin KO cells due to the complete abrogation of HLA class I expression in this cell subset. These data provide evidence that HLA class I might be more stably presented in non-productively infected bystander cells in HIV-1 infection. Furthermore it appears that this effect is more profound in cells where tapasin is not expressed.

6.2.5. HLA class I expression levels in HIV-1 infected tapasin KO HAP1 cells are undetectable after treatment with BFA

We were unable to compare HLA class I complex stability at the cell surface between WT HAP1 cells and tapasin KO cells infected with the Nef-expressing wild-type HIV-1 strains NL4-3-G or CH077-G. HLA class I expression levels are already significantly reduced in tapasin KO cells compared to WT before HIV-1 infection (Figure 4.2 and Figure 6.1A). When HIV-1 downregulation of HLA class I is compounded with tapasin KO and BFA treatment in HAP1 cells, the HLA class I expression is reduced to levels close to the background level of autofluorescence of the unstained HAP1 cells (Figure 6.2A, unstained: dashed grey line compared to Gag+ tapasin KO cells: dashed green lines). This makes it difficult to infer HLA class I complex stability in this assay, as the level of HLA class I expression plateaued around this background level of autofluorescent noise in Gag+ HIV-1 infected tapasin KO HAP1 cells (Figure 6.2A). This meant that when expression of HLA class I molecules on Gag+ tapasin KO

HAP1 cells was calculated as a percentage of the maximum level of expression measured at time 0, the subsequent values produced aberrant results over 100% (Figure 6.2B). These values do not make biological sense in this assay and are indicative of methodological error, as BFA blocks the *de novo* transport of new HLA class I alleles to the cell surface. The statistical comparisons involving the half-lives of HLA class I expressed on Gag+ NL4-3-G and CH077-G infected tapasin KO HAP1 cells cannot be interpreted, as the level of HLA class I expression is far too low to be measured accurately in this system.

6.2.6. The cell surface stability dynamics of HLA-B*44:05 and HLA-B*44:02 do not differ in 721.221-CD4 cells

As we have shown, multifaceted downregulation of HLA class I by both HIV-1 infection and tapasin KO resulted in expression levels that made it very difficult to compare infected with noninfected cells. We therefore sought to interrogate how HIV-1 infection might impact tapasin-dependent and tapasin-independent HLA class I allele stability, respectively.

HLA class I-deficient 721.221-CD4 cells were transduced with mono-allelic HLA class I expression vectors encoding HLA-B alleles that were representative of either end of the tapasin-dependency spectrum. HLA-B*44:02 is highly dependent on tapasin for its cell surface expression whereas HLA-B*44:05 is tapasin-independent. Firstly, 721.221-CD4-HLA-B*44:02/05 cells were treated with BFA for 0, 0.5, 1, 2 and 4 hours and stained for their cell surface HLA class I expression at each time point by flow cytometry.

In agreement with the data previously shown in Chapter 4, HLA-B*44:05 alleles were expressed at higher levels on 721.221-CD4 cells compared to HLA-B*44:02 (Figure 6.3A). When HLA class I expression was normalised by plotting the MFI measured at each time point as the percentage of expression at time 0, we observed similar rates of HLA-B*44:02 and HLA-

B*44:05 decay from the cell surface (Figure 6.3B). When tapasin is expressed; there is no evidence that the cell surface stability of tapasin-dependent HLA-B*44:02 (half-life (6.37 ± 2.08 hours) and tapasin-independent HLA-B*44:05 (half-life 4.97 ± 0.54 hours) alleles differs ($p > 0.05$). We next asked whether tapasin-dependent or tapasin-independent HLA class I stability at the cell surface might be affected differently by HIV-1 infection.

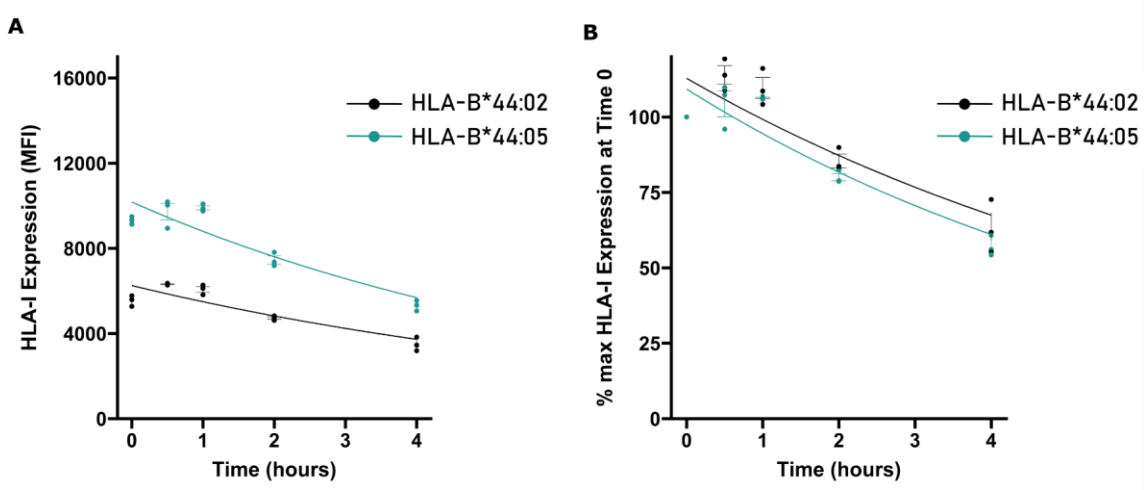


Figure 6.3. **The decay from the cell surface of tapasin-dependent HLA-B*44:02 and tapasin-independent HLA-B*44:05 alleles at steady state.** A) The level of HLA class I surface expression (MFI) measured over time after addition of Brefeldin A (BFA) to cells at Time 0. MFI was measured by flow cytometry after staining with anti-HLA class I antibody W6/32 in HLA-B*44:02 (black) or HLA-B*44:05 (green) cells. B) The level of HLA class I surface expression measured over time after addition of BFA to cells by flow cytometry after staining with anti-HLA class I antibody W6/32. HLA class I measurements plotted as the percentage of the level of expression at time 0 level of HLA class I measured at time 0 on HLA-B*44:02 cells (black) or HLA-B*44:05 cells (green). Individual points correspond to the mean value of technical duplicates from independent experiments (n=3).

6.2.7. HLA-B*44:02, but not HLA-B*44:05, molecules are less stably expressed on HIV-1 infected cells, compared to uninfected controls

We next questioned how the tapasin-dependency of HLA class I molecules might affect the HIV-1 peptide repertoire presented at the cell surface. We hypothesised in this thesis that HIV-1 might interfere with tapasin in order to downregulate HLA class I. If this were the case and HIV-1 were to interfere with tapasin function to prevent the loading of high affinity peptides, this would be more apparent in tapasin-dependent HLA class I alleles.

721.221-CD4-HLA-B*44:02 and 721.221-CD4-HLA-B*44:05 cells were infected with VSV-G pseudotyped NL4-3-G, CH077-G or NL4-3ΔNef-G at an MOI of 1.0 for 48 hours. Cells were cultured with RPMI only as an uninfected control. Infected and noninfected cells were then treated with BFA for 0, 0.5, 1, 2 and 4 hours and stained at each time point for their cell surface HLA class I expression and intracellular Gag expression by flow cytometry. HLA class I expression levels in Gag+ and Gag- cells were calculated to follow the rate of decay of HLA class I molecules at the cell surface after blockage of *de novo* transport of new HLA class I molecules from the ER.

As previously shown in Chapter 4, HIV-1 infection of 721.221-CD4-HLA-B*44:02 and 721.221-CD4-HLA-B*44:05 cells with NL4-3-G and CH077-G, but not NL4-3ΔNef-G, downregulates HLA class I molecules (Figure 4.6 and Figure 6.4A dashed lines). HIV-1 downregulation of HLA class I was more effective for the tapasin-dependent HLA-B*44:02 allele compared to the independent HLA-B*44:05 in 721.221-CD4 cells (Figure 4.7). This result indicated that the ability of HIV-1 to downregulate HLA class I may be impeded when HLA molecules are not reliant on tapasin for the cell surface expression.

The dynamics of HLA class I expression measured over time after BFA treatment in both HIV-1 infected and noninfected cells was analysed and calculated as the percentage of expression

at time 0 (Figure 6.4B). Unlike the results described above in HIV-1 infection of WT and tapasin KO HAP1 cells, we found no evidence that the stability of either HLA-B*44:02 or HLA-B*44:05 was significantly different in Gag- bystander cells after infection with NL4-3-G, CH077-G or NL4-3ΔNef-G compared to uninfected controls ($p > 0.05$, Figure 6.4B and Table 6.2).

However, we observed that the stability of HLA-B*44:05 molecules in Gag+ cells infected with both NL4-3-G and CH077-G was significantly greater compared to both the Gag- bystander cell population and noninfected cell controls (NL4-3-G $p = 0.00485$; CH077-G $p = 0.00000159$) (Figure 6.4B and Table 6.2). This result indicated that the tapasin-independent HLA-B*44:05 alleles were more stably expressed when they could derive peptides from the HIV-1 peptidome in infected cells than when presenting self-derived peptides. We did not find similar evidence for increased tapasin-dependent HLA-B*44:02 stability in Gag+ cells compared to Gag- bystander cells ($p > 0.05$, Figure 6.4B and Table 6.2). This may indicate that the tapasin-dependent HLA-B*44:02 allele is better at presenting self-peptides compared to the tapasin-independent HLA-B*44:05.

When we directly compared the stability of each HLA-B*44 allele in NL4-3-G or CH077-G infected cells, we found the half-life of HLA-B*44:05 was longer than the half-life of HLA-B*44:02 in Gag+, but this result was not statistically significant (Table 6.2). Again, although not statistically significant, we observed the opposite trend in NL4-3ΔNef-G Gag+ infected cells, pointing again to the possibility of a Nef-tapasin interaction. NL4-3ΔNef-G infected cells more stably expressed HLA-B*44:02 compared to HLA-B*44:05 (Table 6.2). These results would be interesting to follow up with further experiments and other HLA class I alleles with varying dependencies on tapasin for the cell surface expression to clarify this association.

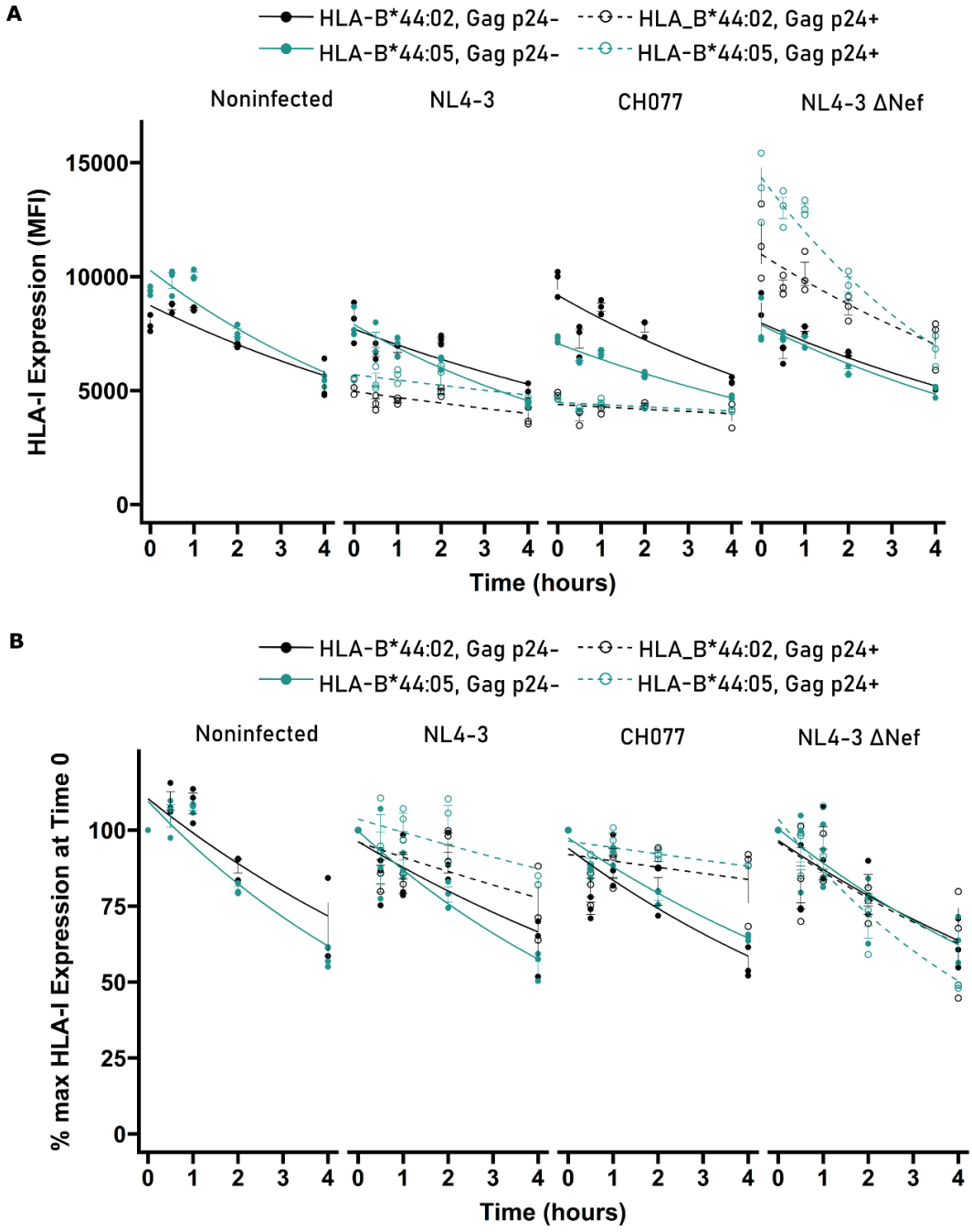


Figure 6.4. The decay from the cell surface of tapasin-dependent HLA-B*44:02 and tapasin-independent HLA-B*44:05 alleles after infection with HIV-1.

Figure 6.4. **The decay from the cell surface of tapasin-dependent HLA-B*44:02 and tapasin-independent HLA-B*44:05 alleles after infection with HIV-1.** A) The level of HLA class I surface expression (MFI) measured over time after infection with VSV-G pseudotyped NL4-3, CH077 or NL4-3ΔNef and addition of Brefeldin A (BFA) over 4 hours 48 hours post infection. HLA class I expression was measured by flow cytometry after staining with anti-HLA class I antibody W6/32.HLA class I expression was measured after gating HIV-1 positive cells as Gag+ (dashed lines) or HIV-1 negative bystander cells as Gag- (Gag-, solid lines) on HLA-B*44:02 (black) or HLA-B*44:05 (green) 721.221-CD4 cells. B) The level of HLA class I surface expression was measured over time after addition of BFA to cells by staining with anti-HLA class I antibody W6/32. Measurements plotted at the percentage of the level of HLA class I measured at time 0 on HLA-B*44:02 (black) or HLA-B*44:05 (green) 721.221-CD4 cells. HLA class I expression was measured after gating HIV-1 positive cells as Gag+ (dashed lines) or HIV-1 negative bystander cells as Gag- (solid lines). Individual points correspond to the mean value of technical duplicates from independent experiments (n=3).

Sample	721.221-CD4-HLA-B*44:02				721.221-CD4-HLA-B*44:05			
	Gag p24+		Gag p24-		Gag p24+		Gag p24-	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Noninfected	*NA*	*NA*	7.2	6.22	*NA*	*NA*	5.06	0.55
NL4-3	9.37	8.59	5.86	1.8	14.91	1.77	4.74	0.66
CH077	15.43	13.56	4.75	0.78	22.52	0.35	6.36	0.24
NL4-3ΔNef	6.23	4.44	5.82	1.78	3.85	0.07	6.18	1.73

Table 6.2. Half-life of HLA class I molecules expressed on 721.221 tapasin-dependent and independent cell lines infected with HIV-1. Half-life of HLA class I molecules expressed on Gag+ and Gag- (bystander) 721.221-CD4-HLA-B*44:02 and 721.221-CD4-HLA-B*44:05 cells after mock infection (noninfected) and infection for 48 hours with VSV-G pseudotyped NL4-3, CH077 or NL4-3ΔNef. *NA* denotes non-applicable data for Gag+ cells in noninfected group. Data expressed as the mean and standard deviation (SD) of independent experiments (n=3).

It has been well characterised by us and others that HIV-1 ΔNef strains do not downregulate HLA class I. We have found that in 721.221-CD4 cells, infection with NL4-3ΔNef-G results in significant upregulation of HLA-B*44:05 in Gag+ cells compared to Gag- bystander cells ($p = 0.00699$, Figure 6.4A green lines). HLA-B*44:02 was also upregulated by NL4-3ΔNef-G infection, but we did not find any statistically significant evidence for this effect ($p > 0.05$, Figure 6.4A black lines). Even though we measured HLA-B*44:05 at significantly higher levels in NL4-3ΔNef-G productively infected cells compared to bystander cells, we did not measure any significant difference in the stability of this HLA-B*44:05 in the same cell subsets ($p > 0.05$, Figure 6.4B and Table 6.2). Nor did we measure any significant change in HLA-B*44:02 stability in NL4-3ΔNef-G infected cells (Table 6.2).

Lastly, we did observe that HLA-B*44:05 alleles expressed on Gag+ cells had longer half-lives compared to HLA-B*44:02 alleles expressed on Gag+ cells in wild-type HIV-1 infection with either NL4-3-G or CH077-G (Table 6.2). This association was reversed in NL4-3ΔNef-G infection: HLA-B*44:02 expressed on Gag+ cells was more stable and had a longer half-life then HLA-B*44:05 expressed on Gag+ cells (Table 6.2). These relationships between HLA-B*44 allele half-life and HIV-1 infection were not statistically significant, but nevertheless interesting to further investigate. These data provide tentative evidence that when HIV-1 strains express Nef, tapasin-independent HLA class I alleles can be more stably expressed at the cell surface whereas tapasin-dependent HLA class I alleles are more stably expressed in cells infected with Nef-deficient HIV-1.

These results indicate a preferential ability of HIV-1-infected cells expressing tapasin-independent HLA class I alleles to present high affinity HIV-1-derived peptides compared to uninfected cells also expressing tapasin-independent HLA class I alleles as well as HIV-1-infected cells expressing tapasin-dependent HLA class I alleles. Here we show for the first time

that HIV-1 infection may influence the stability of HLA class I presentation of viral peptides to CD8+ T cells directly in the context of the tapasin-dependency phenotype.

6.3. Chapter 6 Discussion

Determining the cellular and immunological factors that contribute to the optimal presentation of HIV-1-derived peptides at the cell surface is crucial in the design of T cell-based vaccines. Immunodominant HIV-1 epitopes stabilise the presentation of HLA class I molecules and HLA class I stability directly correlates with the anti-HIV-1 CD8+ T cell response (Kaseke et al., 2021). Identification of alternative aspects of the cellular landscape that might contribute to CD8+ T cell epitope optimisation can help to inform the development of anti-HIV vaccine strategies.

We used a BFA decay assay to block transport of newly synthesised HLA class I molecules from the ER and use measures of HLA class I half-life at the cell surface as an indication of HLA class I stability at the cell surface after HIV-1 infection. HLA class I stability was compared between tapasin-competent and tapasin-knockout HAP1 cells as well as 721.221-CD4 cells that expressed either the tapasin-dependent HLA-B*44:02 allele or tapasin-independent HLA-B*44:05.

We found that although HLA class I expression was very low on tapasin KO HAP1 cells compared to WT HAP1 cells, HLA class I expression was equally stable between each cell line at a steady state (Figure 6.1). HAP1 cells express HLA-A*02:01, HLA-B*40:01 and HLA-C*03:04 (de Waard et al., 2021). These HLA class I allotypes are common among Caucasians and moderate to strongly tapasin-independent for their cell surface expression (Bashirova et al., 2020). Given these cells express HLA class I alleles that do not appear to be reliant on tapasin expression for their stable presentation, it is striking how drastically the expression level of HLA class I reduces when tapasin is knocked out (Figure 4.2). Further investigation into other allotypes more commonly expressed among African populations and with different tapasin-dependencies are warranted.

We then investigated how HIV-1 infection might impact the stability of HLA class I presentation on HAP1 cells by measuring HLA class I expression by flow cytometry after the addition of BFA. HIV-1 infected cell populations were gated on productively infected (Gag+) and bystander (Gag-) cells in both the WT HAP1 and tapasin KO cell line. We found that HLA class I alleles were more stably expressed on CH077-G infected Gag+ WT HAP1 cells compared to noninfected cells, but HLA class I was more stable in tapasin KO HAP1 cells when infected with NL4-3ΔNef-G. The role of Nef in stabilising HLA class I expression in cells that express tapasin is still unclear. This study was limited as it became apparent that tapasin KO cells may not be good model to study HLA class I stability in the context of HIV-1 infection. HIV-1 downregulates HLA class I in tapasin KO cells to almost undetectable levels (Figure 6.2A). This limited the analyses that could be performed to compare HLA class I stability in productively infected (Gag+) tapasin KO HAP1 cells. Analysis of HLA class I stability in the Gag+ population of tapasin KO cells was constrained to NL4-3ΔNef-G infection only, as Nef-deficient HIV-1 does not downregulate HLA class I. HIV-1 infection of different cell lines with deficient and abundant tapasin expression would be interesting to further examine HLA class I stability *in vitro*. Further investigation of HLA class I stability in the other PAKC HAP1 cell lines (TAP1 KO, TAP2 KO, ERp57 KO, Calreticulin KO) would also be interesting to examine the impact of the PLC on HIV-1 peptide presentation by HLA class I.

We also found evidence that HLA class I alleles expressed on uninfected bystander cells (Gag-) after infection with CH077-G were significantly more stable in WT compared to tapasin KO cells (Figure 6.2B). HLA class I alleles expressed on Gag- bystander tapasin KO cells infected with NL4-3-G, CH077-G or NL4-3ΔNef-G were also more stably expressed compared to noninfected tapasin KO cells. The effects of viral infection on bystander cells have been well described in the literature, including for HIV-1 (McNally et al., 2001, Sprague et al., 2018). HIV-1 Nef is secreted via exosomes and has been shown to trigger apoptosis of neighbouring

uninfected bystander cells (Lenassi et al., 2010, James et al., 2004). HLA class I upregulation in bystander cells has been observed after influenza A, influenza B virus and rotavirus infection (Koutsakos et al., 2019, Holloway et al., 2018). It is unclear whether the increased stability of HLA class I molecules observed in HIV-1 uninfected bystander cells is as a result of Nef or IFN release from infected cells, or a resultant artefact from the assay methodology. BFA is commonly used to block the extracellular transport of cytokine molecules so that intracellular cytokine levels can be measured by flow cytometry. The addition of BFA to HIV-1 infected cells was delayed until 48 hours post infection to allow for productive viral infection and processing of viral antigen for display on HLA class I molecules, consistent with previous experiments conducted in this thesis. A previous study showed that the delayed addition of BFA after stimulation of CD8+ T cells with *Plasmodium falciparum* sporozoites resulted in an aberrant detection of IFN-γ production in bystander cells due to the release of pro-inflammatory cytokines into the cell supernatant (Martin et al., 2019). It should be considered that increased HLA class I stability observed in HIV-1 uninfected bystander cells may be as a result of the release of type I IFN from infected HAP1 cells that trigger the upregulation of ISGs in neighbouring cells. To clarify the results from our study of HIV-1 in HAP1 cells, further optimisation in the methodology should be conducted. This should be done with the aim to reduce the period of time between HIV-1 infection and BFA addition that allows for efficient processing of viral peptides for presentation on HLA class I molecules, but would mitigate any potential effect on bystander cells that might skew the results.

We also aimed to evaluate the impact of HIV-1 infection on the stability of tapasin-dependent and independent HLA class I alleles. HLA-B*44:02 and HLA-B*44:05 are commonly used in the study of the tapasin dependency spectrum, as they are highly dependent and independent, respectively, on tapasin. HLA-B*44:02 and HLA-B*44:05 only differ at residue 116 of the F-pocket of the peptide binding groove and present similar peptide repertoires (> 95% similarity)

(Thammavongsa et al., 2006, Macdonald et al., 2003). Consistent with previous studies, we showed that tapasin-independent HLA-B*44:05 alleles were expressed at higher levels compared to tapasin-dependent HLA-B*44:02, but we did not detect any significant differences between the stability of their expression at the cell surface (Figure 6.3) (Rizvi et al., 2014).

We infected 721.221-CD4-HLA-B*44:02/05 cells with different HIV-1 strains for 48 hours and measured HLA class I stability at the cell surface by flow cytometry after addition of BFA over a period of 4 hours. The results revealed that the tapasin-independent HLA-B*44:05 allele was more stably expressed compared to HLA-B*44:02 in NL4-3-G and CH077-G infected Gag+ cells (Table 6.2). Tapasin-independent HLA class I alleles are more likely to exit the ER and present at the cell surface at higher levels but sub-optimally when peptide supply is limited (Rizvi et al., 2014, Thammavongsa et al., 2009, Geng et al., 2018). It is unclear whether this increased stability of tapasin-independent HLA class I alleles in HIV-1 infection might be due to potential viral interference with tapasin or TAP, as the TAP-dependency spectra of HLA-B*44:02 and HLA-B*44:05 mimic their tapasin-dependency phenotype. TAP-dependency correlates with tapasin-dependency but there is not complete overlap between the spectra of tapasin and TAP-dependency for individual HLA class I alleles (Geng et al., 2018). High abundance of peptides supplied to the ER by TAP has been shown to drive peptide presentation via a tapasin-independent pathway in cases where tapasin expression is limiting (Boulanger et al., 2022). Therefore further investigation into the effect of HIV-1 infection on HLA class I stability in cell lines expressing alleles with different dependencies on tapasin and TAP such as HLA-B*18:01 (tapasin-independent, TAP-dependent) and HLA-B*51:01 (more tapasin dependent, less dependent on TAP) would be interesting to start to explain potential contributions of TAP and tapasin to HLA class I stability in HIV-1 infection (Geng et al., 2018).

When 721.221-CD4 cells were infected with Nef-deficient HIV-1, we saw an opposite relationship between tapasin-dependency and HLA class I stability to that described for CH077-G. NL4-3ΔNef-G infected Gag+ cells expressed HLA-B*44:02 alleles with increased stability on the cell surface compared to Gag+ HLA-B*44:05 (Figure 6.4B and Table 6.2). These data suggests that HLA class I stability and optimisation of the peptide cargo is greater in tapasin-dependent alleles compared to tapasin-independent alleles infected with NL4-3ΔNef-G. In the presence of tapasin, tapasin-dependent HLA class I alleles have been shown to acquire a more optimal peptide cargo compared to tapasin-independent alleles (Williams et al., 2002). We showed some preliminary evidence in Chapter 5 that could suggest that NL4-3ΔNef-G infection upregulates tapasin protein expression, albeit we only showed this in HEK293FT cells (Figure 5.7). Increased tapasin expression has been shown to enhance peptide filtering on HLA class I and the presentation of a more refined repertoire (Boulanger et al., 2022).

The upregulation of these peptide-loading chaperone proteins might work to promote more rigorous peptide sampling on tapasin-dependent alleles. More tapasin-dependent HLA-B alleles have been shown to have longer half-lives in lymphocytes compared to monocytes (Yarzabek et al., 2018). Tapasin and HLA class I are upregulated in monocytes compared to lymphocytes, but the ratio is such that less tapasin is actually available to stabilise a disproportionately greater upregulation of HLA class I molecules (Fischbach et al., 2015). It would be interesting to repeat our experiments looking at the expression levels and stability of tapasin-dependent and tapasin-independent HLA class I alleles in myeloid cells and lymphocytes from HLA class I-typed PBMC donors. Our data could suggest that if NL4-3ΔNef-G upregulates tapasin, then this may promote the optimisation of the peptide cargo and stability of tapasin-dependent HLA class I alleles presented at the cell surface. Conversely, in wild-type HIV-1 infection, viral interference with the PLC may result in more limited peptide

supply or levels of tapasin/TAP and therefore HIV-1 peptide presentation is more stably presented on tapasin-independent, intrinsically stable peptide-receptive HLA class I alleles such as HLA-B*44:05. Further experimental evidence needs to be provided to support these observations.

This study looks for the first time at the impact of the level of and reliance on tapasin expression on the stability of HLA class I expression in HIV-1 infected cells. HLA class I stability is directly associated with HIV-1 epitope immunodominance and the CD8+ T cell response (Kaseke et al., 2021). Our data show that HIV-1 infected cells more stably present tapasin-independent HLA class I alleles compared to tapasin-dependent alleles. Recently, Boulanger *et al*. (2022) described optimal levels of tapasin expression or the "tapasin bonus" for individual peptides presented by HLA class I. It would be interesting to further study the immunodominance hierarchies and stability of expression of individual vaccine candidate HIV-1 epitopes in tapasin KO cells, or by tapasin-dependent and independent alleles alike.

Targeting tapasin function in antiviral vaccine strategies is not a novel concept. Tapasin fusion proteins have been successful in promoting anti-HBV CD8+ T cell responses and viral clearance *in vivo* and *in vitro.* A fusion protein of cytoplasmic transduction peptide (CTP) with the HBV core antigen 18-27 (HBcAg18-27) with tapasin was able to enter the cytoplasm of bone marrow-derived dendritic cells (BMDCs), enhance HLA class I presentation and promote IFN-γ and IL-2 release from T cells (Chen et al., 2014, Tang et al., 2014b, Tang et al., 2014a, Chen et al., 2013). Further exploration into whether this strategy might also benefit HIV-1 epitope presentation would be beneficial in the design of T-cell-based vaccines.

Chapter 7: Discussion and Future Directions

Forty years since the start of the HIV pandemic, an effective vaccine has yet to be developed. Research into the immunological and genetic factors that govern natural control of HIV infection is essential for the development of novel immunotherapies and vaccination strategies. Polymorphisms of HLA class I molecules, which present viral peptides to CD8+ T cells, have been consistently associated with HIV-1 control (Rowland-Jones et al., 1998a, Rowland-Jones et al., 1995, Rowland-Jones et al., 1998b, Ndhlovu et al., 2015, Koup et al., 1994, Goonetilleke et al., 2009, Oxenius et al., 2004, Hersperger et al., 2011, Migueles et al., 2002, Migueles et al., 2008, Monel et al., 2019, Matano et al., 1998, Schmitz et al., 1999, Jin et al., 1999, Kaslow et al., 1996, Martin et al., 2002, Gao et al., 2001). To evade the CD8+ T cell response, HIV-1 has evolved mechanisms to downregulate HLA class I molecules via Nef and Vpu (Kerkau et al., 1989, Schwartz et al., 1996, Roeth et al., 2004, Apps et al., 2016). Many viral pathogens such as HCMV, EBV and adenovirus have evolved similar immune evasion mechanisms to downregulate HLA class I that target various components of the PLC (reviewed by (Roder et al., 2008)). Viral targeting of tapasin in the PLC such as that exerted by HCMV protein US3 has been shown to result in the selective downregulation of tapasin-dependent HLA class I alleles, whilst having little effect on the expression of tapasin-independent alleles (Zernich et al., 2004). We hypothesised that HIV-1 may employ a similar mechanism to target tapasin and avoid HIV-1 peptide loading on HLA class I molecules. This hypothesis was built on an observation made in 2020 by Bashirova *et al.*, which showed that lower HIV-1 VLs and slower disease progression were associated with the expression of tapasin-independent HLA class I alleles (Bashirova et al., 2020). In Chapter 3 of this thesis, we have presented data to corroborate these findings in an independent cohort of older children and adolescents with slow progressing HIV-1 disease from Harare, Zimbabwe. We showed that in a subset of these children, who were defined as having slow progression or viral control of HIV-1 infection, the expression of tapasin-independent HLA class I alleles was associated with lower mean HIV-1

VLs. We identified 31 slow progressors and 13 viral controllers of HIV-1 infection from the wider ZENITH cohort of nearly 400 patients. When the original ZENITH cohort was identified, "test and treat" was not initiated on the basis of an HIV-1 diagnosis; instead, ART was started on the basis of clinical thresholds that had to be met (Ferrand et al., 2017). Furthermore, there were far fewer strategies available for the diagnosis of HIV-1 in older children at the time, meaning that identification of these slow progressing patients was less likely (Simms et al., 2017). Given the unique and time limited opportunity in identifying the original ZENITH cohort, it is remarkable that so many slow progressors (8.07% prevalence, 31/384 patients) and viral controllers (prevalence of 3.39%, 13/384 patients) were identified. The prevalence of EC of HIV-1 infection in the ZENITH cohort was 0.52% (2/384 patients), placing paediatric EC prevalence closer to that of adults and five to ten fold higher than that previously described for children (Olson et al., 2014, Vieira et al., 2019). This observation may have been due to immunogenetic differences in ethnicity, as the patients studied by Vieira and colleagues (2019) were from an ethnically diverse group of children from South Africa, Brazil, the UK, Thailand and the EU. These findings highlight the importance of identifying other populations of patients with slow or long-term non-progression of HIV-1 disease in order to conduct further genetic association studies. In the advent of "test and treat" it would be difficult to study longitudinal clinical parameters without the confounding influence of ART. However, it would be possible to conduct retrospective analyses of HLA class I genotyping data from historical cohorts using the extrapolated tapasin-dependency data published by Bashirova *et al.* (2020). An accessible and updatable compendium of tapasin-dependency data for HLA class I alleles would be useful to perform additional association studies of tapasin-dependency with HIV-1, other viral infections or even cancer.

We aimed to study the cellular mechanisms that might be behind the associations we and others have identified between tapasin-independent HLA class I alleles and slow progression of HIV-

1 disease. We hypothesised that tapasin might be implicated in the mechanism of HLA class I downregulation in HIV-1 infection (Figure 1.5). Consistent with our hypothesis, we found that HLA-B*44:05 alleles were 1.34 times less efficiently downregulated by HIV-1 compared to tapasin-dependent HLA-B*44:02 alleles (Figure 4.7). This finding should be tested in a wider range of HLA class I alleles to confirm that this is a general feature of tapasin-independent HLA class I alleles in HIV-1 infection. Further supporting these observations, we showed that HIV-1 downregulation of HLA class I was 2.05, 1.47 and 2.10 times less efficient in TAP1, TAP2 or tapasin KO cells, respectively, compared to WT cells (Figure 4.4). In order to support the evidence presented in Chapter 4, experiments examining the extent of HLA class I downregulation by HIV-1 infection of tapasin/TAP1/TAP2 KO mutants in other cell lines should be conducted. Single HLA class I allele expression of HLA-B*44:02 and HLA-B*44:05 in the 721.221.CD4 cell line was studied in this thesis, as this pair of alleles exhibit vastly different dependencies on tapasin (Williams et al., 2002, Bashirova et al., 2020). These alleles differ only at position 116 of the peptide binding groove but it has been also shown that mutation of the tryptophan residue at position 147 of HLA-B*44:05 to alanine (HLA-B*44:05^{W147A}) also affects the ability of this normally tapasin-independent allele to present stable peptides at the cell surface in the absence of tapasin, without changing the peptide cargo presented (Bailey et al., 2015). Further experiments studying the expression and stability of HLA-B*44:05 and HLA-B*44:05^{W147A} would be interesting to expose the role of tapasin-dependency in HIV-1 infection, regardless of the potential peptide repertoire that can be presented. Examination of the extent of HIV-1 mediated downregulation of other allele pairs such as HLA-B*57:01 (tapasin-dependent) and HLA-B*35:01 (tapasin-independent) that are less closely related in terms of amino acid sequence, but associated with different outcomes of HIV-1 would also provide further evidence to support the hypothesis that HIV-1 more efficiently downregulates tapasin-dependent HLA class I alleles compared to tapasin-

independent alleles. Experiments in CD4+ T cells isolated from HLA class I-typed PBMCs would be interesting to explore the impact of HIV-1 infection on HLA class I or PLC expression levels in a more natural infection cell model.

The impact of HIV-1 infection on PLC expression levels was explored in Chapter 5, but no evidence was found to support the hypothesis that HIV-1 reduces tapasin mRNA or protein levels in order to downregulate HLA class I presentation of viral peptides. As discussed in Chapter 5, these expression studies were marred by low infection efficiencies of CD4+ T cells during infection of bulk PBMCs, likely due to killing of infected cells by neighbouring CD8+ T cells. However, these experiments provide a good foundation for future studies of PLC expression in isolated CD4+ T cells. Rather than affecting tapasin or PLC expression levels, however, it is possible that HIV-1 targets tapasin in some other way to affect its function instead. We did not conduct experiments to look for potential physical interactions between tapasin or the PLC and HIV-1 in this thesis, but this avenue should be explored. Kasper *et al.* (2005) originally showed the co-IP of HIV-1 Nef and tapasin, but did not look at Nef interactions with any other components of the PLC such as ERp57, TAP1 or TAP2 (Kasper et al., 2005). The findings presented here outline the need for further investigation into specific HIV-1 viral protein interactions with the PLC in different cell types.

It is possible that HIV-1 could interact with tapasin function indirectly, as is the case for adenovirus protein E19 (Jackson et al., 1990). Adenovirus protein E19 physically interacts with the HLA class I binding groove and blocks tapasin bridging of HLA to TAP without affecting the expression of tapasin mRNA or protein levels (Flomenberg et al., 1992, Sester and Burgert, 1994, Sester et al., 2013, Bennett et al., 1999). Whether this is a viral immune evasion strategy used by HIV-1 remains to be seen. Future co-IP analysis of native or epitope-tagged HIV-1 proteins interacting with PLC components by Western Blot or MS would be useful to build on the evidence first presented by Kasper and colleagues in 2005 for HIV-1 Nef and by Luo and

colleagues in 2016 for HIV-1 Env. We found limited evidence to show that HIV-1 infection or exogenous Nef expression affected the level of tapasin mRNA or protein expression (Chapter 5). Therefore, we propose that further experiments should be conducted to test whether HIV-1 co-localises with, binds to or perhaps blocks tapasin's function in the ER similar to adenovirus protein E19. It would also be interesting to investigate this with confocal immunofluorescent microscopy techniques to look at the sub-cellular co-localisation of HIV-1 proteins, including Nef, with tapasin and the PLC during different stages of the HIV-1 replicative cycle. Viral and host protein interaction studies by co-IP, MS or yeast-two-hybrid systems could also be conducted in different cell systems to provide further evidence as to whether a physical interaction between HIV-1 and the PLC occurs.

Understanding HIV-1 viral immune mechanisms and factors influencing viral peptide presentation is important in the design of future anti-HIV T cell-based immunotherapies and vaccines. Exploiting tapasin expression and its functional role in the stable presentation of peptides on HLA class I has been explored previously for different vaccination strategies. Fusion of the hepatitis B virus (HBV) core antigen HBc18-27 to tapasin significantly increased HBV-specific CD8+ T cell responses *in vitro* and in mice *in vivo*, compared to the same antigen expressed without tapasin (Chen et al., 2013, Chen et al., 2014, Tang et al., 2014a, Tang et al., 2014b, Wu et al., 2018). The anti-HPV type 16 (HPV-16) E7 CD8+ T cell response was enhanced when mice were vaccinated with HPV-16 E7 DNA fused to tapasin or calreticulin, compared to vaccination with wild-type HPV-16 E7 DNA alone (Lin et al., 2005, Cheng et al., 2001). Vaccination with tapasin-fused HPV-16 E7 DNA also resulted in a reduction in tumour growth *in vivo* (Lin et al., 2005). It has also been shown that expression of an adenovirus vector encoding tapasin can restore CD8+ T cell killing and improve cross-presentation of tumour antigens by dendritic cells for metastatic carcinoma *in vitro* and in mice *in vivo* (Lou et al., 2008). These vaccination strategies either exogenously expressing tapasin in HIV-1 infected

cells or fusing tapasin and other components of the PLC to HIV-1 antigens have not been explored. However, the expression of even a soluble form of tapasin lacking the C-terminal domain has been shown to stabilise the HLA class I peptide binding groove and restore the presentation of the HIV-1 Gag epitope SL9 *in vitro* (Gao et al., 2004, Lehner et al., 1998). Using computational models, tapasin expression has also been suggested to promote the stable binding of high affinity HIV-1 Gag epitopes on tapasin-independent and dependent HLA class I alleles alike (Dalchau et al., 2011, Eccleston et al., 2017). As such, it is feasible that if HIV-1 infection were to interfere with tapasin expression or function and the efficiency of HIV-1 peptide presentation, then vaccination strategies utilising tapasin such as those investigated with HBV and HPV may be beneficial to combat this viral immune evasion mechanism.

It should be considered that the findings described in Chapter 3 may have been impacted by the high prevalence of both HCMV and EBV infection in HIV-1 infected individuals (Gronborg et al., 2017, Gianella and Letendre, 2016, Mentzer and Kreuz, 1999, Adland et al., 2015a, Lichtner et al., 2015, Yindom et al., 2019, Shindiapina et al., 2020, Nazim et al., 2022, Whitehurst et al., 2022). As previously mentioned, evidence has shown that both HCMV and EBV target tapasin and TAP to downregulate the expression of viral peptides on HLA class I molecules to CD8+ T cells (Roder et al., 2008). In the case of HCMV, this has been shown to result in the specific downregulation of tapasin-dependent HLA class I alleles, which has also now been noted to occur in HIV-1 infection ((Bashirova et al., 2020, Halenius et al., 2011, Park et al., 2004a, Zernich et al., 2004) and Chapter 3). We hypothesise that the expression of tapasin-independent HLA class I alleles may be protective in HIV-1 infected individuals as they are resistant to downregulation by co-infecting HCMV. It would be interesting to perform further experiments to try and interpret the specific benefit in the expression of tapasin-independent HLA class I alleles in HIV-1 infected individuals that are also infected with HCMV. This may be difficult to analyse *ex vivo* due to the high levels of co-infection of HIV-

1 and HCMV (Yindom et al., 2019). Dual infection experiments of primary human cells and cell lines expressing tapasin-independent and tapasin-dependent HLA class I alleles with both HIV-1 and HCMV may provide interesting insight into the specific benefit of tapasin-dependency in HIV-1 infection. These questions could also be similarly investigated by co-transfecting permissible cell lines with HIV-1 Nef and HCMV US3.

In conclusion, the data presented in this thesis have provided important evidence for the first time to show that HIV-1 preferentially downregulates tapasin-dependent HLA class I alleles and HLA class I in cells that express tapasin, compared to tapasin-independent alleles or tapasin KO cells. These data indicate that HIV-1 may target the PLC in order to reduce presentation of viral peptides on HLA class I molecules at the cell surface. Further work is required to fully elucidate the cellular mechanisms behind this association and explain the correlation between the expression of tapasin-independent HLA class I alleles and control of HIV-1 infection.

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