

The role of dendritic cells in determining the breadth and epitope specificity of the initial CD8⁺ T cell response during acute HIV-1 infection



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Abstract

During acute HIV-1 infection (AHI), CD8⁺ T cell responses are initially induced to only 1-3 viral epitopes in many patients, and Nef-derived epitopes are very commonly targeted. The earliest CD8⁺ T cell responses decrease in magnitude as viraemia begins to decline, and are followed by delayed expansion of subsequent waves of responses of broader epitope-specificity. In this thesis, the hypotheses that prevalent presentation of Nef-derived epitopes by dendritic cells (DCs) favours priming of Nef-biased CD8⁺ T cell responses in AHI, and that impairments in DC function after the first responses are primed delay expansion of responses of additional specificities, were explored. Mechanisms of impairment of DC function in AHI were not definitely identified, although pre-exposure of monocyte-derived DCs to IFN α , which is rapidly induced during AHI, was shown to reduce TLR3-induced co-stimulatory molecule expression and affect the quality of the CD8⁺ T cell response primed *in vitro*. Using mass spectrometry-based immunopeptidomics, novel HLA class I-restricted HIV-1 epitopes presented by HIV-1-infected CD4⁺ T cells were characterised, including epitopes derived from the 5' "untranslated" region of the HIV-1 genome. High ratios of Nef/Gag protein were found associated with extracellular vesicles (EVs) in the plasma of HIV-1⁺ patients during early, chronic and treated infection. Rather than CD4⁺ T cells, HIV-1-infected macrophages were identified as a potential source of these Nef-containing EVs. Utilising an *in vitro* DC cross-presentation assay incorporating immunopeptidomics, cross-presented epitopes were found to derive preferentially from proteins that were more abundant or membrane-associated. Multiple cross-presented HIV-1 (mainly Gag-, Pol- and Env-derived) epitopes were identified, but HIV-1-infected CD4⁺ T cells were not found to be a source of Nef for cross-presentation. Collectively, these results extend understanding of how DCs control CD8⁺ T cell immunodominance and expansion during AHI, and could potentially be exploited in future development of novel prophylactic and therapeutic strategies against HIV-1.

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Table of Contents

Abstract	i
Acknowledgements	ii
List of Figures	viii
List of Tables.....	x
List of Abbreviations.....	xi
Author's contributions.....	xiv
Chapter 1: Introduction.....	1
1.1 Human Immunodeficiency virus (HIV-1) pandemic.....	1
1.2 HIV-1 structure.....	1
1.3 HIV-1 genome	2
1.4 Overview of the HIV-1 lifecycle	3
1.5 Natural history of HIV-1 infection	4
1.5.1 HIV-1 transmission and acute HIV-1 infection (AHI).....	5
1.6 Overview of innate and adaptive immunity	6
1.6.1 Innate immune responses against HIV-1	7
1.7 Adaptive immune responses against HIV-1	9
1.7.1 B cell responses and antibodies against HIV-1	9
1.7.2 T cell responses against HIV-1.....	10
1.7.2.1 CD4 ⁺ T cell responses against HIV-1.....	11
1.7.2.2 CD8 ⁺ T cell responses against HIV-1.....	12
1.8 The Major Histocompatibility Complex (MHC) gene region	14
1.8.1 HLA class I structure.....	15
1.8.2 HLA class II structure	18
1.9 Antigen processing and presentation.....	18
1.9.1 HLA class I antigen processing pathway overview.....	18
1.9.2 HLA class II antigen processing pathway overview	21
1.9.3 Sources of peptides for HLA class I presentation	21
1.9.4 Antigen processing in the cytoplasm.....	22
1.9.5 Antigen processing and peptide loading in the ER.....	24
1.9.6 Recycling/degradation of HLA class I complexes	25
1.9.7 Cross-presentation	25
1.9.7.1 Antigen uptake for cross-presentation.....	26
1.9.7.2 Mechanisms of cross-presentation.....	27
1.9.8 Cross-dressing	30

1.10	Dendritic cells as orchestrators of the adaptive immune system	30
1.10.1	CD8 ⁺ T cell priming	31
1.11	CTL immunodominance.....	32
1.11.1	CTL immunodominance hierarchy in AHI	34
1.12	HIV-1 Nef.....	36
1.12.1	Nef genetics and structure	37
1.12.2	The varied functions of HIV-1 Nef	37
1.12.2.1	Modulation of cell surface receptors	37
1.12.2.2	Modulation of cell signalling, activation and apoptosis by Nef	39
1.12.2.3	Nef increases virion infectivity.....	39
1.12.3	Nef induces its own secretion in EV	40
1.12.4	Effects of extracellular Nef on cells	41
1.13	Research questions	43
1.14	Hypotheses	43
1.15	Objectives.....	44
Chapter 2:	Materials and Methods	46
2.1	Materials	46
2.1.1	Plasticware and general reagents.....	46
2.1.2	Cell lines and tissue culture reagents.....	46
2.1.3	Cytokines.....	48
2.1.4	Peptides	48
2.1.5	Chemical reagents	49
2.1.6	Enzyme-linked immunospot assay reagents (ELISPOT)	50
2.1.7	Antibodies for flow cytometry and stimulation.....	51
2.1.8	Antibodies for immunoblotting	52
2.2	Methods	53
2.2.1	Human samples and isolation of PBMC and plasma.....	53
2.2.2	Cell culture	54
2.2.3	Monocyte-derived dendritic cells (MDDC)	54
2.2.4	Dextran-FITC uptake assay	55
2.2.5	Monocyte-derived macrophages.....	55
2.2.6	<i>In vitro</i> priming assay	55
2.2.7	Enzyme-linked immunospot (ELISPOT) assay	57
2.2.8	Intracellular cytokine staining	57
2.2.9	HIV-1 IIIB propagation.....	58
2.2.10	NL4.3 BaL <i>env</i> plasmid production	59
2.2.11	HIV-1 strain NL4.3 BaL <i>env</i> production.....	60

2.2.12	HIV-1 titration.....	61
2.2.13	HIV-1 IIIB genome sequencing	61
2.2.14	HIV-1 infection of cells.....	63
2.2.15	Intracellular p24 staining.....	64
2.2.16	Dendritic cell cross-presentation assay.....	64
2.2.17	Acid elution of surface MHC-bound peptides.....	65
2.2.18	Antibody purification for use in MHC isolation.....	65
2.2.19	Antibody resin preparation	66
2.2.20	HLA isolation from cells	66
2.2.21	Reverse-phase high performance liquid chromatography (RP-HPLC) peptide purification	67
2.2.22	Tryptic digest of cellular/subcellular proteomes	67
2.2.23	Liquid chromatography-tandem mass spectrometry (LC-MS/MS).....	68
2.2.24	Mass spectrometry data analysis	70
2.2.25	T2 HLA-E stabilisation assay.....	71
2.2.26	Isolation of EVs.....	71
2.2.27	Separation of HIV-1 virions from exosomes by iodixanol gradient.....	71
2.2.28	Acetylcholinesterase activity assay	72
2.2.29	Separation of HIV-1 virions from exosomes by CD45 depletion	72
2.2.30	TZM-B1 luciferase assay	73
2.2.31	Trichloroacetic acid (TCA) protein precipitation.....	73
2.2.32	Immunoblotting	73
2.2.33	Exosome staining and uptake assays	74
2.2.34	Nanoparticle tracking analysis (NTA).....	75
2.2.35	LABScreen single antigen HLA class I antibody cross-reactivity	75
2.2.36	Statistics and data presentation.....	76
Chapter 3: <i>In vitro</i> modelling of CD8⁺ T cell priming during AHL.....		77
3.1	Introduction	77
3.2	Results	81
3.2.1	Establishment of an <i>in vitro</i> CD8 ⁺ T cell priming assay and evaluation of its utility for <i>in vitro</i> priming of HIV-1-specific CD8 ⁺ T cell responses.....	81
3.2.2	Consistent CD8 ⁺ T cell priming against epitopes with high precursor frequency in the naïve repertoire.....	90
3.2.3	Plasma from acutely-infected HIV-1 patients around the time of peak viral load does not impair <i>in vitro</i> CD8 ⁺ T cell priming	92
3.2.4	Effects of IL-4-DC exposure to IFN α on <i>in vitro</i> CD8 ⁺ T cell priming	98
3.3	Discussion	104
Chapter 4: Novel classes of MHC I epitopes presented by HIV-1-infected CD4⁺ T cells		112

4.1	Introduction	112
4.2	Results	118
4.2.1	HLA immunoprecipitation allows specific and reproducible elution of HLA class I-bound peptides.....	118
4.2.2	Extended Gag-derived peptides are non-specifically eluted after HLA immunoprecipitation.....	125
4.2.3	Novel epitopes derived from the HIV-1 5' leader sequence translated and presented by MHC class I on infected CD4 ⁺ T cells	137
4.2.4	HIV-1 epitopes presented by the non-classical MHC molecule HLA-E.....	140
4.2.4.1	Elution of peptides from naturally expressed HLA-E	140
4.2.4.2	An unconventional HLA-E binding motif is revealed by elution of peptides from HLA class Ia negative CD4.221 cells.....	148
4.2.4.3	Stabilisation of HLA-E by peptides on the surface of TAP-deficient T2 cells	150
4.3	Discussion	155
Chapter 5: Characterisation of extracellular vesicles secreted by HIV-1-infected cells		169
5.1	Introduction	169
5.2	Results	174
5.2.1	Validation of a differential ultracentrifugation method for isolation of exosomes ...	174
5.2.2	Nef is abundant in the plasma of HIV-1 ⁺ patients.....	178
5.2.3	Gag is the most abundant HIV-1 antigen secreted by infected CD4 ⁺ T cells.....	181
5.2.3.1	Separation of exosomes and virions using an iodixanol gradient.....	181
5.2.3.2	Separation of exosomes and virions using CD45 depletion: Nef is predominantly contained within CD45 ⁺ exosomes.....	186
5.2.3.3	Gag is the most abundant antigen in vesicles secreted by infected CD4 ⁺ T cells and cell lines	189
5.2.4	Characterisation of exosomes secreted by HIV-1-infected macrophages	191
5.3	Discussion	195
Chapter 6: The cross-presented HIV-1 immunopeptidome of DCs.....		204
6.1	Introduction	204
6.2	Results	208
6.2.1	Development of a method to investigate the dendritic cell cross-presented immunopeptidome.....	208
6.2.2	Comparison of the immunopeptidomes presented by DC and CD4 ⁺ T cells	230
6.2.3	Analysis of peptides and proteins cross-presented by IFN-DCs	236
6.3	Discussion	242
Chapter 7: General discussion		253
8.0	References	260
9.0	Appendix	299
9.1	Primer sequences.....	299

9.2	Peptide pool sequences.....	299
9.3	Peptides identified by immunopeptidomics	305
10.0	List of Publications.....	308

List of Figures

Figure 1.1. <i>Structure of the mature HIV-1 infectious virion.</i>	2
Figure 1.2. <i>Structure of the HIV-1 genome.</i>	3
Figure 1.3. <i>Acute HIV-1 infection.</i>	Error! Bookmark not defined.
Figure 1.4. <i>The MHC gene locus on chromosome 6</i>	14
Figure 1.5. <i>Schematic structure of HLA class I molecules</i>	16
Figure 1.6. <i>Overview of the HLA class I antigen processing and presentation pathway</i>	20
Figure 1.7. <i>Intracellular pathways of cross-presentation</i>	29
Figure 1.8. <i>Asynchronous expansion of CD8⁺ T cell responses during AHI</i>	34
Figure 1.9. <i>Extracellular vesicles; exosomes and microvesicles</i>	41
Figure 3.1 <i>Phenotype of monocyte-derived IL-4-DC and IFN-DC</i>	84
Figure 3.2 <i>Uptake of dextran-FITC by immature and mature monocyte-derived DCs</i>	85
Figure 3.3 <i>In vitro priming assay protocol schematic</i>	86
Figure 3.4. <i>Isolation of naïve CD8⁺ T cells and expansion during the in vitro priming assay</i>	87
Figure 3.5 <i>Priming of HIV-1-specific CD8⁺ T cells by DC pulsed with optimal-length epitope peptides</i>	88
Figure 3.6 <i>Intermittent priming of HIV-1-specific CD8⁺ T cells by DCs pulsed with optimal-length epitope peptides but not HIV-1 protein</i>	89
Figure 3.7 <i>Consistent priming of CD8⁺ T cells in vitro against epitopes with a high frequency in the naïve repertoire</i>	91
Figure 3.8 <i>Effect of plasma from AHI on in vitro CD8⁺ T cell priming</i>	96
Figure 3.9 <i>Effect of plasma from AHI on IL-12 production by IL-4-DC</i>	97
Figure 3.10 <i>Reduced levels of costimulatory markers on IL-4-DC after IFNα exposure</i>	101
Figure 3.11 <i>Effects of IFNα treatment of DCs on in vitro CD8⁺ T cell priming</i>	102
Figure 3.12 <i>Effects of IFNα treatment of DCs on in vitro CD8⁺ T cell priming against long peptide Melan-A16-40(A27L).</i>	103
Figure 4.1 <i>Workflow for identification and analysis of MHC-bound peptides by mass spectrometry</i>	121
Figure 4.2 <i>Immunoprecipitation is superior to mild acid elution in terms of specificity of eluted MHC-bound peptides</i>	122
Figure 4.3 <i>Immunoprecipitation and identification of MHC class I peptides from human cells is highly reproducible</i>	124
Figure 4.4 <i>Low background of peptides eluted from W6/32 immunoprecipitate of the MHC class Ia negative cell line CD4.221</i>	130
Figure 4.5 <i>HLA class I-bound peptides are eluted from CD3 immunoprecipitate of C8166 cells</i>	132
Figure 4.6 <i>HIV-1 peptides are eluted from CD3 immunoprecipitate of C8166 cells</i>	134
Figure 4.7 <i>HIV-1 peptides are eluted from CD4 immunoprecipitate of HIV-1-infected CD4.221 cells</i>	135
Figure 4.8 <i>HLA class I detected in OKT3 and W6/32 IP from C8166 cells and in OKT4 IP from CD4.221 cells</i>	136
Figure 4.9 <i>The HIV-1 5' leader region</i>	138
Figure 4.10 <i>Peptides derived from the HIV-1 5'leader sequence are presented with MHC class I in infected CD4⁺ T cells</i>	139
Figure 4.11 <i>Peptide elution from HIV-1-infected PMI cells using DT9 immunoprecipitation</i>	145
Figure 4.12 <i>Peptide elution from HIV-1-infected C8166 cells using HLA-E-specific 4D12 immunoprecipitation</i>	147
Figure 4.13 <i>Peptide elution from HLA-E expressed by HIV-1-infected CD4.221 cells using W6/32 immunoprecipitation</i>	149
Figure 4.14 <i>HLA-E stabilisation by synthetic peptides on TAP-deficient T2 cells</i>	154

Figure 4.15 <i>Proteolytic processing of Gag antigen within maturing virions</i>	160
Figure 5.1 <i>Validation of a differential ultracentrifugation protocol to isolate extracellular vesicles</i>	177
Figure 5.2 <i>Nef is abundant in the plasma of HIV-1+ patients</i>	180
Figure 5.3 <i>Substantial acetylcholinesterase activity associated with foetal bovine serum</i>	184
Figure 5.4 <i>Iodixanol gradients do not appear to separate Nef exosomes from virions secreted by the CD4⁺ T cell line PM1</i>	185
Figure 5.5 <i>Nef is predominantly contained within CD45⁺ exosomes released into HIV-1-infected cell supernatants</i>	188
Figure 5.6 <i>Gag is the most abundant antigen produced and secreted by HIV-1 IIIB-infected CD4⁺ T cells</i>	190
Figure 5.7 <i>Characterisation of vesicles secreted by HIV-1-infected macrophages</i>	193
Figure 5.8 <i>Longitudinal characterisation of vesicles secreted by HIV-1-infected macrophages</i>	194
Figure 6.1 <i>IFN-DCs cross-present and are cross-dressed with HIV-1 epitopes from EVs secreted by HIV-1 IIIB-infected MT-2 cells</i>	212
Figure 6.2 <i>Elution of peptides from IFN-DC co-cultured with primary CD4⁺ T cells</i>	217
Figure 6.3 <i>Elution of peptides from IFN-DCs co-cultured with HIV-1 IIIB-infected PM1 cells</i>	220
Figure 6.4 <i>IFN-DC co-culture with CD4.221 cells</i>	224
Figure 6.5 <i>Effects of AZT on IFN-DC dextran-FITC endocytosis</i>	228
Figure 6.6 <i>Optimisation of IFN-DC cross-presentation assay</i>	229
Figure 6.7 <i>IFN-DCs and CD4⁺ T cells present qualitatively and quantitatively similar immunopeptidomes</i>	234
Figure 6.8 <i>Analysis of peptides/proteins cross-presented by IFN-DC</i>	240

List of Tables

Table 2.1	List of plasticware and general reagents	46
Table 2.2	Hybridomas expressing immunoglobulins targeting human proteins used	47
Table 2.3	List of cell culture reagents and buffers	47
Table 2.4	List of cytokines used <i>in vitro</i>	48
Table 2.5	List of chemical reagents and drugs used	49
Table 2.6	List of ELISPOT assay reagents	50
Table 2.7	Antibodies for flow cytometry or cell stimulation	51
Table 2.8	List of antibodies used for immunoblotting detection	52
Table 4.1	Studies using immunopeptidomics to identify viral epitopes naturally presented on virally-infected human cells	114
Table 6.1	HIV-1 epitopes identified in IFN-DCs cross-presenting MT-2 EVs	213
Table 6.2	HIV-1 peptides identified in BDCA4 ⁻ and BDCA4 ⁺ populations of IFN-DC/PM1 co-cultures	221
Table 6.3	HIV-1 peptides cross-presented by IFN-DCs co-cultured with HIV-1 IIIB-infected CD4.221 cells	225
Table 6.4	HIV-1 epitopes identified in autologous IFN-DCs and primary CD4 ⁺ T cells	235
Table 6.5	Prediction of HLA class I restriction of peptides identified in IFN-DC cross-presentation pathway	241

List of Abbreviations

AchE	acetylcholinesterase
ADCC	antibody-dependent cellular cytotoxicity
Ag	antigen
AHI	acute HIV-1 infection
AIDS	acquired immunodeficiency syndrome
APC	antigen-presenting cell
ART	antiretroviral therapy
AZT	azidothymidine
BCR	B cell receptor
β 2m	beta-2-microglobulin
bNAbs	broadly neutralising antibody
CA	capsid protein
cDNA	complementary DNA
CDP	common DC precursor
CDR3	complementarity-determining region 3
cGAMP	cyclic GMP-AMP
cGAS	cyclic GMP-AMP synthase
CHIP	C-terminus of Hsc70-interacting protein
CHX	cycloheximide
CLIP	class II-associated Ii peptide
CP	core particle (proteasome)
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DCIR	dendritic cell immunoreceptor
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DFOSx	days following onset of symptoms
DIA	data-independent acquisition
DIG-POD	anti-digoxigenin-POD
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DRiP	defective ribosomal product
DTT	dithiothreitol
EBV	Epstein-Barr virus
EC	elite controllers
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot assay
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERAP1/2	endoplasmic reticulum aminopeptidase 1/2
ESI	electrospray ionisation
EtHCD	electron-transfer and higher-energy collisional dissociation
EV	extracellular vesicles
FBS/FCS	foetal bovine serum/foetal calf serum
FDR	false discovery rate
FEC	Flu, EBV, CMV (peptides)
GP	glycoprotein
HBV	hepatitis B virus
HCD	higher-energy collisional dissociation
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HRP	horse radish peroxidase
HSV	herpes simplex virus

iBAQ	intensity-based absolute quantitation
ICS	intracellular cytokine staining
IFN	interferon
Ig	immunoglobulin
Ii	invariant chain
INT	integrase
IP	immunoprecipitation
IRAP	insulin responsive aminopeptidase
kDa	kilodalton
KIR	killer-cell immunoglobulin-like receptors
KLD	Kullback Leibler distance
LANL	Los Alamos National Laboratories
LC-MS/MS	liquid chromatography tandem-mass spectrometry
LCMV	lymphocytic choriomeningitis virus
LSEC	liver sinusoidal endothelial cell
LTNP	long-term non-progressors
LTR	long terminal repeat
MA	matrix protein
MAE	mild acid elution
mDC	myeloid DC
MDDC	monocyte-derived DC
MDM	monocyte-derived macrophages
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MIP3 α	macrophage inflammatory protein-3 alpha
miRNA	microRNA
MM	Mortimer Market cohort
MPER	membrane proximal region
MR	mannose receptor
Mtb	<i>Mycobacterium tuberculosis</i>
MV	microvesicles
MVB	multivesicular body
MWCO	molecular weight cutoff
NC	nucleocapsid protein
NK	natural killer cells
NP	nucleoprotein
NTA	nanoparticle tracking analysis
ORF	open reading frame
OVA	ovalbumin
PAS/PGS	protein A Sepharose/protein G Sepharose
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pDC	plasmacytoid dendritic cells
PLC	peptide loading complex
PR	protease
PRR	pattern recognition receptor
RhCMV	rhesus cytomegalovirus
RNA	ribonucleic acid
RP	regulatory complex (proteasome)
RP-HPLC	reversed phase high performance liquid chromatography
RPMI	Roswell Park Memorial Institute medium
RRE	Rev response element
RT	reverse transcriptase
SAA	serum amyloid A
SBBC	Sydney Blood Bank Cohort
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SERINC5	serine incorporator 5

SFU	spot-forming units
SILAC	stable isotope labelling with amino acids in cell culture
SIV	simian immunodeficiency virus
SMR	secretion modification region
SOC	super optimal broth with catabolite repression
SRK	Src family kinase
ssRNA	single-stranded RNA
STING	stimulator of interferon genes
T/F	transmitted/founder virus
TAP	transporter associated with antigen processing
TAR	trans-activation response element
TCA	trichloroacetic acid
TCID ₅₀	50% tissue culture infective dose
TCR	T cell receptor
TEMRA	effector memory T cells re-expressing CD45RA
TFA	trifluoroacetic acid
Tfh	T follicular helper cell
TLR	toll-like receptor
TNF α	tumour necrosis factor alpha
TPPII	tripeptidylpeptidase II
Treg	regulatory T cell
Tscm	stem cell-like memory T cell
UTR	untranslated region
VL	viral load
VSV	vesicular stomatitis virus

Author's contributions

This thesis is original and independent work performed and written by myself (Tom Partridge) with the following exceptions:

- Dr Nicola Ternette (University of Oxford, UK) operated mass spectrometers for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of all peptide samples
- Dr Simon Brackenridge (University of Oxford, UK) assisted with the LABScreen Single Antigen HLA class I protocol described in section 2.2.35 in order to generate data displayed in Figure 4.12D.
- Dr Rebecca Dragovic (University of Oxford, UK) assisted with operation of the NS500 nanoparticle analysis instrument for data presented in Figure 5.1A.
- Dr Anna Kliszczyk (University of Oxford, UK) produced the pNL4.3 BaL.ecto plasmid, produced/titrated infectious NL4.3 Bal *env* viral stocks, and performed the experiment described in Figure 5.8.

Chapter 1: Introduction

Human immunodeficiency virus (HIV) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS), a disease characterised by progressive dysfunction of the immune system which eventually leads to potentially fatal infection with opportunistic pathogens. HIV is classified as a member of the primate *Lentivirus* genus within the *Retroviridae* family. Two main types of HIV have been described: HIV type 1 (HIV-1) and HIV type 2 (HIV-2). Although both types of HIV cause AIDS, HIV-1 constitutes the vast majority of HIV infections globally whereas HIV-2 is largely restricted to western Africa. HIV-1 can be further classified into 3 distinct groups (M, N and O) which are thought to represent distinct transmission events from primates to humans¹. Group M (major) is the group which has caused the most infections globally.

1.1 Human Immunodeficiency virus (HIV-1) pandemic

Worldwide, approximately 36.7 million people are currently living with HIV and at least 25 million people have died as a result of the HIV pandemic since its discovery in 1981². Although the number of people receiving antiretroviral therapy has increased from 7.5 million in 2010 to 17 million in 2015, 2.1 million new HIV infections and 1.1 million AIDS-related deaths occurred in 2015. An estimated 10% of these annual deaths were in children under the age of 15. At the epicentre of the HIV-1 pandemic in Africa, where 25.8 million people are living with HIV, only about 12 million people are receiving antiretroviral therapy. As many individuals either are not aware of their HIV serostatus or do not have access to antiretroviral therapy, a prophylactic or therapeutic vaccine against HIV is widely considered to be the most effective method to end the HIV pandemic.

1.2 HIV-1 structure

In the mature HIV-1 virion (depicted in Figure 1.1), a cell-derived lipid bilayer is closely associated with a HIV-1-encoded matrix (MA) protein layer which ensures efficient incorporation of the gp120 and gp41 envelope glycoproteins into the membrane at the site of assembly³. These glycoproteins facilitate infection by forming trimeric complexes which bind to surface-expressed CD4 on CD4⁺ T cells, macrophages and dendritic cells (DCs)⁴. In the interior of the particle, hexagonal lattices of capsid (CA) proteins form a conical capsid which surrounds two copies of the HIV-1 genome. Nucleocapsid (NC) proteins ensure packaging of the two RNA genomes into the conical CA shell

whilst also protecting the RNA from degradation by nucleases³. Viral reverse transcriptase (RT), integrase (INT) and protease (PR) enzymes as well as accessory proteins Nef, Vif and Vpr are also incorporated into the nascent virion and are important for viral replication in target cells and immune evasion.

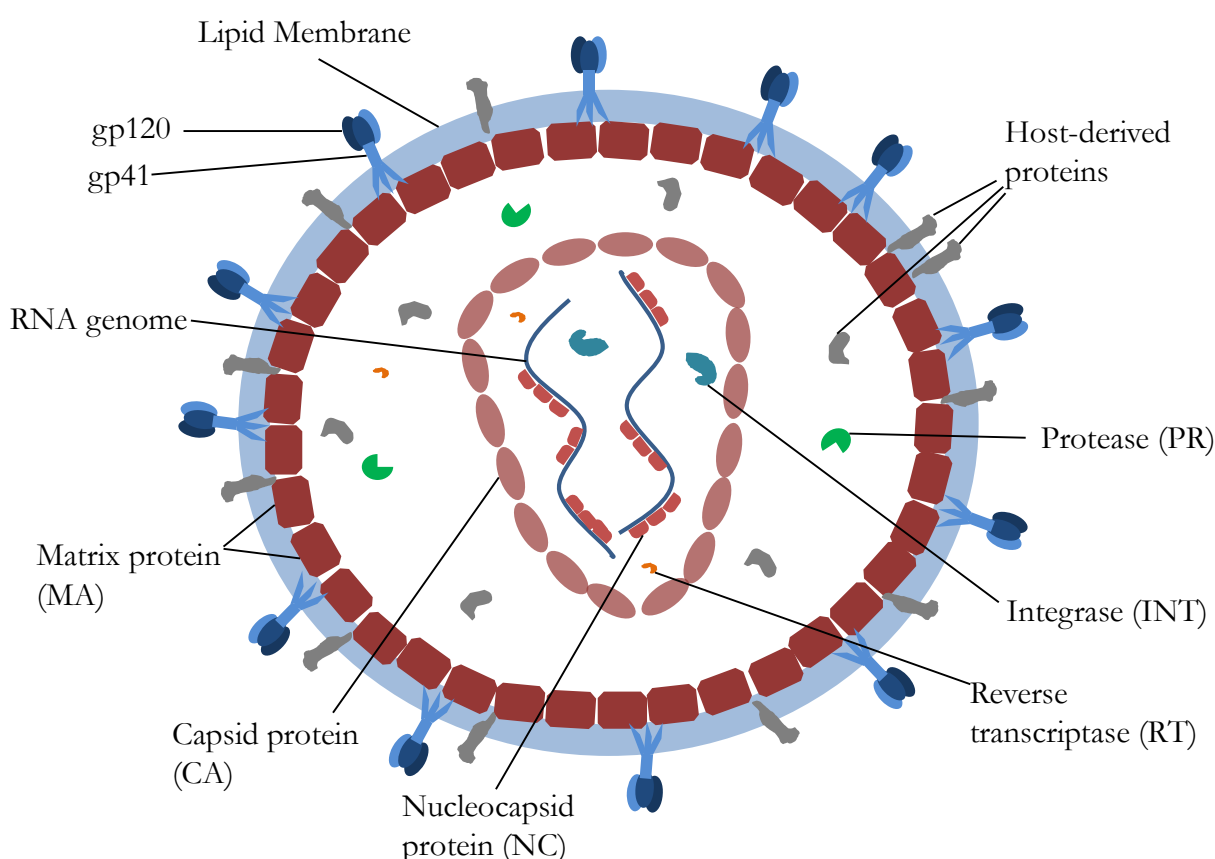


Figure 1.1. Structure of the mature HIV-1 infectious virion.

1.3 HIV-1 genome

HIV-1 has 9 genes (*gag*, *pol*, *env*, *nef*, *rev*, *tat*, *vif*, *vpu* and *vpr*) encoded within a positive sense single-stranded RNA (+ssRNA) genome⁵ (genome depicted in Figure 1.2). The *gag*, *pol* and *env* genes are encoded by all retroviruses whereas the regulatory genes *rev* and *tat* in addition to the so-called ‘accessory genes’ *nef*, *vif*, *vpu* and *vpr* distinguish primate lentiviruses such as HIV-1⁶. The *gag* (group specific antigen) gene encodes the precursor Gag protein which associates with membranes after co-translational myristoylation at the N-terminus³. Gag is then cleaved by the viral PR to form the MA, CA, NC structural elements of the mature virion. The *pol* gene encodes the viral enzymes RT, INT and PR which facilitate reverse transcription and integration of the genome into

host DNA as well as proteolytic cleavage of viral precursor proteins. The *env* gene produces the gp160 envelope precursor which is processed into the external gp120 and transmembrane gp41 proteins. The transcriptional transactivator protein Tat (encoded by *tat*) is essential for HIV-1 replication. It acts in the nucleus by binding to the trans-activation responsive region (TAR) stem-loop element in the long terminal repeat (LTR) located at the 5' end of all viral RNAs, thereby preventing premature transcription termination⁷. The Rev protein (encoded by *rev*) also regulates gene expression by binding to the Rev response element (RRE) present in the *env* region of unspliced genomic and singly-spliced HIV-1 RNAs and facilitating their export to the cytoplasm for ribosomal translation or packaging into virions. The accessory genes are not essential for viral replication in many *in vitro* systems, but perform a variety of functions involved in immune evasion and enhancement of viral replication *in vivo*.

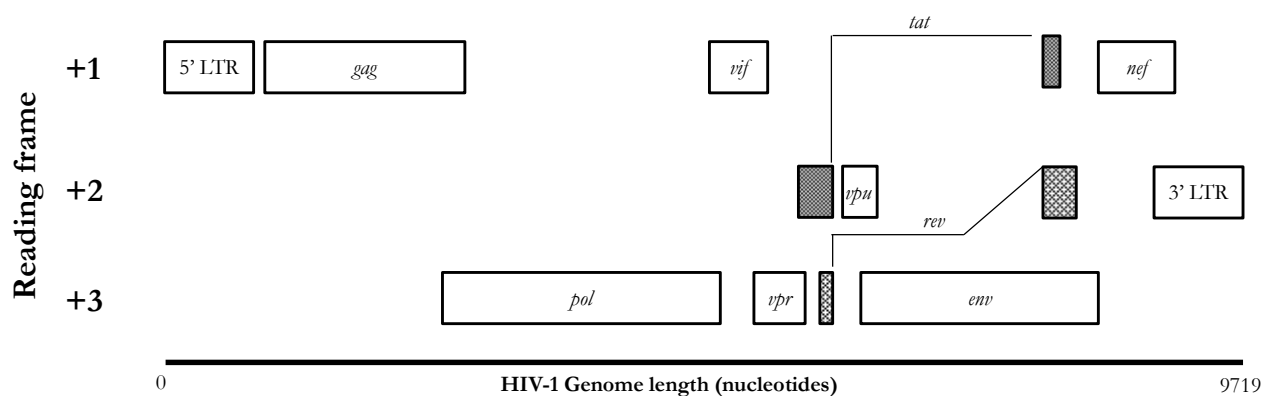


Figure 1.2. Structure of the HIV-1 genome. Diagram modified from Los Alamos National Laboratories (LANL) HIV Database at hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html. LTR; long terminal repeat.

1.4 Overview of the HIV-1 lifecycle

The complete HIV-1 lifecycle in CD4⁺ T cells (the main target of HIV-1 infection) has been estimated to take on average 52 hours⁸. Mature virions displaying gp120/gp41 envelope proteins first bind to CD4 and either CCR5 or CXCR4 co-receptors expressed by target cells, which triggers fusion of the virion membrane with the cellular plasma membrane. The viral capsid is then released into the cytoplasm. The rate limiting step of reverse transcription takes place within the capsid in the cytoplasm, where viral RT transferred in the virion reverse transcribes the RNA into cDNA. The

cDNA then forms a multimeric pre-integration complex to be transported through the nuclear pores into the nucleus⁹. Once there, the HIV-1 cDNA is integrated into the host genome with the aid of the viral INT protein. Early expression of accessory proteins such as Nef or Vpu allows modulation of surface receptors and cell signalling¹⁰. Transcription of HIV-1 RNAs is enhanced by Tat, and Rev enhances export of full-length or singly-spliced RNAs to the cytoplasm where they can be used to produce HIV-1 proteins or be packaged into nascent virions. Gag multimerises at the plasma membrane where developing virions incorporate envelope glycoprotein, two copies of the HIV-1 RNA genome, viral enzymes and accessory proteins¹¹. A budding process releases the virion from the membrane, then the viral PR cleaves the Gag precursor to form the conical CA, generating a mature virion that is able to infect another cell¹². Importantly, many of the stages of this lifecycle are amenable to targeting by drugs, which forms the basis for antiretroviral therapy.

1.5 Natural history of HIV-1 infection

Acquisition of HIV-1 infection is typically associated with non-specific symptoms characteristic of many acute viral infections such as fever, fatigue, lymphadenopathy, headache and rash^{13,14}. During this symptomatic stage of infection, the number of viral RNA copies present in plasma increases exponentially to a peak typically in excess of 10^6 /ml, after which it decreases to a set point viral load which remains relatively constant throughout the asymptomatic chronic infection stage¹⁵ (Figure 1.3). This set point viral load is predictive of the rate of disease progression to AIDS, which is

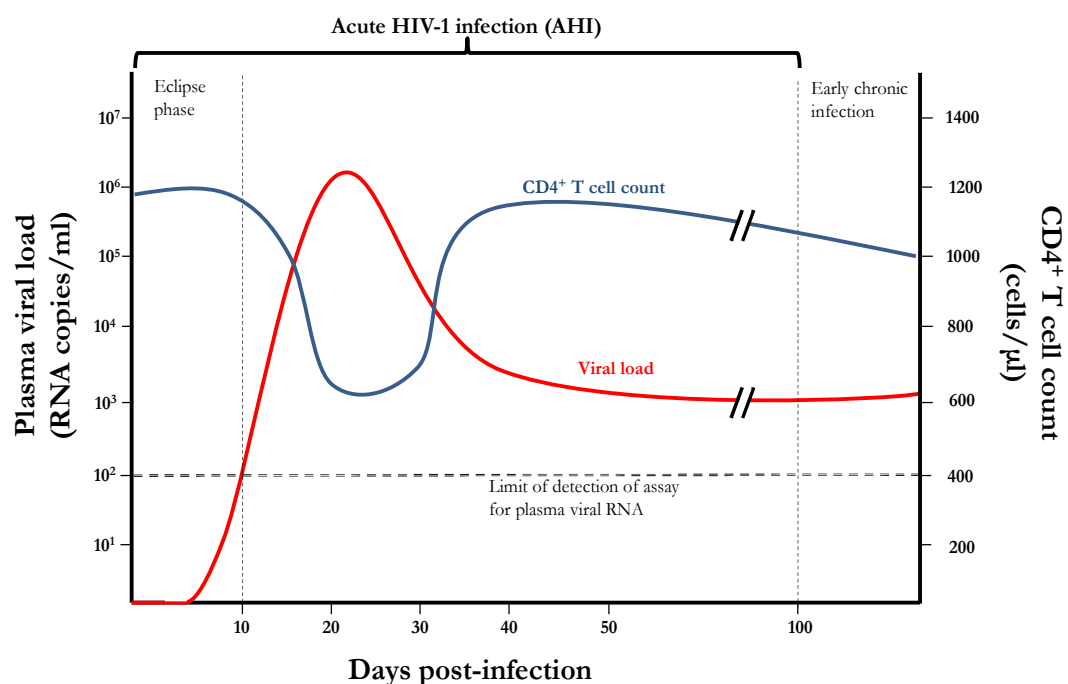


Figure 1.3. *Acute HIV-1 infection.* Modified from³⁰.

defined as a CD4⁺ T cell count <200 cells/ μ l or based on clinical criteria^{16,17}. In untreated infection, a clinically latent stage of several years typically follows primary symptomatic infection before onset of AIDS. Although most untreated individuals eventually progress to AIDS, rare individuals called long-term non-progressors (LTNPs) or elite controllers (ECs) are able to maintain low viral loads for long periods of time or indefinitely in the absence of antiretroviral therapy¹⁸.

1.5.1 HIV-1 transmission and acute HIV-1 infection (AHI)

HIV is most commonly transmitted at mucosal surfaces which come into contact with infected fluids during sexual intercourse or through infected needles/syringes, although can be transmitted by other routes, including vertically from mother to child. In ~80% of heterosexual transmissions studied, systemic infection was shown to entail a transmission bottleneck whereby just one ‘transmitted/founder’ (T/F) virus established the infection^{19,20}. This mucosal bottleneck is thought to result from physical and innate immune barriers which select for a highly transmissible HIV-1 quasispecies²¹. Relatively high 1) resistance to the antiviral effects of type I interferons (IFNs) 2) Env content and 3) replicative capacity have been associated with the inferred consensus sequence T/F virions^{22,23}.

During the initial ‘eclipse’ phase of AHI, viral RNA remains undetectable in the plasma of patients. Although this phase of infection has been difficult to characterise in HIV infection, studies of simian immunodeficiency virus (SIV) infection of rhesus macaques have suggested that mucosal infection at vaginal or rectal surfaces is followed by rapid systemic dissemination to local and more distal lymphoid tissues²⁴⁻²⁶. One study recently contended that SIV infection is disseminated to distal tissues within 24 hours of mucosal exposure, with an eclipse phase of about 7 days, although a relatively high number (median=5) of T/F viruses initially seeded these infections²⁷. Similar studies utilising low viral inoculum doses resulting in establishment of infection by a single T/F virus are associated with longer eclipse phases of up to a few weeks^{28,29}.

The eclipse phase of infection is followed by the viral ramp up phase where exponential replication in secondary lymphoid tissues, in particular the gut-associated lymphoid tissues (GALT), culminates in a peak plasma viral load at approximately 3-4 weeks following initial infection. Throughout the exponential phase of viral replication, systemic innate and adaptive immune responses (discussed

below) are mounted against HIV-1. Host responses subsequently reduce the viral load to the relatively stable set point, demarcating the beginning of chronic HIV-1 infection³⁰.

1.6 Overview of innate and adaptive immunity

Under normal circumstances, the innate and adaptive immune systems together defend the body against infectious pathogens, but are regulated to limit pathology caused by an overzealous immune response. Innate immune defences are comprised of anatomical barriers (such as epithelial cell layers or mucous membrane), immune cell subsets including neutrophils, eosinophils, macrophages, DCs and natural killer (NK) cells, and soluble mediators of inflammation such as cytokines, chemokines or complement proteins, which directly mediate pathogen control, attract innate cell subsets to sites of infection and activate their activity, and mark foreign material for clearance by specialised phagocytic cells³¹. The innate immune system is also known as the non-specific immune system, because innate recognition of microorganisms is mediated by a relatively small repertoire of pattern recognition receptors (PRRs) which recognise molecules conserved across large groups of bacteria, viruses or parasites. Furthermore, the innate system generally performs its function rapidly but typically does not become more efficacious on a second exposure to the same pathogen (although certain innate cell subsets, including macrophages and NK cells, sometimes retain an altered activation state following infection that enables a more effective subsequent response to certain pathogens^{32,33}).

In contrast, the adaptive immune system is known as the specific immune system because it is comprised of populations of lymphocytes (T and B cells) which are able to recognise individual pathogens in an antigen-specific manner through T cell receptors (TCR) or B cell receptors (BCR)³⁴. TCRs generally consist of an α and β chain (T cells with a γ and a δ chain also exist; $\gamma\delta$ T cells) which both contain variable (V), constant (C) regions, joining (J) segments and diversity (D) gene segments (TCR β only). During T cell development in the thymus, these gene segments are somatically rearranged such that each T cell contains a unique TCR and the entire pool of T cells are together able to recognise and respond to any pathogen encountered by the host. Each TCR recognises surface-expressed major histocompatibility complex (MHC; see section 1.9) proteins in association with specific short peptides.

BCRs are also somatically rearranged but additionally undergo a somatic hypermutation process which introduces point mutations within V segments to substantially increase receptor diversity³⁵. This hypermutation process allows ‘affinity maturation’ of the BCR to occur, whereby B cells expressing receptors with higher affinity for their cognate antigen are selected to produce antibodies. These antibodies (immunoglobulins; Ig) perform a variety of functions in immunity, including binding to and neutralisation (inhibition of infection) of pathogens, formation of immune complexes for recognition by phagocytic cells and triggering of antibody-dependent cellular cytotoxicity (ADCC).

The adaptive immune response is generally slower to develop than the innate response because it requires activation and expansion of antigen-specific cells. However, the adaptive system has ‘memory’, which enables T and B cell responses to a second exposure to a pathogen to be mounted rapidly and confer better protection. This immunological memory is the practical basis for vaccination, as immunogens train the immune system to be prepared for rapid elimination of pathogens upon subsequent exposure.

1.6.1 Innate immune responses against HIV-1

In vivo, the first cells to encounter HIV-1 are likely to be epithelial cells at mucosal surfaces. Primate models of infection have elucidated a rapid macrophage inflammatory protein-3 (MIP3 α /CCL20), MIP1 α /CCL3 and IL-8/CXCL8 chemokine response by cervical epithelial cells sensing HIV-1^{36,37}. These chemokines subsequently attract plasmacytoid dendritic cells (pDCs) to the site of infection, where they sense HIV-1 nucleic acids through endosomal toll-like receptor 7 (TLR7) and TLR9³⁸ and secrete abundant amounts of type I IFNs, MIP1 α and MIP1 β ³⁶. In turn, these chemokines recruit CD4⁺ T cells which serve as a substrate for HIV-1 infection. Subsets of myeloid DCs (mDCs) located in mucosa bind to HIV-1 virions through two main receptors 1) DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) interacts with HIV-1 gp120 and 2) the IFN-induced CD169 (sialoadhesin) interacts with the glycosphingolipid GM3 within the virion membrane envelope. A large body of work has demonstrated the importance of these two receptors in facilitating trans-infection of CD4⁺ T cells after migration to lymphoid tissues³⁹⁻⁴¹.

The first measurable systemic response to HIV-1 infection is the upregulation of acute phase proteins such as serum amyloid A (SAA) and the alpha-1-antitrypsin-derived peptide VIRIP, which inhibit HIV-1 replication *in vitro*⁴². This acute phase response begins 5-7 days before detectable virus is present in plasma. Subsequently, a cascade of cytokine induction coincides with the increase in viral load. Cytokines such as type I interferons, IFN γ , IL-10, IL-15, IL-18, IL-22, CXCL10 and TNF α are all induced with distinct kinetics during AHI⁴³. A recent analysis of SIV-infected rhesus macaques and African green monkeys found that production of IL-15, IL-18, IFN γ , G-CSF, MCP-1, MIP1 β and SAA in acute infection correlated with viral load at 3 months post-infection in progressive infection⁴⁴, suggesting that although innate cytokines may contribute to viral control via both direct and indirect mechanisms, the early cytokine storm has a detrimental role in long-term control of viral replication. The systemic cytokine response also recruits large number of CD4⁺ T cells to lymphoid tissues, which augments the available pool of target cells for HIV-1. Subsequent apoptosis of both infected and bystander uninfected CD4⁺ T cells results in the systemic release of immunosuppressive extracellular vesicles (EVs)⁴⁵. These EVs alter DC innate immune responses *in vitro*⁴⁶. The marked decrease in frequencies of mDCs and pDCs in blood during AHI⁴⁷ is likely explained by DC migration to lymph nodes and subsequent apoptosis.

Another cell type of the innate immune system, NK cells, becomes activated and expands during AHI⁴⁸. Genetic evidence suggests a role for NK cells in executing innate antiviral functions during HIV-1 infection, as the co-expression of particular killer-cell immunoglobulin-like receptors (KIRs) (a family of activating and inhibitory receptors expressed predominantly by NK cells) with the human leukocyte antigen (HLA)-B alleles that constitute their ligands (HLA introduced in section 1.9) has been associated with delayed disease progression^{49,50}. NK cells monitor the HLA class I environment and survey for expression of stress-related proteins by means of KIRs and other NK receptors which bind to HLA class I molecules or stress-related proteins on the surface of neighbouring cells⁵¹. Depending on the balance of signals they receive through inhibitory receptors (which engage peptide-HLA complexes expressed by normal cells) and activating receptors (which engage peptide-HLA complexes and other ligands expressed on abnormal cells), NK cells can exert cytotoxic function to kill infected or stressed cells. Although NK cell responses may be important in

control of HIV-1 replication *in vivo*, they are not discussed in detail here; instead more focus is placed on adaptive immune responses.

1.7 Adaptive immune responses against HIV-1

The ultimate aim of a prophylactic vaccine for HIV-1 would be to induce a combination of long-lasting antibody responses and memory T cell responses in order to enforce sterilising immunity or early clearance of HIV-1 infection. Recent vaccination efforts have mainly focused on the induction of protective antibody responses, in large part due to the discouraging outcomes of initial T cell-based vaccine efficacy trials^{52,53}, and the protection demonstrated in the large RV144 antibody-based vaccine trial in low-risk individuals in Thailand which showed 31.2% efficacy at 4 years post-vaccination^{54,55}. A colossal amount of work has been done to characterise genetic, structural and B cell lineage aspects of the generation of HIV-1-specific antibody responses during infection and after vaccination⁵⁶. However, the importance of HIV-1-specific CD8⁺ T cell responses in controlling viral replication are well characterised and innovative vaccines which principally induce strong CD8⁺ T cell responses have shown promise in pre-clinical trials⁵⁷. As B cell responses are beyond the scope of this thesis, I will only provide a summary of HIV-1-specific B cell responses and antibodies before focusing in particular on HIV-1-specific CD8⁺ T cell responses.

1.7.1 B cell responses and antibodies against HIV-1

The first detectable B cell responses to HIV-1 were studied in plasma donors for whom longitudinal plasma samples cryopreserved at timepoints before, during and after AHI were available⁵⁸. This first response was identified as early as 5 days after the detection of plasma viraemia and consisted of immune complexes of non-neutralising IgM or IgG antibodies bound to virions. Subsequently, free anti-gp41 IgM/IgG antibodies are produced whilst anti-gp120 antibody responses are delayed until about 2 weeks later.

Neutralising antibodies are first detected a few months after infection and place selective pressure on the autologous virus which drives the accumulation of escape mutants⁵⁹⁻⁶². In about 20% of individuals, antibodies which recognise multiple heterologous HIV-1 strains (called broadly neutralising antibodies; bNAbs) are developed years into infection^{63,64}. These bNAbs have been

shown to target a limited number of sites on the envelope glycoprotein, including the CD4 binding site, the membrane proximal region (MPER), V1/V2/V3 loops and the gp120/gp41 bridging region⁶⁵. In addition to neutralising virions in the blood, Abs may also be able to mediate ADCC to clear HIV-1-infected cells. Antibodies capable of triggering ADCC have been found in LTNP and were also associated with protection in the RV144 vaccine trial^{66–68}. HIV-specific IgA responses have been detected at mucosal sites early after HIV-1 infection⁶⁹, although relatively few studies have been conducted to further characterise the activity of such mucosal antibodies.

Isolation and characterisation of an ever-growing number of bNAbs has triggered a host of promising passive antibody therapies in animal models^{70–74}. Recent clinical trials have also shown that infusion of bNAbs can reduce viraemia in HIV-1⁺ antiretroviral-untreated patients or patients undergoing treatment interruption^{75–77}. Although passively-transferred bNAbs could be useful in short-term prophylactic or therapeutic settings, inducing such bNAbs through vaccination has proved to be exceedingly difficult.

The difficulty in inducing HIV-1 bNAbs is largely due to the fact that the few conserved sites on gp160 that constitute targets for bNAbs resemble self-epitopes, as most are comprised in whole or in part of carbohydrates and/or lipids⁷⁸. Analysis of bNAbs isolated from chronically-infected individuals has shown that they all exhibit extensive somatic hypermutation, long heavy chain complementarity determining region 3 (CDR3) regions and/or autoreactivity, features of antibodies whose production is constrained by host tolerance controls⁷⁹. However, induction of bNAb-producing B cells by vaccination is thought to be possible through engagement of rare B cell germline precursors and extensive somatic hypermutation/affinity maturation⁸⁰. The B cell developmental pathways which ultimately culminate in the generation of bNAbs in HIV-1-infected individuals are the subject of extensive investigation. A complex interplay of virus evolution and B cell helper lineages seem to be important in this respect^{81,82}. Furthermore, T follicular helper (Tfh) cells are instrumental in supporting the development of B cell responses⁸³. Thus induction of bNAbs by vaccination, which would require both arms of the adaptive immune system, is a monumentally difficult task.

1.7.2 T cell responses against HIV-1

T cells play a crucial role in monitoring cells for the presence of viruses or other intracellular pathogens by recognising foreign peptides (epitopes) in association with HLA class I/II. Although cytotoxic CD4⁺ T cells are present *in vivo*, CD4⁺ T cells predominantly play a ‘helper T cell’ role by regulating or enhancing both innate and adaptive immune responses. On the other hand, CD8⁺ T cells predominantly act by inducing infected cells to undergo apoptosis through cytotoxic mechanisms, or mediating virus control by non-cytolytic mechanisms.

1.7.2.1 CD4⁺ T cell responses against HIV-1

Following priming in lymph nodes, CD4⁺ T cells differentiate into a variety of T helper cell subsets such as Th1, Th2, Th17, Treg or Tfh. These subsets are characterised by differential expression of particular transcription factors, homing markers and cytokines impacting on their downstream function⁸⁴.

Researchers have not traditionally focused on the role of CD4⁺ T cells in controlling HIV-1 infection as expansion of CD4⁺ T cells by vaccination is generally thought to increase the availability of targets for HIV-1 infection. However, evidence from various studies indicates that CD4⁺ T cell responses could be beneficial for control of HIV-1 replication. Strong or polyfunctional HIV-1-specific CD4⁺ T cell responses have been associated with viral control^{85–88}, and non-progressive infection with HIV-2 may be distinguished from progressive HIV-1 infection by the presence of polyfunctional CD4⁺ T cells⁸⁹, although whether this is a consequence or cause of low viraemia remains unclear. Furthermore, escape mutations in CD4⁺ T cell epitopes have been observed (although these are much rarer than escape mutations in CD8⁺ T cell epitopes)^{90,91}. Cytotoxic CD4⁺ T cells expressing granzyme A were shown to correlate with delayed HIV-1 disease progression⁹², leading some to the hypothesis that this population is capable of eliminating HLA class II-expressing infected macrophages⁹³.

The helper functions of CD4⁺ T cells are also thought to be crucial for HIV-1 immunity, particularly in the context of B cell development. Although Tfh are thought to be a significant reservoir of HIV-1 infected cells^{94,95}, their ability to guide the development of antibody-producing B cells is being investigated for vaccination strategies aiming to induce bNAbs^{83,93}. Lastly, CD4⁺ T cell help is also

thought to be important for effective CD8⁺ T cell responses, as CD8⁺ memory formation is impaired in the absence of CD4⁺ T cell help^{96–98}.

1.7.2.2 CD8⁺ T cell responses against HIV-1

CD8⁺ T cells are able to recognise infected cells by engaging peptide-HLA class I complexes on the cell surface through TCRs. Cytotoxic CD8⁺ T cells (CTL) can then induce cell death through 3 mechanisms⁹⁹: 1) lysis of infected cells by releasing granules containing granzyme and perforin, 2) apoptosis induction through engagement of death receptors such as Fas on the surface of infected cells and 3) secretion of cytokines such as TNF α and IFN γ that are known to induce antiviral gene expression in addition to enhancing apoptosis in recipient cells.

A body of evidence suggests that CD8⁺ T cells are important in controlling HIV-1 replication. Firstly, the expansion of CD8⁺ T cells in AHI is temporally associated with control of viremia^{100,101}; furthermore, CD8⁺ T cells rapidly drive selection for escape mutations in HIV-1 during AHI^{102–104}. Elite controllers, who maintain low viral load in the absence of antiretroviral therapy, maintain polyfunctional CD8⁺ T cell responses to HIV-1 epitopes throughout infection¹⁰⁵. In addition, certain HLA class I alleles (i.e. B35, B27, B57) are linked to fast or slow disease progression^{106–109}. Lastly, when CD8⁺ T cells are depleted in the macaque SIV challenge model, control of viremia during acute infection is hindered¹¹⁰.

Many studies have attempted to dissect aspects of the HIV-1-specific CD8⁺ T cell response that influence the control of HIV-1 replication. HIV-1 Gag-specific responses seem to consistently associate with delayed disease progression and lower viral loads, whereas responses targeted towards variable proteins such as Nef or Env tend to associate with poor prognosis^{111–115}. However, it should be noted that in some studies polyfunctional Nef-specific immune responses were associated with control^{116–118}. These conflicting results may be partially explained by the apparent importance of targeting particular conserved epitopes which are unable to escape without a significant fitness cost to the virus^{119–122}. Alternatively, some have hypothesised that Gag epitopes are protective because Gag (and Pol) epitopes from incoming virions are presented early before Nef-mediated downregulation of HLA class I occurs^{123–127}. Polyfunctionality has also been associated with viral

control in various studies^{105,116,128}. Lastly, resistance to apoptosis has been suggested as a factor involved in protection by CD8⁺ T cells from EC¹²⁹.

On the whole however, CD8⁺ T cells display poor function during chronic HIV-1 infection and an 'exhausted' phenotype. CD8⁺ exhaustion entails loss of effector functions and decrease in proliferative capacity as well as the upregulation of markers such as PD-1, CTLA-4 and LAG-3 which dampen immune activation¹³⁰. These receptors are upregulated on CD8⁺ T cells during chronic HIV-1 infection and this dysfunction is thought to contribute to eventual loss of viral control^{131–133}.

On the other hand, the limited data available suggest that CD8⁺ T cells induced during AHI are not exhausted to the same extent as they are during chronic HIV-1 infection^{134,135}, although Eller *et al* identified a functionally impaired CD8^{dim} population which emerged coincidentally with the cytokine storm during AHI¹³⁶. These initial CTL responses during AHI were shown to determine the set point viral load and subsequent CD4⁺ T cell decline in chronic infection¹³⁷. More recent studies additionally suggest that the magnitude, kinetics and specificity of the CTL response during AHI are predictive of set point viral load^{138,139}. Hence the first HIV-1-specific CTL responses during AHI are crucial for subsequent viral control. For vaccination purposes, there is a window of opportunity for efficacious CTL responses to eliminate or effectively contain virus replication during the initial stages of AHI, preventing the high-level virus replication and associated damage to the host immune response that subsequently undermine viral control.

Aspects of the CTL response during AHI are revisited in section 1.12 after MHC molecules, antigen processing and presentation as well as DCs and their role in CD8⁺ T cell priming have been introduced.

1.8 The Major Histocompatibility Complex (MHC) gene region

In vertebrates, the MHC gene region encodes a set of proteins which are essential for adaptive immune recognition of foreign antigens and related immune functions. In humans, the MHC region is located on the short arm of chromosome 6 and contains at least 224 gene loci, 128 of which are predicted to be expressed¹⁴⁰. Amongst the most studied genes in the region are MHC proteins which bind peptides derived from cellular and foreign proteins for recognition by TCRs expressed by T cells (and also receptors expressed on other cell types such as NK cells). MHC molecules, in humans called human leukocyte antigens (HLA), are some of the most polymorphic proteins known, with >15,000 unique HLA allele sequences listed in the IPD-IMGT/HLA Database¹⁴¹. The MHC gene region is subdivided into 3: MHC class I, MHC class II and MHC class III (Figure 1.4). The MHC class I region encodes the ‘classical’ HLA class I (Ia) proteins HLA-A, HLA-B, HLA-C, the ‘non-classical’ HLA class I (Ib) proteins HLA-E, HLA-F and HLA-G as well as proteins such as transporter associated with antigen processing (TAP) and tapasin which ensure optimal loading of

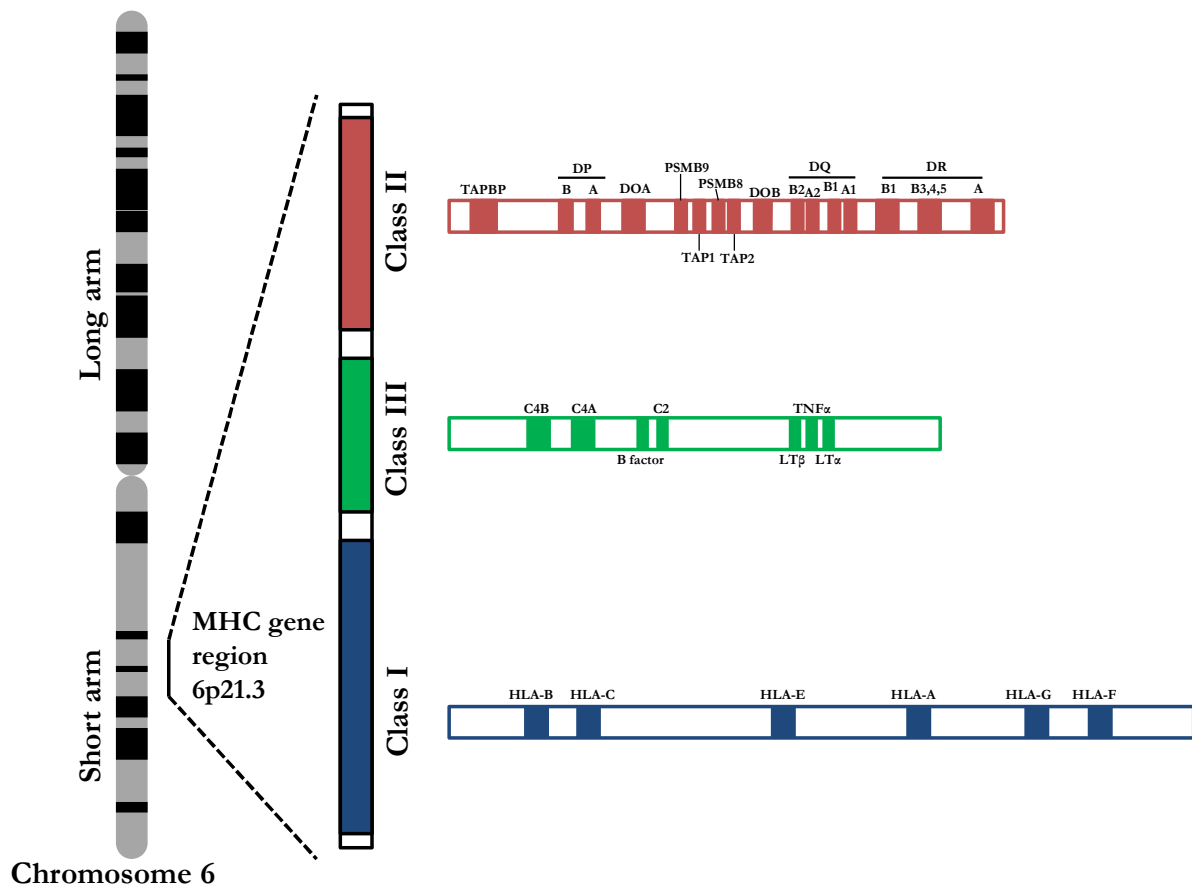


Figure 1.4. The MHC gene locus on chromosome 6. A non-exhaustive list of some of the genes encoded within the MHC gene region are shown for the Class I, Class II or Class III regions. Diagram modified from⁸⁹⁶.

HLA with peptide antigens in the endoplasmic reticulum (ER). The MHC class II region encodes classical HLA class II proteins HLA-DR, HLA-DP, and HLA-DQ, and the non-classical HLA-DM and HLA-DO. The MHC class III region generally encodes proteins unrelated to antigen presentation molecules, such as complement factors, cytokines (e.g. TNF α ,) and heat shock proteins.

There are another group of molecules which are related to HLA in structure that are encoded on chromosome 1. The CD1 family and MR1 proteins associate with particular lipids or small metabolites and are recognised by invariant/semi-invariant T cells of various types¹⁴². As it is beyond the scope of this thesis, I will not describe this field of antigen presentation and T cell immunology further.

1.8.1 HLA class I structure

The classical HLA-A, -B and -C molecules are expressed co-dominantly on all nucleated cells, meaning that up to 6 classical alleles can be expressed by one individual. The mature HLA class I complex is formed as a heterodimer between the polymorphic membrane-anchored 45 kilodalton (kDa) heavy chain and an invariant 12 kDa light chain called β 2-microglobulin (β 2m) (Figure 1.5A). The heavy chain is encoded by 8 exons¹⁴³. Exon 1 encodes the signal sequence peptide required for targeting to the secretory pathway, exons 2, 3 and 4 encode the α 1, α 2 and α 3 domains, exon 5 encodes the transmembrane region and the first 5 amino acids of the cytoplasmic domain and exons 6 and 7 encode the rest of the cytoplasmic domain. Exon 8 encodes the 2nd and 3rd nucleotide of the C-terminal residue and the 3' untranslated region (UTR) of the mature RNA in HLA-A and HLA-C, whilst in HLA-B exon 8 encodes only the 3' UTR. β 2m interacts with all 3 extracellular domains of the heavy chain to stabilise the mature protein once a receptive peptide is bound. The cytoplasmic tail of the heavy chain is important for intracellular trafficking of the peptide-HLA complex.

The α 1 and α 2 extracellular domains are similar in structure and together contribute 4 strands to form an 8-stranded antiparallel β -sheet which is neighboured on either side by an α -helix structure to form the peptide binding groove of approximately 30 angstroms (Å) in length and 12 Å in width¹⁴⁴. The binding cleft narrows at either end where bulky amino acid side chains close the groove, meaning that peptides >11 amino acids in length are generally thought not to bind unless they adopt an

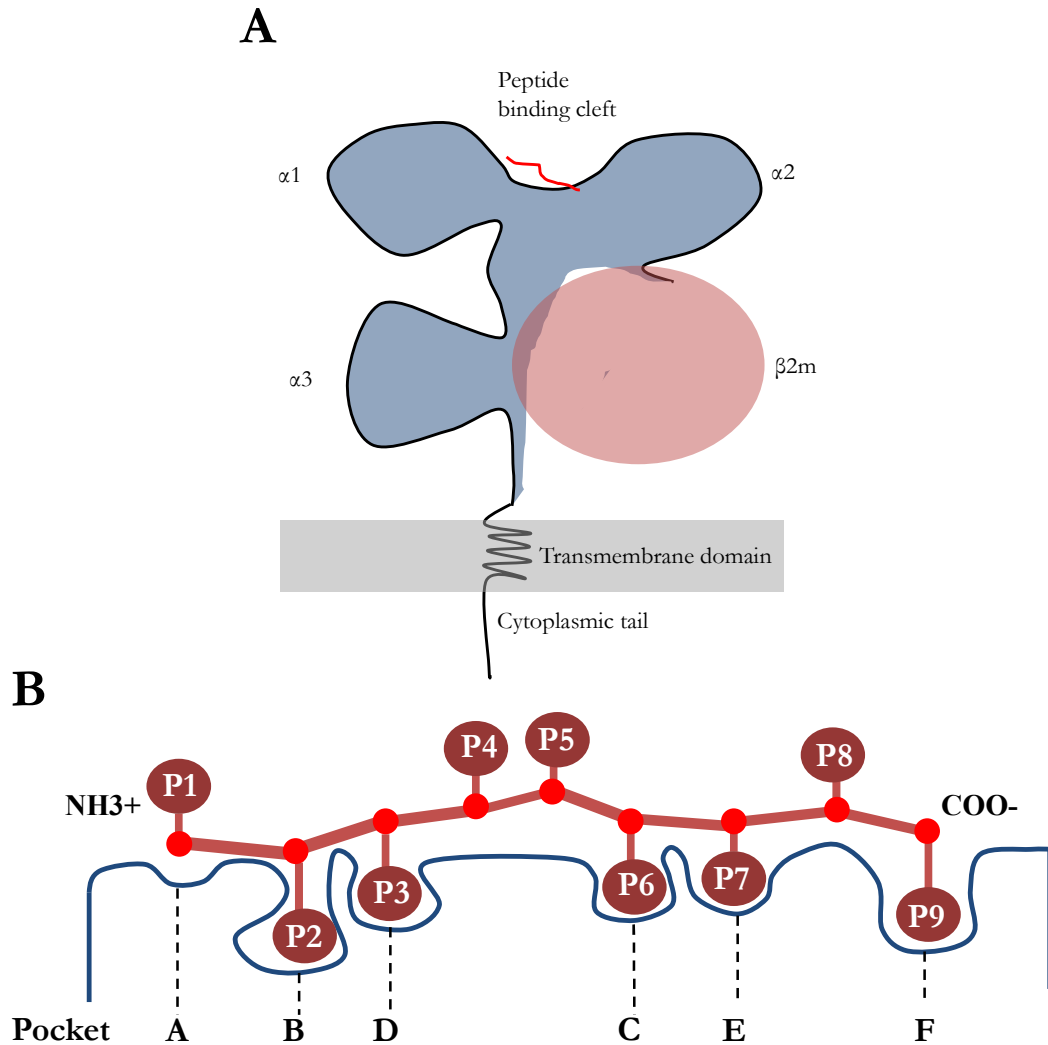


Figure 1.5. *Schematic structure of HLA class I molecules.* **A.** Schematic structure of the HLA class I heavy chain (with $\alpha 1$, $\alpha 2$ and $\alpha 3$ domains) and $\beta 2m$ heterodimer in complex with a peptide on the cell surface. **B.** Section of the HLA class I peptide binding groove. Peptide side chains face inwards or outwards from the binding groove and interact with the side pockets A-F which are of variable depth. Diagram modified from⁸⁹⁷.

unusual centrally-bulged conformation, which may be less stable than the configuration of conventional shorter peptides of 8-11 amino acids.

Most of the extreme polymorphism reported in HLA class I molecules is located within residues pointing into the groove or outwards from the α -helices, which gives each allele distinctive peptide- and/or TCR-binding properties^{143,144} (Figure 1.5B). The uneven binding groove surface resulting from HLA residues protruding into the cleft gives rise to distinct ‘pockets’ where peptide amino acid side chains can interact with complementary side chains in the HLA molecule. In addition to the A and F pockets, which respectively contact the N- and C-terminus of the peptide, other pockets are located along the central portion of the groove. These polymorphic pocket residues therefore impose

characteristic amino acid binding preferences at particular positions within the peptide sequence called 'binding motifs'. Peptide residues which are restricted to the usage of one or a few specific amino acid side chains are termed 'anchor residues'. Most commonly, these anchor residues are at position 2 and at the C-terminus (position 9 for a 9 amino acid peptide) of the peptides (corresponding to the B and F pockets), although particular HLA class I alleles may require anchor residues at position 3 or 5 (e.g. HLA-B*08:01 prefers basic amino acid side chains at position 5). Although the motifs restrict peptide diversity at anchor residues, binding motifs are in fact quite promiscuous, as thousands of peptides are able to bind any single HLA class I allele.

Although computational studies have predicted that particular HLA class I alleles vary in the size of their potential peptide repertoire¹⁴⁵, limited experimental data exist which directly compare the repertoires of different alleles and their subsequent immunogenicities¹⁴⁶. Individual HLA alleles are also associated with varying surface expression levels, as HLA-C is thought to be expressed on the cell surface at levels about 15 times lower than HLA-A and HLA-B¹⁴⁷.

The non-classical HLA class Ib molecules such as HLA-E are surface-expressed at significantly lower levels than HLA class Ia proteins¹⁴⁷. Like HLA class Ia, HLA-E, -F and -G are all found associated with β 2m as heterodimers and HLA-E and HLA-G are known to bind peptides. Whilst HLA-G is primarily expressed in the developing placenta, HLA-E is expressed at low levels by all nucleated cells and plays a specialised role in recognition by NK cells. Mature HLA-E complexes predominantly binds hydrophobic peptides derived from the signal sequence of HLA class Ia molecules (e.g. VMAPRTLTL and variants) which, when displayed on the cell surface, interact with NK-expressed CD94/NKG2A heterodimers to inhibit NK cytotoxic function¹⁴⁸. In the absence of these peptide-HLA-E complexes (e.g. following infection of a cell with pathogens which downregulate HLA class Ia), NK cells are activated to release cytotoxic granules to kill infected/stressed cells. Thus HLA-E represents a mechanism for NK cells to monitor the HLA class I environment and detect infections or immune perturbations. In fact, alternative peptides bound to HLA-E (e.g. QMRPVSRVL from Hsp60) are not recognised by CD94/NKG2A and instead activate NK cells for cytotoxic function¹⁴⁹.

The structure of HLA-E is remarkably similar to that of HLA class Ia molecules, with the extracellular $\alpha 1$ and $\alpha 2$ domains forming a peptide binding groove of similar dimensions¹⁵⁰. In contrast to HLA class Ia proteins, signal sequence-derived peptides seem to utilise all 6 hydrophobic pockets within the HLA-E binding groove, resulting in a deep positioning of the signal peptide. Conversely, recent simulations of HLA-E in complex with non-canonical peptides reveals that these peptides sit considerably higher in the binding cleft than the hydrophobic signal peptide¹⁵¹ (although crystal structures to confirm this have not yet been reported).

1.8.2 HLA class II structure

The mature classical HLA class II proteins (HLA-DR, HLA-DP and HLA-DQ) are formed as heterodimers of an α and a β chain which together create the peptide binding groove. Although the HLA class II binding groove bears structural similarities to HLA class I, the ends of the class II groove are open due to secondary structure alterations and replacement of large amino acids with smaller side chains¹⁴⁴. This characteristic allows HLA class II to accommodate peptides (predominantly between 14-20 amino acids in length) which overhang either side of the binding groove.

1.9 Antigen processing and presentation

The remarkable diversity in HLA peptide binding motifs within individuals and across populations favours the hypothesis that ever-changing pathogens have forced the HLA system to co-evolve in order to allow recognition of diverse antigens by T cells¹⁵². Before this recognition occurs, a multitude of steps must take place to partially degrade proteins into antigenic fragments, which are then efficiently loaded onto peptide-receptive HLA molecules to be transported to the cell surface. Generally speaking, HLA class I presents peptides derived from endogenous proteins (for recognition by CD8⁺ T cells) whereas HLA class II is specialised for presentation of exogenous proteins on professional antigen presenting cells (APCs) such as DCs, macrophages and B cells (for recognition by CD4⁺ T cells). The process by which these molecules come to acquire complementary peptides is termed 'antigen processing and presentation'.

1.9.1 HLA class I antigen processing pathway overview

HLA class I molecules typically acquire their peptide ligands in the ER. These peptides are generated by degradative processes within the cell cytoplasm or organelles. Subsequently, antigenic fragments are transported into the ER where peptide-receptive HLA class I/β2m heterodimers await the arrival of peptide species capable of stabilising the complex (pathway shown in Figure 1.6). Various peptide exchange/editing and trimming processes may then occur before the mature peptide-HLA class I complex is transported through the Golgi network to the cell surface for recognition by CD8⁺ T cells or NK cells. The HLA class I antigen processing/presentation pathway is described in detail in subsequent sections (below) after a brief overview of the HLA class II presentation pathway is given.

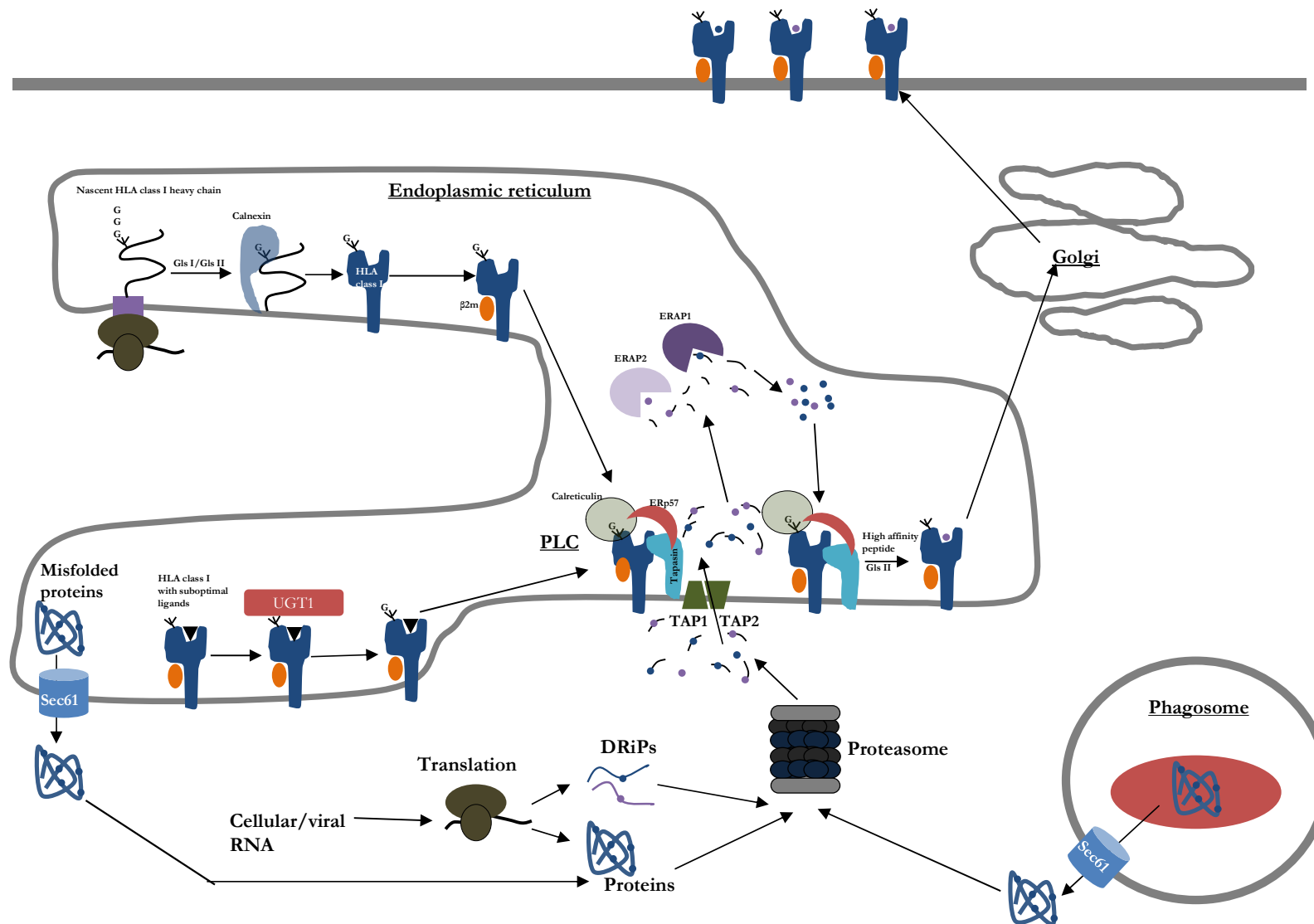


Figure 1.6. Overview of the HLA class I antigen processing and presentation pathway. Nascent HLA class I heavy chains are translocated into the ER lumen and bound by chaperones then incorporated into the peptide loading complex (PLC). Peptides processed in the cytoplasm by the proteasome are transferred to the ER by the action of TAP then further trimmed by ERAP1/ERAP2. Binding of high affinity peptides allows fully formed HLA class I complexes to move to the cell surface via the Golgi secretory pathway. Diagram modified from¹⁵³. Glis; glucosidase, PLC; peptide loading complex, DRiPs; defective ribosomal products, ERAP; endoplasmic reticulum aminopeptidase, TAP; transporter associated with antigen processing, $\beta 2m$; beta-2-microglobulin.

1.9.2 HLA class II antigen processing pathway overview

HLA class II molecules are assembled in the ER and then acquire their peptide ligands within the endosomal system¹⁵³. The HLA class II α and β chains are first translocated into the ER by virtue of an N-terminal signal sequence which directs ribosomes to the ER. The two subunits are then stabilised in the ER by interaction with the invariant chain chaperone (Ii; also known as CD74 when surface-expressed). Ii prevents peptides from binding to HLA class II in the ER, but a di-leucine motif in the cytoplasmic N-terminal domain of Ii allows sorting of the complex to endosomal compartments¹⁵⁴. Once in late endosomes, Ii is processed by non-cysteine proteases, cysteine proteases and finally cathepsin S to yield a minimal class II-associated Ii peptide (CLIP) of about 20 amino acids in length which lies in the peptide binding groove¹⁵⁵. The conserved non-classical HLA class II molecule HLA-DM then interacts with CLIP-associated HLA class II to promote dissociation and exchange of CLIP for another peptide present in the late endosome^{156,157}. The biological function of the other non-classical HLA class II molecule HLA-DO in antigen presentation remains unclear, although HLA-DO is known to competitively inhibit HLA-DM activity^{158,159}.

The protein precursors of peptides binding to HLA class II are typically acquired by phagocytosis of particulate matter, receptor-mediated endocytosis or macropinocytosis then transported to early/late endosomes or lysosomes where cathepsins and other proteases cleave the precursors into peptide fragments capable of stabilising mature HLA class II complexes. Alternatively, endogenously-expressed proteins processed in endosomes/lysosomes can be substrates for HLA class II presentation. Furthermore, cytoplasmic and nuclear proteins can be delivered to the class II pathway through the process of macroautophagy, whereby autophagosomes formed by engulfment of organelles and cytoplasmic/nuclear material are fused with endosomes¹⁶⁰.

1.9.3 Sources of peptides for HLA class I presentation

The source of peptides for MHC class I presentation has been controversial. The leading hypothesis states that rapidly degraded defective ribosomal products (DRiPs) are a major source of peptide ligands for the HLA class I presentation pathway¹⁶¹. The finding that ~30% of newly synthesised proteins are rapidly degraded supported this hypothesis¹⁶² and multiple studies have confirmed that rapidly degraded proteins seem to be a significant source of MHC class I ligands^{163–165}. However,

critics of the DRiP hypothesis suggest that a true DRiP has never been unequivocally identified and that the short 30 minute window for DRiP presentation implies a precursor half-life of around 15 minutes which does not fit with current knowledge of kinetics of protein quality control¹⁶⁶. In fact, other studies have suggested that mature and stable proteins can also be a significant source of HLA class I peptides^{167–169}. Thus it appears that both rapidly-degraded proteins/DRiPs and mature/retired proteins can be used as substrates for HLA class I presentation, and further research may be able to delineate the exact contribution of each pathway under different circumstances.

HLA class I peptides can also be derived from non-conventional translation of short open reading frames (ORFs) within 5' and 3' 'untranslated' regions (UTRs), introns, intergenic regions or even from alternative initiation of translation at non-AUG start codons within mature RNAs^{170,171}. In fact, a proteogenomic study of peptides presented on HLA class I by B cell lines showed that ~10% of all peptides were derived from so-called 'non-coding' sequences¹⁷². Another recent study demonstrated that the cellular integrated stress response altered translation such that peptides derived from short ORFs within the 5' UTR of particular genes were produced and could be presented on HLA class I for recognition by CD8⁺ T cells¹⁷³. In HIV-1-infected cells, generation of CTL targeting cryptic epitopes derived from alternative ORFs within the HIV-1 genome has been demonstrated^{174–176}. Lastly, there is some evidence to suggest that HLA class I-bound peptides arise preferentially from abundant transcripts or transcripts containing miRNA response elements¹⁷⁷.

1.9.4 Antigen processing in the cytoplasm

Proteins synthesised in the cytoplasm are constantly fed into the HLA class I presentation pathway. Additionally, misfolded proteins in the ER are retrotranslocated into the cytoplasm by the ER-associated degradation (ERAD) pathway¹⁷⁸. This creates a paradoxical situation: misfolded ER proteins must be transported to the cytoplasm before their degradation products are then relocated to the ER for loading on HLA class I.

In the cytoplasm, intact or misfolded proteins are bound by Hsp70/Hsp90 α chaperones which recruit the E3 ubiquitin ligase C-terminus of Hsc70-interacting protein (CHIP) to ubiquitinate Hsp-associated protein and transfer them to the proteasome for degradation^{153,179}. The 26S proteasome is a complex consisting of at least 30 subunits which recognises and proteolytically cleaves

polyubiquitinated protein into oligopeptides¹⁸⁰. The 26S proteasome is comprised of a 20S core particle (CP) and two 19S regulatory complex (RP) caps. The 20S CP is a cylindrical structure composed of multiple α and β subunits which exert caspase-, trypsin- and chymotrypsin-like activities on peptide substrates, whereas the 19S RP serves to translocate precursors to the active sites¹⁸¹. The cleavage specificities can be further altered by the incorporation of 3 different subunits (LMP2, LMP7 and MECL-1) into ‘immunoproteasomes’, which promotes the generation of peptides containing hydrophobic C-terminal anchors for more diverse HLA class I presentation^{182–184}. Immune cells constitutively express immunoproteasomes and IFN γ is also known to upregulate expression of immunoproteasome subunits^{185,186}. Additionally, thymoproteasomes containing subunits specific to thymic epithelial cells have been reported, which may enrich for low affinity TCR ligands during positive selection in the thymus^{187,188}.

Although the proteasome initially seemed to be crucial for the generation of most HLA class I ligands¹⁸⁹, proteasome-independent production of peptide precursors has also been shown to occur. For example, certain HLA alleles such as HLA-A*03:01 were shown to present normally after proteasome inhibition¹⁹⁰, indicating that proteasome inhibition could be compensated for by other cytosolic proteolytic mechanisms¹⁹¹. However, pharmacological inhibition of the proteasome often predominantly affects the chymotrypsin-like activity, and it can still cleave particular peptides through caspase- and trypsin-like activities for HLA class I presentation¹⁹². Nevertheless, cytosolic proteases such as tripeptidylpeptidase II (TPPII) have been shown to be required for production of particular peptides^{193,194}. However the balance of evidence suggests that TPPII does not play a major role in bulk epitope production¹⁹².

Other cytosolic proteases such as insulin-degrading enzyme, nardilysin, thimet oligopeptidase, DPP9, caspases, leucine aminopeptidase (LAP), bleomycin hydrolase (Blmh), and dipeptidylpeptidase III (DPPIII) have been implicated in the generation of HLA class I ligands *in vitro*^{195–199}. However, after knocking out many of these aminopeptidase genes in mice, a neutral or negative effect on MHC class I complex production was observed^{200–202}. Thus protection of proteasomal products from degradation by chaperonins such as TRiC may be important for antigen presentation²⁰³, but a more recent study suggests that TRiC only binds a limited repertoire of peptides *in vivo*²⁰⁴. Although the vast majority

of peptides will be rapidly degraded by cytosolic proteases²⁰⁵, some peptide species are translocated into the ER where further processing and peptide loading can take place.

1.9.5 Antigen processing and peptide loading in the ER

Peptide fragments generated in the cytosol are transported into the ER by TAP. TAP is a heterodimer composed of two subunits; TAP1 and TAP2. Cytosolic loops in each subunit mediate peptide binding and ATP hydrolysis allows peptide translocation through the channel (6 transmembrane α -helices)²⁰⁶. TAP displays a binding preference for peptides between 8-16 amino acids in length^{207,208}, but can transport longer peptides with greater selectivity^{209,210}. Once transported into the ER, peptides can interact immediately with the peptide loading complex (PLC) if they contain the correct binding motif.

Before this can happen however, many peptides require trimming by ER aminopeptidase-1 (ERAP1) or ERAP2 in order to generate peptides of the correct length for binding to HLA²¹¹. ERAP1 and ERAP2 trim peptides at the N-terminus to optimal lengths of 8-10 amino acids²¹². ERAP1/2 are thought to exert their actions primarily on free peptides, but peptides bound to HLA class I can also be trimmed at a slower rate by ERAP1/2²¹³. The significance of ERAP for the peptide repertoire presented by MHC class I has been highlighted by studies showing that ERAAP (the mouse homologue of ERAP1) knockout mice present significantly longer and diverse immunogenic peptides²¹⁴⁻²¹⁶. Peptides which are not subsequently bound to HLA in the PLC can be degraded further by aminopeptidases²¹⁷, protected by chaperones²¹⁸, or translocated to the cytoplasm through the aforementioned ERAD pathway.

The PLC is composed of the HLA class I heavy chain, β 2m, calnexin, tapasin, ERp57, calreticulin and TAP¹⁵³. After translocation into the ER lumen, the HLA class I heavy chain is glycosylated and bound by the chaperone GRP-78/BiP²¹⁹, then glucosidases I and II trim two glucose molecules off the heavy chain to allow calnexin to bind the monoglucosylated form²²⁰. After initial folding, β 2m associates with the heavy chain, allowing calnexin to be replaced by calreticulin²²¹. Tapasin facilitates recruitment of ERp57, which is essential for PLC stability²²². Tapasin also promotes the association of HLA heavy chain/ β 2m to TAP which locates HLA in the vicinity of the peptide source²²³. Furthermore, tapasin plays a crucial role in peptide editing by replacing low affinity

peptides with higher affinity peptides^{224–226}. The mechanism for tapasin-mediated peptide editing has remained elusive until recently when Fiset *et al* used computational simulations to suggest that tapasin exerts forces on the HLA molecules to maintain the groove in an open conformation²²⁷. Fiset *et al* suggested that these forces could only be overcome when high affinity peptides bound the groove and induced dissociation of tapasin and the PLC. Furthermore, various HLA polymorphisms contribute to differential tapasin dependency and therefore altered peptide repertoire or surface expression²²⁸. Another molecule structurally related to tapasin, TAPBPR, has also recently been shown to have peptide-editing activities, although TAPBPR does not associate with the PLC and may perform its activities in cellular compartments other than the ER^{229,230}. A final quality control mechanism in the ER is mediated by UGT1 which recognises peptide-HLA class I complexes containing suboptimal peptides and reintroduces them to the PLC²³¹. Fully formed HLA class I complexes are then allowed to reach the cell surface through the secretory pathway for recognition by CD8⁺ T cells and NK cells.

1.9.6 Recycling/degradation of HLA class I complexes

At the cell surface, acidic pH or low affinity peptides can result in the dissociation of the HLA class I complex and subsequent internalisation of the free heavy chain²³². HLA class I can be targeted for lysosomal degradation by the MARCH4 and MARCH9 ubiquitin ligases²³³. On the other hand, some of these so-called ‘open-conformers’ can access the endosomal system and be re-loaded with peptides²³⁴. This type of HLA class I recycling may have importance for the process of cross-presentation.

1.9.7 Cross-presentation

On most cell types, MHC class I presents peptides from endogenously-synthesised proteins which are recognised by activated CD8⁺ T cells and NK cells. Naïve CD8⁺ T cells must be activated by professional APCs (i.e. DCs; see section 1.10). When such APCs are not directly infected with a pathogen they must present exogenous antigen on HLA class I through a specialised process called cross-presentation²³⁵. Introduction of protein antigens to the body during vaccination must also rely on the process of cross-presentation, as otherwise CD8⁺ T cells specific for such antigens would

never be activated. Although progress has been made in elucidating the mechanisms of cross-presentation in recent years, much still remains unknown.

1.9.7.1 Antigen uptake for cross-presentation

Exogenous antigen must be ingested by the APC before cross-presentation can occur. Phagocytosis of particulate antigens, receptor-mediated endocytosis and pinocytosis may all contribute to cross-presentation²³⁶. Macropinocytosis was shown to promote antigen export to the cytosol in both macrophages and bone marrow-derived DCs in mice^{237,238}. Numerous receptors have been reported to capture exogenous antigen leading to efficient cross-presentation. For example, HIV-1 p24 antigen fused to an anti-DEC-205 antibody was shown to be cross-presented more efficiently by monocyte-derived DCs (MDDCs) than p24 fused to anti-DC-SIGN antibody, despite both receptors mediating antigen internalisation²³⁹. DEC-205 is a receptor for apoptotic or necrotic antigen²⁴⁰ and has generated substantial interest as a target for inducing strong T cell responses in both mice and humans²⁴¹. Similarly, targeting of antigen to Clec-9a, which is known to be important for uptake of necrotic antigen by binding to exposed actin filaments^{242,243}, shows promise in inducing both strong humoral and cellular immune responses *in vivo*^{241,244}. Other receptors such as the mannose receptor (MR), DC immunoreceptor (DCIR), LOX-1 and LDLR1 have also been reported to play a role in cross-presentation^{245–248}.

Differential expression of these receptors is thought to reflect the relative efficiencies with which DC subtypes cross-present antigen. In mice, it is generally accepted that CD8⁺/CD103⁺ DC which express Clec-9a and XCR-1 are the major cross-presenting cell subset²⁴⁹. The equivalent subset in humans, XCR1⁺BDCA3⁺ DC, was shown to induce greater activation of a HIV-1 Pol-specific T cell line than other DC cell subsets²⁵⁰. However, the differential ability to cross-present antigen by various DC subsets in humans has been controversial. Some studies support the idea that BDCA3⁺ DC are more efficient cross-presenters^{251,252}, whereas others conclude that human DC subsets are equally equipped to cross-present antigen^{253,254}. These conflicting results may be partially explained by the different types of antigen used in these studies. For example, the selective expression of Clec-9a on BDCA3⁺ DC explains their reported superiority in capturing and cross-presenting necrotic antigen^{251,252}. On the other hand, soluble antigen may be taken up through alternative endocytic

routes and the maturation status of DC subtypes is also likely to play a role^{235,255}. Indeed, a direct comparison of different DC subsets demonstrated that MDDC efficiently cross-presented soluble antigen whilst BDCA3⁺ DCs were specialised in cross-presenting cellular antigen²⁵⁶.

1.9.7.2 Mechanisms of cross-presentation

Two pathways for loading of exogenous antigen onto MHC class I are proposed; the ‘cytosolic’ and the ‘vacuolar’ pathways²³⁵ (Figure 1.7). In the former, antigen is exported from phagosomes to the cytosol where it enters the classical MHC class I pathway. Thus the cytosolic pathway is sensitive to proteasomal inhibitors and TAP-dependent. In contrast, the ‘vacuolar’ pathway is insensitive to proteasomal inhibitors and TAP-independent, and peptide-MHC complexes may be formed from MHC class I present in phagosomes and transported directly to the cell surface.

Limited antigen degradation in phagosomes/lysosomes seems to be a requirement for cross-presentation^{257,258}. In fact, the superior cross-presentation capacity of DC in comparison to macrophages is thought to be partly explained by the higher endosomal pH observed in DC, which protects antigens from excessive degradation^{257,259}. Additionally, particular antigens such as ovalbumin may be targeted to early endosomes with higher pH through engagement of the mannose receptor, therefore enhancing cross-presentation²⁶⁰. Even antigens targeting distinct binding sites on the same receptor can be routed to different cellular compartments, as was shown for DC-SIGN²⁶¹.

The cytosolic pathway also requires a mechanism for antigen to be translocated from the phagosome to the cytosol. Although numerous mechanisms have been hypothesised, recruitment of ERAD components to phagosomes for export of antigens to the cytosol is thought to be involved in the cytosolic pathway^{262–264}. In fact, the ERAD component Sec61 was recently shown to be required for efficient cross-presentation²⁶⁵. The presence of ER-associated machinery in phagosomes also has implications for the vacuolar pathway, which requires HLA class I loading within the phagosomes.

Many components of the PLC have been detected in phagosomes, and are transported there in a Sec22-dependent manner, indicating that HLA class I could be loaded in such a compartment^{263,266}. However, HLA class I does not seem to absolutely require Sec22 for transport to endosomal/phagosomal compartments. Instead, HLA class I could be recycled from the cell surface

into endosomes²⁶⁷ or even shuttled by association with Ii/CD74 from the ER to endosomes²⁶⁸. Antigens in the vacuolar pathway may be degraded by proteases present in the endosomal system such as insulin-regulated aminopeptidase (IRAP) or cathepsin S^{269,270}.

Although the relative contribution of the cytosolic and vacuolar pathways to cross-presentations is not well known, the cytosolic pathway may be more dominant as evidenced by experiments where proteasome inhibition significantly abrogates immune responses against exogenous antigen in mice²⁷¹.

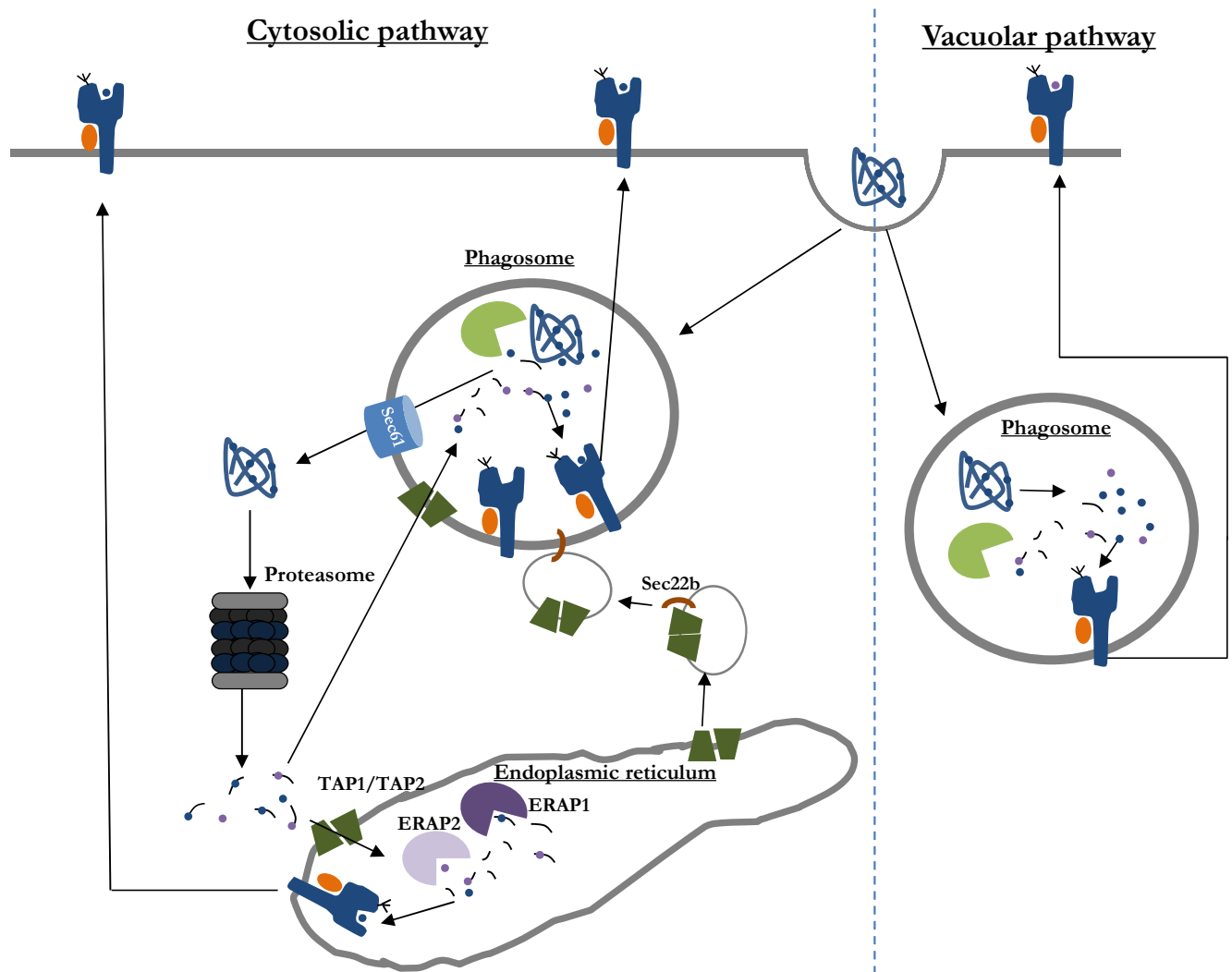


Figure 1.7. Intracellular pathways of cross-presentation. Internalised antigen within phagosomes can be processed via one of two pathways. In the cytosolic pathway antigen is exported to the cytoplasm via Sec61 then digested by the proteasome. Peptide fragments are transported into the ER or the phagosome via TAP1/TAP2 then loaded onto HLA class I and transported to the cell surface for presentation to CD8⁺ T cells. In the vacuolar pathway, antigen is degraded by proteases/cathepsins within the phagosome and loaded onto HLA class I then transferred to the cell surface. Diagram adapted from²³⁵. TAP; transporter associated with antigen processing, ERAP; endoplasmic reticulum aminopeptidase.

1.9.8 Cross-dressing

Another unusual form of antigen presentation which could potentially be harnessed for inducing T cell responses is ‘cross-dressing’²⁷². Cross-dressing involves the transfer of mature peptide-HLA complexes between cells, such that the recipient ‘cross-dressed’ cell can present these complexes to T cells. Although the relevance of this process to development of immunity *in vivo* is still debated, a number of studies in mice have confirmed that cross-dressing can occur²⁷³.

Cross-dressing may be mediated through three separate, mutually compatible mechanisms. First, cells may ‘nibble’ membrane fragments from neighbouring cells in a process called ‘trogocytosis’ first characterised in T cells^{274–276}. Secondly, HLA-containing vesicles released by donor cells may be captured by recipient cells for HLA transfer^{277,278}. Lastly, large protruding structures called tunnelling nanotubes have been shown to transfer MHC complexes between cells²⁷⁹. Given enough antigen, cross-dressed DC are capable of activating naïve T cells, a crucial process for the induction of immunity against viruses^{280,281}.

1.10 Dendritic cells as orchestrators of the adaptive immune system

DCs are a relatively rare and diverse population of cells that play a crucial role in linking innate and adaptive immunity. DCs are specialised in sensing pathogens through various PRRs and play an essential role as professional APC during T cell priming²⁸². Immature DCs sample antigen from the environment until they are matured by exposure to PRR ligands. Mature DCs then move to lymph nodes where they upregulate co-stimulatory molecules and HLA class I/II presenting antigenic peptides in order to prime naïve T cells.

Broadly speaking, DCs arising from common DC precursors (CDPs) are classified as mDCs (myeloid DCs) or pDCs (plasmacytoid DCs), and DCs arising from monocytes are called MDSCs²⁸³. pDCs are specialised for sensing of pathogens through TLR7 and TLR9 and production of type I IFNs to promote antiviral functions²⁸⁴. mDCs are a remarkably diverse population of cells located in lymph nodes or distal non-lymphoid sites throughout the body. mDCs are able to efficiently ingest antigen, home to lymph nodes in a CCR7-dependent manner²⁸⁵ then cross-prime naïve T cells^{286–288}. mDCs are further classified into XCR1[−] and XCR1⁺ DCs, both of which are thought to be able to

prime naïve T cells in humans²⁴⁹. Although pDCs²⁸⁹, neutrophils²⁹⁰ and liver sinusoidal endothelial cells (LSECs)²⁹¹ have been shown to prime naïve T cells under certain circumstances, the balance of evidence (in particular, the observations that *in vivo* ablation of DCs abrogates cross-priming and selective expression of MHC class I on DCs is sufficient for cross-priming^{288,292}) indicates that DCs are the most important cell type involved in activation of T cell immunity *in vivo*.

1.10.1 CD8⁺ T cell priming

When a naïve CD8⁺ T cell encounters its cognate peptide-HLA class I complex in lymphoid organs, it can undergo one of two processes; 1) priming and expansion into a clonal population of effector cells or 2) tolerisation, acquisition of a state of impaired responsiveness to stimulation or deletion from the T cell repertoire. This cell fate decision is dictated by a series of molecular signals transmitted by the interacting APC which are integrated by the naïve T cell.

To be able to prime optimal expansion of naïve CD8⁺ T cells, DCs must be matured following engagement of PRR or detection of inflammatory molecules in order to upregulate HLA class I and co-stimulatory molecules^{293–295}. Matured DCs then migrate to the subcapsular sinus or interfollicular regions of lymph nodes, where they interact with naïve T cells^{296,297}. In addition to TCR signalling after engagement with specific peptide-HLA complexes, CD8⁺ T cells also require co-stimulation primarily through interaction of the constitutively-expressed CD28 with CD80 or CD86 on DCs²⁹⁸. CD27 is also expressed on naïve CD8⁺ T cells and, by binding to APC-expressed CD70, can enhance the effects of CD28 costimulation²⁹⁹.

A third co-stimulatory cytokine signal is required if the CD8⁺ T cell is to avoid tolerisation^{300,301}. IL-12, IL-15 and type I IFNs have been identified as the predominant sources of this third costimulatory signal. Evidence for the involvement of these cytokines came from *in vitro* experiments showing that IL-12/type I IFNs could replace adjuvant in enhancing naïve CD8⁺ T cell proliferation^{302,303}. *In vivo* administration of IL-15 or type I IFN enhanced the primary expansion of CD8⁺ T cells as well as memory formation following vaccination and in mice infected with vesicular stomatitis virus (VSV) or lymphocytic choriomeningitis virus (LCMV)^{304–306}. Similarly, adoptively transferred type I IFN receptor-deficient CD8⁺ T cells in mice exhibited severely abrogated expansion and memory formation in LCMV infection of mice relative to wildtype cells, showing that a direct effect of IFNs

on CD8⁺ T cells was involved^{307,308}. Furthermore, *in vivo* peptide immunisation in the presence of the IL-12/IFNs promoted activation rather than tolerisation of CD8⁺ T cell responses^{309,310}.

What factors allow DCs to modulate expression of these costimulatory molecules and cytokines? Help from CD4⁺ T cells primarily through APC-expressed CD40 signalling induced by CD40L interaction has been demonstrated to condition DCs to promote measurable CTL responses^{311,312}. A recent investigation of T cell help-dependent herpes simplex virus (HSV)-1 infection demonstrated that CD40 signalling amplifies innate IFN-induced signalling in DCs to allow production of IL-15 which acts directly on the naïve CD8⁺ T cell³¹³. CD4⁺ T cell help seems to be necessary for priming against non-inflammatory agents, such as ovalbumin or allogeneic protein³¹⁴⁻³¹⁶. During inflammatory infections however, a differential requirement for CD4⁺ T cell help has been observed. For example LCMV-specific responses may not necessarily require CD4⁺ T cell help³¹⁷, and HIV-1-specific CTL responses could be expanded independent of CD4⁺ help but instead required IL-2 secreted by neighbouring CD8⁺ T cells³¹⁸. On the other hand, effective expansion and memory programming of CTL responses during influenza, HSV-1 and vaccinia virus infections or adenovirus vaccination has been shown to require CD4⁺ T cell help³¹⁹⁻³²². The mechanisms promoting differential requirement for CD4⁺ T cell help during distinct infections are not entirely understood, although viral immune evasion strategies to dampen type I IFN responses or costimulatory molecules have been hypothesised to modulate their helper T cell dependency³¹¹. Interestingly, two studies recently demonstrated through intravital-2-photon imaging of HSV-1- or vaccinia virus-infected mice that antigen-bearing migratory DCs first interact with CD4⁺ T cells and not CD8⁺ T cells^{323,324}. These primed CD4⁺ T cells then interacted with lymph node-resident XCR1⁺ DCs clustered with CD8⁺ T cells, presumably helping prime naïve CTL through DC licensing. Although this process may differ in lymphborne infections (where infected cells reach the lymph node), these studies provided important information about the temporal and spatial separation of CD4⁺ and CD8⁺ T cell priming.

1.11 CTL immunodominance

‘Immunodominance’ is the term used to describe the hierarchy with which the peptides available to the immune system are targeted by T cells during an immune response. Often one epitope is targeted

with a high magnitude CTL response (immunodominant) whilst other epitopes are targeted by CTL with lower frequency (subdominant) and thousands more potential epitopes are not targeted at all³²⁵. Immunodominance hierarchies are thought to place significant immune pressure on pathogens by heavily targeting particular antigenic regions. For example in HIV-1 infection the immunodominance hierarchy (together with epitope variability) was found to be a key factor determining the emergence of HIV-1 immune escape mutations (selection for which is driven by immune pressure exerted by epitope-specific CD8⁺ T cells) in one study³²⁶.

Although the factors contributing to immunodominance remain incompletely understood, many steps of the antigen processing and presentation pathway as well as T cell-intrinsic and pathogen-specific factors may be involved^{325,327}. Immunisation of mice with tumour cells expressing LCMV NP or GP proteins resulted in immunodominant responses only when antigen was cross-presented by professional APC³²⁸. The route of infection can also influence immunodominance hierarchies during influenza and LCMV infections^{329,330}. In addition, mutations within or around HIV-1 epitopes which abrogate epitope processing or binding to HLA have been shown to affect immunodominance hierarchies^{331,332}. Multiple factors in the antigen processing pathway (e.g. proteasome, TAP, ERAP1, calnexin and HLA peptide binding affinity) which influence the level of presentation of particular epitopes may impact on response immunodominance. However, a quantitative mass spectrometry study of vaccinia antigen presentation by APC *in vitro* showed that *in vivo* response immunodominance does not necessarily correlate with epitope abundance - with the caveat that *in vitro* APC may not be representative of conditions *in vivo*³³³.

On the T cell side of the equation, naïve T cell precursor frequency has been shown to correlate with response immunodominance in both mice³³⁴⁻³³⁶ and hepatitis C virus (HCV) infection of humans³³⁷, although other studies contend that the naïve T cell recruitment and expansion phase³³⁸ or the presence of high-avidity CTL³³⁹ is more important for generation of immunodominant responses. Other T cell factors which could shape immunodominance are TCR affinity or pre-existing cross-reactive memory T cells, the presence of which has been demonstrated in studies of HIV-1-reactive T cells in HIV seronegative individuals^{340,341}. Factors which may influence the immunodominance hierarchy of CD8⁺ T cell responses during AHI are discussed in the next section.

1.11.1 CTL immunodominance hierarchy in AHI

CD8⁺ T cell responses expand asynchronously during AHI³⁴². The first CD8⁺ T cell responses in AHI reach peak frequencies within a few days of peak viremia and are frequently focused on just 1-3 epitopes^{103,342} (Figure 1.8). These initially-immunodominant responses are commonly directed towards epitopes within Nef and, to a lesser extent, Gag and Env^{103,137,342–344}. These responses typically undergo a sharp decrease in magnitude as viraemia begins to decline, with responses that have driven the rapid emergence of viral escape mutations often declining to very low frequencies. They are followed by subsequent waves of responses that are typically broader in epitope-specificity and expand in a delayed fashion from 6-12 weeks following onset of symptoms³⁴². Some of these responses also drive viral escape and decline in relative immunodominance over time – hence immunodominance hierarchies are highly dynamic throughout acute and early infection (and continue to undergo shifts in immunodominance throughout chronic infection³⁴⁵).

The limited breadth of the initial response (1-3 epitopes) is thought to allow for an increased rate of selection for escape mutations during AHI. Mathematical models based on patient data predicted that the rate of evolution of viral escape mutations decreases as the number of T cell responses increases^{346,347}. Methods to induce broad initial responses would therefore be beneficial for vaccine

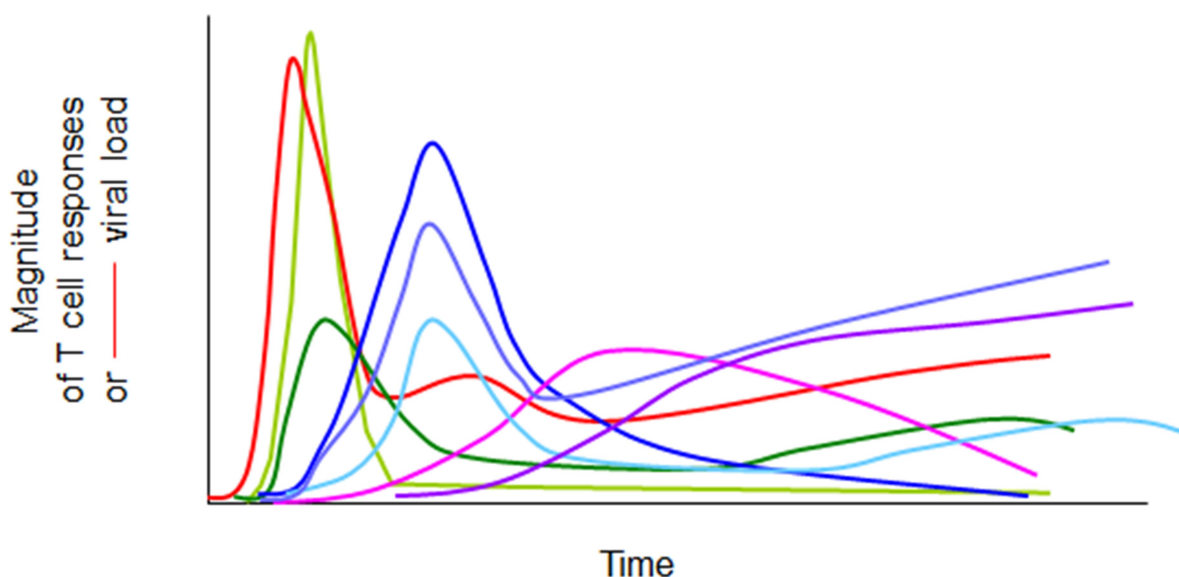


Figure 1.8. *Asynchronous expansion of CD8⁺ T cell responses during AHI.* Shown is a depiction of the kinetics of expansion of HIV-1-specific CD8⁺ T cell responses during AHI. The red line indicates the viral load. The two green lines represent the first narrow wave of HIV-1-specific CD8⁺ T cell responses which commonly target Nef epitopes. The blue lines represent the delayed second wave of HIV-1-specific CD8⁺ T cell responses of broader specificity. Note that the x and y axis units are arbitrary.

design. In fact, a novel vaccine expressing SIV proteins in a rhesus macaque CMV (rhCMV) vector was recently shown to induce extraordinarily broad CD8⁺ T cell responses which effectively eliminated virus following SIV challenge in 50% of infected rhesus macaques^{348,349}.

The biased specificity of the initial immunodominant CD8⁺ T cell response in AHI may have detrimental consequences for later viral control. As Nef is an intrinsically variable protein, mutations that confer escape from early responses directed against Nef epitopes are often rapidly developed at a low fitness cost to the virus^{350,351}. The factors which contribute to this marked bias towards Nef epitopes in AHI are of yet unknown. One hypothesis states that the early expression kinetics of Nef are responsible for Nef's immunodominance³⁵², as has been observed for early expressed viral antigens during infection with LCMV³⁵³. Given that other early proteins such as Rev and Tat are typically not targeted by immunodominant responses (with some prominent exceptions such as the HLA-C*12:01-restricted Tat epitope³⁴⁴), and Gag/Env are commonly targeted, early viral gene expression does not adequately explain Nef immunodominance.

The HLA class I alleles expressed by patients certainly have a considerable effect on the HIV-1-specific CTL immunodominance hierarchy during AHI³⁵⁴. For example, the HLA-A*03:01-restricted Gag epitopes RK9 (RLRPGGKKK) and KK9 (KIRLRPGGK) were targeted during AHI by >50% of individuals expressing HLA-A*03:01^{354,355}. The HLA-B*07:02-restricted Env-IL9 (IPRIRRQGL), HLA-A*11:01-restricted Nef-QK10 and HLA-B*08:01-restricted FL8 (FLKEKGGL) responses are all also commonly observed to be immunodominant during AHI³⁵². In HLA-B*57⁺ HIV-1-infected individuals a characteristic pattern of immunodominance arises, where the earliest HLA-B*57-restricted response is directed at the Gag TW10 (TSTLQEQIGW) epitope which typically drives rapid emergence of escape mutations and there is subsequent expansion of Gag IW9- (ISPRTLNAW) then Gag KF11 (KAFSPEVIPMF)-specific CTL responses in chronic infection³⁵⁶. On the other hand, the well-described HLA-A*02:01-restricted SL9 (SLYNTVATL) epitope is often the immunodominant response during chronic HIV-1 infection but is rarely targeted during AHI³⁵⁷. In fact, an intrinsic property of HLA-A*02:01 seems to be a lack of responses during AHI. In an analysis of 20 HIV-1-specific HLA-B*42:01-restricted responses, immunodominance correlated with public TCR clonotypes (TCR sequences shared by multiple people)³⁵⁸. Thus at the individual epitope level, TCR

bias could partially explain immunodominance. Overall however, this allele-specific immunodominance during AHI does not adequately explain the general Nef-biased hierarchy. Another possibility is that the repertoire of naïve CD8⁺ T cells is enriched in Nef-specific clones. Alternatively, memory CD8⁺ T cells previously induced by infection with a different pathogen encoding a protein homologous to Nef could theoretically result in a Nef-biased T cell repertoire. These hypotheses are unlikely to be true as they would require substantial T cell cross-reactivity towards one protein containing hundreds of potential epitopes with considerable variation across the viral population within individuals of diverse HLA binding preferences and immunological history. Therefore it seems most likely that the Nef-biased immunodominance hierarchy during AHI results from an intrinsic property of the HIV-1 Nef protein.

It is important to note that in contrast to HIV-1, HIV-2 Nef is rarely targeted by CTL by HIV-2-infected patients^{359,360}. Furthermore, in contrast to adults, HIV-1-infected children have been shown to mount infrequent and low magnitude responses towards Nef^{361–363}, although one study of treated children of different demographic suggested otherwise³⁶⁴. The HIV-1 Nef protein and its functions/properties are described in the following section.

1.12 HIV-1 Nef

Nef is an accessory protein encoded by all primate lentiviruses. Originally described as a factor which negatively regulates viral transcription and replication (*negative factor*; *nef*)³⁶⁵, Nef is now thought to play an important role in HIV-1 immunopathogenesis through a plethora of molecular functions³⁶⁶. Evidence for the effect of Nef on disease progression arose from Nef-transgenic and humanised mouse models^{367,368} as well as rhesus macaques and human patients infected with Nef-deleted SIV/HIV-1 strains^{369–372}. Kestler *et al* found that infection of macaques with a SIV strain containing a premature stop codon within the *nef* coding region rapidly resulted in reversion of such mutants. Furthermore, infection with a *nef*-deleted strain resulted in significantly reduced viral loads and pathogenic potential relative to *nef*-competent strains. The most notable examples demonstrating the contribution of Nef to disease progression arose from study of a number of patients in the ‘Sydney Blood Bank Cohort’ (SBBC) who received blood transfusions containing Nef-defective HIV-1^{370–372}. In the absence of antiretroviral therapy these patients maintained low viral load and

high CD4⁺ counts for >20 years^{373,374}. A decreased ability of Nef to enhance viral infectivity and modulate surface levels of HLA class I, CD4 and CD74 has also been associated with viral control in EC, although the relative contribution of protective HLA alleles and variation in Nef sequence to this effect remains unclear³⁷⁵. Nef function in ECs has also recently been linked to development of a common sequela of infection, Kaposi's sarcoma³⁷⁶. Evidently, the effects of Nef are behind many of the pathological consequences of HIV-1 infection, and a large body of research has focused on resolving the mechanisms through which Nef functions.

1.12.1 Nef genetics and structure

The *nef* gene, which encodes the 206 amino acid (27-35 kDa) Nef protein in a single exon, is situated at the 3' end of the HIV-1 genome and partially overlaps with the 3' LTR. Nef protein is translated early in the replication cycle in the cytoplasm from multiply-spliced RNAs transcribed from the HIV-1 provirus. A co-translational myristoylation modification at its N-terminus in addition to two clusters of basic residues imparts Nef with its ability to associate with membranes³⁷⁷⁻³⁷⁹. This N-terminal arm is a polymorphic and flexible linker region of approximately 70 amino acids which is trailed by a relatively conserved and compact core domain (residues 56-206) composed of a four-stranded antiparallel β -sheet flanked by four α -helices³⁸⁰⁻³⁸³. Projecting away from the core domain is a disordered loop (residues 148-178) containing motifs required for endocytic internalisation (di-leucine motif at residues 160-165) and association with adaptor proteins. As the presence of these flexible linker regions is unusual for cytoplasmic proteins, Arold and Baur hypothesised that Nef cycles between 'open' and 'closed' conformations at the membrane or cytoplasm to interact with its myriad of known binding partners³⁸³.

1.12.2 The varied functions of HIV-1 Nef

Nef does not display any enzymatic activity, instead exerting its effects through multiple motifs located in the core folded domain or in either of the two flexible linker/loop regions. The sheer number of reported binding partners is mirrored in the multitude of functions ascribed to Nef over the years.

1.12.2.1 Modulation of cell surface receptors

One of the first described functions of Nef was downregulation of the CD4 receptor^{384,385}, which is thought to increase virion infectivity by reducing incorporation of CD4 into particles which saturate gp120 binding sites, thereby increasing the amount of functional Env molecules available to bind to CD4 expressed on target cells³⁸⁶. Alternatively, CD4 downregulation may inhibit superinfection of target cells, which could enhance viral spread or prevent the cytopathic effects of viral DNA accumulation³⁸⁷. Other potential consequences of CD4 downregulation may be impaired T helper cell responses and altered cell signalling. Similarly, Nef has been shown to downregulate the HIV-1 co-receptors CCR5 and CXCR4 which may inhibit superinfection^{388,389}.

HLA class I is also downregulated from the cell surface by Nef³⁹⁰, presumably as a mechanism of immune evasion from CD8⁺ T cells³⁹¹. Indeed, infection of monkeys with SIV strains harbouring Nef mutations which selectively abrogate MHC class I downregulation resulted in higher magnitude T cell responses and rapid reversion of Nef mutations to allow MHC class I downregulation^{392,393}. Interestingly, Nef-mediated MHC downregulation may be most important during AHI, as animals infected with viruses showing reversion of Nef mutants by 1 month post-infection did not progress to AIDS-like disease as rapidly as monkeys infected with wildtype SIV³⁹³. Furthermore, studies of HIV-1 Nef have indicated that immunocompetent individuals harbour viruses with greater ability to downregulate HLA than individuals with AIDS, supporting a role in immune evasion³⁹⁴. Interestingly, Nef downregulates HLA-A and HLA-B (albeit to varying extents in an allele- and strain-dependent manner) but not HLA-C, HLA-E or HLA-G³⁹⁵⁻³⁹⁷. Instead, HIV-1 Vpu has recently been shown to downregulate HLA-C in a strain-dependent manner, possibly as a result of the balance between CTL and NK cell pressure³⁹⁸.

HLA class II surface expression is also affected by Nef³⁹⁹. Mature HLA class II is downregulated whilst immature HLA class II presenting Ii is upregulated on infected cells⁴⁰⁰. A recent study used the lymphoblastoid cell line 721.221 to show that Nef clones from patients with AHI downregulated HLA-DR more than Nef clones from chronic infection, suggesting an important role for HLA class II downregulation in early infection⁴⁰¹. Although the mechanisms behind this HLA class II modulation have been partially delineated and may hold significance for evasion of CD4⁺ T cell responses *in vivo*, the data so far have only been generated using artificial cell line models⁴⁰².

Nef has also been shown to strongly downregulate CD8^{403,404}. Although CD8 downregulation seems to be a conserved activity of primate lentivirus Nef proteins⁴⁰⁵, the relevance of this downregulation remains unclear because studies were performed using cell lines or PBMCs transduced with Nef. It is conceivable that infection of double-positive T cells or endocytosis of extracellular Nef could cause this effect and result in dysfunctional CD8⁺ T cell responses, although further work is required to determine the relevance to infections *in vivo*. Lastly, Nef has been shown to modulate CD28^{404,406}, CD1 family molecules^{407–409}, and the co-stimulatory molecules CD80 and CD86^{410,411}.

1.12.2.2 Modulation of cell signalling, activation and apoptosis by Nef

Nef also has profound effects on T cell signalling processes which presumably allow increased viral gene expression and replication⁴¹². Activation of T cells by Nef has been well-documented^{413–415}, and may involve multiple interactions with serine-threonine kinases (SRKs) such as PAK-2^{416,417}, protein kinase C⁴¹⁸, MAPK⁴¹⁹ and components of the calcium signalling pathway^{413,420}. Nef also enhances its own expression by inducing the transcription factors NFAT, NFκB, c-fos, Jun-d and IRF1/2 which transactivate the HIV-1 LTR^{412,421}.

The effect of HIV-1 Nef on apoptosis is contentious. Some studies show that Nef upregulates CD95/CD95L whilst downregulating Bcl-2 and Bcl-XL, leading to apoptosis^{422,423}. In contrast, other studies showed no effect or an anti-apoptotic effect of Nef on apoptosis^{424–426}, possibly indicating the involvement of indirect effects of Nef on survival, as was shown in CD4⁺ T cell/macrophage co-cultures⁴²⁷. Alternatively, CD4⁺ T cell subsets could be affected differently by Nef as was indicated in a Nef-transgenic mouse model where Nef-expressing Tregs preferentially survived⁴²⁸, although the relative expansion or depletion of Tregs in HIV-1 infection is not clear (absolute numbers of peripheral Tregs drop in untreated HIV-1 infection)^{429–431}. Nef seems to protect against apoptosis when expressed in monocyte-derived macrophages or cell lines by inducing anti-apoptotic Bcl-XL and inhibiting pro-apoptotic Bad protein^{432,433}.

1.12.2.3 Nef increases virion infectivity

Nef has long been known to increase the infectivity of HIV-1 virions⁴³⁴. Although the infectivity increase was recognised as a Nef-dependent effect on the virus producer cell and not the recipient

cell⁴³⁵⁻⁴³⁷, the mechanism by which Nef exerted its effects was elusive for many years. This enigma was recently solved independently by two groups^{438,439}. They discovered that serine incorporator 5 (SERINC5) and to a lesser extent SERINC3, localise to the plasma membrane where they are incorporated into nascent virions and block an early stage of viral replication in target cells. To counteract the antiviral effect of SERINC5, Nef seemingly induces the clathrin-mediated endocytosis of SERINC5 into late endosomes through an AP-2-dependent mechanism. This activity of Nef seems to be a conserved function as SERINC5 was counteracted by all primate lentiviruses and the prevalence of particular strains in the wild correlated with their ability to counteract the antiviral effects of SERINC5⁴⁴⁰.

All of the effects described in this section (1.11.2) are caused by endogenously-expressed Nef. Equally important, however, may be the effects caused by extracellular Nef. In fact, Baur argues that because we have been too focused on the effects of Nef on receptor downmodulation and endocytosis in the producer cell, we have ignored the significant effects of extracellular Nef on exocytosis and immunopathogenesis⁴⁴¹. He contends that induction of cellular secretion could be the most important pathogenesis-inducing function of HIV-1 Nef.

1.12.3 Nef induces its own secretion in EV

Cells contain a complex network of vesicles, many of which are trafficked to the extracellular environment. EVs play an important role in intercellular signalling, immunity²⁷⁷, and pathogen spread and are implicated in a number of diseases⁴⁴².

The study of EVs is hampered by an inconsistent application of nomenclature, with terms such as microparticles, shedding vesicles, microvesicles and ectosomes all being used by investigators⁴⁴³. The terms 'exosomes' and 'microvesicles' are often broadly used to delineate the two main classes of EVs (Figure 1.9). Exosomes are described in electron microscopy studies as 40-100 nm vesicles released by fusion of multi-vesicular bodies (MVBs) with the plasma membrane⁴⁴². However, a larger size (90-160 nm) for exosomes is becoming apparent, as fixing and dehydrating vesicles for electron microscopy causes shrinkage artefacts⁴⁴⁴. Microvesicles are defined as EVs that bud directly from the plasma membrane and tend to be larger than exosomes (up to 1000 nm). Yet another class of understudied EVs are apoptotic bodies which are large vesicles (>1000 nm) originating from dead

cells. Originally thought of as cellular garbage, EVs are now known to play a major role in immunity²⁷⁷. Exosomes are known to contain peptide-MHC complexes and can directly activate T cells *in vitro*^{445,446}. Intact protein encapsulated within exosomes can also be transferred between cells for cross-presentation. For example, tumour antigens^{278,447}, mycobacterial proteins⁴⁴⁸ or CMV antigens⁴⁴⁹ transferred by EV all induce T cell responses *in vitro*.

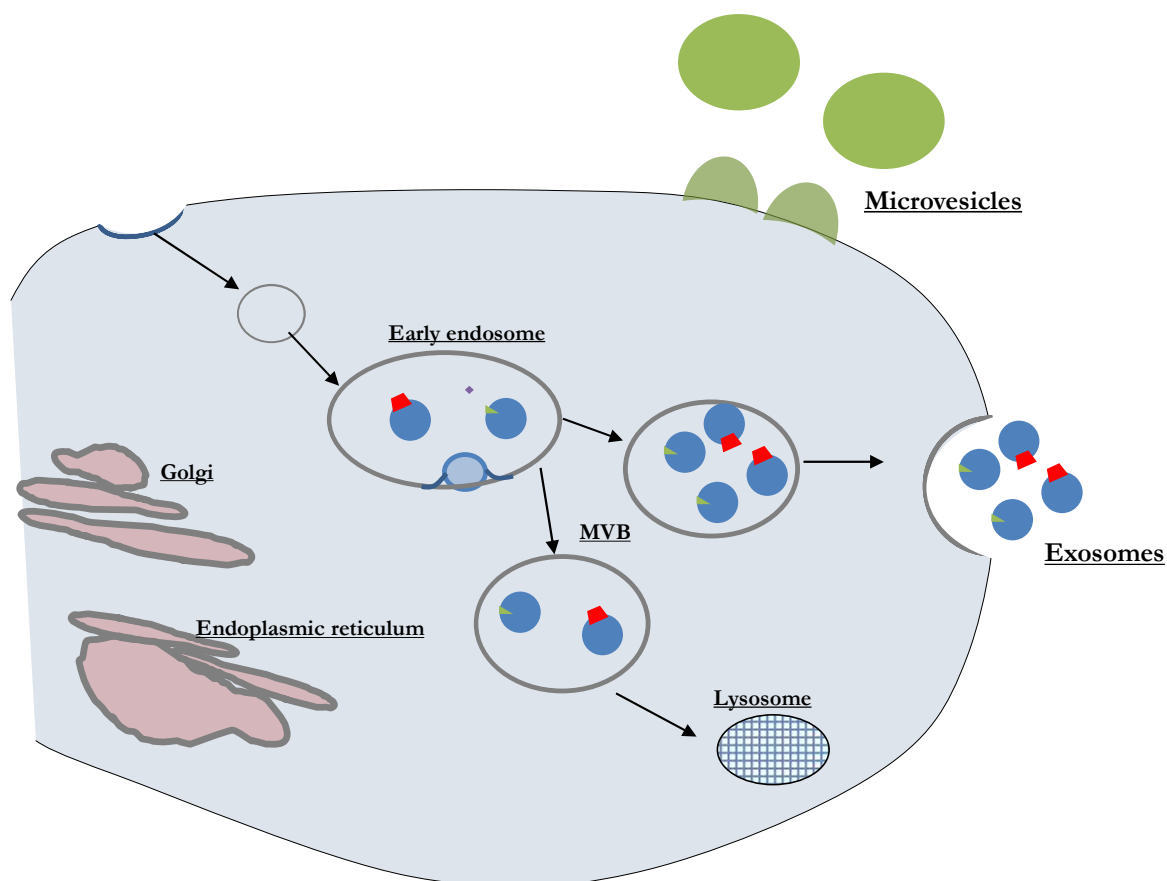


Figure 1.9. *Extracellular vesicles; exosomes and microvesicles.* Exosomes bud into multivesicular bodies (MVB) and are then released into the extracellular space once the MVB fuses with the plasma membrane. Microvesicles bud directly from the plasma membrane into the extracellular space.

HIV-1 Nef is also secreted in extracellular vesicles by HIV-1-infected cells *in vitro*^{450–453}, and extremely large amounts (1-10 ng/ml) of Nef are present in the plasma of HIV-1-infected patients^{453,454}. The next section summarises the effects that extracellular recombinant Nef exerts particularly on myeloid cells and the source of and functions that Nef-containing EVs might play during HIV-1 infection are discussed in Chapter 5.

1.12.4 Effects of extracellular Nef on cells

Much of the previous work to elucidate the effects of exogenous Nef on cells has been performed using 'soluble' Nef recombinantly expressed in bacteria which could therefore differ in route of uptake and downstream consequences to vesicular Nef. Nonetheless, like endogenous Nef, recombinant exogenous Nef also exerts potent modulatory effects on receptors and cell signalling. Uptake of Nef by macrophages results in CD36 and CD4 downregulation^{455,456}. In DCs, addition of recombinant Nef downregulated HLA class I and the mannose receptor whilst upregulating HLA-DR, CD1a, CXCR4, CD40 and CD83⁴⁵⁷. Exogenous Nef also induces many cell signalling changes in macrophages and DCs, including NFκB and STAT1 activation, IRF-3 phosphorylation and Vav phosphorylation which can collectively result in increased IFN-β and IL-12p70 secretion⁴⁵⁸⁻⁴⁶¹. In B cells Nef could be transferred through macrophage tunnelling nanotubes⁴⁶² to induce the regulatory factors IκBα, SOCS1 and SOCS3 which inhibit downstream CD40-dependent antibody class switching⁴⁶³. In one study, recombinant Nef was also able to stimulate HIV-1 transcription in chronically infected U937 monocytic cells by virtue of NFκB activation⁴⁶⁴. Apoptosis also seems to be an effect of extracellular Nef on neurons⁴⁶⁵ and CD4⁺ T cells but not myeloid cells^{451,453,454,466}. James *et al* proposed that extracellular Nef induces apoptosis in CD4⁺ T cells by interacting with surface-expressed CXCR4⁴⁶⁷. Nef-containing exosomes secreted by monocytes were also shown to harbour mRNAs encoding proteins associated with apoptosis and could hypothetically represent another mechanism by which Nef can induce apoptosis in bystander cells⁴⁶⁸. Bystander cells are also known to be affected by pro-inflammatory cytokines secreted by Nef-treated macrophages/monocytes^{458,469-471}.

Exogenous Nef has been demonstrated to increase immunological synapse formation and clustering of DCs with CD4⁺ T cells⁴⁶⁰. Furthermore, Nef-treated DCs are more adept at stimulating bystander CD4⁺ T cells⁴⁵⁷, which may help explain the reported requirement for Nef in enhancing viral replication in co-cultures of DCs and CD4⁺ T cells^{472,473}. The role of Nef in CD8⁺ T cell dysfunction during HIV-1 infection is relatively unexplored. In one study, Nef-treated DCs were impaired in their ability to stimulate proliferation of CD8⁺ T cells in allogeneic mixed lymphocyte reactions⁴⁷⁴. In this setting, DCs induced apoptosis of CTL through a TNFα- and FasL-dependent mechanism. In another study, it was suggested that addition of exogenous Nef to co-cultures of DCs and CTL modulated

CTL cytotoxic activity and proliferation, although the large variability observed between donors casts doubt on the conclusions of the authors⁴⁷⁵. In summary, HIV-1 Nef evidently dramatically alters DC and T cell function but the contribution of extracellular/vesicular Nef to the induction of Nef-specific CD8⁺ T cell responses has, to my knowledge, not been examined before.

1.13 Research questions

Although a vast amount of longitudinal CTL epitope mapping and functional studies have elucidated a clear pattern of narrowly-restricted and Nef epitope-biased immunodominance hierarchies in CTL responses during AHI, no suitable explanation for this phenomenon has been proposed. Moreover, the mechanistic basis underlying the delay between the first and second waves of CTL responses is unknown. Given that DCs are instrumental in priming antiviral responses during acute infections, a closer examination of how antigen presentation and CTL priming by DCs is altered during AHI was warranted. Therefore, in this thesis I explored the following questions:

1. Why are CD8⁺ T cell responses typically induced to only a very limited number of viral epitopes in AHI, with a delay prior to the expansion of responses of additional specificities? Does DC function become impaired shortly after priming begins, resulting in initial expansion of responses to just a small number of the initially most efficiently presented epitopes?
2. Why does the first wave of CD8⁺ T cell responses induced in AHI preferentially target epitopes derived from Nef? Are Nef epitopes preferentially cross-presented by DCs during AHI? What is the source of cross-presented Nef?

Two major factors described in the literature influenced the hypotheses I postulated when addressing these research questions:

- AHI is accompanied by a cytokine storm⁴³ and a massive release of apoptotic EV⁴⁵.
- About 10 ng/ml Nef is present within the plasma of HIV-1-infected patients and much of it is vesicle-associated^{453,454}.

1.14 Hypotheses

As DCs are infected more rarely and substantially less productively by HIV-1 than in CD4⁺ T cells⁴⁷⁶, it is likely that cross-priming plays a more important role in induction of CD8⁺ T cell responses during AHI than direct priming via infected DCs. Since Nef-containing EVs are found abundantly in the plasma of HIV-1-infected patients and DCs are known to use EVs as a source of antigen for cross-presentation, I based my work to address the preferential targeting of CD8⁺ T cell responses in AHI to Nef epitopes on the following hypothesis:

During AHI, abundant Nef-containing EVs released from HIV-1-infected cells are ingested by DCs. Subsequently, DCs process and cross-present Nef epitopes and preferentially prime Nef-specific naïve CD8⁺ T cells.

Furthermore, to explain the delay between the first and second wave of CD8⁺ T cell responses during AHI, I worked on the following hypothesis:

Shortly after initial antigen presentation by DC during the eclipse and viral ramp phases of infection, priming of CD8⁺ T cells by DC is impaired by factors present during AHI.

The difficulty in obtaining samples from individuals acutely infected with HIV-1, and the low frequency of DCs present in the peripheral blood during AHI⁴⁷, have limited studies of DCs at this stage of infection, hence little is known about changes in DC functionality in AHI.

1.15 Objectives

Specifically, I aimed to fulfil the following objectives:

1. To develop an *in vitro* CD8⁺ T cell priming assay in order to a) characterise the specificities of CD8⁺ T cells primed by DCs pulsed with various antigen and b) determine whether soluble and particulate factors present in the plasma of acutely-infected patients impair naïve CD8⁺ T cell priming *in vitro* (**Chapter 3**).
2. To gain insight into the repertoire of HIV-1 epitopes presented by HIV-1-infected CD4⁺ T cells by characterising the immunopeptidome using a mass spectrometry approach and to define novel epitope targets for CD8⁺ T cells (**Chapter 4**).
3. To characterise vesicles secreted from HIV-1-infected cells and to provide evidence for their likely cellular source *in vivo* (**Chapter 5**).

4. To characterise the repertoire of HIV-1 peptides cross-presented by *in vitro*-generated DCs ingesting HIV-1-infected cells or EVs (**Chapter 6**).
5. To a) compare the immunopeptidomes of DCs and CD4⁺ T cells and b) to compare the cross-presented and directly-presented subsets of the DC immunopeptidome (**Chapter 6**).

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Plasticware and general reagents

Table 2.1 <i>List of plasticware and general reagents</i>		
Item	Company, Country	Catalogue number
0.1 µm Stericup filters	Millipore, US	SCVPU02RE
0.22 µm filters	Millipore, US	SLGP033RS
0.45 µm filters	Millipore, US	SLHA033SS
0.5 ml, 1.5 ml Eppendorf tubes	Thermo Fisher Scientific, US	FB74023/FB74031
15, 50 ml falcon tubes	Corning Inc, US	430791/430829
175 cm ² tissue culture flasks	Corning Inc, US	431080
2 ml screwtop tubes	Sarstedt, DE	72.694.005
24-well plates; tissue culture-treated	Corning Inc, US	3526
25 cm ² tissue culture flasks	TPP	90025
48-well plates; tissue culture-treated	Corning Inc, US	3548
5, 10, 25 and 50 ml pipettes	Corning Inc, US	4487/4488/4489/4490
6-well plates; tissue culture-treated	Corning Inc, US	3516
70 µm cell strainers	BD Biosciences	352350
75 cm ² tissue culture flasks	Corning Inc, US	430641
96-well flat-bottom plates; tissue culture-treated	Corning Inc, US	3596
96-well round-bottom plates; tissue culture-treated	Corning Inc, US	3799
96-well V-bottom plates; tissue culture treated	Corning Inc, US	3894
Amicon Ultra 10kDa MWCO	Millipore, US	UFC801008
Cell scrapers	BD Biosciences, US	353085
Cryovials 2ml, free-standing	Elkay Laboratory Products	127-T311-200
Econo-Column® Chromatography Columns, 1.0 × 10 cm	BioRad, US	7374011
FACS tubes	BD Biosciences, US	352063
Protein Lo-Bind Eppendorf tubes; 1.5 ml/2 ml	Thermo Fisher Scientific, US	13-698-794/13-698-795
Stericup-GP, 0.22 µm, polyethersulfone, 500 mL, radio-sterilized	Millipore, US	SCGPU05RE
UltraClear 13.2 ml tubes, 14 x 89 mm	Beckman Coulter, US	344059
UltraClear 38.5 ml tubes, 25 x 89 mm	Beckman Coulter, US	344058
LCGC Certified Clear Glass 12 x 32mm Screw Neck Total Recovery Vial, with Cap and PTFE/silicone Septum, 1 mL Volume	Waters, US	186000384C
Immobilon-FL PVDF membrane	Millipore, US	IPFL00010
5 kDa MWCO centrifugal filters	Sartorius, DE	VS0414

2.1.2 Cell lines and tissue culture reagents

C8166, PM1 and U937 cell lines were obtained from the National Institute for Biological Standards and Control (NIBSC) Centre for AIDS Reagents (CFAR). The MHC class I negative 721.221 cell line expressing CD4 (CD4.221) was a kind gift from the laboratory of Dr Masafumi Takaguchi (Kumamoto University, JP). The TAP- and MHC II-deficient T2 cell line stably transfected with

lentiviral constructs expressing the HLA-E*01:01 (T2E1) or HLA-E*01:03 (T2E3) alleles were a kind gift from the Christina Bade-Doeding laboratory (Hannover Medical School, DE). The D10/HuT-78 cell line was a kind gift from the laboratory of Dr Maurizio Federico (National AIDS Center, IT). 293FT cells were obtained from Invitrogen. Hybridomas used for antibody production were kindly provided by Alastair Waugh at the Human Immunology Unit of the Weatherall Institute for Molecular Medicine (WIMM) (listed in Table 2.2).

Table 2.2 Hybridomas expressing immunoglobulins targeting human proteins used				
Name	Specificity	Isotype	Culture media	ATCC Reference
DT9	HLA-C, HLA-E	IgG2a	R10	N/A
L243	HLA-DR	IgG2a	R10	HB-55
OKT3	CD3	IgG2a	R20	CRL-8001
OKT4	CD4	IgG2b	IMDM + 20% FBS	CRL-8002
W6/32	Pan MHC class I (HLA-A, -B, -C, -E)	IgG2a	R10	HB-95

Cell culture reagents are listed in Table 2.3. Culture medium was stored at 4°C protected from light.

Table 2.3 List of cell culture reagents and buffers		
Item	Company, Country	Catalogue number
0.9% NaCl w/v solution	Aguetant, UK	600019
4% paraformaldehyde (PFA)	Santa Cruz Biotechnology, US	sc-281692
AIM V medium	Thermo Fisher Scientific, USA	12055091
Benzonase	Millipore, USA	70746
Brefeldin A	eBioscience, US	00-4506-51
CD4 microbeads	Miltenyi Biotec, DE	130-045-101
CD4 ⁺ T cell isolation kit	Miltenyi Biotec, DE	130-096-533
CD45 microbeads	Miltenyi Biotec, DE	130-045-801
Cytofix Fixation Buffer	BD Biosciences, US	554655
Foetal bovine serum (FBS); heat-inactivated	APS, US	S-001
Golgistop containing monensin	BD Biosciences, US	554724
Heparin sodium	Leo Pharma, UK	-
HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Thermo Fisher Scientific, US	15630080
Histopaque-1077	Sigma-Aldrich, US	10771
L-ARGININE:HCL (13C6, 99%; 15N4, 99%)	Cambridge Isotopes, US	CNLM-539-H-PK
L-glutamine	ThermoFisher Scientific, US	25030081
L-LEUCINE (13C6, 99%; 15N, 99%)	Cambridge Isotopes, US	CNLM-281-H-PK
L-LYSINE:2HCL (13C6, 99%; 15N2, 99%)	Cambridge Isotopes, US	CNLM-291-H-PK
LD columns	Miltenyi Biotec, DE	130-042-901
LS Columns	Miltenyi Biotec, DE	130-042-401
Male human serum AB	Sigma-Aldrich, US	H4522
MEM Non-Essential Amino Acids	Thermo Fisher Scientific, US	11140035

Naïve Pan T cell isolation kit	Miltenyi Biotec	130-097-095
OptiMem I reduced Serum Medium	Thermo Fisher Scientific, US	31985-062
Penicillin/streptomycin	Thermo Fisher Scientific, US	15140122
Perm/Wash Buffer	BD Biosciences, US	554723
Phosphate-buffered saline (PBS)	Sigma-Aldrich, US	D8537
RPMI 1640 medium	Thermo Fisher Scientific, US	11875093
RPMI containing L-Glutamine without L-leucine, L-Lysine, L-Arginine and phenol red	Sigma-Aldrich, US	R1780
SILAC dialysed FBS (MWCO 5 kDa)	Dundee Cell Products, UK	DS1008
Trypsin/EDTA	Lonza, CH	BE17-161E
X-VIVO15 medium	Lonza, CH	BE02-060F
IMDM		

2.1.3 Cytokines

All cytokines (listed in Table 2.4) were resuspended in distilled water or PBS then stored at -20°C for up to 12 months. Aliquots were thawed no more than once before addition to culture media.

Table 2.4 List of cytokines used in vitro				
Cytokine	Company, Country	Stock concentration	Working concentration	Catalogue number
GM-CSF	Miltenyi Biotec, DE	500 U/μl	100-500 U/ml	130-093-864
IFNα	Peptotech, US	10,000 U/μl	500-10,000 U/ml	300-02A
IL-1β	R&D Systems, US	10 μg/ml	5 ng/ml	201-LB
IL-2 (ProLeukin)	Novartis, CH	10,000 U/ml	10-20 U/ml	N/A
IL-4	Miltenyi Biotec, DE	500 U/μl	300 U/ml	130-093-920
IL-6	R&D Systems, US	100 μg/ml	150 ng/ml	206-IL
IL-7	Miltenyi Biotec, DE	100 μg/ml	5 ng/ml	130-095-361
IL-15	Miltenyi biotec, DE	100 μg/ml	10 ng/ml	130-095-762
M-CSF	Miltenyi Biotec, DE	100 μg/ml	20 ng/ml	130-096-485
PGE ₂	R&D Systems, US	50 μg/ml	1 μg/ml	2296
TNFα	R&D Systems, US	10 μg/ml	5 ng/ml	210-TA

2.1.4 Peptides

Peptides were synthesised by Sigma Aldrich or GenScript. Lyophilised peptides were resuspended in DMSO at 40 mg/ml and stored at -80°C. Pools of 18-mers overlapping by 10 amino acids representing the HIV-1 Clade B consensus (2001, LANL) proteome were prepared at a concentration of 20 μg/ml in R10. 3 Gag pools, 5 Env pools, 5 Pol pools and 4 Nef/Vif/Vpu/Vpr/Tat/Rev pools were assembled and stored at -80°C (sequences are listed in Appendix 9.2; Tables 9.3, 9.4, 9.5 and 9.6). Flu, EBV and CMV optimal HLA-restricted peptides used in ELISPOT assays are listed in

Appendix 9.2, Table 9.7. A list of peptides used to prime naïve CD8⁺ T cells in *in vitro* priming assays is detailed in Appendix 9.2, Table 9.8.

2.1.5 Chemical reagents

All chemical reagents were resuspended and stored according to the manufacturer's instructions or as indicated in the section Methods 2.2. When used in tissue culture experiments, reagents were kept sterile by handling in a class II biosafety cabinet and where needed were sterilized by passing through a 0.22 µm filter unit.

Chemical	Company, Country	Stock concentration	Working concentration	Catalogue number
10X Sample reducing agent	Thermo Fisher Scientific, US	500 mM	50 mM	NP0004
12% Mini-Protean TGX Pre-cast protein gels, 10 well, 50 µl	BioRad, US	-	-	4561044
12% Mini-Protean TGX Pre-cast protein gels, 15 well, 50 µl	BioRad, US	-	-	4561046
3'-Azido-3'-deoxythymidine (AZT)	Sigma-Aldrich, US	93.5 mM	10 µM	A2169
4X Laemmli buffer	BioRad, US	4X	1X	1610747
5,5-dithio-bis(2-nitrobenzoic acid)	Sigma-Aldrich, US	20.16 mM in ethanol	0.1 mM	D8130
Acetic acid	Sigma-Aldrich, US	100%	10%	A6283
Acetone	Thermo Fisher Scientific, US	100%	-	A/0560/PC 17
Acetonitrile LC grade	Thermo Fisher Scientific, US	100%	1-65%	A/0627/17
Acetylthiocholine iodide	Sigma-Aldrich, US	345.8 mM	1.25 mM	01480
Aldehyde/sulfate latex beads, 4% w/v, 4 µm	Thermo Fisher Scientific, US	-	-	A37304
Bodipy Maleimide	Thermo Fisher Scientific, US	2 mM	0.2 mM	B10250
Boric acid	Sigma-Aldrich, US	-	0.1 M	B0394
Chloroform	Sigma-Aldrich, US	100%	-	650498
Cycloheximide	Sigma-Aldrich, US	10 mg/ml	100 µg/ml	
Dextran-FITC	Sigma-Aldrich, US	10 mg/ml	1 mg/ml	F0405
Dimethyl pimelimidate	Sigma-Aldrich, US	-	40 mM	D8388
EDTA	Invitrogen, US	0.5 M	5 mM	15575-020
Emetine dihydrochloride	Sigma-Aldrich, US	50 mg/ml	100 µg/ml	E2375
Ethanol	Sigma-Aldrich, US	100%	Various	32221
Glycine	Sigma-Aldrich, US	-		G7126
IGEPAL CA630	Sigma-Aldrich, US	10%	0.5%	I8896
Iodoacetamide	Sigma-Aldrich, US	0.5 M	50 mM	I1149
Lipofectamine 2000	Invitrogen, US	-	-	11668-030
Lysolecithin	Sigma-Aldrich, US	-	20 µg/ml	L4129

Methanol	Sigma-Aldrich, US	100%	20%	32213
NP-40 substitute	Sigma-Aldrich, US	100%	0.1-1%	74385
nProtein A Sepharose 4 Fast Flow	GE Healthcare, UK	-	-	17-5280-02
Odyssey Blocking Buffer (PBS)	Li-Cor, US	1X	1X	927-40000
OptiPrep Density Gradient medium (60% iodixanol w/v in water)	Sigma-Aldrich, US	60%	6-18%	D1556
PageBlue protein staining solution	Thermo Fisher Scientific, US	-	-	24620
Phenol red sodium salt	Sigma-Aldrich, US	-	5 mg/l	P4758
Polyinosinic:polycytidilic acid (poly I:C)	Sigma-Aldrich, US	2 mg/ml	2 µg/ml	P1530
Protease inhibitor cocktail tablets, EDTA-free	Roche, CH	Tablets	1 tab per 10 ml lysis buffer	118361700 01
Protein G Sepharose 4 Fast Flow	GE Healthcare, UK	-	-	17-0618-05
Sodium chloride	BDH, DE	-	-	102415K
Sodium dodecyl sulfate (SDS)	BDH, DE	-	-	444464T
Sodium hydroxide	Sigma-Aldrich, US	-	-	S5881
Sucrose	Sigma-Aldrich, US	-	20%	S0389
Trichloroacetic acid	Sigma-Aldrich, US	100%	20%	T6399
Triethanolamine	Sigma-Aldrich, US	-	0.2 M	90278
Trifluoroacetic acid	Sigma-Aldrich, US	100%	0.01-1%	302031
Tris-HCl	Sigma-Aldrich, US	-	Various	857645
Tween-20	Sigma-Aldrich, US	100%	0.05-0.1%	P2287
Urea	Sigma-Aldrich, US	-	6 M	U6504

2.1.6 Enzyme-linked immunospot assay reagents (ELISPOT)

Reagents for ELISPOT were stored as recommended by the manufacturer's instructions (Table 2.6).

N-N-dimethylformamide was aliquoted under a laminar flow hood.

Table 2.6 List of ELISPOT assay reagents				
Reagent	Company, Country	Stock concentration	Working concentration	Catalogue number
3-amino-9-ethylcarbazole (AEC)	Sigma-Aldrich, US	Tablets	1 tablet per 50 ml	A6926
Acetic acid	Sigma-Aldrich, US	0.1 M	7 mM	A8976
anti-human IFN-γ mAb 7-B6-1, biotinylated	Mabtech, SE	1 mg/ml	1 µg/ml	3420-6-250
anti-human IFNγ mAb 1-D1K, purified	Mabtech, SE	1 mg/ml	10 µg/ml	3420-3-250
Avidin-HRP conjugate	Vector laboratories, UK	-	1 drop per 5 ml PBS/0.1% Tween	
Bovine serum albumin	Sigma-Aldrich, US	7.5%	0.5%	A8412

Hydrogen peroxide	Sigma-Aldrich, US	30%	25 µl in 50 ml	H1009
MultiScreen _{HTS} IP filter plate, 0.45 µm, clear, sterile	Millipore, US	N/A	N/A	MSIP4510
<i>N-N</i> -dimethylformamide for molecular biology >99%	Sigma-Aldrich, US	100%	5%	D4551
Sodium acetate	Sigma-Aldrich, US	3 M	280 µl in 50 ml	S2404

2.1.7 Antibodies for flow cytometry and stimulation

All antibodies (listed in Table 2.7) were stored at 4°C or -20°C as recommended by the manufacturer. Optimal quantities of antibody for flow cytometry were determined by titration on PBMC or cell lines of interest. Antibodies not conjugated to fluorochromes ('Pure') were used for stimulation of T cells at the concentration indicated.

Table 2.7. Antibodies for flow cytometry or cell stimulation					
Specificity	Clone	Fluorochrome	µl/ sample	Catalogue number	Company, Country
CD3	S4.1	PE-Texas-Red	1	MHCD0317	Thermo Fisher Scientific, US
CD3	UCHT1	Pure	50 ng/ml	MAB100	R&D Systems, US
CD3	SK7	APC-Cy7	1	341100	BD Biosciences, US
CD4	RPA-T4	PE	1	555347	BD Biosciences, US
CD8	SK1	APC-Cy7	1	557834	BD Biosciences, US
CD8	SK1	APC	1	345775	BD Biosciences, US
CD9	HI9a	FITC	2.5	312103	BioLegend, US
CD11c	-	APC-HiLyte 750	0.5	Custom conjugate	ReaMetrix, US
CD14	M5E2	PB	1	558121	BD Biosciences, US
CD28	CD28.2	Pure	1 µg/ml	16-0289-85	eBioscience, US
CD40	LOB7/6	PE	3	MCA1590PE	Bio-Rad, US
CD45RA	HI100	APC	1	550855	BD Biosciences, US
CD49d	L25	Pure	1 µg/ml	340976	BD Biosciences, US
CD63	H5C6	FITC	2.5	353005	BioLegend, US
CD81	5A6	FITC	2.5	349503	BioLegend, US
CD83	HB15e	FITC	3	556910	BD Biosciences, US
CD86	2331 (FUN-1)	APC	3	555660	BD Biosciences, US
HIV-1 p24	KC57	FITC	2	6604665	Beckman Coulter, US
HLA-DR	L243	PerCP	1	347402	BD Biosciences, US
IFNγ	B27	APC	5 of 1:100 dilution	554702	BD Biosciences, US
IL-10	JES3-9D7	PerCP-	3	46-7108-42	eBioscience, US

		efluor710			
Isotype control	MOPC-21	FITC	2.5	400109	BioLegend, US
Live/Dead Aqua	-	-	20 of 1:200 dilution	L34957	Thermo Fisher Scientific, US
MIP1β	24006	FITC	10	IC271F	R&D Systems, US
TNFα	Mab11	efluor450	3	48-7349	eBioscience, US

2.1.8 Antibodies for immunoblotting

Antibodies were stored at 4°C or -20°C as indicated by the manufacturer's instructions. Antibodies were diluted in Odyssey PBS Blocking Buffer (Cat:927-40000, Li-Cor, UK) for immunoblotting. The anti-V5 tag antibody was a kind gift from the laboratory of Rick Randall (University of St. Andrew's, UK).

Table 2.8 List of antibodies used for immunoblotting detection					
Specificity	Clone	Species	Working Dilution	Company	Catalogue number
Alix	3A9	Mouse	1:1000	Cell Signalling Technology, US	2171S
Anti-mouse IgG IRDye 680RD	Polyclonal	Goat	1:15000	Li-Cor, US	926-68070
Anti-rabbit IgG IRDye 800CW	Polyclonal	Goat	1:15000	Li-Cor, US	926-32211
B-actin	Polyclonal	Rabbit	1:1000	Abcam, UK	ab8227
Calnexin	Polyclonal	Rabbit	1:1000	Cell Signalling Technology, US	2433S
CD45	69/CD45	Mouse	1:250	BD Biosciences, US	610265
HIV-1 Gag	05-009	Mouse	1:1000	NIBSC, UK	ARP3243.3
MHC class I heavy chain	HC10	Mouse	1.5 μ g/ml	N/A	N/A
HIV-1 Nef	JR6	Mouse	1:2500	Abcam, UK	ab42358
Nef (HIV-1 IIIB)	AG11	Mouse	1:1000	Millipore, US	MAB899
Syntenin-1	Polyclonal	Rabbit	1:1000	Genetex, US	GTX108470
Tsg101	Polyclonal	Rabbit	1:1000	Abcam, UK	ab30871
V5 tag	236	Mouse	1.5 μ g/ml	N/A	N/A

2.2 Methods

2.2.1 Human samples and isolation of PBMC and plasma

Leukocyte cones derived from healthy platelet apheresis donors were purchased from NHS Blood & Transplant Services at the John Radcliffe Hospital, Oxford and used as a source of PBMC. To isolate mononuclear cells, leukocyte cones (~10 ml volume) were diluted in 80-90 ml saline solution then overlaid onto 15 ml Histopaque-1077 and spun at 2200 rpm in a Sorvall Legend RT centrifuge (Heraeus Rotor 75006445) for 25 minutes at room temperature with a slow deceleration program. Mononuclear cells at the interface were removed using a Pasteur pipette and washed twice in 50 ml 0.9% NaCl w/v solution. PBMC were frozen in 18 vials in 10% DMSO/90% foetal bovine serum (FBS), each of which typically contained $5-7 \times 10^7$ cells. DNA was extracted from 5×10^6 PBMC using the QIAamp DNA Mini Kit (Cat:51304, Qiagen, DE) according to the manufacturer's instructions. Four-digit typing of MHC class I alleles was performed by Tim Rostron at the WIMM HLA typing and Sequencing facility using Sanger sequencing on a 48 capillary ABI-3730 DNA analyser (Applied Biosystems, US).

Plasma samples collected at serial timepoints from US plasma donors who were found on subsequent testing to have acquired HIV-1 infection during the time-frame of plasma donation were purchased from Zeptometrix, US. The anticoagulant used when these samples were collected was 4% citrate. For some experiments, a pool of pre-viraemic plasma and pool of plasma taken from the same individuals just prior to the peak of viraemia were used. Equal amounts of samples 9016-01, 9018-01, 9020-01, 9021-01, 9023-01 and 9030-01 were included in the pre-infection pool. Equal amounts of samples 9016-10, 9018-10, 9020-21, 9021-16, 9023-21 and 9030-15 were included in the AHI pool.

Plasma samples from patients recruited into an acute infection cohort at the Mortimer Market (MM) Centre in London and healthy UK control subjects were also used. HIV-infected subjects were mostly male Caucasians who presented with symptoms of acute retroviral illness. They were sampled at serial timepoints from acute into chronic infection; some subjects chose not to receive anti-retroviral therapy (ART) whereas others started ART either in acute infection or subsequently. Study approval was obtained from The National Health Service Camden and Islington Community local

Research Ethics Committee and blood samples were drawn with written informed consent. Blood from HIV-infected subjects was drawn into ethylene diamine tetra-acetic acid (EDTA)-containing tubes and plasma was isolated by spinning blood at 2000 x g for 10 minutes at room temperature. Plasma was aliquoted and stored at -80°C until use. Blood was drawn from healthy donors into heparinized blood collection tubes and plasma was isolated and stored at -80°C in a similar way.

2.2.2 Cell culture

C8166, PM1, HuT-78, D10/HuT-78, F12/HuT-78, MT-2, Jurkat, U937, T2, T2E1 and T2E3 cells were cultured in RPMI 1640 medium containing 10% FBS, 100 µg/ml penicillin, 100 IU/ml streptomycin and 10 mM HEPES (hereafter called 'R10'). 293FT cells were cultured in Dulbecco's Modified Eagle Medium containing 10% FBS, 100 µg/ml penicillin, 100 IU/ml streptomycin, 2 mM L-glutamine and 1% non-essential amino acids (hereafter called 'D10'). All cells were routinely cultured in a humidified incubator at 37°C with 5% CO₂.

Frozen PBMC were thawed quickly at 37°C in a waterbath and diluted slowly with 30-50 ml R10. Primary CD4⁺ T cells were negatively isolated from PBMC using a CD4⁺ T cell isolation kit according to the manufacturer's instructions. Prior to infection with HIV-1, CD4⁺ T cells were activated by culture for 3 days in RPMI 1640 medium containing 20% FBS, 100 µg/ml penicillin, 100 IU/ml streptomycin, 10 mM HEPES (hereafter called 'R20'), 50 ng/ml anti-CD3 antibody (clone: UCHT1), 100 ng/ml anti-CD28 (clone:CD28.1) and 20 U/ml IL-2.

2.2.3 Monocyte-derived dendritic cells (MDDC)

Monocytes were isolated by adherence of 10⁶ PBMC/cm² to tissue culture flasks for 2 hours in RPMI 1640 medium containing 5% male human serum AB, 100 µg/ml penicillin, 100 IU/ml streptomycin and 10 mM HEPES (hereafter called RAB5) at 37°C and 5% CO₂. Non-adherent cells were removed by 3-5 washes with 5 ml RAB5. Monocytes were then differentiated into IL-4-DC or IFN-DC by culturing in 10 ml of RAB5 containing 300 IU/ml IL-4 and 100 U/ml GM-CSF or 10,000 U/ml IFN- α and 500 U/ml GM-CSF, respectively. Fresh IL-4/GM-CSF-containing media was added to IL-4-DCs on day 3 post-isolation and cells were harvested for experiments on days 5-6. IFN-DCs were harvested for experiments on day 3 post-isolation. In some experiments, 2 x 10⁵ DC per well of a 96-

well round-bottom plate were treated with 10% plasma in X-VIVO15 medium containing 1 U/ml heparin. In some experiments, plasma was treated at 56°C for 10 minutes before culture with MDDC. To determine MDDC purity and analyse their phenotype, IL-4-DC and IFN-DC populations were phenotyped using a panel containing CD11c-AH7, CD40-PE, CD83-FITC, CD86-APC and HLA-DR-PerCP antibodies. 2×10^5 MDDCs per tube were first washed in 1 ml PBS then pelleted at 400 x g in a benchtop centrifuge and supernatant was discarded. Cells were stained with antibodies (for amounts see Table 2.7) for 10 minutes in the dark at room temperature then washed once in 3 ml PBS. Cells were then pelleted (as above) and resuspended in 200 µl 4% PFA. Data were acquired on a CyAn ADP Analyser with Summit Software and analysis was performed on FlowJo software version 8.8.6 (FlowJo LLC, US). The AbC Anti Mouse Bead Kit (Invitrogen, US, Cat:A10344) was used for compensation purposes according to the manufacturer's instructions.

2.2.4 Dextran-FITC uptake assay

To gain insight into the antigen uptake capacity of *in vitro*-generated MDDC populations, their ability to take up FITC-labeled dextran was assessed⁴⁷⁷. Dextran-FITC was added to 2×10^5 DC in 200 µl PBS at a final concentration of 1 mg/ml in a 5 ml FACS tube. Samples were incubated at 37°C (or at 4°C as a negative control) for 5-60 minutes then washed 4 times in 3 ml PBS containing 2% FCS, pelleting cells at 1800 rpm for 3 minutes between washes. Sample data were acquired and analyzed as described in section 2.2.3.

2.2.5 Monocyte-derived macrophages

Monocytes were isolated by adherence of 10^6 PBMC/cm² to tissue culture flasks for 2 hours in R10 at 37°C and 5% CO₂. Non-adherent cells were removed by 3 washes with 10 ml R10. Monocytes were differentiated into monocyte-derived macrophages (MDM) by culturing in 20 ml R10 containing 20 ng/ml M-CSF for 10 days in a T175 cm² flask. Half of the medium was removed and replaced on day 3 followed by a full medium replacement on day 7.

2.2.6 *In vitro* priming assay

To gain insight into the processes which govern the efficiency of priming of antigen-specific CD8⁺ T cells by MDDC, *in vitro* priming assays were optimized. IL-4-DC or IFN-DC were generated from

HLA-A*02:01⁺ (and in some experiments HLA-A*03:01⁺ and HLA-B*07:02⁺) donors as described in section 2.2.3. In some experiments, $3-4.5 \times 10^6$ DCs were pre-treated in a 6-well plate with 2-3 ml 10% plasma from acutely-infected HIV-1 patients or control plasma in AIM V medium containing 1 U/ml heparin to prevent clotting; and/or patient/control plasma was included in the medium during the T cell priming step. DCs were then pulsed in the 6 well plates at 10^6 cells/ml in a 2-3 ml volume with known HLA-A*02:01/HLA-A*03:01/HLA-B*07:02-restricted peptides (see Appendix 9.2, Table 9.8) for 6-8 hours in serum-free AIM V medium at a final peptide concentration of 10 µg/ml. After incubation, DCs were harvested and then washed in PBS and co-cultured with naïve CD8⁺ T cells from the same donor.

Naïve CD8⁺ T cells were isolated from approximately 10^9 PBMC using a two-step depletion protocol to avoid positive selection. Firstly, naïve T cells were isolated by negative selection using the Naïve Pan T Cell Isolation kit according to the manufacturer's instructions. Naïve CD4⁺ T cells were then depleted using CD4 microbeads according to the manufacturer's instructions with the following modification: the quantity of CD4 microbeads used was adjusted to be sufficient for 7.5 times the actual number of naïve T cells to account for the pre-enrichment of the T cell population from PBMC.

For T cell phenotyping, CD4-PE, CD8-APC, CD3-APC-Cy7 and CD45RA-APC were used. 2×10^5 T cells were washed once in 3 ml PBS then pelleted by spinning at 400 x g in a benchtop centrifuge and supernatant was discarded. Cells were then stained by resuspension in antibodies (for details of antibodies, see Table 2.7) for 15 minutes in the dark at room temperature. Cells were then washed in 3 ml PBS and pelleted at 400 x g for 3 minutes in a benchtop centrifuge. Cells were then fixed in 200 µl 4% PFA. Data were analysed as described in section 2.2.3.

Naïve CD8⁺ T cells were resuspended at 2×10^6 cells/ml in AIM V medium containing 5% human serum AB (hereafter called AIMVAB5) and 500 U/ml IFNα, then plated at 100 µl /well into 96-well round-bottom plates. DC were resuspended at 4×10^5 cells/ml in AIMVAB5 containing 500 U/ml IFNα then 100 µl /well was added to naïve CD8⁺ T cells to give a final volume of 200 µl per well. On day 3, 100 µl of supernatant was replaced with fresh AIMVAB5 supplemented with IL-2 and IL-7 to give a final concentration of 10 IU/ml and 5 ng/ml, respectively. On day 5, non-adherent T cells

were split 1:2 and transferred to fresh 96-well round-bottom plates with fresh AIMVAB5 containing IL-2, IL-7 and IL-15 at final concentrations of 10 IU/ml, 5 ng/ml and 10 ng/ml, respectively. On day 8 cells were split 1:2 with fresh AIMVAB5 containing IL-2, IL-7 and IL-15. On day 10, T cells were restimulated with 4×10^4 fresh peptide and/or plasma-pulsed DC/well. On day 15, T cells were harvested for intracellular cytokine staining and ELISPOT assays.

2.2.7 Enzyme-linked immunospot (ELISPOT) assay

To determine the antigen-specificity of expanded CD8⁺ T cells following *in vitro* priming, IFN γ secretion in response to synthetic peptides was assessed by ELISPOT. Multi-screen_{HTS} IP filter 96-well plates were activated with 70 μ l/well 70% ethanol for 5 minutes at room temperature then washed 3 times with 200 μ l/well sterile PBS. Activated plates were coated with 70 μ l of 5 μ g/ml anti-human IFN γ (Clone:1-D1K) in sterile PBS at 4°C overnight then washed three times with 200 μ l/well sterile PBS. Plates were then blocked with 200 μ l/well AIMVAB5 for 3 hours at 37°C. Primed CD8⁺ T cells were plated at 5×10^4 - 10^5 cells/well and stimulated with peptides at a final concentration of 10 μ g/ml (or 10 μ g/ml PHA or medium only as positive and negative controls, respectively) in AIMVAB5 for 16-20 hours. Plates were then washed six times with PBS/0.05% Tween20 (PBS/T) and incubated with 70 μ l/well 0.22 μ m-filtered 1 μ g/ml biotinylated anti-human IFN γ mAb (Clone:7-B6-1-biotin) in PBS/0.5% BSA for 4 hours at room temperature. After six washes with 200 μ l/well PBS/T, 100 μ l /well of avidin-horse radish peroxidase (HRP) conjugate in PBS/T was added at room temperature for 1 hour. Wells were washed three times with PBS/T and three times with PBS then 100 μ l/well of 0.45 μ m-filtered substrate in buffer was added (0.33 mg/ml 3-amino-9-ethylcarbazole, 5% N,N-dimethylformamide, 7 mM acetic acid, 168 mM sodium acetate and 0.015% hydrogen peroxide) and the reaction was developed for 3 minutes. Washing plates in tap water stopped the reaction. Plates were dried overnight then spots counted using an AID image analysis system with AID ELISPOT software version 2.5 (Autoimmun Diagnostika GMBH, DE).

2.2.8 Intracellular cytokine staining

To determine the antigen-specificity and functional characteristics of expanded CD8⁺ T cells following *in vitro* priming, cytokine responses to synthetic peptides were assessed using an

intracellular cytokine staining flow cytometry panel. Primed CD8⁺ T cells were harvested, washed and resuspended at 10⁶ cells/ml in PBS then 1 ml/tube aliquoted into clear FACS tubes. Cells were pelleted (this and subsequent spin steps were done for 3 minutes at 1800 rpm in a Heraeus Megafuge 40R BIOLiner 75003667 rotor (Thermo Fisher Scientific) at room temperature) then resuspended in 5 µl tetramer (MBL International; HLA-A*02:01-MART1 ELAGIGILTV) and incubated in the dark for 30 minutes at room temperature. Cells were washed once with 500 µl R10 then resuspended in 1 ml R10 containing 2 µg/ml peptide with 1 µg/ml anti-CD28 and 1 µg/ml anti-CD49d for 30 minutes at 37°C. Cells stimulated in 1 ml R10 containing 2 µg/ml SEB were used as a positive control, and cells incubated in R10 medium only as a negative control. Brefeldin A and monensin were added at a concentration of 3 µg/ml and 2 uM respectively to each tube then the cells were incubated at 37°C for 5.5 hours. Cells were then pelleted, resuspended in 200 µl PBS and transferred to a V-bottom 96-well plate, then washed once more with 100 µl PBS. Cells were then stained with 20 µl of a 1:200 dilution of Live/Dead Aqua dye in PBS for 10 minutes at room temperature. Cells were washed once in 100 µl PBS then stained with CD8-APC-Cy7 for 15 minutes at room temperature. Cells were washed once in 100 µl PBS then fixed in 100 µl fixation buffer for 15 minutes at room temperature in the dark. Cells were permeabilised by 2 washes with 100 µl PermWash then stained for 15 minutes at room temperature in the dark with MIP-1β-FITC, CD3-PETR, IL-10-PerCP-eFluor750, TNF-α-eFluor710 and IFNγ-APC (see Table 2.7 for details). Flow cytometry data were acquired and analyzed as described in section 2.2.3.

2.2.9 HIV-1 IIIB propagation

The lab-adapted X4-tropic clade B strain HIV-1 IIIB was obtained from the Centre for AIDS Reagents (CFAR) at the National Institute for Biological Standards and Controls (NIBSC). HIV-1 IIIB stocks were prepared by propagation in the CD4⁺ T cell line C8166. Approximately 7 x 10⁶ C8166 cells were infected with HIV-1 IIIB in a minimal volume for 90 minutes at 37°C in a 50 ml falcon tube. C8166 cells were then resuspended in 20 ml R10 and cultured for 24 hours when a further 20 ml R10 was added. Virus-containing supernatant was harvested 5-6 days post-infection then concentrated by underlaying a 5 ml sucrose cushion (20% sucrose, 0.2 mM EDTA in PBS) under 30 ml supernatant in 38 ml Ultraclear tubes and spinning at 23,000 rpm for 2 hours at 4°C in a

swinging bucket SW28 rotor placed in a Beckman XL-80K ultracentrifuge. The virus-containing pellet in each tube was resuspended in 500 µl R10, aliquoted and frozen at -80°C.

2.2.10 NL4.3 BaL *env* plasmid production

A recombinant version of the lab-adapted X4-tropic clade B HIV-1 strain NL4.3 expressing a R5-tropic Env derived from the HIV-1 BaL strain was produced from an infectious molecular clone. The pNL4.3 BaL.ecto plasmid was a kind gift from Drs John Kappes and Christina Ochsenbauer at the University of Alabama at Birmingham, USA. Plasmid stocks were prepared by adding 30 ng plasmid to Stbl2 competent bacterial cells (Invitrogen, US, Cat:10268019) on ice for 30 minutes. Competent cells were heat shocked at 42°C for 70 seconds then placed on ice for 2 minutes. Under sterile conditions, 450 µl RT SOC (Super Optimal broth with Catabolite repression; Cat: 15544034, Thermo Fisher Scientific) media was added then the tube was shaken at 225 rpm in an Innova 4000 orbital shaker (New Brunswick Scientific, US) at 30°C for 90 minutes. 150 µl of cell solution was plated onto warm lysogeny broth (LB) agar plates containing 100 µg/ml ampicillin overnight at 37°C. The next day, growing colonies were picked and cultured in 5 ml LB media containing 100 µg/ml ampicillin for 16 hours. Plasmid was purified using the Qiaprep Spin Miniprep kit (Qiagen, Cat:27104, UK) or the Nucleobond Xtra Midi Plus kit (Macherey-Nagel, Cat:740412.50, UK) as follows. For the Qiagen kit, bacterial cells were pelleted at 8000 rpm (all spin steps were performed in a Eppendorf centrifuge 5414C) for 3 minutes at room temperature and supernatant was discarded. The cell pellet was resuspended in 250 µl Buffer P1 then cells were lysed by addition of 250 µl Buffer P2, tube inversion, and incubation at room temperature for 5 minutes. 350 µl Buffer N3 was added then the tube was immediately inverted 5 times and centrifuged at 13,000 rpm (the remaining spins were also at this speed) for 10 minutes. Supernatant from the spin step was applied to a QIAprep 2.0 column then centrifuged at 13,000 rpm for 1 minute. Flowthrough was discarded and the column was washed by addition of 500 µl Buffer PB and spun for 1 minute. Flowthrough was discarded and the column was washed with 750 µl Buffer PE, then spun for 1 minute. Flowthrough was discarded and the column was spun again for 1 minute to remove residual buffer. DNA was eluted from the column by addition of 50 µl Buffer EB, incubated at room temperature for 1 minute and spun for 1 minute into 1.5 ml microcentrifuge tubes. For the Nucleobond kit, bacterial cells were

pelleted at 5000 x g in a Avanti J-26S XP ultracentrifuge (Beckman Coulter, US, J-Lite series rotor-JLA 16.250) for 10 minutes at 4°C then resuspended in 8 ml Buffer RES then lysed by addition of 8 ml Buffer LYS, gently inverting the tube 5 times then incubating at room temperature for 5 minutes. 8 ml of Buffer NEU was added to the lysate then inverted 5 times and immediately loaded into an equilibrated Nucleobond Xtra Column/Filter. The column/filter was then washed with 5 ml Buffer EQU and the filter was removed. Then the column was washed with 8 ml Buffer WASH and plasmid DNA was eluted into a 15 ml falcon tube by addition of 5 ml Buffer ELU to the column. To precipitate DNA, 3.5 ml isopropanol was added to the eluate. The mixture was vortexed and incubated for 2 minutes at room temperature. DNA was concentrated using a NucleoBond Finalizer. Precipitation mixture was applied to a 30 ml syringe with NucleoBond Finalizer attached to the outlet. The mixture was slowly pressed through the NucleoBond Finalizer in a dropwise manner and the flow-through was discarded. Similarly, the NucleoBond Finalizer was washed with 2 ml of 70% ethanol. To minimise ethanol carry-over, the NucleoBond Finalizer was dried by passing air through it. Finally, the NucleoBond Finalizer was attached to the outlet of a fresh 1 ml syringe and DNA was eluted dropwise using 800 µl of Redissolving Buffer TRIS (5 mM Tris/HCl, pH 8.5). The first eluate was applied again into the syringe and eluted a second time into the same collection tube. The procedure was repeated 3 times in total.

DNA concentration was measured by Nanodrop (NanoDrop 2000 spectrophotometer, Thermo Fisher Scientific, US). To confirm the plasmid identity, 0.5 µg of newly-prepared plasmid DNA and the original plasmid were digested with KpnI and BstBI enzymes in 1X CutSmart buffer (Cat:B7204S, NEB, USA) for 2 hours at 37°C in a heating block then the digestion products were run on a 1% agarose gel.

2.2.11 HIV-1 strain NL4.3 BaL *env* production

Virus was produced from the NL4.3 BaL *env* infectious molecular clone by transfection into 293FT cells. Per T75 flask, 12 µg plasmid DNA was mixed gently in 1 ml OptiMem I. Separately, 25 µl Lipofectamine 2000 was mixed gently in 1 ml OptiMem I and incubated for 5 minutes at room temperature. Plasmid and Lipofectamine solutions were mixed gently together then incubated at room temperature for 30 minutes. Medium was removed from each flask containing 293FT cells and

replaced with 8 ml fresh pre-warmed D10. The plasmid DNA-Lipofectamine complex solution was added dropwise to the flask and incubated at 37°C for 3 days. At day 3 post-transfection, supernatant was harvested from the cells and spun at 2000 rpm in a Heraeus 75006445 rotor for 5 minutes to pellet cell debris. Supernatant from this step was then concentrated by underlaying a 5 ml sucrose cushion (20% sucrose, 0.2 mM EDTA in PBS) under 30 ml supernatant in 38 ml Ultraclear tubes and spinning at 23,000 rpm for 2 hours at 4°C in a swinging bucket SW32 Ti rotor placed in a Beckman XL-80K ultracentrifuge. Supernatant was poured off and the pellet was resuspended in 2 ml R10, aliquoted then frozen at -80°C.

2.2.12 HIV-1 titration

To determine the tissue culture infectious dose (TCID₅₀) of the HIV-1 IIIB stock, C8166 cells were plated at 2×10^5 /well in wells of a 96-well flat-bottom plate in 100 µl of a 10^1 - 10^7 dilution of concentrated stock and spinoculated by spinning the plate at 2200 rpm in a BIORiner 75003667 rotor for 2 hours at room temperature in a benchtop centrifuge. Cells were washed twice in R10 then cultured in 200 µl R10 for 7 days. At day 7, the p24 concentration in the supernatant was determined using the p24 ELISA kit (Advanced Bioscience Laboratories, US, Cat:5421) according to the manufacturer's instructions. Wells were scored as positive when absorbance was 200% of the average negative control value and TCID₅₀ calculated using the Reed and Muench method⁴⁷⁸.

NL4.3 BaL *env* virus stocks were titrated using a colorimetric Reverse Transcriptase assay kit (Roche, CH, Cat:11468120910) according to the manufacturer's instructions. Briefly, virus stock was diluted in to a total of 40 µl in lysis buffer (solution 4) then incubated at room temperature for 30 minutes. 20 µl of the reaction mixture (solution 3a) was added to each reaction and incubated at 37°C overnight. Samples were then transferred in duplicates to MP module wells and incubated at 37°C for one hour. Wells were then washed 5 times with 250 µl washing buffer (solution 6) for 30 seconds each. After the final wash, 200 µl of anti-DIG-POD (anti-digoxigenin-peroxidase)(solution 5a) was added per well and incubated for 1 hour at 37°C. Wells were then washed 5 times with 250 µl washing buffer (solution 6) for 30 seconds each. 200 µl of ABTS substrate (solution 7) was added to the well and absorbance at 405 nm was immediately read on a FLUOstar Omega platereader.

2.2.13 HIV-1 IIIB genome sequencing

RNA was extracted from 140 µl HIV-1 IIIB concentrated stock using a QiaAmp Viral RNA Mini Kit (Qiagen, UK). Briefly, 560 µl of Buffer AVL was deposited in a 1.5 ml microcentrifuge tube then 140 µl HIV-1 IIIB stock was added and mixed by vortexing for 15 seconds, then incubated at room temperature for 10 minutes. 560 µl of ethanol was added then samples were vortexed for 15 seconds. 630 µl of the solution was applied to a QIAamp Mini column (in a 2 ml collection tube) then centrifuged at 8000 rpm for 1 minute in a MICROSTAR 17R microcentrifuge (and all subsequent spin steps) (VWR, US). This step was repeated for the remaining lysate and flowthrough was discarded. 500 µl of Buffer AW1 was added to the column then the tube was centrifuged at 8000 rpm for 1 minute and flowthrough was discarded. 500 µl of Buffer AW2 was added to the column then centrifuged at 14000 rpm for 3 minutes. Viral RNA was eluted by addition of 60 µl Buffer AVE and a 8000 rpm spin step for 1 minute then frozen at -80°C until cDNA synthesis.

cDNA synthesis and PCR amplification of the 5' and 3' halves of the HIV-1 IIIB genome was performed on a PTC-225 Tetrad Thermal Cycler (MJ Research, US) as described⁴⁷⁹. Firstly, 15 µl RNA and 11 µl of mastermix A (0.5 mM of deoxy nucleoside triphosphate mix (Qiagen, US, Cat:201900) and 0.25 µM of the R3.B3R reverse (for 3' end of the genome) or B5R1 (for 5' end of the genome) primer (synthesized by MWG Biotech, DE, resuspended in dH₂O and stored at -20°C)) were incubated for 5 minutes at 65°C in PCR tubes then placed on ice for 1 minute. 26 µl of the reaction mixture was combined with mastermix B (5 mM DTT, 2 U/µl RNaseOUT (Invitrogen, US, Cat:1077-019), 5 U/µl Superscript III reverse transcriptase and 1X RT buffer (both Invitrogen, US, Cat:18080-093)) then incubated at 50°C for 1.5 hours. 1 µl additional Superscript III enzyme was added and tubes were incubated for another 1.5 hours at 55°C. The reaction was terminated by heating samples to 70°C for 15 minutes then 1 µl RNaseH (Invitrogen, US, Cat:18021-014) was added and samples were incubated at 37°C for 15 minutes. cDNA was stored at -80°C until PCR amplification.

Two rounds of PCR amplification were performed in a 50 µl reaction containing 1X Expand Long Template buffer 1 (17.5 mM MgCl₂), 3.75 U/µl Expand Long Template Enzyme Mix (both Roche, CH, Cat:11681834001), 0.35 mM dNTPs and 0.3 µM of each primer. To amplify the 5' and 3' genome segments, primer pairs U5B1F/B5R1 and B3F1/R3B3R were used, respectively (94°C 2

min; 37 cycles of 94°C [20 sec], 55°C [30 sec], 68°C [5 min 50 sec]; 68°C 5 min). Second round amplification was performed by transferring 1 µl of the first round product into a 50 µl reaction (prepared as above) with primer pairs U5B4F/B5R2 and B3F3/R3B6R for the 5' and 3' segments, respectively (94°C 2 min; 37 cycles of 94°C [20 sec], 58°C [30 sec], 68°C [5 min 50 sec]; 68°C 5 min). To amplify the LTR region, a separate 50 µl reaction (prepared as above) containing the '5'R' and 'gag' primers was performed (94°C 2 min; 37 cycles of 94°C [20 sec], 55°C [30 sec], 68°C [1 min]; 68°C 5 min).

PCR products were purified using the QiaQuick PCR Purification Kit (Cat:28104, Qiagen, DE). Briefly, 250 µl of Buffer PB was added to the PCR products then placed in a QIAquick column in a 2 ml collection tube then centrifuged at 13,000 rpm (same for all subsequent steps) for 1 minute and flowthrough was discarded. 750 µl of Buffer PE was added to the column and spun for 1 minute then flowthrough was discarded and the tube was spun once more to remove residual buffer. DNA was eluted by adding 50 µl Buffer EB into the column, spinning for 1 minute and collecting in a 1.5 ml microcentrifuge tube. DNA concentrations were measured using a Nanodrop 1000 (Thermo Fisher Scientific, US). Sanger sequencing was performed by Tim Rostron using a 48 capillary ABI-3730 DNA analyser (Applied Biosystems, US) using 100 ng PCR product template with 3.2 pM primers covering the HIV-1 clade B consensus (2001; LANL) genome sequence. Primer sequences are listed in Appendix 9.1, Tables 9.1 and 9.2. Assembly of sequence reads was performed using Staden software⁴⁸⁰.

2.2.14 HIV-1 infection of cells

CD4⁺ cell lines and primary CD4⁺ T cells were infected in a minimal volume with HIV-1 IIIB at a MOI of 0.01-0.1 for 90 minutes at 37°C in a 50 ml falcon tube. Immortalised CD4⁺ T cell lines and CD4.221 cells were resuspended in 20 ml R10 (7.5 x 10⁶ cells/flask) and incubated in an upright T175 flask for 24 hours before a further 20 ml R10 was added. On day 2, cells were split 1:2 with fresh R10, and they were cultured for up to 6 days more. The percentage of p24⁺ cells was determined and cell viability assessed at serial timepoints after infection by intracellular p24 staining (see section 2.2.14 below). Based on findings from the p24 staining experiments, cells were harvested for HLA immunoprecipitations on day 3 post-infection. HIV-1-infected primary CD4⁺ T

cells were resuspended at 2×10^6 /ml in R20 containing 10 IU/ml IL-2 and harvested for immunoprecipitations on days 4-6 dependent on percentage of p24⁺ cells as determined by intracellular p24 staining.

MDM were infected with 10 ng RT equivalents per 10^6 cells of the R5-tropic HIV-1 train NL4.3 BaL *env* in 10 ml R10 in a T175 flask for 6 hours at 37°C in 5% CO₂ then topped up with 10 ml R10 containing a final concentration of 20 ng/ml M-CSF. On day 4 post-infection, medium was removed and replaced with fresh exosome-free R10. On day 7, supernatant was collected for exosome isolation (see Section 2.2.26; Isolation of EVs). MDM were harvested for intracellular p24 staining or immunoblotting at different times post-infection by scraping into 10 ml PBS, and were then lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% sodium dodecylsulfate, 0.5% sodium deoxycholate, 1% NP-40 substitute; 0.22 µm filtered).

2.2.15 Intracellular p24 staining

The percentage of infected cells was determined by intracellular p24 staining as previously described⁴⁸¹ with minor modifications. In 15 ml falcon tubes, 10^6 cells were pelleted then washed and resuspended in 200 µl PBS and 1 µl Live/Dead Aqua fluorescent dye for 30 minutes at room temperature in the dark. All wash steps used 5 ml PBS and all centrifugation steps were performed at 2000 rpm in a Heraeus Megafuge 40R centrifuge with a BioLiner rotor (Cat:75003667) for 3 minutes at 4°C. Cells were washed then fixed in 1 ml 20 µg/ml lysolecithin in 4% paraformaldehyde, vortexed and incubated at room temperature for 2 minutes. Cells were pelleted, vortexed then permeabilised in 2 ml cold 50% methanol in PBS (-10 to -20°C) and incubated at 4°C for 15 minutes. Cells were then pelleted and incubated at 4°C for 5 minutes in 1 ml PBS containing 0.1% NP-40 substitute. Cells were pelleted, resuspended in 150 µl PBS, then 2 µl anti-p24-FITC antibody was added and they were incubated at room temperature in the dark for 15 minutes. Cells were washed and resuspended in 200 µl PBS, then were analyzed by flow cytometry as described in section 2.2.3.

2.2.16 Dendritic cell cross-presentation assay

To identify HIV-1 and self-peptides cross-presented by DCs, an assay was developed whereby MDDCs were incubated with exogenous protein antigen then lysed for immunopeptidome analysis

using mass spectrometry. IFN-DC were differentiated from monocytes as described in section 2.2.3. IFN-DC were then harvested by washing with 10 ml PBS per T75 flask with scraping, pelleted, counted and $58-88 \times 10^6$ cells transferred to T175 flasks in 10 ml/flask fresh pre-warmed RAB5. CD4.221 cells were then resuspended in 10 ml/flask fresh pre-warmed RAB5 and added to each flask at a 1:1 or 1:2 ratio with IFN-DC to give a final cell concentration of 8.75×10^6 /ml in a 20 ml volume. In some experiments, DCs were pre-incubated for 30 minutes at 37°C with the RT inhibitor azidothymidine (AZT) at a final concentration of 10 μ M, which was maintained for the duration of the co-culture. For SILAC experiments, CD4.221 cells were pre-labelled for 7-10 days in RPMI 1640 (without leucine, lysine, arginine and phenol red) containing only heavy stable isotopes of 0.382 mM leucine ($^{13}\text{C}_6$, $^{15}\text{N}_1$), 0.274 mM lysine ($^{13}\text{C}_6$, $^{15}\text{N}_2$) and 1.15 mM arginine ($^{13}\text{C}_6$, $^{15}\text{N}_4$), 10% 5 kDa MWCO dialysed FBS, 2.5 mg/l phenol red, 100 μ g/ml penicillin, 100 IU/ml streptomycin and 10 mM hepes (hereafter called 'SILAC R10'). SILAC-labelled CD4.221 were maintained, expanded and infected with HIV-1 IIIB in SILAC R10 for 3 days then washed once in PBS before co-culture with IFN-DC. In some experiments, 100 μ g/ml emetine dihydrochloride or cycloheximide was added to CD4.221 cells for 4 hours then washed out twice with PBS before co-culture.

2.2.17 Acid elution of surface MHC-bound peptides

IL-4-DC were washed twice with 25 ml and 2 ml PBS then incubated with 2 ml citrate phosphate buffer (pH 3.2) for 30 minutes at room temperature. Samples were sequentially spun at 300 x g for 5 minutes in a benchtop centrifuge then at 15,000 x g for 5 minutes in a microcentrifuge and supernatant was collected. Large proteins were removed by filtration through an Amicon Ultra 10kDa MWCO filter. Flowthrough was collected and dried in a Speedvac (SP Scientific, US) then resuspended in 20 μ l 0.1% trifluoroacetic acid (TFA)/1% acetonitrile for LC-MS/MS analysis.

2.2.18 Antibody purification for use in MHC isolation

Hybridomas expressing monoclonal antibodies (see Table 2.2) were cultured in appropriate growth media in T175 flasks (40 ml medium/flask) until the cells became dense and would normally have been split. Another 40 ml medium/flask was then added and cells were then allowed to die off over 10-14 days (until >50% cells were dead according to trypan blue staining). The medium was then harvested and spun at 2500 x g for 25 minutes at 4°C in a refrigerated benchtop centrifuge to pellet

cell debris. Antibody-containing supernatant was filtered through a 0.2 µm Stericup filter and adjusted to pH 8.0 with 1M Tris. Antibody was captured by addition of 2-3 ml pre-washed protein A (PAS) or protein G sepharose (PGS) beads for 30 mins at room temperature on a Celltron orbital shaker (InforsHT, CH) at 100 rpm. Beads were collected by gravity flow through an Econo-Column® chromatography column then washed with 20-30 ml PBS. Captured antibody was eluted by addition of 5 ml 100 mM glycine pH 3.0 to the beads then 1 ml fractions were collected into tubes containing 50 µl 1M Tris pH 9.5. Buffer was exchanged for PBS by concentration through a 5 kDa MWCO centrifugal filter. Antibody concentration was routinely checked by measuring absorbance at 280 nm on a NanoDrop spectrophotometer (Thermo Fisher Scientific, US). Antibody purity was routinely checked via SDS-PAGE by loading 10 µg purified antibody on a 12% TGX gel under reducing conditions and observing the presence of light and heavy chains at ~25 and 50 kDa, respectively (in the absence of other substantial protein bands).

2.2.19 Antibody resin preparation

PAS or PGS were washed in 50 ml PBS then incubated with 1-2 mg purified antibody per ml of beads for 60 minutes on a Celltron orbital shaker (InforsHT, CH) at 4°C. Beads were then transferred to an Econo-Column® chromatography column and washed with 15 ml of borate buffer (0.05 M boric acid, 0.05 M KCl, 4 mM NaOH). Beads were then equilibrated by flowing through 15 ml 0.2 M triethanolamine pH 8.2. Antibody was then cross-linked to beads by flowing through 15 ml 40 mM dimethyl pimelimidate in 0.2 M triethanolamine pH 8.3 and incubating at room temperature for 1 hour. The cross-linking reaction was ended by addition of 15 ml ice-cold 0.2 M Tris pH 8.0. Unlinked antibody was removed by flowing through 15 ml citrate buffer pH 3.0 followed by 2-3 washes with 15 ml PBS to neutralise pH.

2.2.20 HLA isolation from cells

Cells were washed once in PBS then pelleted and 1 ml IGEPAL buffer (0.5% IGEPAL 630, 50 mM Tris pH8.0, 150 mM NaCl and 1 tablet cOmplete Protease Inhibitor Cocktail EDTA-free per 10 ml buffer) was added per $0.5-1 \times 10^8$ cells, and cells were lysed by mixing for 45 minutes at 4°C on a SB3 rotator (Bibby Scientific, UK). Cells were lysed at approximately $0.5-1 \times 10^8$ cells/ml. Lysates were cleared by sequential centrifugation steps at 2000 x g (BIOLiner 75003667 rotor) for 10

minutes and 20,000 x g for 15 min at 4°C (MICROSTAR 17R, VWR, US). Peptide-MHC class I complexes were captured by incubation of lysate with 1-2 mg antibody coupled to PAS overnight at 4°C on a rotator in a final volume of 15-25 ml in a 50 ml Falcon tube. The lysate was flowed through a pre-washed chromatography column then the beads were consecutively washed with 15 ml wash buffer 1 (0.005% IGEPAL, 50 mM Tris pH8.0, 150 mM NaCl, 5 mM EDTA), 15 ml wash buffer 2 (50 mM Tris pH8.0, 150 mM NaCl), 15 ml wash buffer 3 (50 mM Tris pH8.0, 450 mM NaCl) and 15 ml wash buffer 4 (50 mM Tris pH8.0). Peptide-MHC complexes were eluted by addition of 3-5 ml 10% acetic acid and collected in 1 ml fractions. Eluates were dried in a SpeedVac (SP Scientific, US).

2.2.21 Reverse-phase high performance liquid chromatography (RP-HPLC) peptide purification

In order to purify peptides, co-eluted protein material was separated from peptides using RP-HPLC. Immunoaffinity column-eluted peptide-MHC complexes were resuspended by vortexing and sonication in 120 µl 0.1% trifluoroacetic acid (TFA)/1% acetonitrile then loaded into glass mass spectrometry vials (Waters, US, Cat: 186000384C). Samples were injected onto a 4.6 x 50 mm ProSwift RP-1S column (Thermo Fisher Scientific, Cat:064297) and eluted using a 500 µl/min flow rate over 10 min from 3 to 35% buffer B (0.01% TFA in acetonitrile) in buffer A (0.01% TFA in water) using an Ultimate 3000 HPLC system (ThermoFisher Scientific). Detection was performed using a variable wavelength detector at 280 nm. Odd and even fractions of 500 µl up to 12 min that did not contain β2-microglobulin were combined and dried in 2 ml protein Lo-bind Eppendorf tubes in a Speedvac.

2.2.22 Tryptic digest of cellular/subcellular proteomes

10-50 µg of lysates or protein samples were diluted to 200 µl with Milli-Q H₂O then reduced by addition of DTT to a final concentration of 10 mM for 20 minutes at room temperature. Alkylation was achieved by addition of iodoacetamide to a final concentration of 50 mM for 20 minutes at room temperature. Proteins were precipitated via the methanol/chloroform extraction method as follows. 600 µl methanol and 150 µl chloroform were added to samples and tubes were vortexed. 450 µl Milli-Q H₂O was added, then tubes were vortexed again and spun at 20,000 x g for 1 minute at room

temperature (MICROSTAR 17R, VWR, US). The upper aqueous phase was removed then 450 µl methanol were added, and the samples were vortexed and centrifuged at 20,000 x g at room temperature for 2 minutes. Supernatant was removed, then the protein pellet was resuspended in 50 µl urea buffer (6M urea, 0.1 M Tris pH 7.8) by vortexing and sonication (Ultrasonic Cleaner, VWR, US, Cat:142-6005). The concentration of urea was reduced by addition of 250 µl Milli-Q H₂O, then samples were vortexed. Trypsin was added to samples at a 1:50 protein ratio and they were incubated on a 37°C heated shaker (Thermomixer Compact, Sigma, Cat:T1317) at 1200 rpm overnight for digestion. Tryptic peptides were purified the next day on C18 Sep-Pak Light cartridges (Waters, US, Cat: 186000384C) previously equilibrated with 5 ml Sep-Pak Buffer B (65% acetonitrile, 0.1% trifluoroacetic acid) and 10 ml Sep-Pak Buffer A (2% acetonitrile, 0.1% trifluoroacetic acid). Cartridges were washed twice with 10 ml Sep-Pak Buffer A then peptides eluted using 2 x 600 µl Sep-Pak Buffer B into 2 ml Protein Lo-bind Eppendorf tubes and dried down in the Speedvac. Tryptic peptides were resuspended in 10-50 µl loading buffer (1% acetonitrile, 0.1% trifluoroacetic acid) in preparation for LC-MS/MS analysis.

2.2.23 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Purified peptides were resuspended in 20 µl 0.1% TFA/1% acetonitrile by vortexing and sonication then loaded into glass mass spectrometry vials (Waters, US). MHC peptide samples were analysed on either an Orbitrap Elite (ThermoFisher Scientific, US) online coupled to an Acquity nano UPLC (Waters, US), a TripleTOF 5600 (AB SCIEX, US) online coupled to a ekspert nanoLC 400 cHiPLC system (Eksigent) or a Fusion Lumos (ThermoFisher Scientific, US) online coupled to an Ultimate 3000 HPLC system (ThermoFisher Scientific, US). Tryptic digest samples were analysed on a QExactive-HF.

OrbiTrap Elite: Peptides were separated on a nano Acquity UPLC system (Waters, US) supplemented with a 25 cm BEH130 C18 column, 1.7 mm particle size using a linear gradient from 8 to 35% buffer B in buffer A at a flow rate of 250 nL/min for 60 min. Peptides were introduced to an Orbitrap Elite mass spectrometer using a nanoESI source. Subsequent isolation and collision-induced dissociation was induced on the 20 most abundant ions per full MS scan using an isolation width of

1.5 amu. All fragmented precursor ions were actively excluded from repeated selection for 15 seconds.

TripleTOF 5600: Peptides were separated on an ekspert nanoLC 400 cHiPLC system (Eksigent) supplemented with a 15cmx 75µm ChromXP C18-CL, 3 µm particle size using a linear gradient from 8% buffer A to 35% buffer B at a flow rate of 300 nL/min for 60 minutes. Peptides were introduced to the TripleTOF 5600 mass spectrometer and collision-induced dissociation fragmentation using ramped collision energy was induced on the 30 most abundant ions per full MS scan using unit isolation width 0.7 amu. All fragmented precursor ions were actively excluded from repeated selection for 15 seconds.

Fusion Lumos: MHC class I peptides were separated on an Ultimate 3000 HPLC system supplemented with a 75 µm x 50 cm PepMap RSLC C18 Easy Spray column using a linear gradient from 3-25% buffer B at a flow rate of 250 nl/min for 60 minutes. Peptides were introduced to the Fusion Lumos using an Easy-Spray source. Precursors were selected in top-speed mode within a 2 second cycle time (injection time of 120 ms) and an isolation width of 1.2 amu for fragmentation. Higher-energy collisional dissociation (HCD) with a collision energy setting of 28 was performed on the peptides with a charge state of 2-4, whilst a higher collision energy of 32 was applied to singly-charged precursor ions. MS resolution was set at 120,000 and MS² resolution was set at 30,000. All fragmented precursor ions were actively excluded from repeated selection for 30 seconds. Parameters were similar for MHC class II peptides with the exception of a 3-30% buffer B gradient, and only peptides with 2-5 charges were acquired.

Q-Exactive-HF: Tryptic digest samples were separated on an Ultimate 3000 HPLC system supplemented with a 75 µm x 50 cm PepMap RSLC C18 Easy Spray column using a linear gradient from 2-35% buffer B at a flow rate of 250 nl/min for 60 minutes. Peptides were introduced to the QExactive-HF using an Easy-Spray source. HCD with a collision energy setting of 28 was performed on the top 12 most abundant precursor ions per full MS scan (injection time of 45 ms) using an isolation width of 1.2 amu. Full MS resolution was set at 60,000. Only peptides with a charge state of 2-5 were isolated and fragmented. All fragmented precursor ions were actively excluded from repeated selection for 27 seconds.

2.2.24 Mass spectrometry data analysis

TripleTof 5600 data were converted to MASCOT generic files using msconvert⁴⁸² or ProteinPilot 4.5⁴⁸³. Data generated by ThermoFisher instruments was imported into PEAKS 7 software (Bioinformatic Solutions, Waterloo CA)⁴⁸⁴ as .raw files. A database containing all annotated human Swiss-Prot (http://www.uniprot.org/uniprot/?query=*%&fil=reviewed%3Ayes) entries including translations of all six reading frames of the sequenced HIV-1 IIIB genome was used for interpretation of MS/MS spectra (20296 entries). In analyses of samples from CD4.221 cells, the Epstein-Barr virus proteome (strain B95-8, <http://www.uniprot.org/taxonomy/10377>; 113 entries) was included in the database. Precursor and fragment error tolerances were generally set at 10 Da and 0.05 ppm, respectively. A false discovery rate (FDR) of 5% was defined using parallel decoy database searches. Peptide spectrum matches were exported as .csv files. Duplicate peptides and peptides <7 amino acids in length were excluded from all analyses. Where two possible peptides differing only by a single leucine to isoleucine residue were identified, both matches were retained in the dataset. Putative HLA restriction of identified peptides was predicted using the NetMHC4.0 online algorithm (<http://www.cbs.dtu.dk/services/NetMHC/>)^{485,486}. Sequence logos of identified peptides of equal length were produced using Seq2Logo (<http://www.cbs.dtu.dk/biotools/Seq2Logo-1.1/index.php>)⁴⁸⁷. Area-proportional Venn diagrams were created using the online BioVenn tool (<http://www.cmbi.ru.nl/cdd/biovenn/>)⁴⁸⁸. Gibbs Clustering analysis was performed on identified 9-mers with the online GibbsCluster 1.1 server (<http://www.cbs.dtu.dk/services/GibbsCluster/>) using the default settings without alignment, 1-6 number of clusters and a trash cluster with a threshold score of zero to remove outlier peptides⁴⁸⁹. Where intensity values were missing for one or more samples, the corresponding peptides were excluded from peptide abundance analyses. To relatively quantitate proteins present in tryptic digest samples, data were imported into MaxQuant software. Data were searched against the human Swissprot and HIV-1 IIIB genome database as above (20296 entries) with carbamidomethyl fixed modification. A reversed decoy database was used to set a threshold of 1% FDR for identified peptides. Only unique (no razor) peptides were used for protein quantitation. Intensity-based absolute quantitation (iBAQ) values as calculated by MaxQuant were used to compare relative amounts of HIV-1 proteins present within each sample.

2.2.25 T2 HLA-E stabilisation assay

Peptide binding to HLA-E was assessed by analyzing the ability of peptides to stabilize HLA-E expression on the surface of the TAP-deficient T2 cell line⁴⁹⁰. T2E1 and T2E3 cells were cultured in R10 at 26°C and 5% CO₂ overnight to enable higher levels of MHC class I to accumulate at the cell surface, then were washed and incubated with 1-200 uM test peptides for 3-24 hours in serum-free AIM V media at 37°C and 5% CO₂. The canonical HLA-E-binding peptide VMAPRTL_{LL} ('VL9')⁴⁹¹ was used as a positive control whilst cells treated with an equivalent concentration of DMSO served as a negative control. Cells were washed in 200 µl PBS then stained with 1 µl anti-HLA-E-PE for 30 minutes on ice. Cells were washed once in 200 µl PBS then fixed in 4% paraformaldehyde. Sample data were acquired on a Cyan ADP Analyser and analyzed as described in section 2.2.3.

2.2.26 Isolation of EVs

Bovine exosome-free medium was prepared by spinning R20 at 100,000 x g for 16-18 hours at 4°C in 38 ml Ultra-Clear centrifuge tubes in a SW28 or SW32 Ti rotor in a XL-80K ultracentrifuge (Beckman Coulter, US). Supernatant was diluted with RPMI 1640 to yield a final FBS concentration of 10% then filtered through a 0.1 µm SteriCup filter to yield exosome-free R10. Cells were cultured in exosome-free R10 for 24-96 hours. Vesicle-containing supernatant was first cleared of cell debris by spinning at 2000 x g for 10 minutes at 4°C in a benchtop centrifuge. Microvesicles were pelleted by spinning supernatant at 20,000 x g for 30 minutes at 4°C in a SW28 or SW32 Ti swinging bucket rotor placed in a XL-80K ultracentrifuge. Supernatant was harvested and exosomes were isolated by spinning supernatant at 100,000 x g for 2 hours at 4°C in a SW28 or SW32 Ti swinging bucket rotor placed in a XL-80K ultracentrifuge. To isolate vesicles from human plasma samples, neat plasma was diluted 1:7 with PBS then cleared of debris by a 2000 x g spin for 10 minutes at 4°C in a benchtop centrifuge. The supernatant was then spun at 100,000 x g for 2 hours at 4°C in order to isolate microvesicles and exosomes together. Vesicles were often resuspended in 35 ml PBS then pelleted again at 100,000 x g for 2 hours to remove extravesicular soluble protein complexes. Tubes were dried with KimWipes then vesicle pellets were resuspended in PBS for functional assays or lysed in 50 µl RIPA buffer for SDS-PAGE.

2.2.27 Separation of HIV-1 virions from exosomes by iodixanol gradient

In order to separate the relatively dense HIV-1 virions from exosomes secreted by HIV-1-infected cells, iodixanol density gradients were prepared as previously described⁴⁹². Exosomes/virions pelleted at 100,000 x g were resuspended in 1 ml PBS by pipetting 10-15 times. A 6-18% iodixanol/PBS gradient in 1.2% increments was formed by slowly overlaying twelve 1 ml fractions on top of each other in a 13.2 ml UltraClear tube. The exosome/virion pellet was then overlaid slowly on top of the gradient and the tube was spun at 200,000 x g in a SW41 Ti rotor for 1.5 hours at 4°C in a XL-80K ultracentrifuge. Fractions 1-12 were each harvested from the top of the gradient slowly using a 1 ml pipette. In some experiments, reverse transcriptase activity or p24 concentration were measured in collected fractions by the colorimetric Reverse Transcriptase assay (described in section 2.2.12) or the p24 ELISA kit (described in section 2.2.12), respectively. Fraction density was measured by absorbance of 100 µl of fractions at 340 nm in a FLUOstar Omega platereader (BMG Labtech, DE).

2.2.28 Acetylcholinesterase activity assay

Because acetylcholinesterase (AChE) has been reported as a useful marker of exosomes when separating exosomes from virions by iodixanol density gradients⁴⁹³, AChE activity assays were performed on density gradient fractions. 100 µl of iodixanol gradient fractions were mixed with 100 µl Ellman's reagent (prepared by mixing 1.25 mM acetylthiocholine in PBS pH 8 with 0.1 mM 5,5-dithio-bis(2-nitrobenzoic acid) in PBS pH 7) for 10 minutes at room temperature. Endpoint absorption at 450 nm was measured on a FLUOstar Omega platereader.

2.2.29 Separation of HIV-1 virions from exosomes by CD45 depletion

Since virions are reported to exclude CD45 from their membrane envelope, a CD45 depletion method reported by previous studies^{494,495} was optimized in order to separate and characterize exosomes and virions secreted by HIV-1-infected cells. Exosomes/virions pelleted at 100,000 x g were resuspended in 200 µl PBS. A LD depletion column was first washed with 2 ml cold TNE buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA). 200 µl of CD45 microbeads were then added to the LD column under the influence of a magnetic field. Beads on the column were washed 3 times with 0.5 ml cold TNE buffer then the column was removed from the magnetic field and beads were eluted by adding 200 µl TNE buffer. Beads were then added to exosomes/virions sample, mixed and

incubated at 4°C for one hour. The mixture was then applied to a fresh, pre-washed LD depletion column and flowthrough collected with two 0.5 ml washes with TNE buffer. The LD column was then removed from the magnetic field and flushed with 6 ml TNE buffer to elute CD45⁺ vesicle-bead complexes. Flowthrough and eluate fractions were then diluted with PBS to 13 or 35 ml in 13.2 ml Ultraclear or 36 ml Ultraclear tubes and tubes were spun at 100,000 x g or 200,000 x g for one hour at 4°C in a SW28 or SW32 Ti rotor, respectively. For western blotting, pellets were resuspended in 50 µl RIPA buffer on ice, vortexed, sonicated and cleared of debris by spinning at 20,000 x g for 15 mins and supernatant frozen at -20°C.

2.2.30 TzM-B1 luciferase assay

TzM-B1 cells were used in order to titrate relative amounts of infectious HIV-1 particles in iodixanol density gradient fractions. TzM-B1 cells are infectable by diverse HIV-1 isolates and contain integrated copies of the luciferase gene under the control of the HIV-1 promoter, thus serving as a quantitative indicator of infectious HIV-1 concentration⁴⁹⁶. TzM-B1 cells were plated at 1.5×10^4 cells/well of a flat-bottom 96-well plate in 100 µl of D10 then 100 µl of each iodixanol gradient fraction was added. Cells were incubated at 37°C for 48 hours. Luciferase activity within TzM-B1 cells was then measured using the Luciferase Assay System (ProMega, Cat: E4030) according to the manufacturer's instructions.

2.2.31 Trichloroacetic acid (TCA) protein precipitation

In some experiments, 250 µl of 100% (w/v) TCA was added to 1 ml iodixanol fractions and incubated overnight at -20°C to precipitate protein. Samples were then spun at 14,000 rpm in a MICROSTAR 17R microcentrifuge (VWR, US) for 5 minutes at room temperature to pellet the precipitate. Supernatant was discarded and the protein pellet was washed twice with 200 µl cold acetone. The pellet was dried by placing the open tube in a 95°C heating block for 5 minutes, then was resuspended in Milli-Q H₂O for immunoblotting.

2.2.32 Immunoblotting

Cells and EV material were lysed in RIPA buffer for 30 mins on ice then vortexed, sonicated and cleared at 20,000 x g for 15 minutes at 4°C. Supernatant was stored at -20°C. Protein concentration

was measured using the BCA Assay kit (Cat:23225, ThermoFisher Scientific, UK). Briefly, 10 µl of lysate and 200 µl of working reagent (50 parts BCA reagent A, 1 part BCA reagent B) were mixed in a flat-bottomed 96-well plate then incubated at 37°C for 30 minutes. Absorbance was measured at 562 nm on a FLUOstar Omega platereader (BMG Labtech, DE) and blank values were subtracted from sample replicate values then a standard curve was used to calculate protein concentrations.

Protein lysates were denatured in 1x Laemmli Buffer containing 50 mM DTT for 5 minutes at 95°C then put on ice. Protein samples were loaded onto 12% TGX polyacrylamide gels and run for 1-1.5 hours at a constant voltage of 130V in Tris-Glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% sodium dodecyl sulfate (SDS)). Immobilon-FL PVDF membrane was pre-wetted for 30 seconds in methanol then equilibrated in transfer buffer (25 mM Tris, 192 mM Glycine, 0.01% SDS, 20% methanol). Protein was then transferred from the polyacrylamide gel to pre-wetted membrane using a semi-dry transfer system (Trans-blot SD, Bio-Rad, US) run at a constant 15V for 1 hour. Membranes were then washed once in transfer buffer and blocked for 1 hour in Odyssey PBS Blocking Buffer at room temperature on a rocker. Membranes were then cut and incubated overnight at 4°C in a 50 ml Falcon tube with primary antibodies diluted (as detailed in Table 2.8) in Odyssey PBS Blocking Buffer. Membranes were washed 3 times for 10 minutes in PBS containing 0.1% Tween 20 (PBS/T) at room temperature on a rocker. Blots were then incubated with 15 ml Odyssey Blocking Buffer containing a 1:15,000 dilution of anti-mouse-680RD or anti-rabbit-800CW infrared detection secondary antibodies for 1 hour at room temperature on a MAXI rotator (Labline, IN). Membranes were again washed 3 times in PBS/T and once in PBS for 5 minutes each then dried overnight between KimWipe tissue at 4°C. Blots were visualised on a Licor Odyssey CLX instrument according to the manufacturer's settings. Fluorescent bands were quantitated in Li-Cor software.

2.2.33 Exosome staining and uptake assays

For vesicle surface staining, debris (pelleted at 2000 x g), microvesicles (subsequently pelleted at 20,000 x g) or exosomes (subsequently pelleted at 100,000 x g, as described in section 2.2.26) pellets were resuspended in 50 µl particle-free PBS. 12.75 µg protein equivalents debris, 10 µg microvesicles and 0.85 µg exosomes were incubated with 5 µl aldehyde/sulphate latex beads (4 µm,

4% w/v) for 15 minutes at room temperature. 1 ml PBS was added and mixed gently for 1 hour at room temperature. Bead-vesicle complexes were pelleted by spinning at 3000 x g for 10 minutes then blocked by addition of 20 µl FCS for 30 minutes at room temperature. Vesicle-bead complexes were then washed 3 times by addition of 5 ml PBS followed by spins at 3000 x g for 10 minutes. Beads were then resuspended in 100 µl PBS and stained with 2.5 µl each of CD9-FITC, CD63-FITC, CD81-FITC or isotype control-FITC for one hour at 4°C. Vesicle-bead complexes were again washed 3 times with 5 ml PBS between 3000 x g spins for 10 minutes. Samples were resuspended in 200 µl PBS then data were acquired on a Cyan ADP Analyzer as described in section 2.2.3.

For DC exosome uptake assays, exosomes were resuspended in PBS then stained with 0.2 mM Bodipy-FL Maleimide for 20 minutes at room temperature. Exosomes were diluted to 11 ml in PBS then pelleted at 100,000 x g for one hour at 4°C. Pellets were resuspended in PBS then added to 2 x 10⁶ IFN-DC in 24-well plates. After incubation at 4°C or 37°C for 1-20 hours, cells were washed once in 5 ml PBS then fixed in 2% PFA for 10 minutes at room temperature then sample data were acquired on a Cyan ADP Analyser and analyzed as described in section 2.2.3.

2.2.34 Nanoparticle tracking analysis (NTA)

To determine the size distribution and concentration of microvesicles and exosomes in cell supernatants, EV pellets were subjected to NTA. Samples were diluted to 2-10 x 10⁸ particles/ml then 5 sequential videos of 60 seconds were captured at a camera exposure level of 12 on a NS500 nanoparticle analysis instrument (Nanosight, UK). Nanoparticle tracking analysis settings were set according to the manufacturer's instructions.

2.2.35 LABScreen single antigen HLA class I antibody cross-reactivity

LABScreen Single Antigen Class I Combi beads (One Lambda, US)⁴⁹⁷ were used in order to determine the cross-reactivity of the HLA-E-specific antibody 4D12 with HLA class Ia alleles. LABScreen Single Antigen Class I Combi beads were incubated 4D12 antibody for 30 minutes at room temperature in 96-well plates then washed 3 times with 200 µl PBS. Beads were then incubated with goat anti-mouse IgG-R-PE (Invitrogen, US. Cat:P-852) for 30 minutes at room temperature and

washed another three times with 200 µl PBS. Samples were analysed on a LABScan 100 flow analyser (One Lambda, US).

2.2.36 Statistics and data presentation

Statistical tests (Mann-Whitney tests, Wilcoxon ranked-sign tests and Spearman's rank correlations) were performed and graphs were created using GraphPad Prism 7 Software (GraphPad Software Inc, USA).

Chapter 3: *In vitro* modelling of CD8⁺ T cell priming during AHI

3.1 Introduction

HIV-1-specific CD8⁺ T cell responses to individual epitopes expand asynchronously during AHI^{326,342}. The first wave of responses peaks within a few days of peak viraemia then rapidly wanes as viral load decreases¹⁵. These early HIV-1-specific CD8⁺ T cell responses during AHI not only correlate temporally with control of viraemia^{100,101}, but also contribute to its decline. Evidence for CD8⁺ T cell-mediated control of virus replication in AHI comes from studies showing that CD8⁺ T cells drive rapid selection for escape mutants during AHI^{102–104}, CD8-depletion studies in macaques¹¹⁰ and the association of particular HLA class I alleles with fast or slow disease progression^{106–109}. Notably, the first wave of responses are characteristically narrowly directed, each individual typically recognising only 1-3 epitopes at this stage of infection, and a high proportion of these responses target epitopes within the HIV-1 Nef protein^{342–344}. Although some have speculated that these Nef-specific responses become immunodominant due to the early expression kinetics of the Nef protein within infected cells³⁵², the mechanism behind this phenomenon remains ill-defined. The second wave of responses is typically delayed, expanding as the first wave of responses decline and peaking 1-3 weeks later, and targets a broader repertoire of HIV-1 epitopes^{326,342}. The kinetics of responses in AHI differ significantly from those of epitope-specific CD8⁺ T cell responses during acute infections with many other viruses, where broadly-directed responses of differing immunodominance expand synchronously and wane as the virus is cleared, or undergo subsequent changes in immunodominance if virus persists⁴⁹⁸. The mechanisms underlying the development of the narrowly-directed, Nef-biased primary CD8⁺ T cell response in AHI are not clear.

mDCs are thought to be the major cell type involved in T cell priming *in vivo*²⁸², but the relative contribution of direct-presentation and cross-presentation to CD8⁺ T cell response induction in virus infections is not very clear. Productive infection is blocked in HIV-1-infected DCs⁴⁷⁶, hence endogenous presentation of HIV-1 epitopes by DCs is thought not to occur to a significant extent⁴⁹⁹. Therefore priming of naïve CD8⁺ T cells by cross-presenting DCs is likely to be more important than direct presentation by HIV-1-infected DCs.

CD8⁺ T cell response specificity is determined by pre-existing T cell repertoire and by DC-mediated antigen presentation. The Nef bias of primary CD8⁺ T cell responses could be determined by the pre-existing memory T cell repertoire, however this is unlikely given that such a repertoire would require substantial T cell cross-reactivity towards one protein containing hundreds of potential epitopes with considerable variation across the viral population within individuals of diverse HLA binding preferences and immunological history. Although it's common for a single epitope sequence to be present somewhere in the microbiome⁵⁰⁰, sharing a whole protein seems far less likely. This hypothesis could be explored using the T cell library method³⁴⁰. However, in this thesis I focused on the hypothesis that DCs are preferentially presenting and priming CD8⁺ T cell responses to Nef.

To explain the reason for the narrow breadth of the primary HIV-1-specific CD8⁺ T cell response and the delay before expansion of additional response specificities, I worked on the hypothesis that T cell priming is interrupted very soon after it begins because of an impairment in DC functions, hence only responses to a very small number of epitopes are initially primed. Subsequently there could be a delay before DC functionality is restored (potentially because new DCs need to be generated) and responses of additional specificities are expanded.

Very little is known about DC function during AHI, although dysfunction of DCs (particularly reduced IL-12 secretion in response to TLR ligands) has been described at timepoints prior to the peak of viremia⁵⁰¹. A few mechanisms could contribute to impairment of DCs during AHI. Frleta *et al* showed that microvesicles released by apoptotic cells can inhibit DC function through CD44 engagement⁴⁶. Decreased IL-12, TNF α and IL-6 release by DCs was observed in response to TLR3, TLR4, TLR5 and TLR7/8 ligands after exposure to microvesicle-containing plasma from acutely-infected HIV-1⁺ patients. These microvesicles shed from apoptotic CD4⁺ CCR5⁺ cells increase in number concurrently with viral load during AHI⁴⁵. Consistent with this, the magnitude of inhibition was greatest when DCs were pulsed with plasma from Fiebig stages I-II⁴⁶. Furthermore, inhibition of DC function seems to be independent of infectious HIV-1 virions^{46,502}. However, it should be noted that HIV-1 gp120 protein has been shown to inhibit autophagy in monocyte-derived DC through activation of the mTOR pathway⁵⁰³. In this setting, autophagy inhibition by gp120 resulted in decreased TNF α production in response to TLR4 and TLR8 ligands, as well as hindered antigen

processing and presentation to Ag-specific CD4⁺ T cells. Soluble CD40 ligand (sCD40L), is upregulated in both therapy-naïve and treated patients relative to uninfected controls⁵⁰⁴⁻⁵⁰⁶. Recently, exposure to both monomeric and multimeric forms of soluble CD40 ligand were shown to reduce DC IL-12 secretion in response to poly I:C stimulation and ability to induce proliferation of allogeneic naïve CD4⁺ T cells⁵⁰⁷. Soluble HLA class I molecules, which are increased in plasma during HIV-1 infection, inhibit DC function through interaction with LILRB2⁵⁰⁸.

Additionally, the cytokine storm during AHI⁴³ could have a detrimental effect on DC function. IFN α for example, is one of the earliest cytokines to peak at high levels in plasma during AHI⁴³. IFN α has been shown to induce DC activation and maturation followed by apoptotic death in the acute phase of LCMV infection in mice, so may likewise contribute to activation-induced death of DCs in AHI^{509,510}; and in MCMV infection in mice IFN α production by pDCs has been shown to reduce IL-12 production by mDCs, which may impact on the T cell response they induce⁵¹¹. These results beg the so-far unexplored question; do these inhibitory factors impede CD8⁺ T cell priming during AHI?

In vitro T cell priming assays have been extensively used to assess questions such as the requirements for optimal expansion of antigen-specific T cell responses, the relative priming ability of DC subsets, the role of particular cytokines in enhancing priming, and the antigen specificities arising under chosen *in vitro* conditions. The type of APC, maturation stimuli, type of T cell population, antigens used, medium growth factors and cytokines, co-culture ratios, re-stimulation schedules and readouts all differ significantly depending on the aims of each study. Many protocols even include memory T cells in their ‘input’ population and therefore cannot exclude the possibility of re-stimulation of Ag-specific memory cells. Importantly, HIV-1-specific memory T cells are known to be present in the blood of HIV-1 seronegative individuals; these are thought to be cross-reactive cells primed by exposure to microbial antigens^{340,341}.

In order to explicitly examine the process of CD8⁺ T cell priming during AHI, in this chapter I aimed to:

- Establish an *in vitro* CD8⁺ T cell priming assay
- Explore its utility for evaluating the specificity of CD8⁺ T cell responses primed by HIV-1-infected DCs or DCs exposed to viral components

- Use the *in vitro* priming assay to examine the mechanisms contributing to impairment of CD8⁺ T cell priming by DCs during AHL.

3.2 Results

3.2.1 Establishment of an *in vitro* CD8⁺ T cell priming assay and evaluation of its utility for *in vitro* priming of HIV-1-specific CD8⁺ T cell responses

As mDC are present in blood at insufficient numbers for large-scale *in vitro* CD8⁺ T cell priming assays, I employed a well-described method to produce DCs differentiated from blood monocytes. The most commonly used protocol differentiates adherent monocytes into IL-4-DCs by addition of recombinant IL-4 and GM-CSF. IFN-DCs, known for their superior cross-presentation ability⁵¹²⁻⁵¹⁵, were also differentiated from adherent monocytes using IFN α and GM-CSF. IL-4-DCs were comprised of both adherent and non-adherent large/granular cells, whereas the IFN-DC tended to be predominantly non-adherent and smaller in size and granularity (Figure 3.1A). I then confirmed that monocytes isolated from frozen PBMC and differentiated into IL-4-DC or IFN-DC expressed surface markers characteristic of monocyte-derived DCs. IL-4-DC and IFN-DC were treated with or without a maturation cocktail containing IL-6, TNF α , IL-1 β and PGE₂ then the expression of CD11c, CD40, CD83, CD86 and HLA-DR was measured by flow cytometry. Both IL-4-DC and IFN-DC, regardless of maturation state, expressed high levels of the myeloid cell integrin marker CD11c (Figure 3.1B). In contrast, the maturation marker CD83 was only expressed on the surface of matured IL-4-DC. The modest expression of CD40, HLA-DR and CD86 by IL-4-DC and IFN-DC was further increased upon exposure to the maturation cocktail. The majority of isolated DC did not express the monocyte marker CD14 (Figure 3.1C). Additionally, the ability of IL-4-DC and IFN-DC to endocytose antigen was tested by incubating cells with dextran-FITC and measuring uptake by flow cytometry. As expected, immature DC displayed greater endocytosis activity than mature DC (Figure 3.2), although the effect of maturation was more pronounced for IL-4-DC than for IFN-DC. In summary, I was able to differentiate large numbers of monocyte-derived DC characterised by known DC markers and endocytosis ability, which could then be used in *in vitro* priming assays.

For *in vitro* priming of CD8⁺ T cells, these matured antigen-presenting DC were pulsed with antigen (or left unpulsed as a negative control) and then co-cultured with autologous naïve CD8⁺ T cells and primed T cells were expanded over 15 days based on an adapted protocol (Figure 3.3) previously described by another group⁵¹⁶. To isolate untouched naïve CD8⁺ T cells from bulk PBMC, a two-step

method was optimised. Total naïve T cells were first negatively isolated then naïve CD4⁺ T cells were depleted by positive selection. I routinely observed >99% of isolated cells were CD4 negative and expressed the naïve T cell marker CD45RA (Figure 3.4A). The majority of these cells were likely naïve CD8⁺ T cells, although the population isolated would also be expected to contain other naïve CD4⁺ T cells (e.g. double negative T cells) and antigen-experienced CD8⁺ T cells that had re-expressed CD45RA. Although the level of expansion varied between donors, CD8⁺ T cells in co-cultures with antigen-pulsed DCs typically outnumbered by 2-fold CD8⁺ T cells in co-cultures with unpulsed DCs at day 15 (data not shown). After 15 days co-culture, >95% remaining cells typically co-expressed CD3 and CD8 (Figure 3.4B). Having isolated and characterised monocyte-derived DCs and autologous naïve CD8⁺ T cells, I then attempted to expand HIV-1-specific CD8⁺ T cells from the naïve repertoire of HIV-seronegative donors using DCs pulsed with diverse forms of antigen.

Firstly, IL-4-DCs from the HLA-A*02:01⁺ donor 127 were pulsed with a mix of 3 optimal-length HLA-A*02:01-restricted HIV-1 epitope peptides (or unpulsed as a negative control) then co-cultured with autologous naïve CD8⁺ T cells. At day 15, CD8⁺ T cells were harvested and restimulated with each optimal-length epitope in an ELISPOT assay. Strong IFN γ responses of up to 3000 spot-forming units/million cells were observed in the VL10 peptide-treated wells, but not for SL9- or PL10-treated wells (Figure 3.5), thus confirming that the *in vitro* priming assay was capable of priming HIV-1-specific CD8⁺ T cells.

Further *in vitro* priming assays were set up incorporating monocyte-derived DC pulsed with either 1) optimal-length HLA-A*02:01/HLA-A*03:01/HLA-B*07:02-restricted HIV-1 epitope peptides 2) whole p24 protein or 3) HIV-1 IIIB-infected CD4⁺ T cells. In 3/11 experiments using DCs from different donors pulsed with mixes of optimal HIV-1 peptides, strong IFN γ responses to 1-2 peptides per donor were observed (Figure 3.6A). These responses were directed towards HLA-A*02:01, HLA-A*03:01 and HLA-B*07:01-restricted peptides (Figure 3.5, Figure 3.6C). Magnitudes of expanded epitope-specific IFN γ responses ranged between approximately 1000-3000 SFU/10⁶ cells. However, DC pulsed with supraphysiological concentrations of optimal HIV-1 peptides often failed to activate antigen-specific naïve CD8⁺ T cells (Figure 3.6A, B). Both IL-4-DCs and IFN-DCs primed antigen-specific CD8⁺ T cells infrequently (data not shown). Cells expanded from donor 105,

regardless of the DC treatment, exhibited a low magnitude IFN γ response against a mixture of optimal-length epitope peptides restricted by common HLA alleles from influenza virus, EBV and CMV (FEC peptides) despite the absence of reactivity towards HIV-1-specific peptides (Figure 3.6B). This low-level response was probably mediated by contaminating FEC-reactive CD45RA⁺ memory cells in the naïve CD8⁺ T cell fraction. Moreover, DCs pulsed with whole antigen (p24 protein or HIV-1-infected CD4⁺ T cells) were unable to expand detectable levels of IFN γ -producing HIV-1-specific CD8⁺ T cells by day 15 under any conditions (examples in Figure 3.6B, C). In summary, HIV-1-specific T cell responses were only primed sporadically *in vitro*, suggesting that use of this approach for assessment of the HIV-1-specific CD8⁺ T cell repertoire primed during AHI would not be possible.

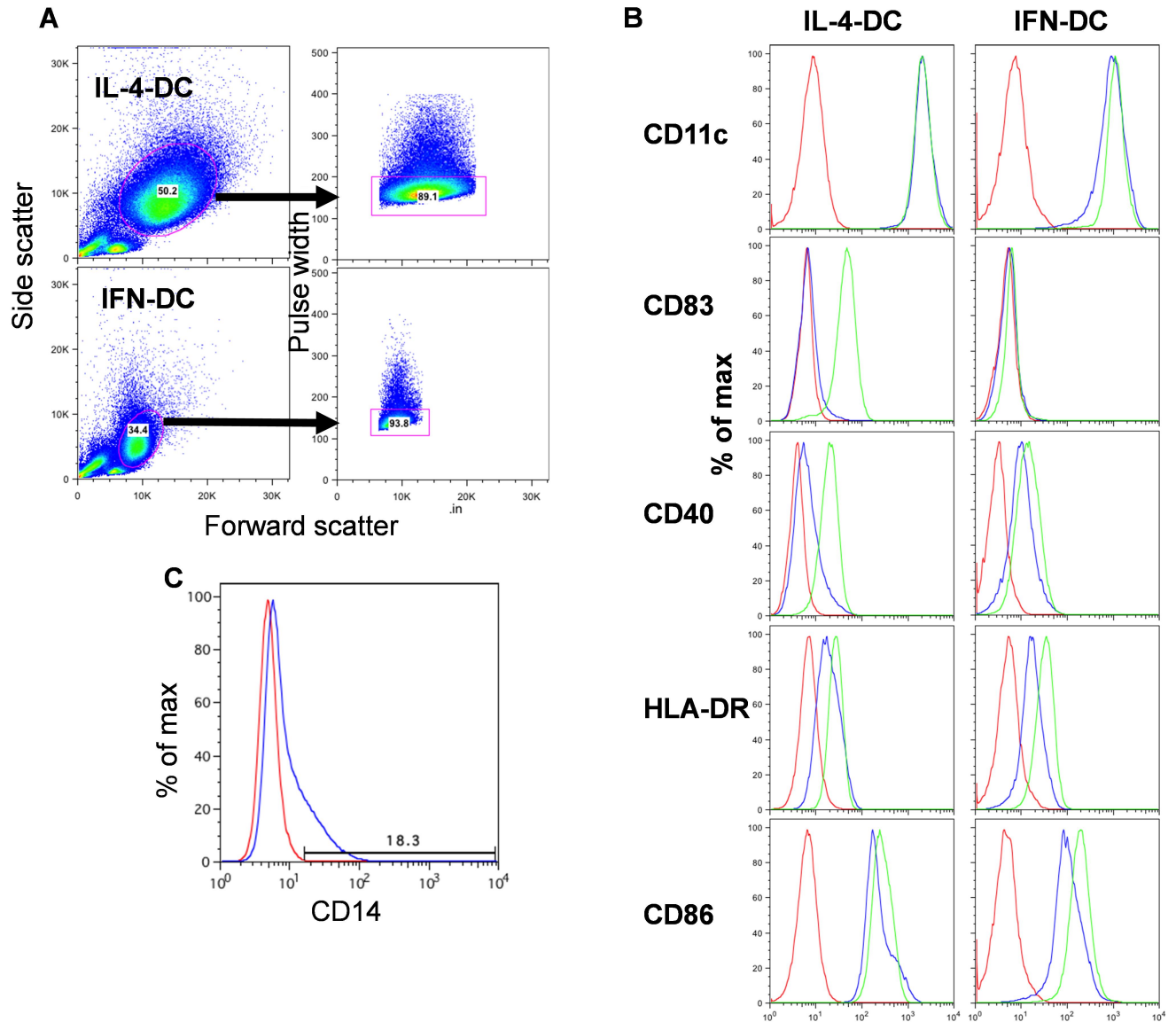


Figure 3.1 *Phenotype of monocyte-derived IL-4-DC and IFN-DC.* IL-4-DC and IFN-DC were differentiated for 6 days with IL-4 and GM-CSF or 3 days with IFN α and GM-CSF, respectively. DC were harvested and stained with the indicated antibodies and data were acquired by flow cytometry. **A.** Gating strategy showing forward scatter and side scatter characteristics of monocyte-derived DC. Doublets were removed from analysis as shown in the panels on the right. **B.** Expression of common DC markers on IL-4-DC and IFN-DC was determined using a flow cytometry panel. DCs were either immature (blue) or matured with a cytokine maturation cocktail (IL-6, TNF α , IL-1 β and PGE $_2$) for one day (green). Unstained control background is indicated in red. **C.** CD14 expression on IL-4-DC after 6 days of differentiation in the presence of IL-4 and GM-CSF. Unstained control is shown in red. The results shown are representative of data obtained in 3 independent experiments using cells derived from different donors.

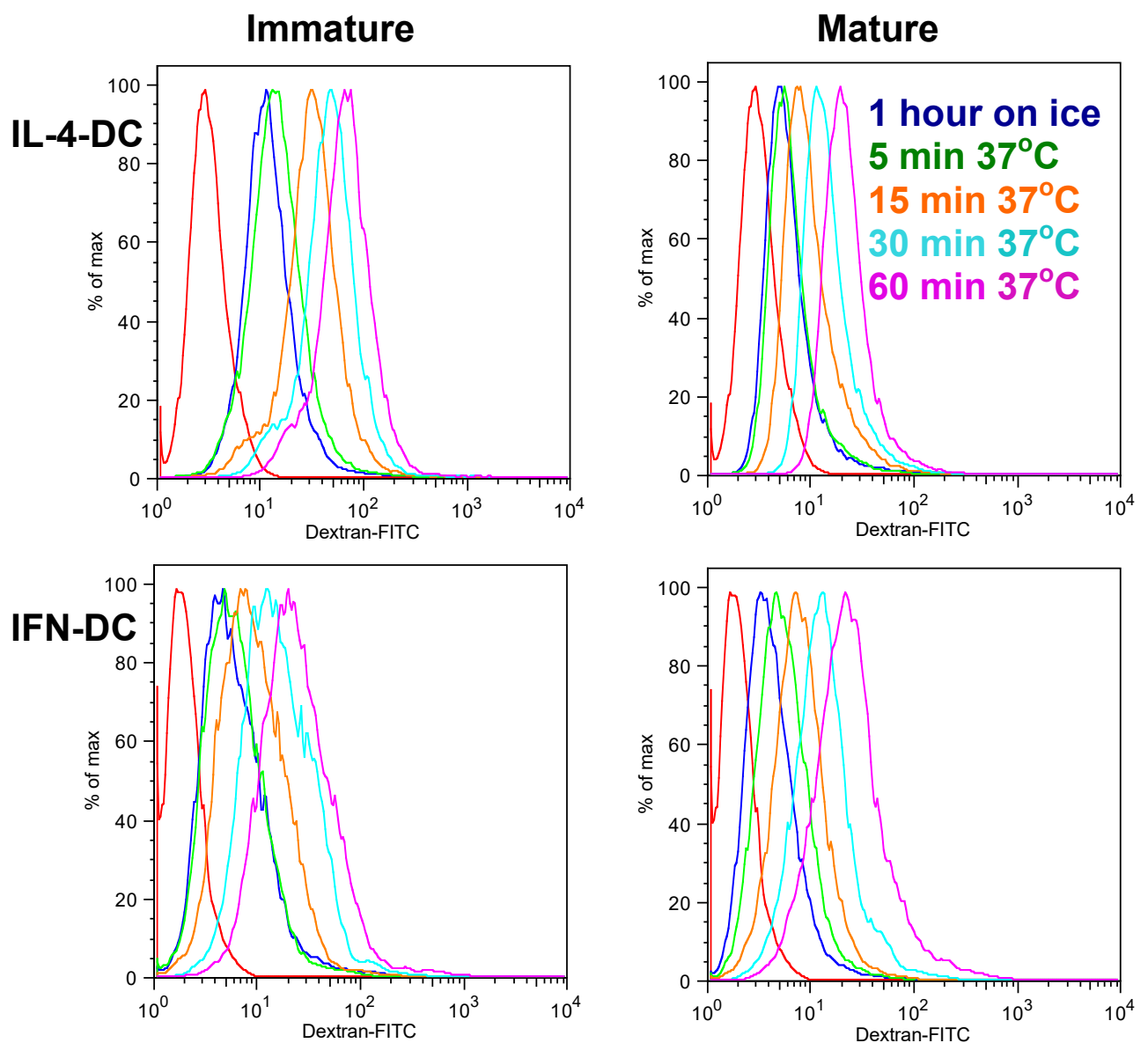


Figure 3.2 Uptake of dextran-FITC by immature and mature monocyte-derived DCs. IL-4-DC and IFN-DC were left immature or matured with a cytokine cocktail (IL-6, TNF α , IL-1 β and PGE $_2$) for 24 hours. DC were then pulsed with 1 mg/ml dextran-FITC for the time indicated in the legend then washed thoroughly and uptake measured by flow cytometry.

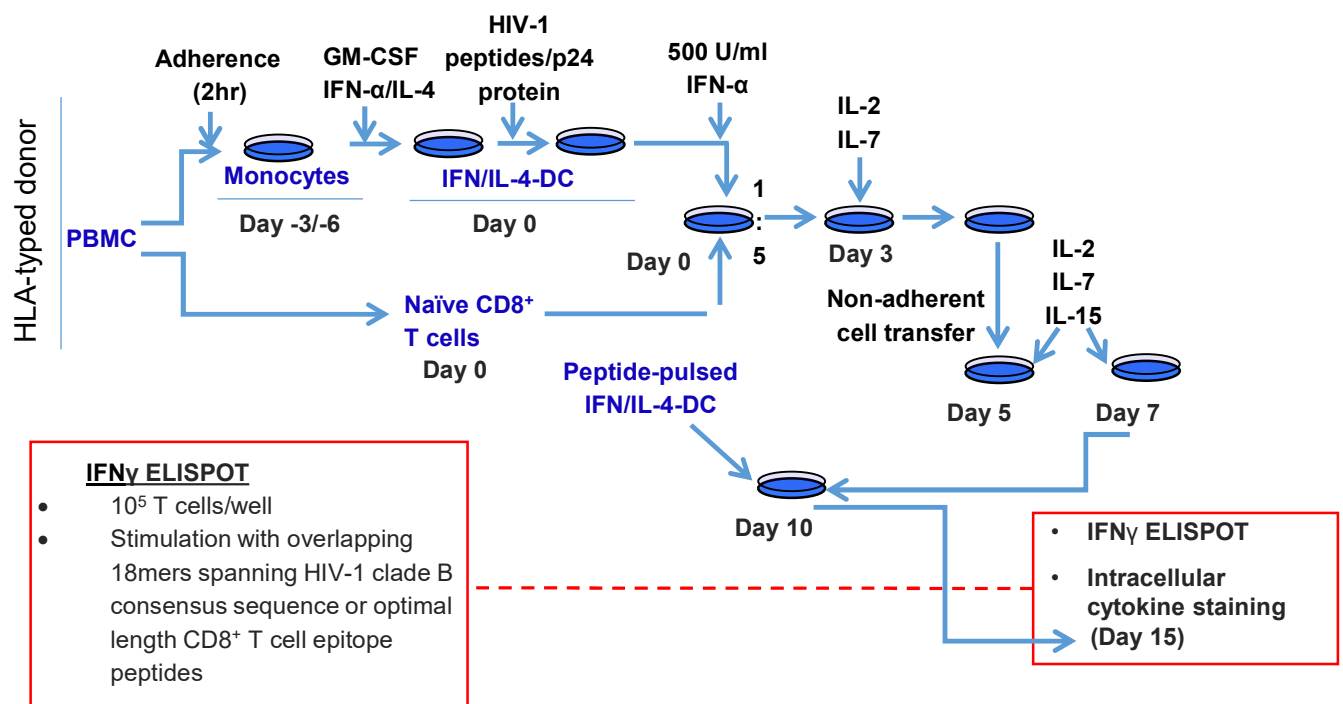


Figure 3.3 *In vitro* priming assay protocol schematic. Naïve CD8⁺ T cells were co-cultured with IL-4-DC or IFN-DC pulsed with peptides corresponding to optimal-length HLA-A*02:01-, HLA-A*03:01- or HLA-B*07:02-restricted CD8⁺ T cell epitope peptides or whole p24 protein at a ratio of 5:1 with 500 U/ml IFNα. Primed antigen-specific T cells were then expanded over 10-15 days in the presence of IL-2, IL-7 and IL-15. At day 10 T cells were restimulated with autologous peptide/protein-pulsed DCs. Then on day 15, T cells were harvested and incorporated into an ELISPOT assay or an intracellular cytokine staining assay to test for expansion of antigen-specific cells. This workflow was adapted from a previously described protocol with some alterations⁵¹⁶.

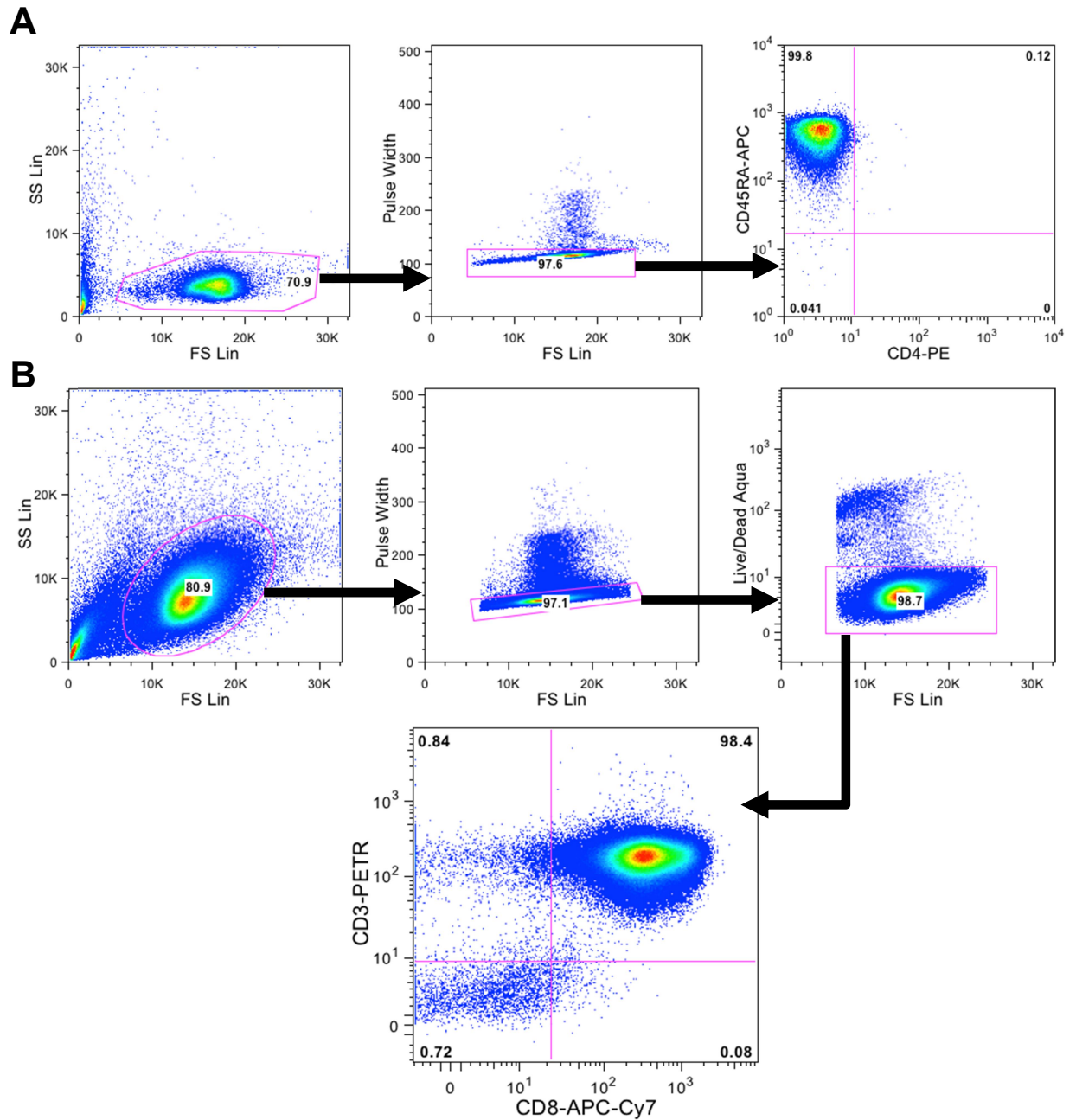


Figure 3.4. Isolation of naïve $CD8^+$ T cells and expansion during the *in vitro* priming assay. **A.** Naïve T cells were isolated from PBMC then naïve $CD4^+$ T cells were depleted in a second step. The remaining cells were stained with CD4-PE and CD45RA-APC. The gating strategy to remove cell debris and doublets is shown. **B.** After 15 days of T cell co-culture with DC and *in vitro* expansion, the remaining cells were harvested and stained for CD3 and CD8 expression. Shown is the gating strategy to exclude cell debris, doublets and dead cells.

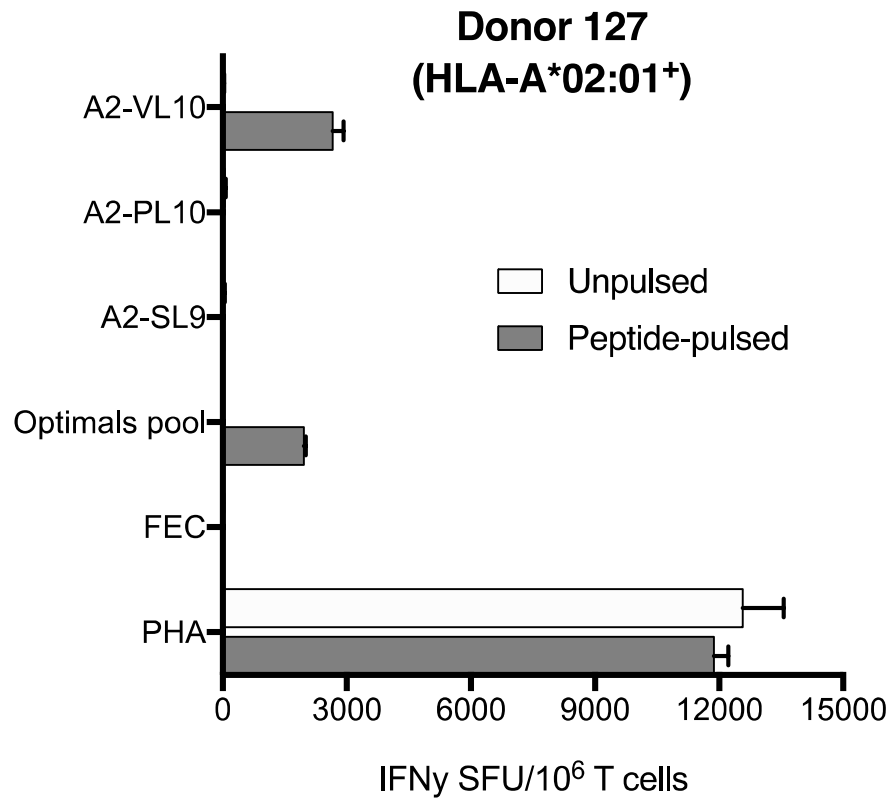


Figure 3.5 *Priming of HIV-1-specific CD8⁺ T cells by DC pulsed with optimal-length epitope peptides.* IL-4-DCs from the HLA-A*02:01⁺ donor 127 were pulsed (or unpulsed as a negative control) with a mix of 10 μ g/ml each of the optimal-length epitope peptides A2-SL9 (SLYNTVATL), A2-VL10 (VLEWRFD SRL) and A2-PL10 (PLTFGW CYKL). IL-4-DCs were then co-cultured with autologous naïve CD8⁺ T cells. On day 15 post-priming, cells were harvested and incubated in an IFN γ ELISPOT assay overnight at 100,000 cells/well with 10 μ g/ml of the indicated peptides. Optimals pool indicates that a mix of 10 μ g/ml each of A2-SL9, A2-VL10 and A2-PL10 were used for stimulation in the ELISPOT assay. FEC peptides (10 μ g/ml) were included as an irrelevant peptide negative control and PHA (10 μ g/ml) was used as a positive control. Each measurement was performed in technical duplicates and results are expressed as the number of IFN γ spot-forming units per 10⁶ cells with non-specific background subtracted $\pm$ standard deviation.

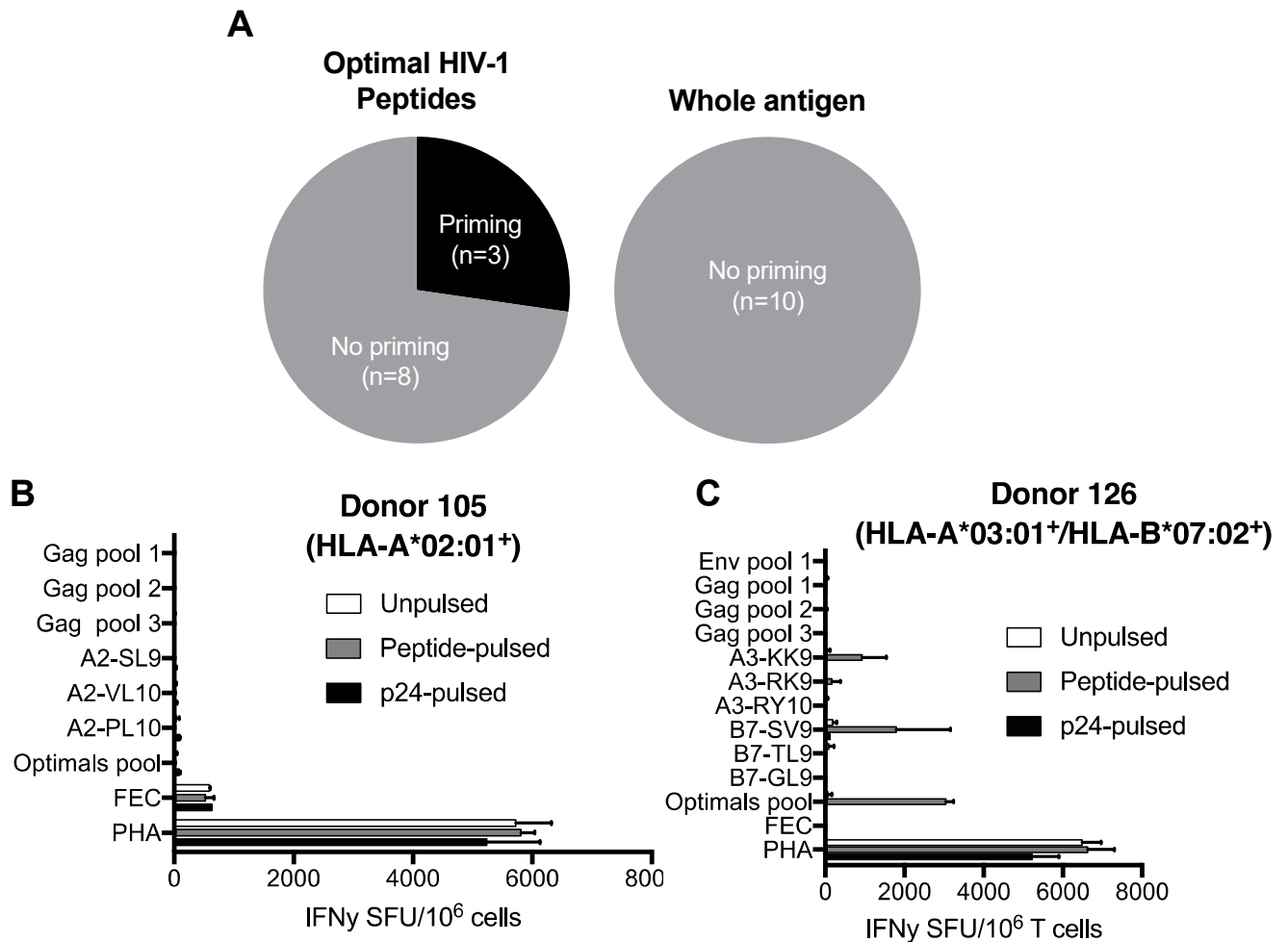


Figure 3.6 Intermittent priming of HIV-1-specific CD8⁺ T cells by DCs pulsed with optimal-length epitope peptides but not HIV-1 protein **A**. Summary of all *in vitro* priming experiments where IFN-DC or IL-4-DC were loaded with optimal length HLA-A*02:01/HLA-A*03:01/HLA-B*07:02-restricted peptides or whole HIV-1 p24 protein or HIV-1 IIIB-infected CD4⁺ T cells. Shown in the pie charts are the proportion of individual donors where antigen-specific IFN γ -producing CD8⁺ T cells were detected (black) or not (grey) by ELISPOT assay after the *in vitro* priming step. **B,C**. Examples of assays where monocyte-derived DC expressing the indicated HLA molecules were pulsed with a mix of 10 μ g/ml each of the listed optimal peptides then co-cultured with autologous naïve CD8⁺ T cells. Donor 105 IFN-DC: A2-SL9 (SLYNTVATL), A2-VL10 (VLEWRFDSRL) and A2-PL10 (PLTFGWCYKL). Donor 126: IL-4-DC with A3-KK9 (KIRLRPGGK), A3-RK9 (RLRPGGKKK), A3-RY10 (RLRPGGKKKY), B7-SV9 (SPRTLNAWV), B7-TL9 (TPQDLNTML), B7-GL9 (GPGHKARVL). A third of the DC from Donor 105 (**B**) and Donor 126 DC (**C**) were also pulsed with 10 μ g/ml clade B consensus HIV-1 p24 protein before co-culture with autologous naïve CD8⁺ T cells. On day 15 post-priming, cells were harvested and incubated in an IFN γ ELISPOT assay overnight at 50,000-100,000 cells/well with 10 μ g/ml of the indicated peptides. Optimals pool indicates that a mix of A2-SL9, A2-VL10 and A2-PL10 or A3-KK9, A3-RK9, A3-RY10, B7-SV9, B7-TL9 and B7-GL9 were used for stimulation. Gag and Env pools include 18-mers overlapping by 10 amino acids representing the clade B consensus HIV-1 sequence. FEC peptides were included as an irrelevant peptide negative control and PHA (10 μ g/ml) was used as a positive control. Each measurement was performed in technical duplicates and results are expressed as the number of IFN γ spot-forming units per 10⁶ cells with non-specific background subtracted $\pm$ standard deviation.

3.2.2 Consistent CD8⁺ T cell priming against epitopes with high precursor frequency in the naïve repertoire

Studies have shown that approximately 1 in $0.5-1 \times 10^6$ naïve CD8⁺ T cells are specific for particular HIV-1 Gag epitopes in HLA-A*02:01⁺ individuals^{517,518}. Given that only $1-5 \times 10^6$ naïve CD8⁺ T cells could be isolated from the blood of an individual donor, I postulated that perhaps fewer than 1-5 HIV-1 peptide/MHC-specific naïve CD8⁺ T cells were being incorporated into each assay, which could explain the failure to prime HIV-1-specific responses in many assays. Therefore, I attempted to prime CD8⁺ T cells by co-culturing with DC pulsed with the HLA*A:02:01-restricted MART1/Melan-A₂₆₋₃₅(A27L), PGT₁₇₈₋₁₈₇ and NS3₁₀₇₈₋₁₀₈₁ peptides, which are known to have high frequencies of approximately 1:5000, 1:5000 and 1:50000 in the naïve CD8⁺ T cell repertoire of HLA-A*02:01⁺ individuals, respectively⁵¹⁸.

In all HLA-A*02:01⁺ donors tested, Melan-A₂₆₋₃₅(A27L)- and PGT₁₇₈₋₁₈₇-specific CD8⁺ T cells expanded and responded to peptide re-stimulation as measured by ELISPOT assay (Figure 3.7A, B). NS3₁₀₇₃₋₁₀₈₁-specific CD8⁺ T cells were also detectably expanded, albeit to a lower magnitude than Melan-A₂₆₋₃₅(A27L) and PGT₁₇₈₋₁₈₇-specific cells, in 7/10 donors (Figure 3.7A, B). The proportion of Melan-A₂₆₋₃₅(A27L)-specific CD8⁺ T cells in each assay was also enumerated using tetramer staining (Figure 3.7C). In different assays, tetramer-positive populations ranged from 16-60% of the live CD3⁺ cells after Ag-specific expansion (Figure 3.7D). In summary, although it did not prove possible to *in vitro* prime CD8⁺ T cell responses to HIV-1 epitopes in a consistent fashion, I was able to establish an *in vitro* priming assay system where monocyte-derived DC consistently activate naïve CD8⁺ T cells against epitopes with a high frequency in the naïve CD8⁺ TCR repertoire.

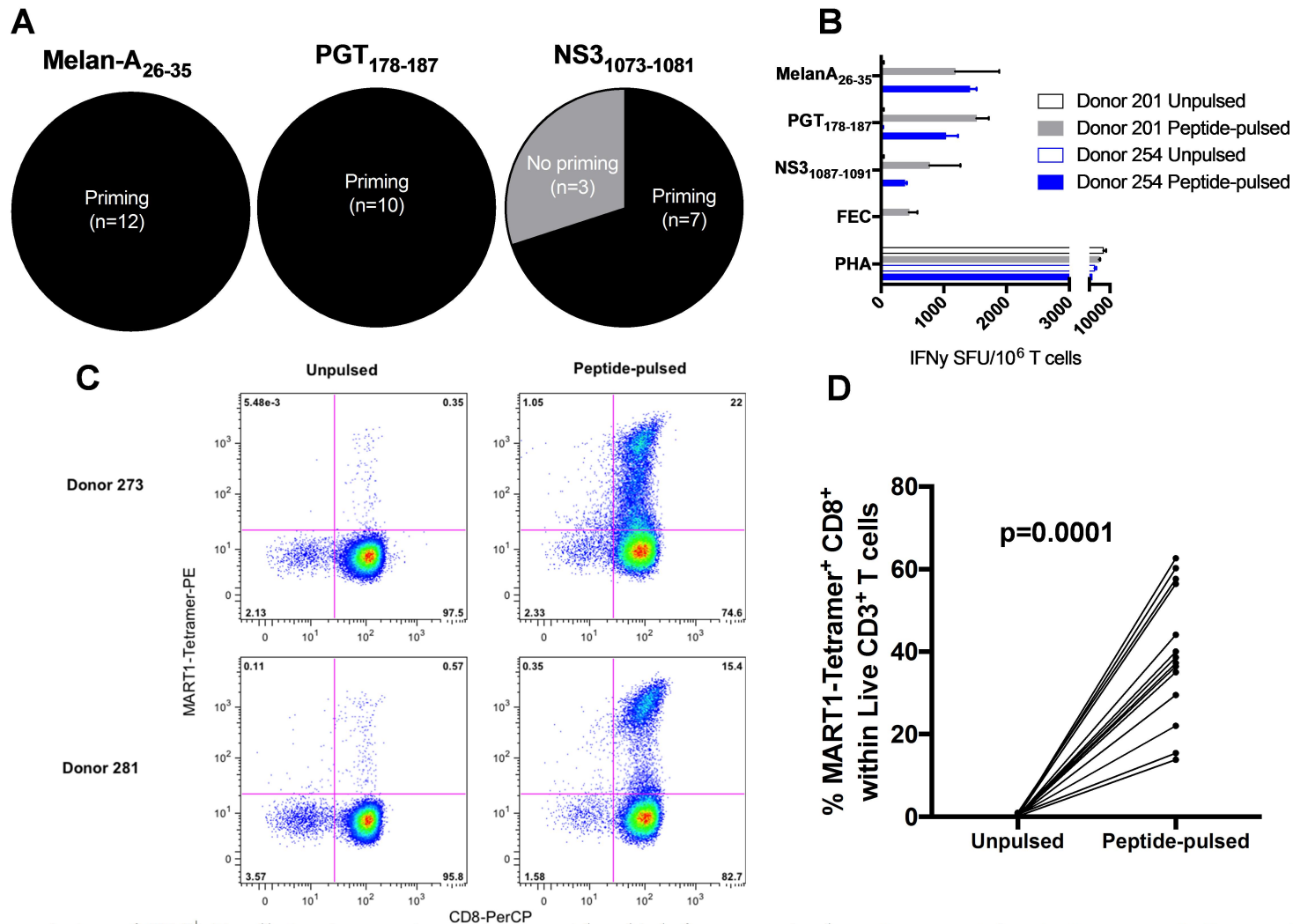


Figure 3.7 Consistent priming of CD8⁺ T cells *in vitro* against epitopes with a high frequency in the naïve repertoire. **A.** Summary of all assays incorporating DC pulsed with Melan-A₂₆₋₃₅(A27L), PGT₁₇₈₋₁₈₇ and NS3₁₀₇₃₋₁₀₈₁. Shown in the pie charts are the proportion of individual donors where antigen-specific IFN γ -producing CD8⁺ T cells were detected (black) or not (grey). **B.** Naïve CD8⁺ T cells from two donors (201 and 254) were primed by autologous DC pulsed with a mix of 10 μ g/ml each of Melan-A₂₆₋₃₅(A27L), PGT₁₇₈₋₁₈₇ and NS3₁₀₇₃₋₁₀₈₁ then restimulated in an IFN γ ELISPOT assay at 100,000 cells/well with the indicated peptides at 10 μ g/ml. FEC peptides were included as an irrelevant peptide negative control and PHA (10 μ g/ml) was used as a positive control. Results are expressed as the number of IFN γ spot-forming units per 10⁶ cells with background subtracted. **C.** Naïve CD8⁺ T cells from two donors (273 and 281) were primed by autologous DC pulsed with or without a mix of 10 μ g/ml each of Melan-A₂₆₋₃₅(A27L), PGT₁₇₈₋₁₈₇ and NS3₁₀₇₃₋₁₀₈₁ then stained at day 15 with CD8-PerCP and Melan-A/MART1-Tetramer-PE. Data were acquired by flow cytometry. **D.** The percentage of MART1-Tetramer⁺CD8⁺ cells within the live CD3⁺ T cells was enumerated for each assay and condition pulsed with or without the peptide mix. Paired data were analysed for statistical significance using a Wilcoxon ranked-sign test.

3.2.3 Plasma from acutely-infected HIV-1 patients around the time of peak viral load does not impair *in vitro* CD8⁺ T cell priming

The difficulties in obtaining sufficient quantities of blood from subjects acutely infected with HIV-1 have precluded comprehensive analysis of DC functions at different stages of AHI. Various studies have shown that DC from HIV⁺ patients during the chronic stage of infection show reduced IL-12 production and response to TLR stimulation⁵¹⁹ as well as diminished ability to stimulate T cells⁵²⁰. Both shed apoptotic microvesicles in AHI⁴⁶ and soluble CD40 ligand in chronic infection⁵⁰⁷ have been proposed as mediators of the inhibitory effect on DCs. As IL-12 is an important facilitator of naïve CD8⁺ T cell activation and memory formation⁵²¹, I hypothesised that factors present in the plasma during AHI would inhibit the activation of naïve CD8⁺ T cells by DCs.

Therefore, monocyte-derived DC were pulsed with plasma pooled from plasma donors who were found on retrospective screening to have acquired HIV-1 infection during the course of plasma donation⁴³. Two pools of plasma were made; one comprised of samples cryopreserved from 6 patients at a timepoint around the peak of viraemia in AHI and another of plasma taken from the same individuals at a pre-viraemic timepoint presumed to be prior to acquisition of infection, then incorporated plasma-treated DC into the *in vitro* priming assay to determine whether CD8⁺ priming was impaired. Based on concentrations found to affect DC functions in previous studies⁴⁶, 10% plasma was used in all assays. When plasma was diluted in media and added to cells, clots formed over the subsequent hours. To inhibit clotting, 1 U/ml heparin was added to media containing 10% plasma.

DC from donors 312 and 321 were pre-incubated with pooled plasma + heparin and washed before co-culture, whereas DCs from donors 315 and 322 were treated with 10% pooled plasma + heparin during the DC:CD8 co-culture (Figure 3.8A). In all 4 donors tested, Melan-A₂₆₋₃₅(A27L)- and PGT₁₇₈₋₁₈₇-specific cells were expanded when co-cultured with IL-4-DC pulsed with pre-infection or AHI plasma in addition to peptides (Figure 3.8B, C). Melan-A₂₆₋₃₅(A27L)-specific responses tended to be higher in magnitude than PGT₁₇₈₋₁₈₇-specific responses. NS3₁₀₇₃₋₁₀₈₁-specific IFN γ -producing cells were detected at low magnitude only in donor 312. No consistent difference was observed between pre-infection and AHI plasma-pulsed co-cultures in the number of IFN γ -producing cells

expanded by peptide-pulsed DC. In agreement with the ELISPOT data, the percentage of MART1-tetramer⁺ cells in the Donor 315 co-culture was slightly higher when IL-4-DCs were pulsed with AHI plasma (Figure 3.8C). On the other hand, 3/4 donors showed a decreased percentage of MART1-tetramer⁺ cells when co-cultured with AHI plasma-pulsed IL-4-DC. As the number of cells available from each subject was limited it was not possible to set up experiments in duplicate or triplicate to enable comparison of the variation in the magnitude of the response observed when T cells from a single donor were primed by autologous DC under equivalent conditions; however the variation in the magnitude of responses observed when priming was carried out with pre-infection or AHI plasma was likely attributable to inter-replicate variation in the assay. Cytokine production in response to recall antigen peptides was also examined (gating strategy shown in Figure 3.8D). Again, no consistent trend was observed in the % of IFN γ -, TNF α -, or MIP1 β -producing cells responding to Melan-A₂₆₋₃₅(A27L) or PGT₁₇₈₋₁₈₇ when CD8⁺ T cells were primed in the presence of plasma from pre-infection or AHI timepoints (Figure 3.8E). In concordance with the ELISPOT results, limited NS3₁₀₇₃₋₁₀₈₁-specific cytokine production was only measured in Donor 312 (data not shown). In summary, exposure of either IFN- or IL-4-DC to AHI plasma was found not to reduce CD8⁺ T cell priming or affect cytokine production in response to antigen recall by the expanded CD8⁺ T cells.

Since heparin and CD44 both interact with the same domains of fibronectin and other heparin-binding proteins⁵²², I wondered whether the heparin added to DC/plasma cultures was inhibiting interactions between immunomodulatory vesicles and DC-expressed CD44. As previous studies had shown that DC exposure to AHI plasma results in decreased TLR responsiveness and in particular, IL-12 production following stimulation with TLR ligands such as poly I:C⁴⁶, I tested whether similar results could be obtained when heparin was added to DC/plasma cultures. IL-4-DCs were treated with 10% pre-infection or AHI plasma overnight then stimulated with TLR3 agonist poly I:C. IL-12p70 production was measured in the supernatant 24 hours after stimulation. Contrary to expectations, exposure to matched plasma samples from 4 patients during pre-infection and AHI timepoints did not cause a discernible difference in DC IL-12p70 production (Figure 3.9A). If anything, some AHI plasma samples seemed to increase IL-12 production relative to pre-infection controls (although the difference between pre-infection and AHI plasma sample-treated groups was

not significant). Extending the number of plasma samples tested, I again measured no change, or slight increases in IL-12p70 production by AHI plasma-exposed IL-4-DC in the presence of heparin (Figure 3.9B).

To determine whether the lack of inhibition was due to the fact that heparin interferes with the effect of inhibitory vesicles on DC, I attempted to arrest the clotting process by other means. Clotting could not be stopped by pre-calcification, pre-clotting in glass or clot-activator serum collection tubes, addition of the thrombin inhibitor argatroban monohydrate, or incubation of plasma diluted in media at 37°C for 2 hours (data not shown). However, I was able to inhibit the clotting process by heat-inactivating plasma at 56°C for 15 minutes. When IL-4-DCs were treated with this heat-inactivated plasma, some variation was observed in IL-12p70 production by DCs treated with plasma from individual timepoints. However, the pool of AHI plasma increased IL-12p70 production by both donor IL-4-DCs tested (Figure 3.9C). I tested additional heat-inactivated plasma samples, including samples from some of the same donors as used by Frleta *et al* and saw no consistent downregulation of IL-12p70 production by AHI-exposed IL-4-DCs (Figure 3.9D). I therefore concluded that the AHI samples I used did not consistently inhibit IL-12 production by IL-4-DCs, indicating that no inhibitory activity was present or that the heparin and heat-inactivation processes were obscuring clear plasma inhibitory effects.

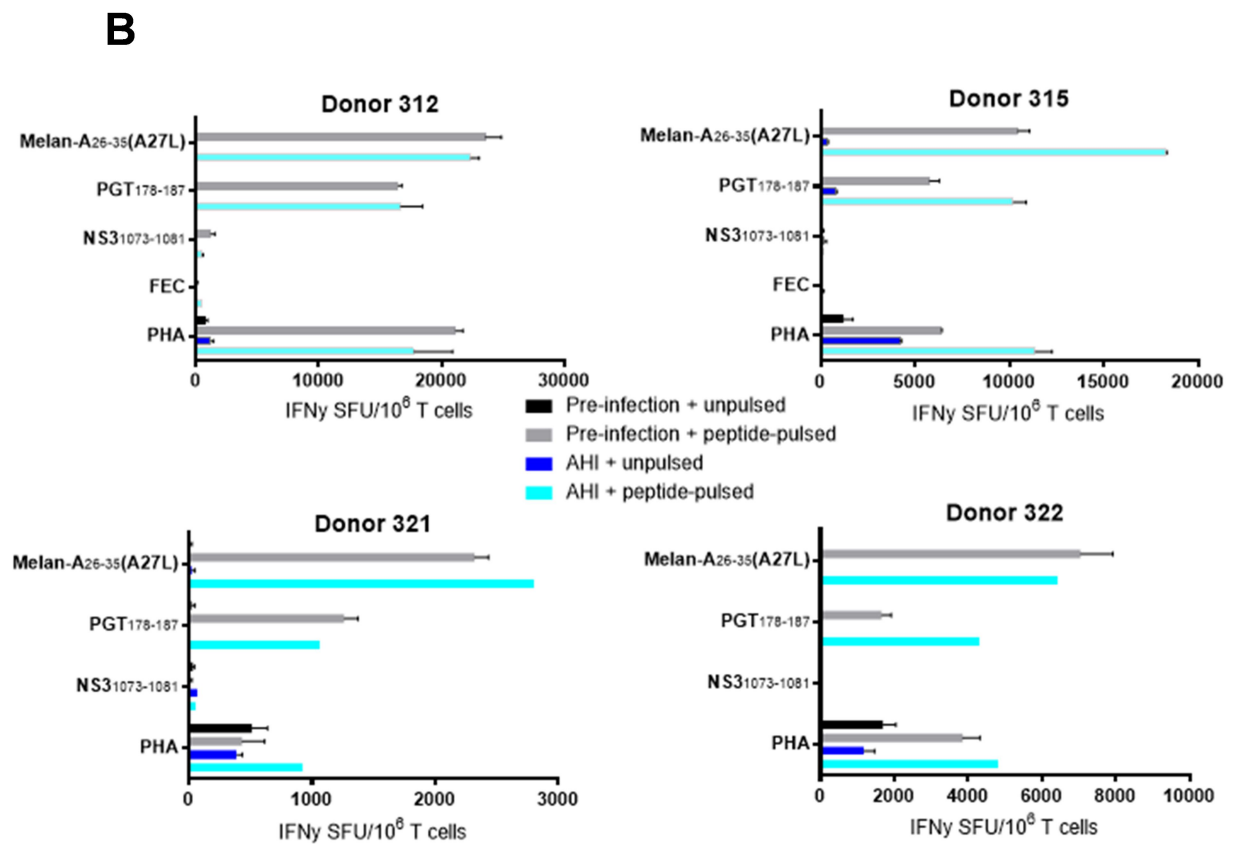
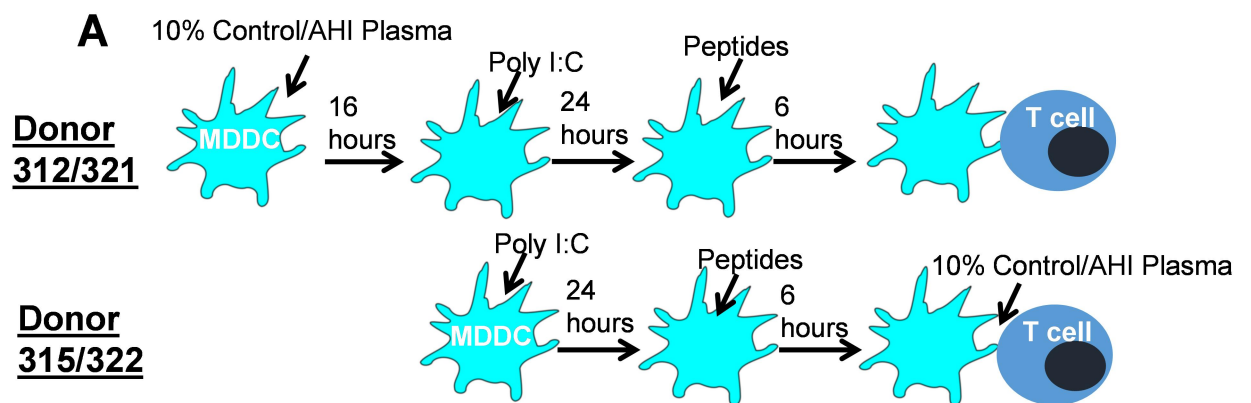


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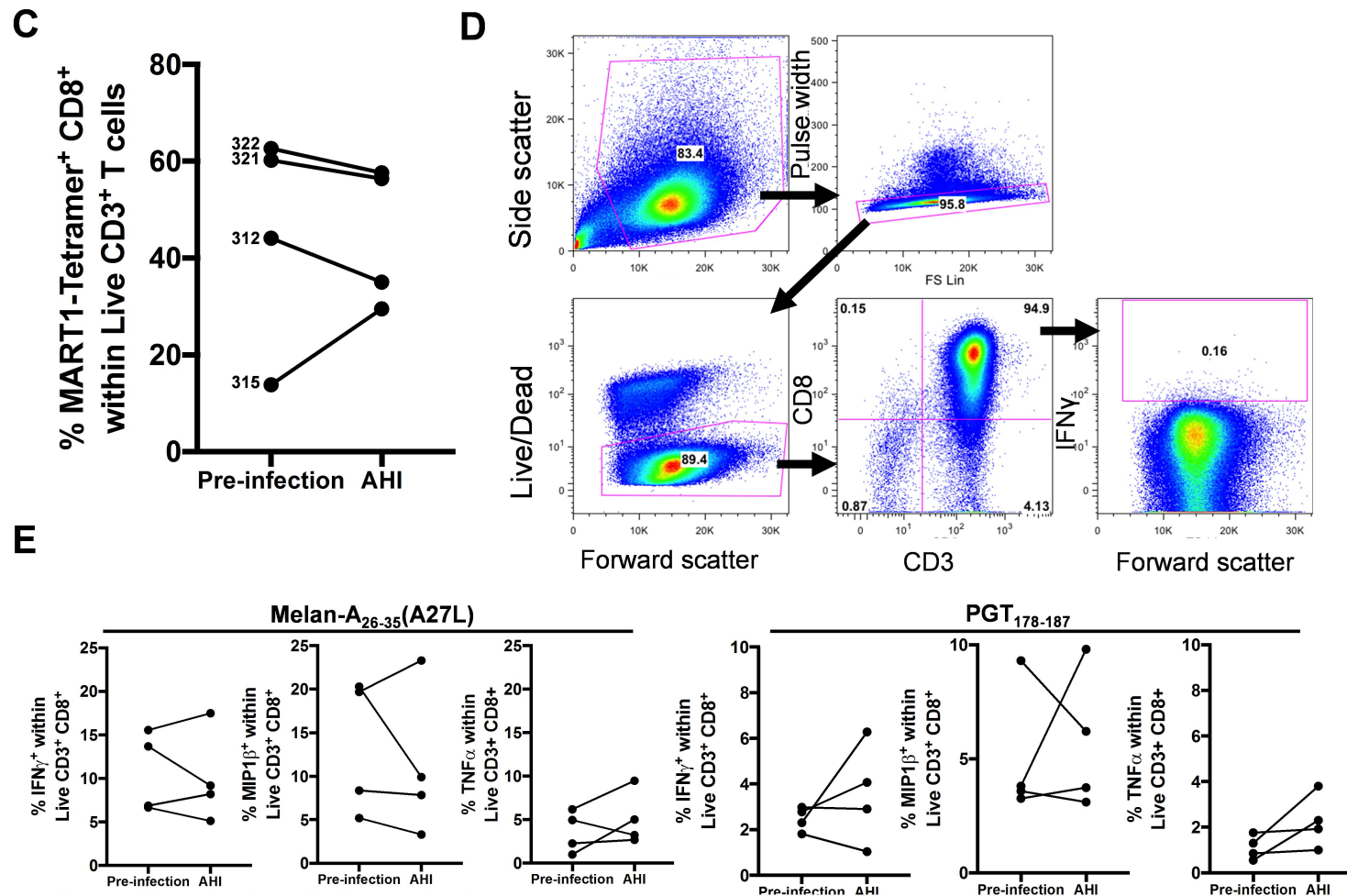


Figure 3.8 Effect of plasma from AHI on *in vitro* CD8⁺ T cell priming. IFN-DCs (Donors 312 and 315) and IL-4-DCs (Donors 321 and 322) were differentiated for 3 and 5 days, respectively. After differentiation, donor 312 and 321 DCs were treated with 10% plasma in AIM V medium containing 1 U/ml heparin for 16 hours. IL-4-DCs were then treated with 2 ug/ml poly I:C for 24 hours before co-culture. DCs were pulsed with/without peptide mix (10 ug/ml each of Melan-A₂₆₋₃₅(A27L), PGT₁₇₈₋₁₈₇ and NS3₁₀₇₃₋₁₀₈₁) for 6 hours before co-culture. DCs from donors 315 and 322 were not pre-treated with plasma, but were co-cultured with naïve CD8⁺ T cells in the presence of 10% plasma and 1 U/ml heparin. ELISPOT assays, tetramer staining and intracellular cytokine staining were performed on day 15 post-priming. **A.** Schematic depicting the timing of plasma, poly I:C and peptide treatment of DCs and DC:CD8⁺ co-cultures. **B.** Depicted are the number of cells producing IFNγ in response to stimulation with the indicated peptides per 10⁶ cells for each donor tested as assessed by the ELISPOT assay. FEC peptides served as an irrelevant peptide control whilst PHA served as a positive control. Data are displayed as the mean ± standard deviation with background subtracted. **C.** After co-culture, cells were also stained with PE-conjugated MART1-Tetramer. Shown is the percentage of tetramer⁺ CD8⁺ cells within live CD3⁺ cells for each donor tested when primed with DC/co-cultures exposed to pre-infection or AHI plasma. **D.** Gating strategy for determining percentage of cytokine-producing cells when stimulated with peptides as measured by intracellular cytokine staining. Shown is an unstimulated control sample. **E.** Shown is the percentage of cytokine producing cells within Live CD3⁺ CD8⁺ cells when stimulated with Melan-A₂₆₋₃₅(A27L) (left panels) or PGT₁₇₈₋₁₈₇ (right panels). Background cytokine production by each unstimulated cell population was subtracted. Cytokines measured were IFNγ, MIP1β and TNFα.

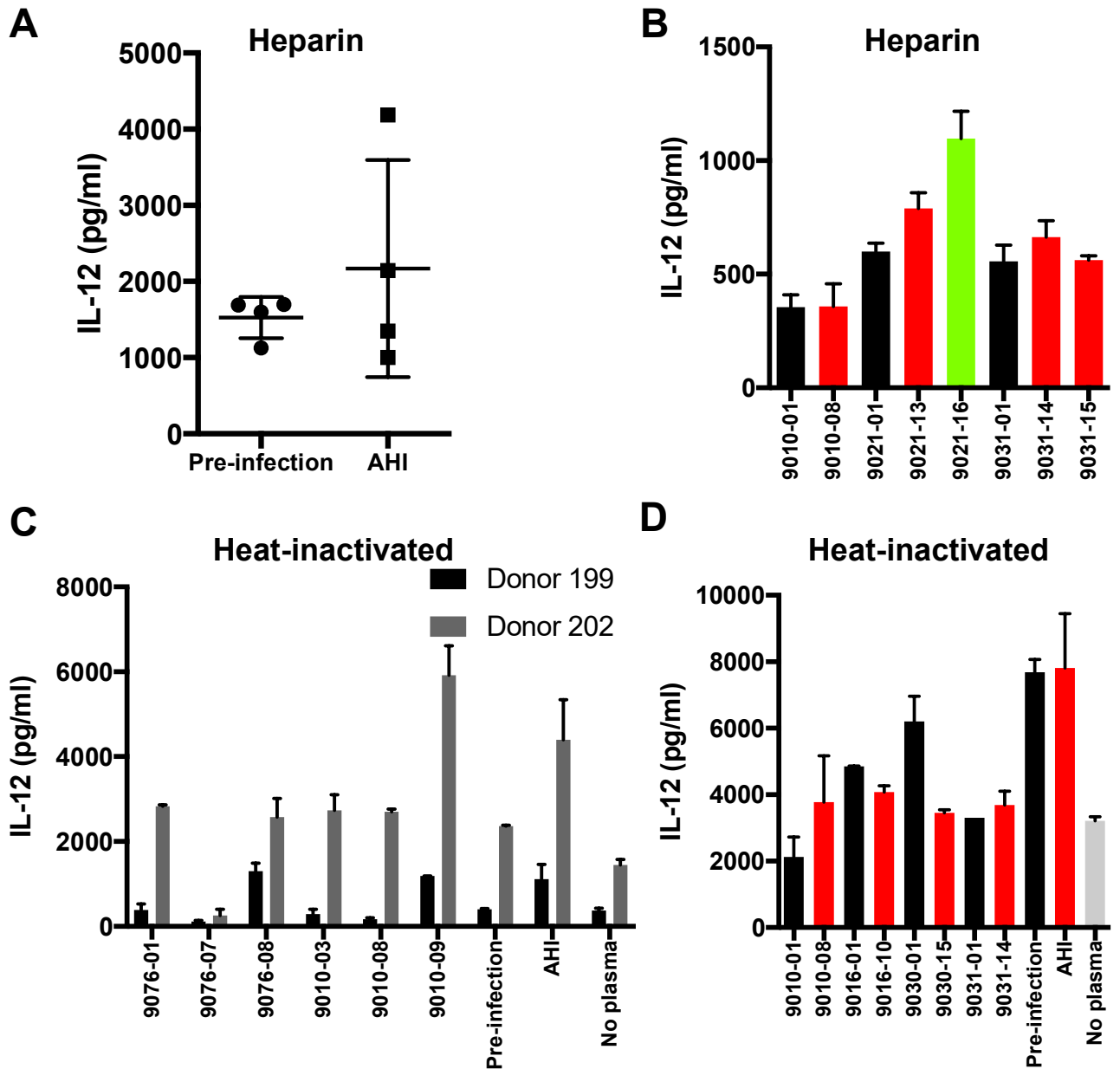


Figure 3.9 Effect of plasma from AHI on IL-12 production by IL-4-DCs. **A.** IL-4-DCs from Donor 221 were differentiated for 6 days then harvested and plated in 96-well round-bottom plates at 2×10^5 cells/well. Cells were treated overnight with 10% plasma cryopreserved from individual donors at timepoints prior to infection (9016-01, 9018-01, 9020-01, 9023-01) or during AHI (9016-10, 9018-10, 9020-21, 9023-21) in AIM V medium containing 1 U/ml heparin sodium. Cells were then treated with 2 μ g/ml poly I:C for 24 hours and IL-12 was measured in supernatant. Individual data points represent the average of two technical replicates. The mean of IL-12 production after plasma exposure for the pre-infection and AHI sample groups is shown $\pm$ standard deviation. **B.** IL-4-DCs from Donor 199 were treated overnight as in **A**) with plasma samples cryopreserved from individual donors at timepoints prior to infection (black bars) or during AHI (red bars pre-peak viraemia; green bar; post-peak viraemia) in the presence of 1 U/ml heparin. IL-12p70 production was measured after 24 hours of poly I:C stimulation. Results shown are indicative of 2 technical replicates $\pm$ standard deviation. **C.** Individual pre-infection plasma timepoints (9076-01, 9010-03,) or AHI plasma (9076-07, 9076-08, 9010-08, 9010-09) was first subjected to heat-inactivation at 56°C for 15 minutes. IL-4-DCs from Donors 199 and 202 were incubated with heat-inactivated plasma as in **A**) and IL-12p70 production measured in supernatant after poly I:C stimulation. Pools of 'Pre-infection' and 'AHI' heat-inactivated plasma samples (6 donors) were also added to IL-4-DCs. **D.** IL-4-DCs from Donor 202 were treated as in **C**) with heat-inactivated plasma from timepoints pre-infection (black bars) or during AHI (red bars). Data are shown as an average of two technical replicates $\pm$ standard deviation.

3.2.4 Effects of IL-4-DC exposure to IFN α on *in vitro* CD8⁺ T cell priming

During the acute phase of HIV-1 infection, an elevation in levels of multiple cytokines (termed ‘cytokine storm’) can be detected in the plasma of HIV-1⁺ patients⁴³. This cytokine storm coincides with the period where I proposed that DC function becomes impaired. Therefore, I hypothesised that cytokines produced during AHI could impair priming of CD8⁺ T cells by DC. In particular, I studied IFN α because it is, along with IL-15, one of the earliest cytokines to peak at high levels in plasma during AHI⁴³. Furthermore, in the acute phase of LCMV infection in mice IFN α has been shown to induce DC activation and maturation followed by apoptotic death, so may likewise contribute to activation-induced death of DCs in AHI^{509,510}; and in MCMV infection in mice IFN α production by pDCs has been shown to reduce IL-12 production by mDCs, which may impact on the T cell response they induce⁵¹¹. Hence, I aimed to test whether initial exposure of DCs to IFN α would affect their ability to prime naïve CD8⁺ T cells later on. IL-4-DCs were differentiated then exposed to 500 U/ml IFN α , an amount equivalent to that detected in plasma during AHI⁴³. I first stained for co-stimulatory markers expressed by DCs in order to determine whether any phenotypic differences were observed when IL-4-DCs were treated with IFN α . When DCs were IFN α -treated then stimulated with poly I:C, slightly lower levels of surface CD40, HLA-DR and CD86 were observed (Figure 3.10). In contrast, CD83 and CD11c were expressed at similar levels whether IL-4-DCs were treated with IFN α or not. No differences in cell death as observed by trypan blue staining were observed at this timepoint (data not shown).

The lower levels of co-stimulatory molecules and any alterations in DC survival over time could impact on the ability of IL-4-DCs to prime naïve CD8⁺ T cells *in vitro*. Therefore, I tested whether CD8⁺ T cells primed *in vitro* by IFN α -treated IL-4-DCs were qualitatively or quantitatively different to those primed by control IL-4-DCs. The proportions of CD8⁺ MART1-Tetramer⁺ cells within live and CD3⁺ cells were roughly similar between the ‘No IFN α ’ and ‘IFN α ’ treatments (Figure 3.11A). However, in some donors IFN γ production in response to Melan-A₂₆₋₃₅(A27L) peptide measured by ELISPOT was lower in CD8⁺ T cells primed by IFN α -treated IL-4-DC (Figure 3.11B), although a similar trend was not observed in all donors tested (Figure 3.11C). A reduction in IFN γ production was also observed by intracellular cytokine staining in the 2 donors tested. Despite similar

proportions of MART1-Tetramer⁺ cells, a lower percentage of tetramer⁺ cells from co-cultures treated with IFN α made IFN γ in response to Melan-A₂₆₋₃₅(A27L) stimulation (41.1% IFN α , 57.9% without IFN α ; Figure 3.11D). Similarly, the percentages of TNF α - (19.2% IFN α ; 24.1% no IFN α) and MIP1 β -producing (54.8% IFN α ; 65.0% no IFN α) tetramer⁺ cells in the CD8⁺ T cells primed by IFN α -treated DCs were also lower.

In vivo, DCs are required to endocytose antigens then move them into the MHC class I presentation pathway where they are proteolytically processed into optimal length peptides for T cell stimulation. To test whether IL-4-DCs exposed to IFN α and pulsed with antigen requiring intracellular processing have a differential ability to prime naïve CD8⁺ T cells, I utilised the Melan-A₁₆₋₄₀(A27L) peptide which contains the optimal length Melan-A₂₆₋₃₅(A27L) used in previous experiments. This extended Melan-A peptide is cross-presented by DCs *in vitro* through a TAP- and proteasome-dependent pathway⁵²³. In experiments performed with cells from two donors (223 and 354), IL-4-DCs pulsed with Melan-A₁₆₋₄₀(A27L) primed a smaller population of Melan-A₂₆₋₃₅(A27L)-specific cells than IL-4-DCs pulsed with Melan-A₂₆₋₃₅(A27L) (Figure 3.12A). Additionally, the median fluorescence intensity (MFI) for MART1-tetramer⁺ cells was observed to be lower in Melan-A₁₆₋₄₀(A27L)-primed cultures (Melan-A₂₆₋₃₅(A27L) MFI=103; Melan-A₁₆₋₄₀(A27L) MFI=42.1). Melan-A₂₆₋₃₅(A27L)-primed cultures exhibited roughly similar percentages of Melan-A₂₆₋₃₅(A27L)-specific CD8⁺ T cells when IL-4-DCs were treated with or without IFN α (35.7% no IFN α ; 36.5% IFN α). The percentage of MART1-tetramer⁺ cells in cultures primed with IFN α -treated, Melan-A₁₆₋₄₀(A27L)-pulsed IL-4-DCs was lower than that in cultures primed by non-IFN α -treated DCs (0.69% no IFN α ; 0.49% IFN α), although as insufficient cells were available from each donor to enable replicate priming assays to be conducted with each type of DCs it was not possible to discern whether this reflected poorer DC-mediated T cell expansion or intra-assay variation. As expected, IFN γ responses as measured by ELISPOT were far greater in cultures expanded with Melan-A₂₆₋₃₅(A27L)-pulsed IL-4-DCs (Figure 3.12B). PHA responses were poor for both donors in all conditions, which was thought to be due to the use of a degraded PHA stock in these experiments. In the two donors tested here, no difference was observed in the number of IFN γ -producing cells detected by ELISPOT assay in cultures expanded with Melan-A₂₆₋₃₅(A27L)-pulsed DCs with or without IFN α treatment. However, the

number of IFN γ SFU was higher in cultures treated with Melan-A₁₆₋₄₀(A27L)-pulsed IL-4-DCs in the absence of IFN α for both donors tested (Figure 3.12B). Moreover, IFN γ and TNF α production as measured by ICS was also lower in IFN α -treated IL-4-DC co-cultures (Figure 3.12C, D).

In summary, the number of donors tested in these experiments was limited due to time constraints, and given the variation in results obtained in experiments using cells from different donors it is difficult to draw robust conclusions about the effects of prior IFN α exposure on DC-mediated CD8⁺ T cell priming. However, these preliminary data show that exposure of IL-4-DCs to IFN α in the *in vitro* conditions used here does not ablate their T cell priming capacity, and suggest that it can blunt co-stimulatory molecule upregulation in response to TLR ligands and may possibly lead to priming of CD8⁺ T cell responses with a slightly reduced capacity to produce IFN γ and TNF α .

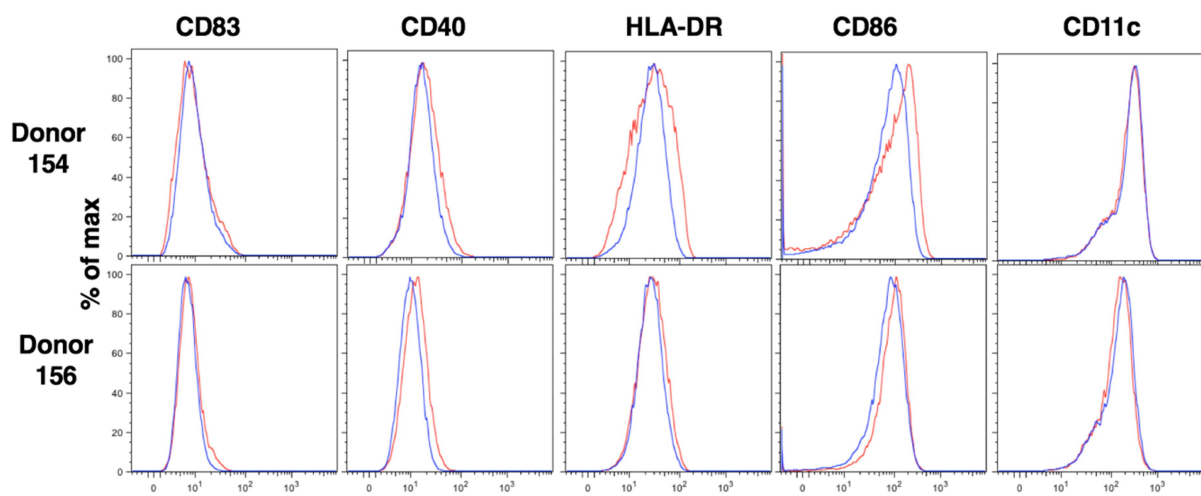


Figure 3.10 *Reduced levels of costimulatory markers on IL-4-DCs after IFN α exposure.* Monocytes from 2 donors were differentiated into IL-4-DCs then cultured with (blue) or without (red) 500 U/ml IFN α in addition to IL-4 and GM-CSF on day 5 post-differentiation. On day 8, IL-4-DCs were treated with 2 ug/ml poly I:C to induce maturation, then they were stained on day 9 with antibodies specific for the indicated markers.

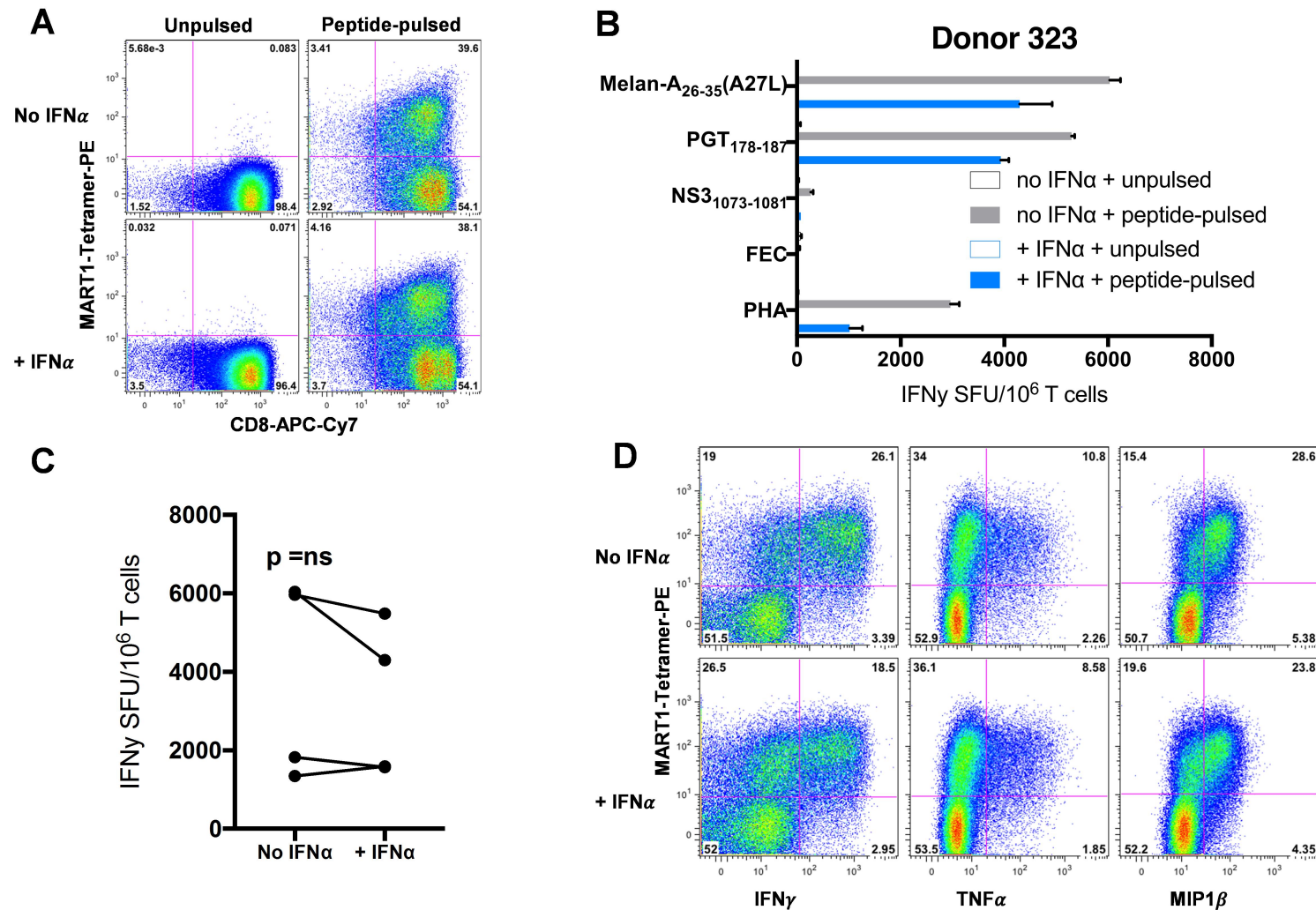


Figure 3.11 Effects of IFN α treatment of DCs on *in vitro* CD8⁺ T cell priming. In 4 donors, IL-4-DCs were differentiated for 5 days then incubated for a further 3 days with or without 500 U/ml IFN α . IL-4-DCs were then incubated with 2 μ g/ml poly I:C for 24 hours and pulsed with Melan-A₂₆₋₃₅(A27L), PGT₁₇₈₋₁₈₇ and NS3₁₀₇₃₋₁₀₈₁ before co-culture with autologous naïve CD8⁺ T cells. On day 15 of co-culture, cells were harvested and incorporated into an IFN γ ELISPOT assay or intracellular cytokine staining assay (with tetramer staining) or using peptides as stimulus. **A.** Plots displaying percentages of MART1-tetramer⁺ and CD8⁺ cells within live CD3⁺ cells. Representative data from 1 of 2 donors analysed is shown. **B.** Example of an IFN γ ELISPOT assay for Donor 323. PHA was used as a positive control and FEC peptides as irrelevant peptides. Results are expressed as the number of IFN γ spot-forming units (SFU) per 10⁶ cells $\pm$ standard deviation with background subtracted. **C.** Summary of 4 donors tested by IFN γ ELISPOT. Displayed are the number of SFU per 10⁶ cells when co-cultures primed with peptide-loaded IL-4-DCs were re-stimulated with Melan-A₂₆₋₃₅(A27L) peptide. Pairwise comparisons are made for each donor when IL-4-DCs were treated with or without IFN α . **D.** Flow plots displaying the percentages of cytokine-producing live CD3⁺ cells from co-cultures primed with peptide-loaded IL-4-DCs when re-stimulated with Melan-A₂₆₋₃₅(A27L) peptide. Representative data from 1 of 2 donors analysed is shown.

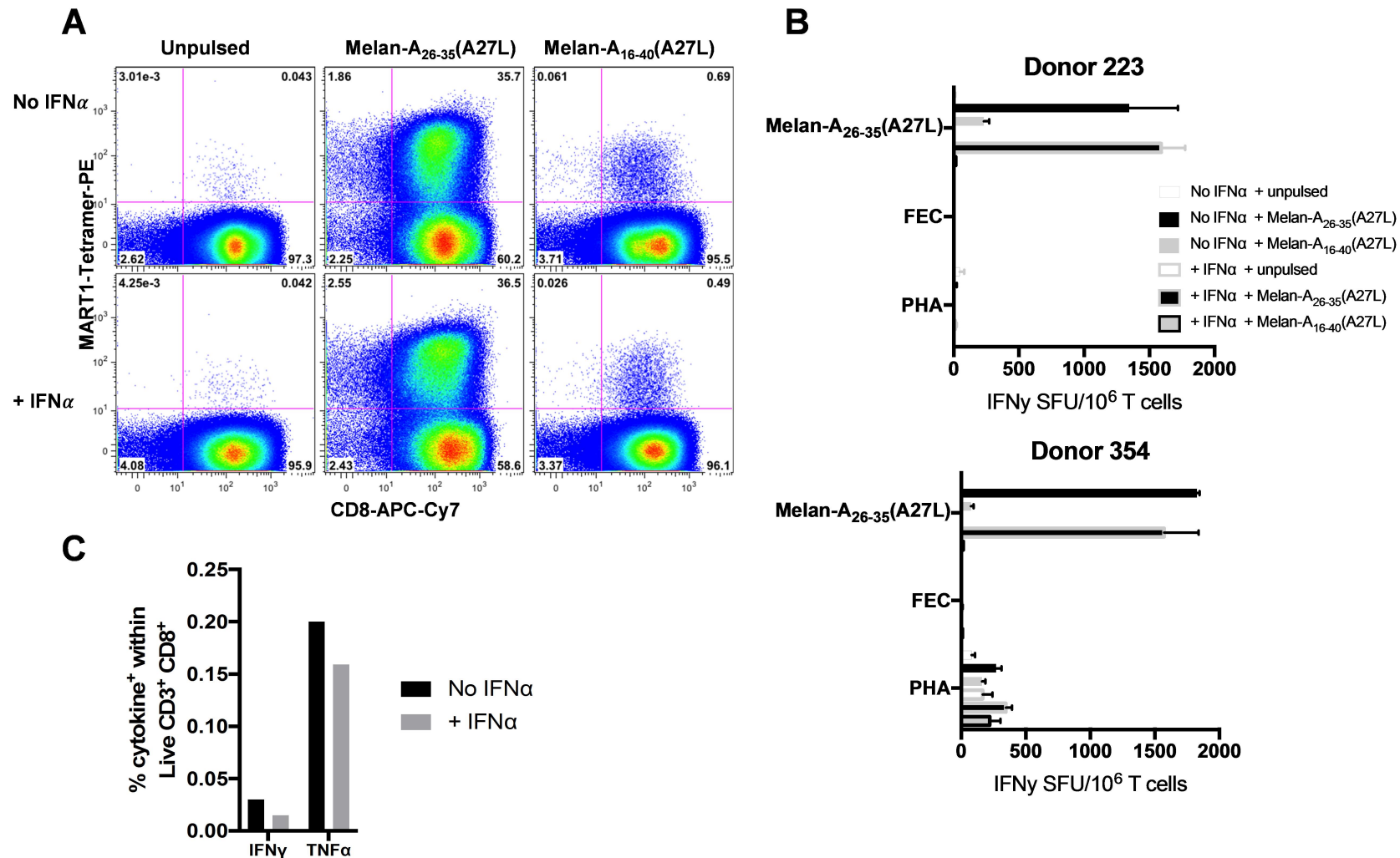


Figure 3.12 *Effects of IFN α treatment of DCs on in vitro CD8⁺ T cell priming against long peptide Melan-A16-40(A27L).* In 2 donors, IL-4-DCs were differentiated for 5 days then incubated for a further 3 days with or without 500 U/ml IFN α . IL-4-DCs were then incubated with 2 μ g/ml poly I:C for 24 hours and pulsed with Melan-A₂₆₋₃₅(A27L), Melan-A₁₆₋₄₀(A27L) or no peptide before co-culture with autologous naïve CD8⁺ T cells. On day 15 of co-culture, cells were harvested and stained with an intracellular cytokine staining panel or incorporated into an IFN γ ELISPOT assay using peptides as stimulus. **A.** Plots displaying percentages of MART1-tetramer⁺ and CD8⁺ cells within the live CD3⁺ cells of Donor 223 **B.** IFN γ ELISPOT assay for Donors 223 and 354. PHA was used as a positive control and FEC peptides as irrelevant peptides. Results are expressed as the number of IFN γ spot-forming units (SFU) per 10⁶ cells $\pm$ standard deviation with background subtracted. **C.** Summary of ICS data displaying the percentages of IFN γ - and TNF α -producing live CD3⁺ CD8⁺ cells from Donor 223 co-cultures primed with Melan-A₁₆₋₄₀(A27L)-loaded IL-4-DCs when re-stimulated with Melan-A₂₆₋₃₅(A27L) peptide. Background from unstimulated control cells was subtracted.

3.3 Discussion

In this chapter I setup and optimised an *in vitro* priming assay capable of consistently priming responses to epitope peptides with high CD8⁺ T cell precursor frequencies in the naïve repertoire (although not to HIV-1 epitope peptides or whole HIV-1 antigens requiring cross-presentation). I used this assay to assess the effects of conditions present during AHI (i.e. plasma from patients during AHI and IFN α) on T cell expansion and function following priming. Neither plasma taken from patients during AHI or IFN α were observed to have any significant effect on the ability of DCs to prime CD8⁺ T cells or the functions of the T cell responses primed, although pre-exposure of IL-4-DCs to IFN α altered co-stimulatory molecule expression and a lower proportion of CD8⁺ T cells primed by IFN α -treated IL-4-DCs produced IFN γ and TNF α in some donors.

Optimisation of the in vitro priming assay

As repeatedly shown in other studies comparing MDDCs, both IFN-DCs and IL-4-DCs expressed CD11c, CD40, CD80, CD86 and HLA-DR (in fact, a greater proportion of IFN-DCs may express CD40 and CD80 at higher levels than immature IL-4-DCs, although that was not examined in this chapter)^{514,524,525}. In contrast to my data (Figure 3.1), Lapenta *et al* showed that CD83 was upregulated on IFN-DCs upon maturation. This difference is likely due to the maturation stimuli utilised; CD40L or the cytokine cocktail I used here. Indeed, CD40L stimulation and IFN α have been shown to synergise in upregulating CD83 expression and mediating DC activation⁵²⁶. Why the cytokine cocktail upregulates CD83 on IL-4-DCs but IFN-DCs is not completely clear, but IFN α is known to drive a distinctive maturation pathway allowing for enhanced antigen presentation and T cell stimulation without reduced antigen processing²⁹⁵. Supporting this, IFN-DCs were capable of endocytosing Dextran-FITC with or without the cytokine cocktail, whereas Dextran-FITC uptake was reduced by the cytokine cocktail maturation in IL-4-DCs (Figure 3.2). I did not directly compare Dextran-FITC uptake by IL-4-DCs and IFN-DCs due to their different size characteristics (and therefore different autofluorescence and surface area per cell available for antigen uptake), although others have demonstrated that both types of MDDC are equally competent antigen scavengers^{513,514}. These studies also demonstrated that IFN-DCs are comparatively specialised for cross-priming due to

their slow endosomal acidification and increased proteasomal activity. For this reason, IFN-DCs were utilised in this chapter and in Chapter 6 for the study of cross-presentation.

A pure naïve CD8⁺ T cell population is important for *in vitro* priming experiments because the fraction of naïve T cells in each donor varies and effector memory T cells may be cross-reactive and/or have a lower threshold for activation (i.e. could be activated by cytokines alone). In routine testing, the isolated naïve CD8⁺ T cells were >99% CD45RA⁺ and CD4⁻ (Figure 3.4A). I did not perform CCR7 staining to investigate whether the CD45RA⁺ cells isolated were all naïve T cells. It is possible that some antigen-experienced T cells re-expressing CD45RA (TEMRA) were present in the isolated population, as no specific antibody to deplete TEMRA is present in the Naïve Pan T cell isolation microbead cocktail currently offered by Miltenyi. However, anti-2B4(CD244) antibody is included in the microbead cocktail (ostensibly to deplete NK cells) and 2B4 may be highly expressed on blood CD8⁺ TEMRA from healthy individuals⁵²⁷. Additionally, preliminary data from a colleague suggest that 80-95% of CD8⁺ TEMRA cells (defined as CD45RA⁺, CCR7⁻) in healthy donors express 2B4 (personal communication, Dimitra Peppas, University of Oxford, UK). Therefore the majority of TEMRA cells may be depleted using the microbead cocktail, although 2B4 negative TEMRA may occasionally be present in the initial isolated 'naïve' population in a donor-dependent manner. The low level FEC responses observed across both unpulsed and antigen-pulsed DC co-culture conditions by IFN γ -ELISPOT assays for Donor 105 could be explained by the presence of FEC-reactive CD8⁺ TEMRA cells in the co-culture (Figure 3.6B). It is also possible that peptide-specific responses observed in this chapter could result from expansion of peptide-specific TEMRA, although the reported predominant CCR7⁺CD45RA⁺ phenotype of circulating Melan-A₂₆₋₃₅(A27L)-specific CD8⁺ T cells in healthy donors makes this unlikely⁵²⁸. Additionally, stem cell-like memory CD8⁺ T cells (Tscm) (CCR7⁺CD45RA⁺CD95⁺) could potentially be present in the isolated population (CD95 antibodies are not included in the Naïve Pan T cell isolation kit)⁵²⁹. However, Gattinoni *et al* showed that virtually all of the Melan-A₂₆₋₃₅(A27L)-specific CD8⁺ T cells were true naïve cells (CD95⁻), in healthy donors, so antigen-specific expansion of Tscm in the assay used here is unlikely. In future assays I could confirm my results by sorting naïve CD8⁺ T cells to high purity at the beginning of the assay (CCR7⁺CD45RA⁺CD95⁻) and showing that Ag-specific responses are not expanded by co-

culture with a Melan-A₂₆₋₃₅(A27L) peptide-pulsed HLA-A*02:01⁺ cell line which isn't a professional APC and shouldn't prime naïve CD8⁺ T cells.

After 15 days co-culture, up to 5% of the cells remaining were CD3⁺CD8⁺ (Figure 3.4B), and could represent a mixture of residual MDDCs or contaminating CD8⁺ NK cells. Interestingly, these contaminating NK cells have been associated with the ability of IFN-DC to prime CD8⁺ T cells *in vitro* through positive cross-talk after IFN α stimulation⁵³⁰. It would be interesting to determine the effects on DC priming capacity of depletion of NK cells (and also CD4⁺ T cells) which are potentially co-purified with IFN-DC by initial negative isolation of CD14⁺ monocytes for IFN-DC differentiation.

Priming HIV-1-specific CD8⁺ T cells in vitro

In just 3/11 donors, naïve HIV-1-specific CD8⁺ T cells were primed when MDDCs were pulsed with supraphysiological amounts of epitope peptides (Figure 3.6A). The low probability of priming HIV-1-specific CD8⁺ T cells is likely due to low frequency of HIV-1-specific CD8⁺ T cells in the naïve repertoire. For example, naïve cells specific for HIV-1 Gag p17₇₇₋₈₅ have been shown to have an average frequency of <1 in 10⁶ naïve CD8⁺ T cells from seronegative donors^{517,518}. Given that only 1-10 x 10⁶ naïve CD8⁺ T cells can be isolated from one donor, this low frequency explains the inability to consistently prime HIV-1-specific responses in my experiments. In addition to the presence of a higher frequency of epitope-reactive cells in the naïve repertoire, priming of responses against particular HIV-1 epitopes may also result from higher affinity for the restricting HLA allele (cross-competing off other peptides).

The inability to prime any HIV-1-specific responses using DCs pulsed with whole p24 protein or infected CD4⁺ T cells could be partially explained by a relatively low frequency of naïve precursors. However, a lower number of specific peptide-HLA class I complexes presented by cross-presenting MDDCs could also be partially responsible for the lack of antigen-specific expansion, as few HIV-1-specific CD8⁺ T cells that may be present would be less likely to encounter their cognate antigen. The use of T cell clones specific for HIV-1 Gag (or other HIV-1 proteins) epitopes could be used to show that MDDCs pulsed with whole antigen can cross-present HIV-1 epitopes to T cells in this setting (although experiments using clones would not give insight into T cell priming). In the case of

MDDCs pulsed with infectious HIV-1 or infected cells, MDDCs could have become impaired in their ability to prime CD8⁺ T cells, as was shown by one group^{531,532}.

The inability to *in vitro* prime HIV-1-specific CD8⁺ T cell responses partially contrasts with the results of previous studies. Lubong Sabado *et al* were able to expand responses by priming by MDDCs pulsed with live or inactivated HIV-1 in 8/11 donors tested⁵³³. However, in this setting, priming usually required at least 3 re-stimulations and just 14% of the confirmed responses were mounted by CD8⁺ T cells (86% were CD4⁺ as total naïve T cell populations were included in the initial co-culture). Furthermore, the purity of naïve T cells used in this study was less stringent (~90% negatively isolated cells were CD45RA⁺CD62L⁺), as only anti-CD14, anti-CD19, anti-CD56 and anti-CD45RO were used for negative isolation of naïve cells, hence leaving a relatively impure population of cells which could alter aspects of priming. Similarly, a recent comprehensive study investigating *in vitro* priming of total naïve T cells (CD4⁺ and CD8⁺; highly pure with TEMRA and Tscm removed) by autologous MDDCs pulsed with autologous HIV-1 isolates from HIV-1⁺ patients on antiretroviral therapy found that CD8⁺ T cell responses against a broad range of HIV-1 epitopes could be primed using this system⁵³⁴. This study matured DCs with CD40L and IFN γ (which was shown to result in the greatest IL-12p70 production by DCs) but otherwise used a similar protocol to the one described in this chapter. Therefore the increased capacity of these studies to prime T cells could be a result of T cell help (which may improve expansion/memory formation) and improved DC licensing by CD40L stimulation. In fact, CD40L was shown to be essential in other studies reporting successful *in vitro* HIV-1-specific T cell priming against recombinant or apoptotic HIV-1 antigens⁵³⁵⁻⁵³⁸, although these studies often used total PBMC or T cells which would certainly include memory T cells, some of which could be HIV-1-specific^{340,341}. Furthermore, Huang *et al* were unable to prime responses (from PBMC) with DCs loaded with HIV-1 proteins unless they were complexed with liposomes⁵³⁵. Thus there is huge variation in the types of *in vitro* priming protocols used by each group. Perhaps optimisation of DC maturation by CD40L/IFN γ stimulation, or incorporation of helper CD4⁺ T cells could license DCs for enhanced priming of low frequency naïve CD8⁺ T cells. In summary, the protocol developed in this chapter did not permit analysis of the repertoire of HIV-1-

specific CD8⁺ T cells primed by MDDCs *in vitro*. Nonetheless, I was able to use it to assess the effects of conditions present during AHI on DC-mediated T cell priming.

Consistent priming of CD8⁺ T cell responses to epitopes with high frequency in the naïve repertoire

For the reasons outlined above, most *in vitro* studies of CD8⁺ T cell priming take advantage of the relatively high frequency of Melan-A₂₆₋₃₅(A27L)-specific CD8⁺ T cells in the naïve repertoire⁵³⁹. This unusually high naïve frequency has been used to study long peptide cross-presentation⁵⁴⁰ or the effects of DC maturation⁵⁴¹ and cytokines such as IL-21⁵⁴² and IFN α ⁵¹⁶ on the process of CD8⁺ T cell priming. In line with the high frequency in the naïve repertoire, consistent priming of Melan-A₂₆₋₃₅(A27L)- and PGT₁₇₈₋₁₈₇-specific CD8⁺ T cells was observed in my system (Figure 3.7A). Furthermore, NS3₁₀₇₃₋₁₀₈₁-specific responses were primed in 7/10 donors tested. This observed pattern of responses as well as the graded magnitudes of each response (MelanA > PGT > NS3) follows the known hierarchy of naïve CD8⁺ T cell frequencies in the naïve repertoire of HLA-A*02:01⁺ individuals^{517,518}.

Assay-dependent variability in the size of the expanded MART1-tetramer-positive population was observed here. Similar variation is observed in other studies, and is expected due to the nature of extended >10 days expansion *in vitro* which can be affected by various undefined factors (up to 20% coefficient of variation calculated by⁵³⁹). The variation observed could be technical in nature (e.g. due to differences in the freezing of cells) or could result from donor-dependent characteristics (e.g. HLA-A*02:01 homozygosity). Nevertheless, I clearly showed that co-cultures with unpulsed DCs did not expand antigen-specific CD8⁺ T cells, hence pairwise or direct intra-assay comparisons can be made.

Effects of plasma from patients with AHI on *in vitro* priming

The experiments performed in this chapter could not confirm a role for plasma from patients with AHI in impairing the capability of MDDCs to prime CD8⁺ T cells. This hypothesis was based on the results of a study in which plasma from pre-peak viral load during AHI reduced IL-12p70 production and allogeneic T cell stimulation by IL-4-DCs⁴⁶. Importantly, I could not recapitulate this reduction in IL-12p70 production caused by factors present in pre-peak AHI plasma. There could be multiple

reasons for this discrepancy. Firstly, the plasma timepoints used here were slightly later during infection than the timepoint at which Frleta *et al* observed an inhibitory effect. This is important, as plasma sampled around the peak of viraemia (Fiebig stage III/IV¹⁵) did not inhibit IL-12p70 production whereas plasma sampled during the early viral ramp phase (Fiebig stage I/II) did inhibit IL-12 production⁴⁶. Secondly, treatment of the plasma with heparin or heat-inactivation may have altered the components causing an inhibitory effect on DCs. Frleta *et al* showed evidence that one of the inhibitory factors in AHI plasma was apoptotic microvesicles which bound DCs through the CD44 receptor. CD44 contains a heparin-sulphate side chain where heparin-binding proteins attach and can engage high affinity receptors for downstream cell signalling effects^{522,543}. Thus it is conceivable that recombinant heparin added to the media bound heparin-binding proteins present on the inhibitory microvesicles and prevented their interaction with CD44 on the surface of DCs. Although heat inactivation inhibited the clotting process successfully, it may have also denatured heparin-binding proteins or other proteins present in the microvesicles which would interact with CD44 and other receptors. Equally, heparin and heat-inactivation could also affect soluble inhibitory factors such as CD40L which has been proposed to inhibit DC function, at least in chronic infection^{505,507}. Lastly, the freeze-thaw history of these commercially-bought plasma samples is unknown. Although some studies suggest that freeze thaw cycles do not affect microvesicle count⁵⁴⁴ or exosome size⁵⁴⁵, other functional properties of microvesicles could potentially be affected.

These methodological caveats could also potentially explain the indiscernible effect of AHI plasma on *in vitro* CD8⁺ T cell priming by MDDCs. Alternatively, conditions during AHI may not actually inhibit DC function but rather inhibit CD8⁺ T cell expansion or function independently of priming. The analysis of more donor DCs treated with plasma from patients during the earliest stages of AHI would potentially elucidate whether inhibitory factors are present, although this may require prevention of plasma clotting by a means other than heparin or heat inactivation.

Effect of MDDC IFN α exposure on *in vitro* CD8⁺ T cell priming

Hypothesising that the increase in plasma IFN α levels during AHI could adversely affect DC function, in this chapter I tested the effects of exogenous IFN α on *in vitro* CD8⁺ T cell priming. My data show that IL-4-DCs pre-exposed to IFN α exhibited a reduced upregulation of co-stimulatory

molecules or activation markers in response to the TLR3 ligand poly I:C (Figure 3.10). Examining the phenotype of IFN α -exposed IL-4-DCs without TLR stimulation, or in response to other TLR ligands (e.g. R848 the TLR7/8 ligand) would also provide useful information about the effect of IFN α on DC function. By trypan blue staining, I did not observe any differences in cell viability at the time of staining, however multiple previous studies have shown that type I IFNs induce activation and subsequent apoptosis of DCs^{546–548}. One study even showed that induction of monocyte apoptosis and impairment of DC generation *in vitro* by inactivated HIV-1 was a result of IFN α production by pDCs, as IFN α alone could mimic the effect of inactivated HIV-1 virions⁵⁴⁹. Furthermore, HIV-1-pulsed MDDCs have been shown to induce upregulation of negative co-stimulatory molecules (e.g. CTLA-4, PD-1, LAG-3, TRAIL) in primed T cells^{531,532}. In the setting of my experiments, it would be interesting to further investigate apoptosis and inhibitory receptor expression of IFN α -treated DCs during the co-culture, as this would impact on T cell phenotype and proliferation following priming.

Although no significant difference was observed, a lower proportion of Melan-A₂₆₋₃₅(A27L)-specific IFN γ - and TNF- α -secreting CD8⁺ T cells were expanded by IFN α -treated, Melan-A₂₆₋₃₅(A27L)-pulsed IL-4-DCs in some donors by ELISPOT/ICS, despite a similar proportion of MART1-tetramer⁺ cells in each co-culture (with or without IFN α). Due to the laborious and time-consuming nature of these experiments, I was only able to perform them on a limited set of donor DCs; more replicates would elucidate whether a true difference in primed T cell phenotype or function exists.

The lower proportion of MART1-tetramer⁺ cells in the Melan-A₁₆₋₄₀(A27L)-primed co-cultures relative to co-cultures primed with the short peptide is likely due to the lower number of Melan-A₂₆₋₃₅(A27L) peptide HLA class I complexes formed on the surface due to relatively inefficient antigen processing, and resulting in fewer naïve antigen-specific CD8⁺ T cells encountering their cognate antigen. Furthermore, a lower MFI of MART1-tetramer⁺ cells in CD8⁺ T cells primed by Melan-A₁₆₋₄₀(A27L)-pulsed IL-4-DCs was observed (Figure 3.12A). This could be due to T cell activation which induces TCR downregulation in proportion to the strength of the initial antigenic stimulus^{550,551}. Therefore the cultures primed with the shorter peptide (higher initial antigen recognition) may have expanded more vigorously initially and were therefore now less stimulated

whilst cultures primed with the longer peptide (lower initial antigen recognition) were still expanding and/or more activated meaning that the TCR was downregulated.

In the comparison of co-cultures with DCs treated with the short or long version of the Melan-A peptide (just 2 donors; Figure 3.12), IFN α treatment showed minimal effect on the size of the MART1-tetramer⁺ population (in accordance with data shown in Figure 3.11). In contrast, the proportion of MART1-tetramer⁺ cells in IFN α -treated Melan-A₁₆₋₄₀(A27L)-pulsed DC co-cultures was >25% lower than in cultures without IFN α . This reduction was mirrored in a lower IFN γ /TNF α response to Melan-A₂₆₋₃₅(A27L) peptide measured by both ELISPOT and ICS. Although more biological replicates are required to determine whether a true difference exists, I speculate that the high antigen dose provided to CD8⁺ T cells through supraphysiological concentrations of Melan-A₂₆₋₃₅ (A27L) peptide presented by IL-4-DCs could mask the immunomodulatory effects of IFN α in this assay. On the other hand, the lower levels of Melan-A₂₆₋₃₅ (A27L) peptide on the surface of IL-4-DCs cross-presenting the extended peptide could potentially mean that the reduced co-stimulatory molecule expression (or other as yet undetermined effects of IFN α exposure) could play a greater role in determining CD8⁺ T cell priming, phenotype and function.

In summary, the *in vitro* priming assay used here was unable to shed light on how the Nef-biased first wave of CTL responses during AHI is developed. Furthermore, no robust conclusions can be made in this chapter about whether priming of CD8⁺ T cells by DCs is impaired by conditions observed during AHI, and whether this is related to the delay between the first and second wave of CTL responses during AHI. However, the preliminary data here provide new avenues for research into the effect of IFN α exposure on the ability of DCs to prime naïve CD8⁺ T cells.

Chapter 4: Novel classes of MHC I epitopes presented by HIV-1-infected CD4⁺ T cells

4.1 Introduction

CD8⁺ T cells constitute a crucial arm of the adaptive immune system capable of recognising and destroying pathogen-infected or cancerous cells in the body. This recognition is conveyed primarily by interaction between the T cell receptor (TCR) and major histocompatibility complex (MHC) molecules in association with antigenic peptides on the cell surface. All nucleated cells express MHC class I which is scanned and recognised by TCRs of T cells expressing the co-receptor CD8. MHC class II, on the other hand, is expressed chiefly by professional APCs for recognition by TCRs of T cells expressing the CD4 co-receptor. Once a T cell encounters its cognate antigenic peptide in association with MHC, signalling through the TCR triggers the activation of an effector response (which may involve cytokine secretion and/or target cell lysis).

The peptides displayed by human MHC, known as human leukocyte antigen (HLA) molecules are influenced by intracellular antigen processing mechanisms such as proteasomes, cathepsins and ERAP1/2¹⁵³. The polymorphisms in HLA molecules located within key peptide-interacting residues within the binding groove also heavily influence the antigenic peptide repertoire ('immunopeptidome') for each individual⁵⁵². Further complexity is added when considering pathogens that have evolved mechanisms to disrupt cellular antigen-processing, peptide-editing and HLA recycling activities, which can all dramatically alter the immunopeptidomic landscape⁵⁵³.

Definition of viral (and potentially also uniquely-expressed self) targets within the 'immunopeptidome' of infected cells can help to facilitate vaccine development against pathogens such as HIV-1. Traditionally, HIV-1 epitope mapping relied on T cell-focused approaches whereby PBMC donated by HIV-1⁺ patients were tested for reactivity to synthetic peptides by IFN γ -ELISPOT assay, intracellular cytokine staining or cell surface phenotyping of antigen-specific markers such as CD154 (CD40L) or CD134 (OX40)⁵⁵⁴. The development of T cell hybridomas, peptide-MHC-specific antibodies and tetramers has also contributed a great deal towards understanding T cell specificities and MHC quantitation. However, these techniques are generally reagent-limited and show varying sensitivity. A huge effort by many groups over the years has allowed the identification

of >1500 unique HLA class I-restricted epitopes listed in the HIV Molecular Immunology database at Los Alamos National Laboratories (LANL). More than 300 of these T cell epitope targets have been well characterised in terms of their optimal peptide length and HLA restriction⁵⁵⁵. Furthermore, these studies provided potential mechanisms for the association of particular HLA alleles with fast or slow disease progression, e.g. particular conserved Gag epitopes seem to favour slow progression to AIDS^{139,556,557}. Despite this substantial body of work, current vaccination approaches being trialled in humans have so far failed to enable clearance or even good control of infection, or clear established HIV-1 infection once established. One explanation among many for this lack of protection may be that CD8⁺ T cell responses induced during HIV-1 infection and after vaccination are rapidly escaped because they target variable epitopic regions within the HIV-1 genome and/or the number of epitopes targeted is too narrow. Therefore, methods to define novel epitopes which are not targeted under normal circumstances would inform the development of innovative vaccines.

In silico prediction of MHC-binding peptides within a known protein sequence is one of the most commonly employed methods by many investigators working in the initial stages of epitope discovery. Numerous online algorithms have been developed over the years, including NetMHC, SYFPEITHI, IEDB, BIMAS, PREDEP, MHCpred and CTLpred among others. The NetMHC server (<http://www.cbs.dtu.dk/services/NetMHC/>), one of the most frequently used online interfaces, is based on an artificial neural network approach trained on experimental data extracted from IEDB and SYFPEITHI databases⁵⁵⁸. Although these programs perform satisfactorily for well-characterised alleles, prediction accuracy suffers for understudied alleles such as HLA-C variants or alleles that are infrequent in the most commonly-studied human populations for which no data exists. *In vitro* HLA peptide binding assays can be used to support *in silico* predictions, however the artificial nature of the system (often including very high peptide concentrations) casts doubt upon whether such peptides would be presented by MHC under natural circumstances. Additionally, *in vitro* peptide binding assays generally do not account for cellular antigen processing mechanisms, which are known to vary widely between different cell types and stimuli^{559–561}. On the other hand, sequencing of peptides naturally processed and presented on MHC using mass spectrometry allows a T cell receptor- and algorithm-independent approach to epitope discovery.

Sequencing of naturally-processed MHC-bound peptides eluted from cultured cells was first described in 1989 when Alessandro Sette and colleagues eluted a mouse MHC class II-restricted peptide derived from hen egg lysozyme⁵⁶². Hans-Georg Rammensee and colleagues then published numerous papers in the early 1990's reporting the identification of MHC class I peptides by mass spectrometry^{563–565}. These early studies required $>10^9$ cells in order to afford confident detection of peptides. Despite this limitation, the method has since been used to refine MHC binding motifs, characterise the functions of antigen processing machinery, as well as for the identification of immunogenic regions of tumour-associated antigens and pathogen-derived proteins. In fact, viral epitopes presented naturally on human cells have been characterised in the context of infection by West Nile Virus, influenza virus, vaccinia virus, measles virus and human papillomavirus among others (Table 4.1).

Table 4.1. Studies using immunopeptidomics to identify viral epitopes naturally presented on virally-infected human cells

Virus	Cell type(s)	HLA	References
West Nile Virus	HeLa	Soluble HLA-A*02:01/HLA-A*11:01	566–568
Influenza Virus	HeLa, JY cells, DC	Soluble HLA-B*07:02, various HLA class I	569,570
Vaccinia virus/Modified Vaccinia Ankara virus (MVA)	Jurkat, HeLa, Lymphoblastoid cells (LCL)	Various HLA class I, HLA-DR	571–574
Measles Virus	Lymphoblastoid cell line (LCL)	HLA-A*02:01, HLA-DR	575–579
Human Papillomavirus	Caski, C66 (cervical keratinocytes)	HLA-A*02:01, various HLA	580
Human Immunodeficiency Virus	Primary CD4 ⁺ T cells, B cells, T cell lines, DC	Soluble HLA-A*11:01, membrane HLA of diverse donors	581–583
Respiratory Syncytial Virus	LCL, C1R cells	HLA-Cw4, various HLA	579,584
Dengue Virus	JY cells, DC, HepG2 cells	HLA-A*02:01, HLA-A*24:02	585,586
Epstein-Barr Virus	LCL	Various, HLA-B*27	587,588
Hepatitis B Virus	Liver tissue from HBV ⁺ patient, HepDE9 cells	Various HLA class I	589,590

In the past 10 years, sensitivity has increased massively with the state-of-the art mass spectrometers, in particular Orbitrap instruments⁵⁹¹. In fact, some of the earliest efforts to identify naturally presented HIV-1 peptides by a proteomics approach were unsuccessful and studies instead focused

on comparing the differences in self immunopeptidomes presented by uninfected and HIV-1-infected cells⁵⁹². Recently however, highly sensitive MS instruments allowed the identification of many naturally presented HIV-1 peptides by infected cells^{581–583}. These studies unexpectedly yielded an abundance of nested sets of peptides extended at the N- or the C-termini. In particular, similar species of unusually long peptides from HIV-1 Gag were detected in multiple donors of diverse HLA types. Although these long HIV-1 peptides showed partial immunogenicity in IFN γ ELISPOT assays with HIV-1⁺ donors, no conclusive evidence that these are MHC class I-restricted peptides has been shown. Other mass spectrometry-based studies have identified unusually long peptides which could potentially bind MHC^{216,593}, and rare crystal structures of MHC class I in complex with 15-mers or 16-mers centrally-bulging out of the binding cleft exist^{594,595}, however the frequency of these complexes in nature remains unclear. Additionally, a recent study of HLA class I-restricted *Toxoplasma gondii* epitopes suggested that *T. gondii* infection may cause a conformational shift in MHC, therefore allowing binding of C-terminally extended peptides protruding through an ‘open’ groove⁵⁹⁶. Further study on these nested sets of extended pathogen-derived peptides is required to determine their suitability for inclusion in innovative vaccines.

Given the recent technological advances, it is now possible to delve deeper into the HIV-1 immunopeptidome than ever before and novel targets for CD8⁺ T cells can be identified for consideration in HIV-1 vaccine development. Current vaccine approaches generally incorporate whole HIV-1 proteins or conserved protein regions. However, T cells targeting cryptic epitopes resulting from translation of alternative reading frames (ARF) within the HIV-1 genome have been described in infected individuals^{174,176,597,598}. These ARF epitopes are thought to be DRiPs, which may contribute extensively to the immunopeptidome¹⁶⁴. However, although T cell responses to some of these epitopes have been shown to exert selective pressure on viral replication during infection, a closer examination of their presentation on infected cells and potential contribution to the repertoire of available T cell targets is warranted.

Two completely unexpected sets of broadly-targeted epitopes were recently highlighted by Hansen *et al* when rhesus macaques were vaccinated with a fibroblast-adapted strain of rhesus cytomegalovirus (RhCMV68.1) encoding SIV proteins. In RhCMV-seronegative macaques, RhCMV68.1 vaccination

consistently induced unusually broad (about 4 targeted epitopes per 100 amino acids) CD8⁺ T cell responses against vector-encoded SIV proteins¹⁵¹. Additionally, two thirds of these CD8⁺ responses were MHC class II-restricted whilst the remaining third was restricted by the MHC class Ib molecule Mamu-E^{151,348}. No discernible binding motif was identified in the set of Mamu-E-restricted optimal-length epitope peptides, revealing an unanticipated broad repertoire of potential Mamu-E binders. Intriguingly, despite genetic complexity and diversity within the Mamu-E gene locus, 2 of the SIV Gag Mamu-E-restricted epitopes were recognised by rhesus macaque CD8⁺ T cells in 100% of vaccinated animals, displaying an unprecedented horizontal immunodominance in primates. Although it is not yet clear whether the MHC-E-, MHC class II-restricted CD8⁺ responses, or other as yet undescribed immune cells are responsible for the 50% SIV clearance observed in vaccinated macaques³⁴⁹, the ability to generate similar broadly-targeted and so-called ‘supertope’-specific CD8⁺ responses in humans could embody a universal vaccine candidate.

Although rare, HLA class II-restricted CD8⁺ T cell responses have been reported recently in HIV-1⁺ patients⁵⁹⁹. Furthermore, HLA-E-restricted CD8⁺ T cell responses have been described in settings of pathogenic infections, cancer and autoimmunity⁶⁰⁰. This new role in adaptive immunity contrasts with the first described function of HLA-E in inhibiting target cell lysis by NK cells through interaction with CD94/NKG2A heterodimers expressed by NK cells¹⁴⁸. The initial indication of this bi-functionality came from studies of *Mycobacterium tuberculosis* (Mtb) infection, where HLA-E-restricted CD8⁺ T cells were found to be immunodominant in individuals latently infected with Mtb⁶⁰¹. Subsequently, similar responses were documented against hepatitis C virus (HCV)⁶⁰², human CMV⁶⁰³ and *Salmonella enterica* antigens⁶⁰⁴. The existence of MHC class Ib-restricted CD8⁺ T cells is not unique among primates, as these studies add to a body of literature on H2-M3 and Qa1 (HLA-E homologue)-restricted CD8⁺ responses during *Listeria monocytogenes* and *Salmonella typhimurium* infection of mice^{605,606}.

Evidently, HLA-E must bind a broader repertoire of peptides than previously thought. *In vitro* HLA-E refolding assays with randomised peptide libraries demonstrated a requirement for leucine at the C-terminal anchor residue and a preference for methionine and leucine at the position 2 anchor position⁶⁰⁷. Although this study extended the list of possible HLA-E-restricted peptides, the results

suggested a far more stringent binding motif than that observed for Mamu-E in RhCMV-vaccinated macaques by Hansen et al. In a TAP-deficient setting, Lampen et al eluted >550 peptides from the MHC class Ia^{low} cell line K562 overexpressing HLA-E*01:03⁶⁰⁸. In this study, enrichment of hydrophobic anchor residues at positions 2 and 9 of the eluted 9-mers conveyed a binding motif resembling that of HLA-A*02:01. More recently, another group has utilised soluble HLA-E*01:01 or HLA-E*01:03 constructs expressed in the MHC class I negative B lymphoblastoid cell line 721.221 to probe the HLA-E peptide repertoire^{609,610}. For HLA-E*01:01 and HLA-E*01:03, 36 and 56 peptides were identified, respectively. The majority of these peptides were >11 amino acids in length and did not contain canonical anchor residues. However, no untransfected control data were presented in these studies, and many of the peptides eluted derived from non-specific contaminants such as ribosomal proteins and histones. Therefore, given that SIV-infected cells are recognised by HLA-E restricted CD8⁺ T cells, a more complete characterisation of the HLA-E peptide repertoire under conditions of HIV-1 infection, stress or in different cell types is merited.

Historically, mild acid elution (MAE) of peptides from the cell surface or MHC immunoprecipitation (IP) with specific antibodies have been used to isolate peptides for sequencing via mass spectrometry. In this chapter, I aimed to 1) compare the specificity of MAE and HLA IP, 2) determine both the qualitative and quantitative reproducibility of the HLA IP method, 3) determine the extent of the non-specific HIV-1/self-peptide background and 4) identify novel CD8⁺ T cell HIV-1 epitope targets on HIV-1-infected CD4⁺ T cells.

4.2 Results

4.2.1 HLA immunoprecipitation allows specific and reproducible elution of HLA class I-bound peptides

To determine the most suitable method for specific purification of MHC-bound peptides, MAE and HLA IP were performed on monocyte-derived dendritic cells differentiated with IL-4 and GM-CSF (IL-4-DC). Peptides were isolated from supernatant of IL-4-DC incubated in acidic buffer whilst equivalent IL-4-DC were lysed for immunoprecipitation of HLA class I complexes using the pan class I-specific antibody W6/32 (workflow shown in Figure 4.1, top panel). An optimised nano liquid chromatography tandem mass spectrometry (LC-MS/MS) method was used to sequence natural peptides eluted from IL-4-DC HLA complexes. Raw spectral data were analysed using PEAKS software against a database containing all annotated human SwissProt entries and identified peptides <7 amino acids in length were excluded. Peptides identified (n=245) in the MAE method were between 7-28 amino acids in length and 45.3% of peptides were between 8-11 amino acids, the typical binding preference for HLA class I alleles (Figure 4.2A). Similarly, 57.1% of peptides identified in a PBS wash (n=240) of IL-4-DC before MAE were between 8-11 amino acids in length. In contrast, 98.5% of the 198 peptides identified in the IP sample were between 8-11 amino acids in length, and 70% were 9-mers (note that this experiment was performed before high-end mass spectrometers were purchased and therefore the number of peptide identifications is considerably lower than observed in later experiments presented in this chapter). Furthermore, a clear enrichment for lysine (K), threonine (T) and glutamine (Q) at position 2 and tyrosine (Y) and phenylalanine (F) at the C-terminal residue of 9-mer peptides eluted from the W6/32 IP sample could be observed in constructed sequence logos (Figure 4.2B). Importantly, these enriched residues were indicative of the known peptide binding motifs for HLA-A*01:01, HLA-A*24:02 and HLA-B*15:03, all of which were expressed by the IL-4-DC of the donor used. No such enriched residues were observed in either the MAE or the PBS wash sample (Figure 4.2B). These results indicated that the IP method specifically isolated HLA-bound peptides whereas the MAE method suffered from a lack of specificity.

To characterise the reproducibility of the HLA IP and LC-MS/MS methods, HLA class I peptides were isolated from uninfected C8166 cells (a CD4⁺ T cell line) or C8166 cells infected with the lab-adapted X4-tropic HIV-1 strain IIIB. Because HIV-1 IIIB is not derived from a molecular clone of known sequence (IIIB has been passaged through cells multiple times), I decided to sequence my viral stock to ensure that the sequences of HIV-1 IIIB peptides produced by infected cells were present in the analysis database. Viral RNA was extracted, then reverse transcribed into cDNA then amplified by two rounds of PCR and sequenced as described in the methods section 2.2.13. The nucleotide sequence complete genome of my viral stock (GenBank KJ925006.1⁵⁸¹) differed from the originally described sequence of HIV-1 IIIB (GenBank U12055.1⁶¹¹) at 27 locations, some of which would alter amino acid sequence (data not shown).

To characterise HLA IP reproducibility, replicate peptide samples (using C8166 cells) prepared on separate days (biological replicates) were sequenced by LC-MS/MS. Additionally, data for one HIV-1-infected C8166 peptide sample were acquired in duplicate to address the magnitude of instrumental variability (technical replicates). Between 7673-10012 and 6753-8766 unique peptides were identified in uninfected and HIV-1-infected samples, respectively (Figure 4.3A). For each sample, about two thirds of peptides were doubly charged and approximately 15% of peptides were singly charged (example of IIIB-infected replicate#2 in Figure 4.3B). 6774 peptides were identified in both technical replicates, representing 77.3-82.9% of the total peptides identified in each sample (Figure 4.3C). Regarding biological replicates, 69.7 or 73.0% of peptides identified in the IIIB-infected (#2) sample were also identified in the IIIB-infected (#1A) and IIIB-infected (#1B) samples. Thus, one can reasonably expect that ~80% of identified peptides will be re-identified in a second LC-MS/MS analysis of the same sample, whilst ~70% peptides will be shared between biological replicates. Although these data suggest that the HLA IP and LC-MS/MS method is qualitatively reproducible, whether similar quantities of peptides were measured in replicate samples was not clear. Therefore, the measured intensities of peptides identified in replicate C8166 samples were plotted against each other to determine whether the amount of a peptide detected in one replicate predicted the quantity of the same peptide in a separate measurement. As expected, the measured peptide intensity in one sample was highly predictive of its quantity in a technical replicate ($r=0.8797$, $p<0.0001$; Figure

4.3D). Similarly, the quantity of peptides measured in one biological replicate positively correlated with the peptide intensity in a sample prepared on a different day ($r=0.6535$, $p<0.0001$) (Figure 4.3E).

HLA class I alleles bind peptides containing sequence motifs favourable for stabilisation of the HLA complex. The extent to which the method specifically sampled HLA-bound peptides was determined by clustering identified 9-mers into groups of peptides with shared sequence motifs using the online GibbsCluster 1.1 server (found at <http://www.cbs.dtu.dk/services/GibbsCluster/>). Based on the greatest information content (Kullback Leibler distance; KLD) for each number of clusters made, 3 clusters were found to be optimal for the eluted C8166 9-mer peptides identified (Figure 4.3F). Upon manual inspection and comparison to known binding motifs, these clusters were found to represent ligands for the HLA class I alleles HLA-A*01:01, HLA-B*08:01 and HLA-B*44:02 expressed by C8166 cells (Figure 4.3G). No distinct HLA-C*05:01 and HLA-C*07:01 (expressed by C8166 cells) clusters could be identified through this method. 131 outlier peptides not conforming to any of the three clusters were excluded by the algorithm. Almost 3 times as many 9-mers containing the HLA-B*44:02 motif (2319 peptides; position 2 glutamate, position 9 aromatic amino acids) were clustered in comparison to HLA-B*08:01 (840 peptides; position 5 basic anchor residue, position 9 hydrophobic anchor). Similarly, despite expressing 2 HLA-A*01:01 alleles, only 849 peptides conforming to the known binding motif (position 2 serine/threonine anchor, position 3 glutamate/aspartate, position 9 tyrosine anchor) were identified. Altogether, these results show that the HLA W6/32 IP and LC-MS/MS approach to characterise the HLA class I immunopeptidome is reproducible and highly specific.

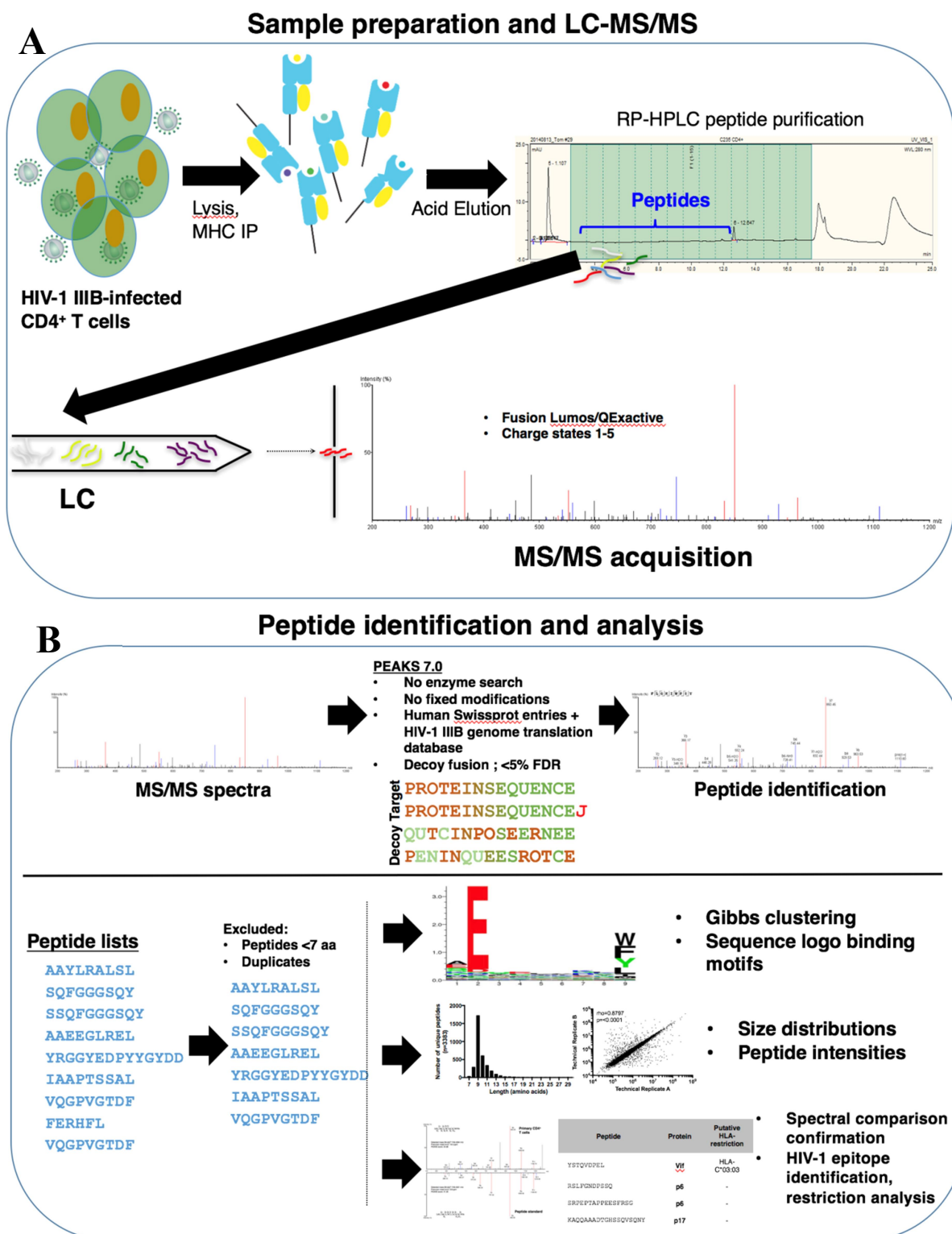


Figure 4.1 Workflow for identification and analysis of MHC-bound peptides by mass spectrometry. A. Sample preparation and LC-MS/MS. Cells of interest (typically primary CD4⁺ T cells from healthy donors or CD4⁺ T cell lines) were first infected with the lab-adapted X4-tropic HIV-1 strain IIB until the percentage of p24⁺ cells was >15% or >40%, respectively. Cells were then lysed and HLA class I or II immunoprecipitated using specific antibodies (e.g. W6/32 for MHC class I). Captured peptide-MHC complexes were washed then dissociated under acidic conditions. Peptides were then purified by RP-HPLC and β 2m, HLA heavy chain and co-eluted protein complexes removed. Purified peptides were again separated by RP-HPLC then injected into an online coupled mass spectrometer by electrospray ionisation (ESI). **B. Peptide identification and analysis.** Acquired spectra were imported into PEAKS software and searched against a database containing all annotated human SwissProt entries and the 6 frame translation of the sequenced HIV-1 IIB genome. Searches were performed with no enzyme specificity or fixed peptide modifications and a 5% false discovery rate (FDR) was set as determined by a parallel decoy-fusion database search using randomised protein sequences. Identified peptides were exported, duplicates and peptides less than 7 amino acids were then excluded from analysis. Consensus binding motifs for identified 9-mers were depicted using sequence logos before and after clustering of similar peptides (using Gibbs Cluster 1.1 server). In some experiments, synthetic peptide spectra were used to confirm correct assignment of sample spectra to protein sequences. Putative HLA restrictions were predicted using the online NetMHC4.0 algorithm.

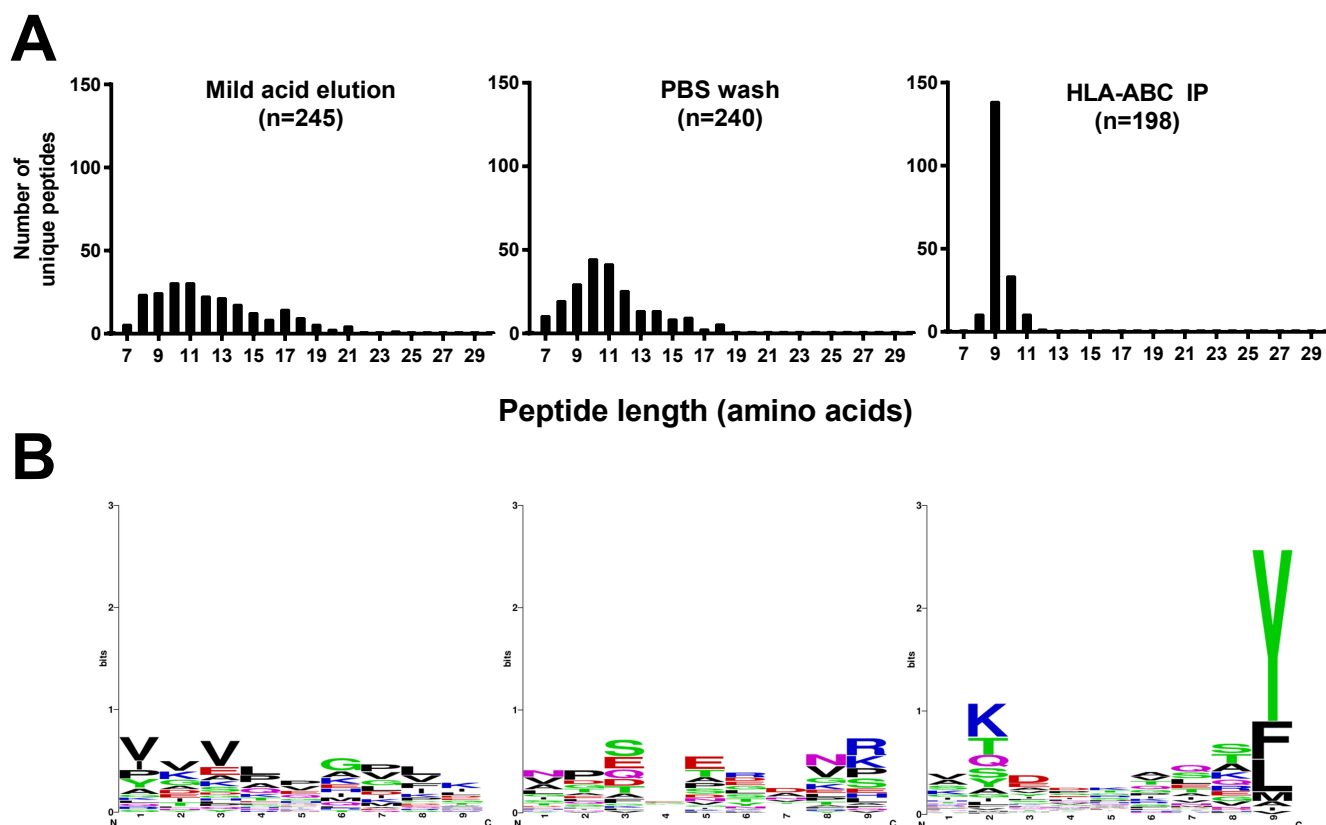


Figure 4.2 *Immunoprecipitation is superior to mild acid elution in terms of specificity of eluted HLA-bound peptides.* IL-4-DCs (HLA type: A*01:01, A*24:02, B*08:01, B*15:03, C*02:10, C*07:01) were differentiated for 6 days in the presence of IL-4 and GM-CSF then matured with a cytokine cocktail of IL-6, TNF α , IL-1 β and PGE2. Cells were then harvested and equal numbers of cells (3×10^7 each condition) were either lysed for HLA immunoprecipitation with W6/32 or washed with PBS then incubated in citrate phosphate buffer. For the latter, peptides were isolated from the PBS wash and the acid eluate. For the former, peptides were isolated from purified W6/32 IP eluates. Eluted peptide samples were analysed by LC-MS/MS on an Orbitrap Velos. **A.** The number of unique peptides of the indicated length (number of amino acids) is shown for each sample. **B.** Sequence logos created in WebLogo⁸⁹⁸ depicting the enrichment of particular amino acids at each position in identified 9-mer peptides are shown for the mild acid elution sample (left), PBS wash (middle) or the HLA immunoprecipitation (IP) sample (right). Polar amino acids are green, basic amino acids are blue, acidic amino acids are red and hydrophobic amino acids are black.

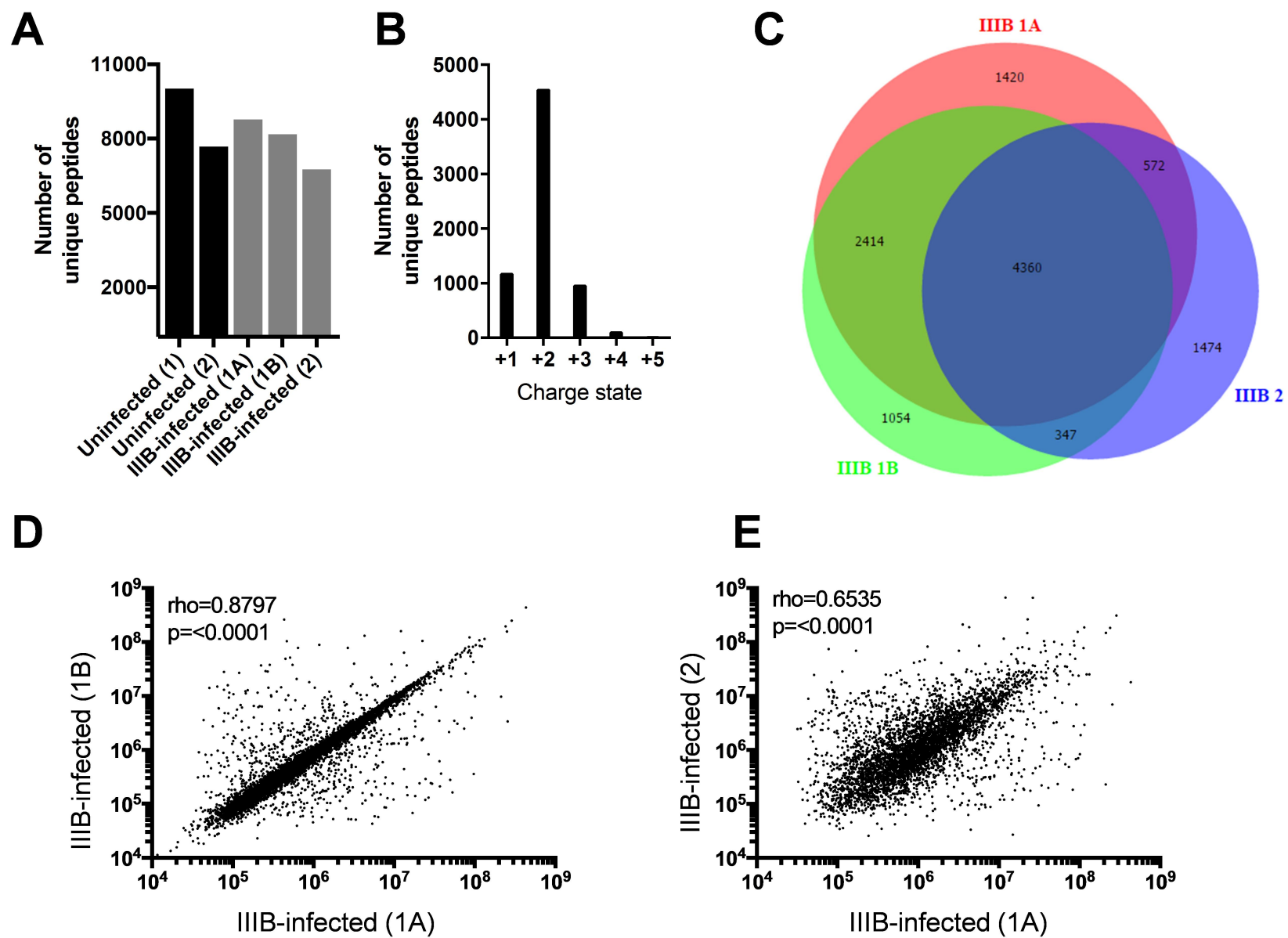


Figure 4.3

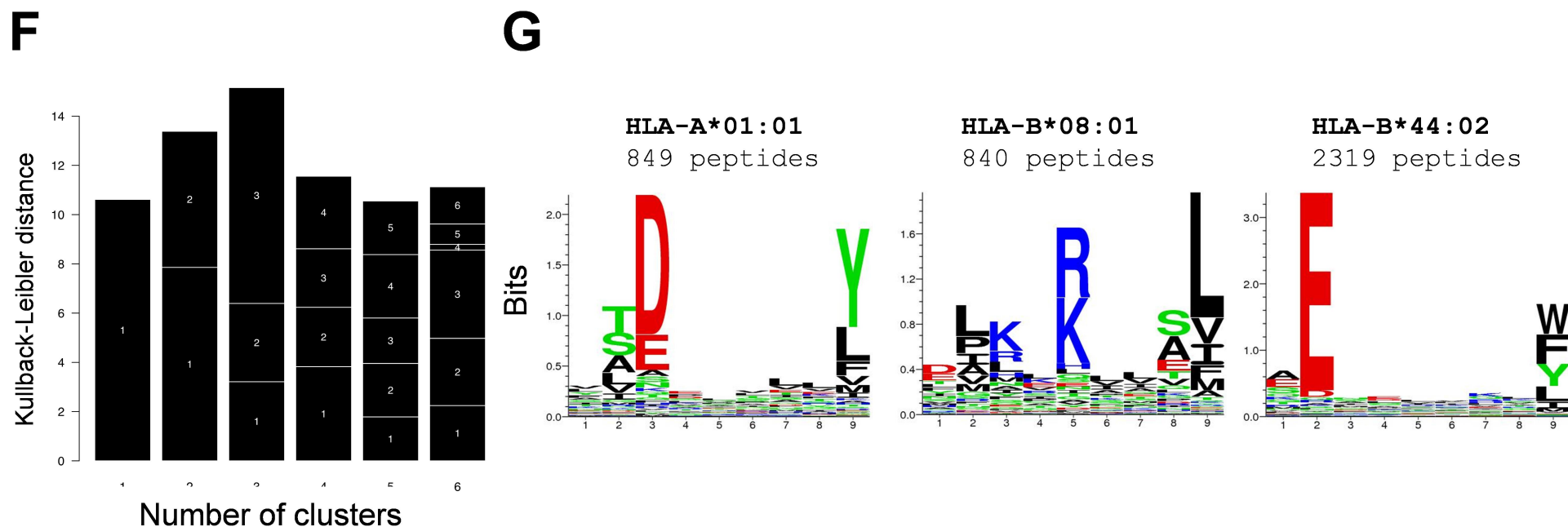


Figure 4.3 *Immunoprecipitation and identification of MHC class I peptides from human cells is highly reproducible.* 10^8 C8166 cells (HLA type: A*01:01 homozygous, B*08:01, B*44:02, C*05:01, C*07:01) were infected with HIV-1 IIIB for 3 days until $>50\%$ cells were $p24^+$ as determined by intracellular $p24$ staining. HLA class I-bound peptides were purified by immunoprecipitation using W6/32 antibody. Sample spectra were acquired on the Fusion Lumos mass spectrometer followed by analysis using PEAKS software. Samples numbered 1 and 2 indicate separate biological replicates prepared on different days. Samples 1A and 1B indicate technical replicates of the same sample run twice on the mass spectrometer. **A.** The total number of peptides identified in each sample analysed is shown for uninfected (black bars) and HIV-1 IIIB-infected samples (grey bars). **B.** Charge states of peptides identified in C8166 cells infected with HIV-1 IIIB (biological replicate 2). **C.** Overlap in peptides identified in HIV-1 IIIB-infected C8166 cells in the biological and technical replicates. **D.** Correlation of measured peptide intensities shared between the HIV-1 IIIB-infected C8166 sample technical replicates (1A on x-axis and 1B on y axis). **E.** Correlation of measured peptide intensities shared between the HIV-1 IIIB-infected C8166 sample biological replicates (1A on x-axis and 2 on y axis). Spearman's correlation co-efficient and p value are shown for each correlation. **F.** 9-mers identified in IIIB-infected C8166 cells (sample 1A) were subjected to Gibbs Clustering. The size of each block within a column is proportional to the number of peptides in one cluster and the height of the column indicates the Kullback-Leibler distance (KLD) sum of the alignments as a measure of the fitness of the system. When 3 clusters were produced (optimal KLD fit), 131 outliers were not clustered. The sequence logos for the three peptide clusters are shown in **G.** The motifs were assigned to 3 alleles expressed by C8166 cells by comparison to known motifs in MHC Motif Viewer. The number of peptides in each cluster is shown above each logo. HLA-C*05:01 and HLA-C*07:01 expressed by C8166 cells were not clustered by the algorithm. Polar amino acids are green, basic amino acids are blue, acidic amino acids are red and hydrophobic amino acids are black.

4.2.2 Extended Gag-derived peptides are non-specifically eluted after HLA immunoprecipitation

Having established the feasibility of the methodology to specifically isolate peptide-HLA class I complexes, the next step was to determine the repertoire of HIV-1 peptides presented by infected CD4⁺ T cells and the extent to which peptides non-specifically eluted during the immunoprecipitation contributed to the HIV-1 immunopeptidome. To examine whether protein degradation products not bound to HLA class I may form a significant portion of the eluted peptides, a W6/32 IP was performed on the lysate of the HLA class Ia negative cell line expressing CD4 (CD4.221) either uninfected or infected with HIV-1 IIIB. Infections of CD4.221 cells with HIV-1 IIIB typically resulted in >50% p24⁺ cells at day 3 post-infection as measured by intracellular p24 staining (data not shown). Just 57 and 110 unique peptides were eluted from uninfected and HIV-1 IIIB-infected CD4.221 cells, respectively (Figure 4.4A). Note that these samples were run on an Orbitrap Fusion Lumos mass spectrometer, which was able to identify with high accuracy upwards of 10,000 peptides in C8166 samples as shown in section 4.2.1. Although 9-mers were the most abundant peptide length detected in IIIB-infected cells, only 9 and 19 unique 9-mers were detected in the uninfected and IIIB-infected CD4.221 cell W6/32 immunoprecipitates, respectively. No HIV-1 peptides were detected in the HIV-1 IIIB-infected CD4.221 sample. Furthermore, enrichment of particular amino acids at canonical peptide anchor residues was not observed when sequence logos incorporating detected 9-mers were constructed (Figure 4.4B). Additionally, other detected peptides originated from proteins such as S100-A9, ribosomal proteins, Annexin-A2 and keratin, which are known as common contaminants introduced during sample preparation or proteins which bind non-specifically to Sepharose beads⁶¹². These data demonstrate that very few peptide/protein species non-specifically bind to W6/32 cross-linked Sepharose when using lysates in the absence of HLA class Ia.

Although the non-specific peptide background was remarkably low for an IP in which the antibody target is not expressed (HLA class I for W6/32), another possibility is that proteins/peptides associated with membrane pulled down by membrane protein-specific antibodies may contaminate sample preparations. To test this possibility, CD3 was immunoprecipitated from lysates of C8166

cells infected with HIV-1 IIIB to ascertain whether HIV-1 or self-peptides can non-specifically enter the relatively pure IP samples. As a positive control, W6/32 antibody-crosslinked Sepharose was used for half the lysate whilst the other half was incubated with OKT3 (anti-CD3) antibody. 1405 and 1625 unique peptides were detected in the uninfected and HIV-1 IIIB-infected C8166 OKT3 IP samples, respectively. The most common peptide length detected in all samples was 9-mers, with 52.4% (736/1405) and 72.4% (1171/1625) of all peptides between 8-11 amino acids in length in the uninfected and HIV-1 IIIB-infected OKT3 IP samples, respectively (Figure 4.5A). Comparatively, the W6/32 IP samples contained a greater proportion of 8-11-mers (84.0% uninfected, 87.7% IIIB-infected). To establish whether any of the peptides detected in the OKT3 immunoprecipitate originated from mature peptide-HLA complexes, 9-mers were clustered using the Gibb's clustering algorithm to elucidate HLA binding motifs. Three distinct clusters were separated for each of the W6/32 and OKT3 IP samples (IIIB-infected OKT3 IP clusters shown in Figure 4.5B). Upon manual comparison to known HLA binding preferences, these clusters were found to represent the sequence motifs for HLA-A*01:01, HLA-B*08:01 and HLA-B*44:02 expressed by C8166 cells. 27.4% and 61.7% of peptides detected in the uninfected and IIIB-infected C8166 OKT3 IPs were also observed in the matched W6/32 IP samples, respectively (Figure 4.5C). No correlation was found between the measured intensity of these peptides common to the W6/32 and OKT3 IPs in uninfected C8166 cells (Figure 4.5D). However, a weak positive correlation was observed between the measured intensities of peptides common to W6/32 and OKT3 IPs in HIV-1 IIIB-infected cells ($r=0.116$, $p=0.0004$). In both correlation analyses, distinct groups of peptides where the peptide abundance was relatively greater in the W6/32 IP sample were noted.

22 and 55 HIV-1 peptides were identified in the OKT3 and W6/32 IP samples, respectively. 14 of the 22 HIV-1 peptides in the OKT3 IP were also detected in the W6/32 IP sample, with the majority of these common peptides being >11 amino acids in length. More than half of the HIV-1 peptides identified in the W6/32 IP were between 8-11 amino acids in length (31/55, Figure 4.6A, example spectra shown in Figure 4.6B). In contrast, just 7/22 HIV-1 peptides were between 8-11 amino acids in length in the OKT3 IP sample, including only one 9-mer. The extended peptides existed in nested sets extended at either the N- or the C-terminus. Of the peptides <14 amino acids in length in the

OKT3 IP sample, just 1 was predicted to bind to any HLA class Ia allele by NetMHC4.0. This AEQASQEVKNW 11-mer is predicted to bind HLA-B*44:02 expressed by C8166 cells and has been previously reported as a HLA-B*44 epitope^{613,614}. Based on the assumption underlying the NetMHC4.0 algorithm that longer peptides bind MHC by bulging out of the cleft and/or extending out of the binding groove at the N- or C-termini, 6/9 of the peptides >14 amino acids in length were predicted to have an interaction core with an affinity for binding to one of the alleles expressed by C8166 cells (data not shown). However, on the advice of the creators of NetMHC, predictions for peptides >14 amino acids should be treated with caution. On the other hand, 21 of the 8-11-mers in the W6/32 IP sample were predicted to bind at least one of the HLA class I alleles expressed by C8166. Furthermore, 6 exact matches for known optimal epitopes in the Los Alamos National Laboratories HIV molecular immunology database were amongst those eluted by W6/32 IP (e.g. HLA-B*08:01-restricted Nef-FLKEKGGL and Gag-EIYKRWII or HLA-C*05:01-restricted Rev-SAEPVPLQL). Many of these identified peptides and their putative HLA restrictions were characterised in detail by myself and colleagues recently⁵⁸¹. Almost all of the peptides identified in the OKT3 IP were derived from Gag (21/22) whilst the other 14-mer originated from Nef. Although Gag peptides still predominated in the W6/32 IP (28 unique peptides), 17 Pol, 3 Env, 2 Nef, 2 Vpu, 2 Vif and 1 Rev peptide were also detected. Altogether, these data suggest that 1) abundant peptide-HLA class I complexes can contaminate CD3 IPs and 2) particular abundant and unusually long Gag-derived peptides are non-specifically eluted from CD3 IPs.

These extended nested sets of HIV-1 Gag-derived peptides were detected in multiple donors of diverse HLA types⁵⁸¹⁻⁵⁸³. To determine whether these abundant extended Gag peptides were HLA class I-associated, CD4.221 cells were infected with HIV-1 IIIB then membrane CD4 was immunoprecipitated using the OKT4 antibody. The size distribution of the 611 unique peptides eluted from CD4 immunoprecipitate was not indicative of canonical HLA class I peptides, as 9-mers were not significantly enriched (Figure 4.7A). Furthermore, no discernible motif was observed when the 51 9-mers were incorporated into a sequence logo (Figure 4.7B). Ten peptides from the HIV-1 Gag protein were also found in the OKT4 IP (Figure 4.7C). Similarly to the OKT3 IP, only one 9-mer was identified and the majority of the Gag peptides were >13 amino acids in length.

Furthermore, most of the peptides originated from the p2p7p1p6 sub-protein of Gag. In fact, the AEAMSQVTNSATIM and FLGKIWPSYKGRPGNF peptides represent the entire length of the p2 and p1 fragments of Gag, respectively. Given that CD4.221 cells do not express surface HLA class Ia, and W6/32 IP from CD4.221 cells did not yield peptides with features indicative of ability for HLA class I binding, any length variants of the extended Gag species were excluded from further analyses as non-specific background peptides.

To further confirm that peptide-HLA complexes contaminate membrane IPs, fractions 10 and 11 of RP-HPLC runs for C8166 W6/32 IP, C8166 OKT3 IP and CD4.221 OKT4 IP were pooled and protein was digested with trypsin. The number of identified proteins (or protein groups where redundant peptides were detected) and number of beta-2-microglobulin (β 2m) peptides identified in each sample is shown in Figure 4.8A. The greatest sequence coverage of the β 2m protein was observed for the C8166 W6/32 sample (21 peptides; 68%), whereas just 5 and 3 peptides were seen in the OKT3 and OKT4 samples, respectively. Many of the detected proteins were contaminants introduced during sample preparation such as keratin. Other contaminants such as ribosomal or S-100 proteins may have originated from non-specific binding to Sepharose beads. Supporting this notion, the overlap in protein identifications was considerable between the 3 different IPs (Figure 4.8B). HLA class I proteins were also identified in the W6/32 protein fractions (Figure 4.8C). Given redundancy in sequence between different alleles present in the SwissProt database, many alleles with up to 32% sequence coverage by peptides were identified by PEAKS. On the other hand, only up to 14% sequence coverage was detected for 4 assigned HLA class Ia antigens and the HLA class Ib antigen HLA-E in the OKT3 IP sample. Surprisingly, HLA class Ia antigens (up to 17% sequence coverage) were detected in addition to HLA class II antigens in the OKT4 IP protein fractions from CD4.221 cells. These data further confirm that peptide-HLA complexes are co-purified with membrane IPs, a finding which has implications for isolation of peptides restricted by lowly expressed alleles such as HLA-E (discussed in section 4.4.4).

The two peptide background issues associated with HLA IP from HIV-1-infected cells can be summarised as follows:

- 1) Abundant peptide-HLA complexes can contaminate membrane IPs of non-HLA antibody targets
- 2) Peptides which may not be HLA class I-associated and derived from abundant HIV-1 Gag proteins can contaminate membrane IPs

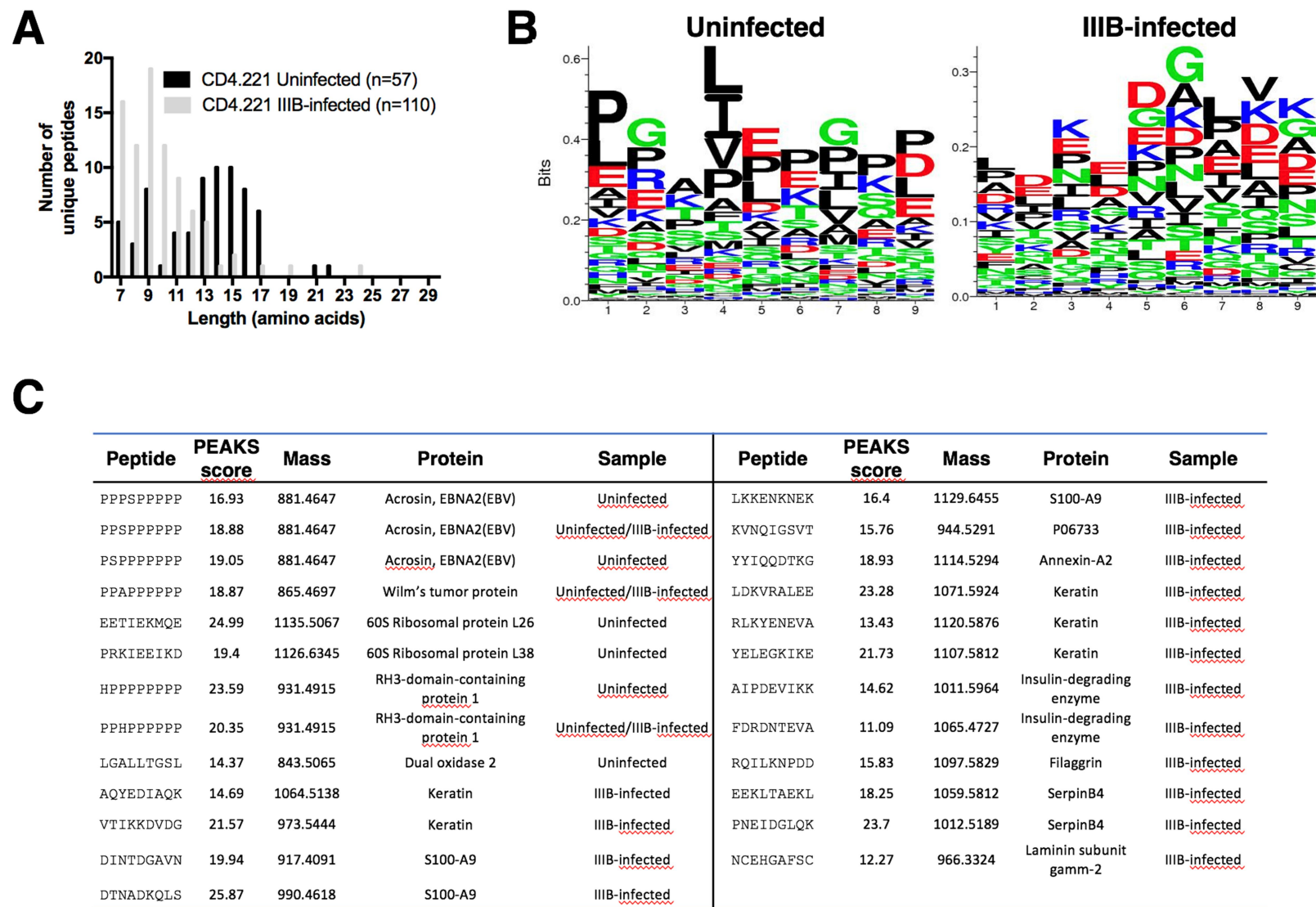


Figure 4.4 Low background of peptides eluted from W6/32 immunoprecipitate of the MHC class Ia negative cell line CD4.221. 10^8 CD4.221 cells were infected with HIV-1 IIIB or left uninfected for 3 days then lysed for immunoprecipitation with the W6/32 antibody. Resultant peptides were sequenced on an Orbitrap Fusion Lumos tandem mass spectrometer. **A.** Size distribution of identified unique peptides in either uninfected (black bars) or HIV-1 IIIB-infected (grey bars) CD4.221 cells. **B.** Sequence logos of all 9-mers identified in uninfected (n=9) or HIV-1 IIIB-infected CD4.221 cells (n=26). Polar amino acids are green, basic amino acids are blue, acidic amino acids are red and hydrophobic amino acids are black. **C.** Table listing examples of some of the peptides identified along with their PEAKS score, mass and the protein from which the peptide

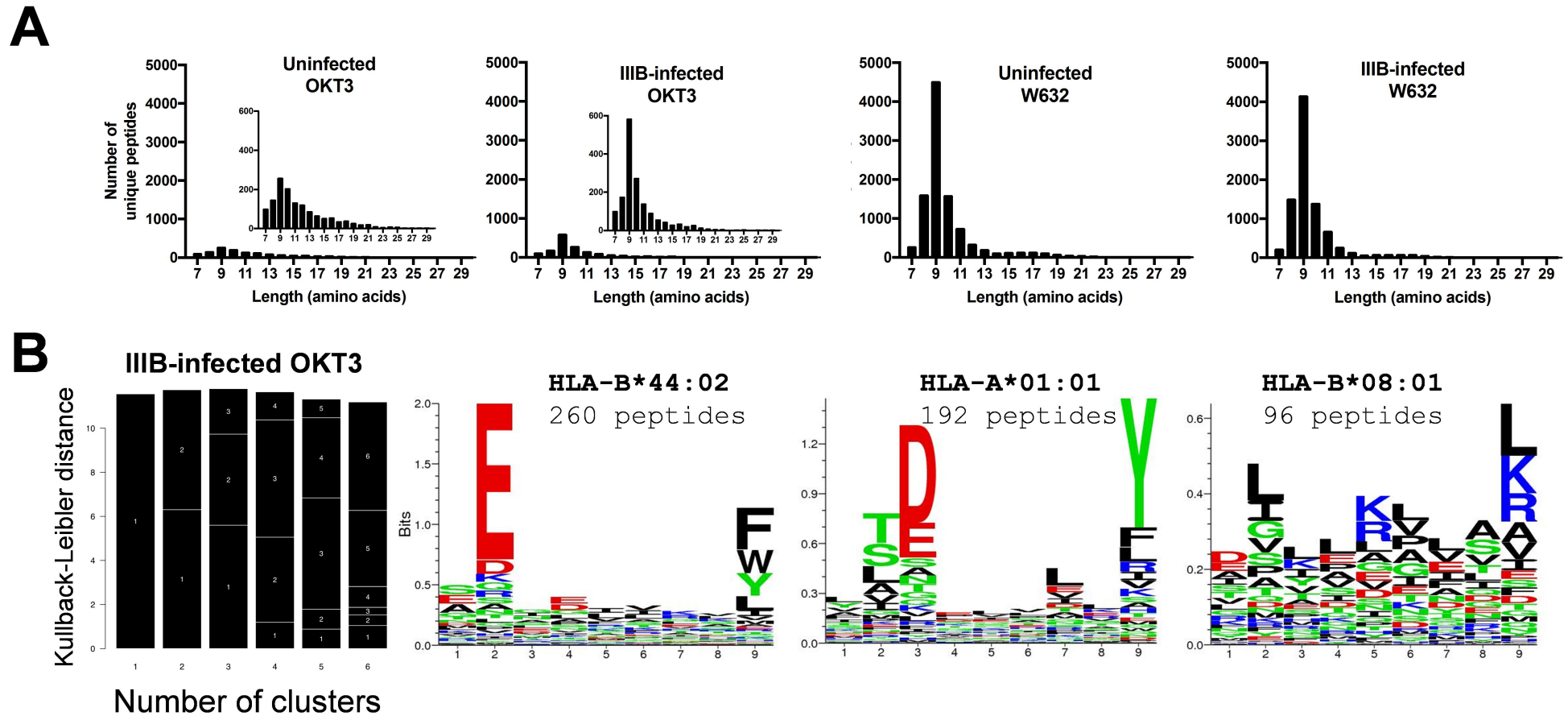


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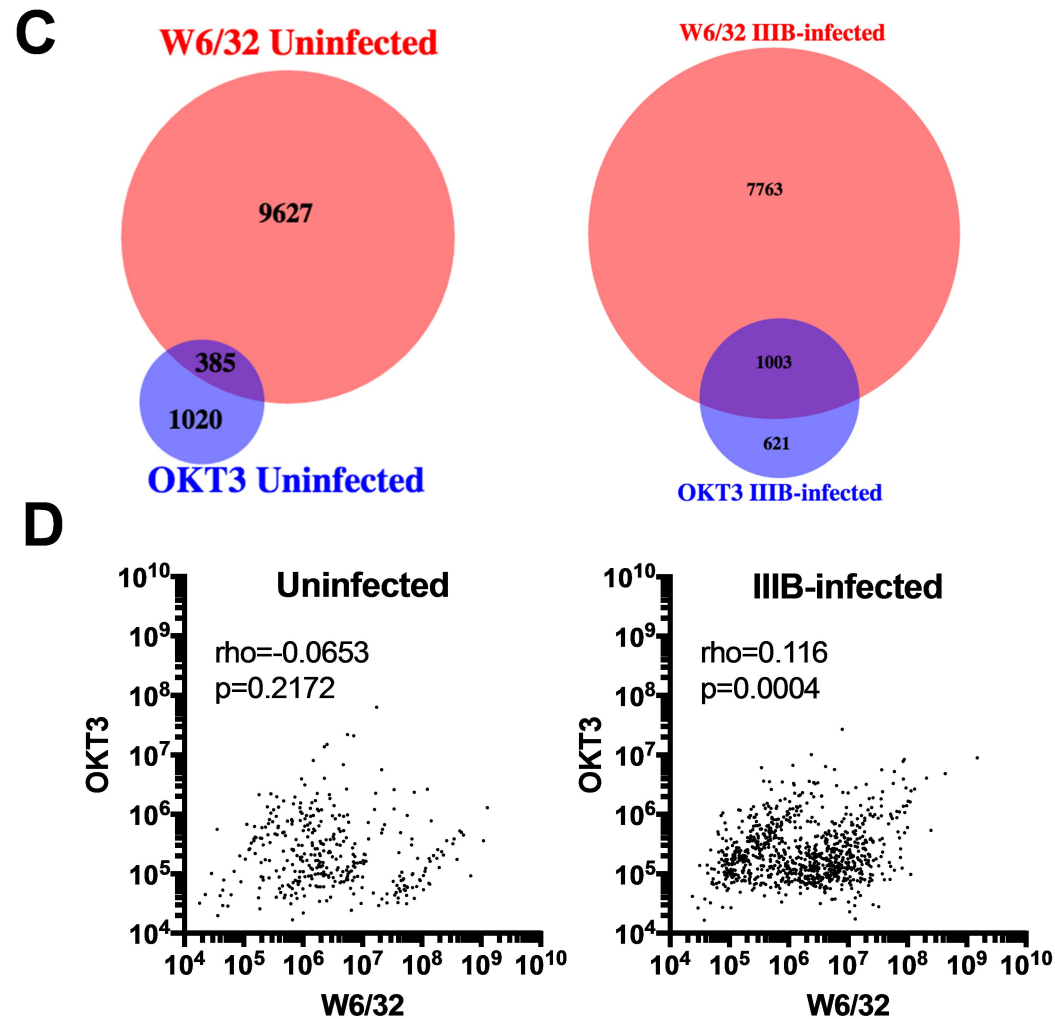
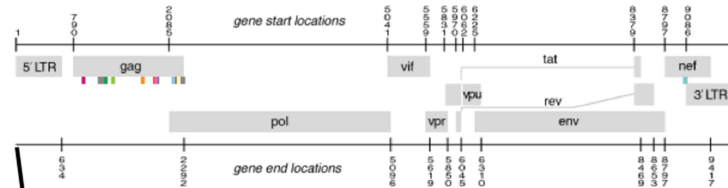


Figure 4.5 *HLA class I-bound peptides are eluted from CD3 immunoprecipitate of C8166 cells.* 10^8 C8166 cells were mock-infected or infected with HIV-1 IIIB for 3 days until cells were $>50\%$ p24⁺ as measured by intracellular p24 staining. Cells were then lysed and lysates were divided equally for immunoprecipitation with either W6/32 or OKT3 (anti-CD3) antibody. Peptides were purified then spectral data were acquired on an Orbitrap Fusion Lumos mass spectrometer. **A.** Size distributions of identified peptides. Total numbers of peptides identified is shown below each sample. Inset graphs show the size distribution plotted on a smaller y-axis for clarity. **B.** Gibbs clustering of 9-mer peptides identified in the HIV-1 IIIB-infected OKT3 sample was performed (left panel). The other three panels show sequence logos of each peptide cluster and the putative HLA type for each cluster determined by comparison to known motifs in MHC motif viewer. 36 outlier peptides were not grouped into any of the clusters. For sequence logos, polar amino acids are green, basic amino acids are blue, acidic amino acids are red and hydrophobic amino acids are black. **C.** Overlap in identified peptides between the OKT3 and W6/32 IP for uninfected and IIIB-infected samples. **D.** Measured intensities of shared peptides for OKT3 and W6/32 IPs were plotted against each other. Rho values and p values for Spearman's correlation are shown.

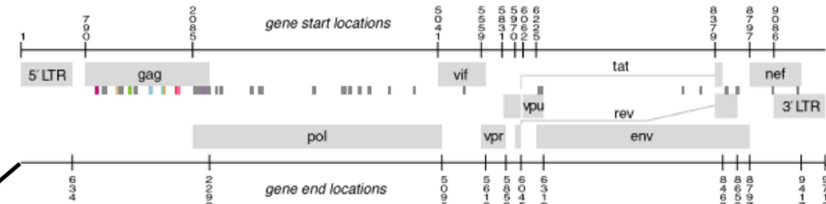
A

OKT3



Peptide sequence	Polyprotein	start	end
AEAMSQVTSATIM	Gag (p2)	364	377
AEQASQEVKNW	Gag (p24)	306	316
LQSRPEPTAPPFLQ	Gag (p2p7p1p6)	449	462
LSEGATPQDLNT	Gag (p24)	175	186
PIVQNIQGMVHQAI SPRTLNA	Gag (p24)	133	154
PLTSLRSLFGND	Gag (p2p7p1p6)	485	496
PLTSLRSLFGNDP	Gag (p2p7p1p6)	485	497
PLTSLRSLFGNDPSSQ	Gag (p2p7p1p6)	485	500
RFAVNPGLE	Gag (p17)	43	52
SQVTSATIM	Gag (p2)	368	377
SRPEPTAPPEESFRSG	Gag (p2p7p1p6)	451	466
SRPEPTAPPFLQ	Gag (p2p7p1p6)	451	462
TGHSSQVSQNY	Gag (p17)	122	132
VETTTTPQKQEPIDKELY	Gag (p2p7p1p6)	467	484
AADTGHSSQVSQNY	Gag (p17)	119	132
DLSHFLKEKGGLG	Nef	86	99
LQSRPEPTAPP	Gag (p2p7p1p6)	449	459
MQRGNFR	Gag (p7)	378	384
RSLFGNDPSSQ	Gag (p2p7p1p6)	490	500
SKKKAQAAADTGHSSQVSQNY	Gag (p17)	111	132
SLRSLFGND	Gag (p2p7p1p6)	488	496
SLRSLFGNDPSSQ	Gag (p2p7p1p6)	488	500

W6/32



Peptide sequence	polyprotein	start	end	Peptide sequence	polyprotein	start	end
AEAMSQVTSATIM	Gag (p2)	364	377	MQMLKETI	Gag (p24)	198	205
AEQASQEVKNW	Gag (p24)	306	316	PIVQNIQGMVHQAI SP	Gag (p24)	133	149
LQSRPEPTAPPFLQ	Gag (p2p7p1p6)	449	462	PLTSLRSLFG	Gag (p2p7p1p6)	485	494
LSEGATPQDLNT	Gag (p24)	175	186	RLAFHHVA	Nef	188	195
PIVQNIQGMVHQAI SPRTLNA	Gag (p24)	133	154	SAEPVPLQL	Rev	67	75
PLTSLRSLFGND	Gag (p2p7p1p6)	485	496	VEMGHHPW	Vpu	69	77
PLTSLRSLFGNDP	Gag (p2p7p1p6)	485	497	VEMGVEMGHHPW	Vpu	65	77
PLTSLRSLFGNDPSSQ	Gag (p2p7p1p6)	485	500	AEIQKQGQGG	Pol (RT)	482	490
RFAVNPGLE	Gag (p17)	43	52	AEIQKQGQGGW	Pol (RT)	482	492
SQVTSATIM	Gag (p2)	368	377	AEIQKQGQGGWTY	Pol (RT)	482	494
SRPEPTAPPEESFRSG	Gag (p2p7p1p6)	451	466	EEMSLPGRW	Pol (protease)	90	98
SRPEPTAPPFLQ	Gag (p2p7p1p6)	451	462	ELKNSAVSL	Gp160 (gp41)	806	814
TGHSSQVSQNY	Gag (p17)	122	132	FIKVRQYDQIL	Pol (protease)	109	119
VETTTTPQKQEPIDKELY	Gag (p2p7p1p6)	467	484	FSVPLDEDFRKY	Pol (RT)	271	282
ADQLIHLY	Vif	103	110	HLKTAVQM	Pol (integrase)	886	893
AEQASQEVKN	Gag (p24)	306	315	IAEIQKQGQGGWTY	Pol (RT)	481	494
EIYKRWII	Gag (p24)	260	267	LTNTTNQKTELQAIY	Pol (RNase)	624	638
ETTTTPQKQEPIDKELY	Gag (p2p7p1p6)	468	484	LVDFRELNK	Pol (RT)	229	237
FLGKIWP	Gag (p1)	433	439	QELLELDKW	Gp160 (gp41)	658	666
FLGKIWPSY	Gag (p1)	433	441	RELNKRQDFW	Pol (RT)	233	243
FLGKIWPSYK	Gag (p1)	433	442	SAGIRKIL	Pol (RNase)	708	715
FLGKIWPSYKGRPGNF	Gag (p1)	433	448	SESELVNQI	Pol (RNase)	668	676
FLKEKGGL	Nef	90	97	VILVAVHV	Pol (integrase)	787	794
GSEERRSLYNTVATLY	Gag (p17)	71	86	VLDVGDAY	Pol (RT)	263	270
LADQLIHLY	Vif	102	110	VSAGIRKIL	Pol (RNase)	707	715
LGKIWPSYKGRPGNF	Gag (p1)	434	448	YLKDQQLL	Gp160 (gp41)	586	593
LQSRPEPTAPPFLQSRPEPTAPPEESFRSG	Gag (p2p7p1p6)	441	466	YVDGAANRETKLGAGY	Pol (RNase)	596	612
LRSLFGNDPSSQ	Gag (p2p7p1p6)	489	500				

Figure 4.6 continued next page

B

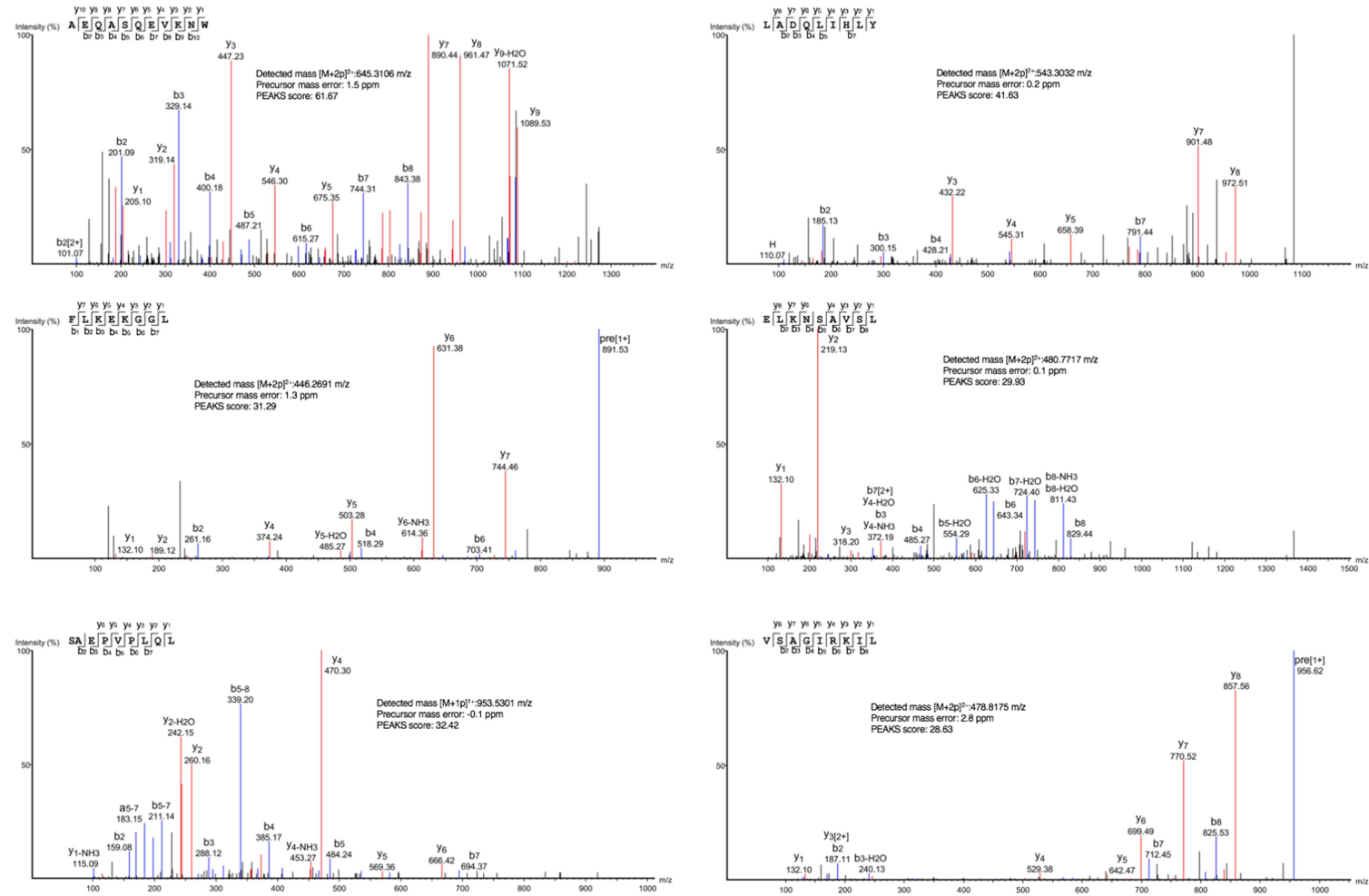


Figure 4.6 HIV-1 peptides are eluted from CD3 immunoprecipitate of C8166 cells. 10^8 C8166 cells were mock-infected or infected with HIV-1 IIIB for 3 days until cells were $>50\%$ p24⁺ as measured by intracellular p24 staining. Cells were then lysed and lysates were divided equally for immunoprecipitation with either W6/32 or OKT3 (anti-CD3) antibody. Peptides were purified then spectral data were acquired on an Orbitrap Fusion Lumos tandem mass spectrometer. **A.** HIV-1 peptides identified in OKT3 or W6/32 IPs are represented as coloured blocks on the depicted HIV-1 genome and tables showing locations within HIV-1 proteins are listed below each schematic. **B.** Examples of spectra assigned to HIV-1 peptides in W6/32 IP samples. For each spectrum, the detected mass over charge ratio $[M+np]^+/n$ (where n is the number of charges and p is the mass of a proton), the PEAKS software peptide score and the measured precursor mass error (in parts per million) are shown. The detected fragment ions in each spectrum are displayed in the peptide sequence where b and y represent N- and C-terminal fragment ions, respectively.

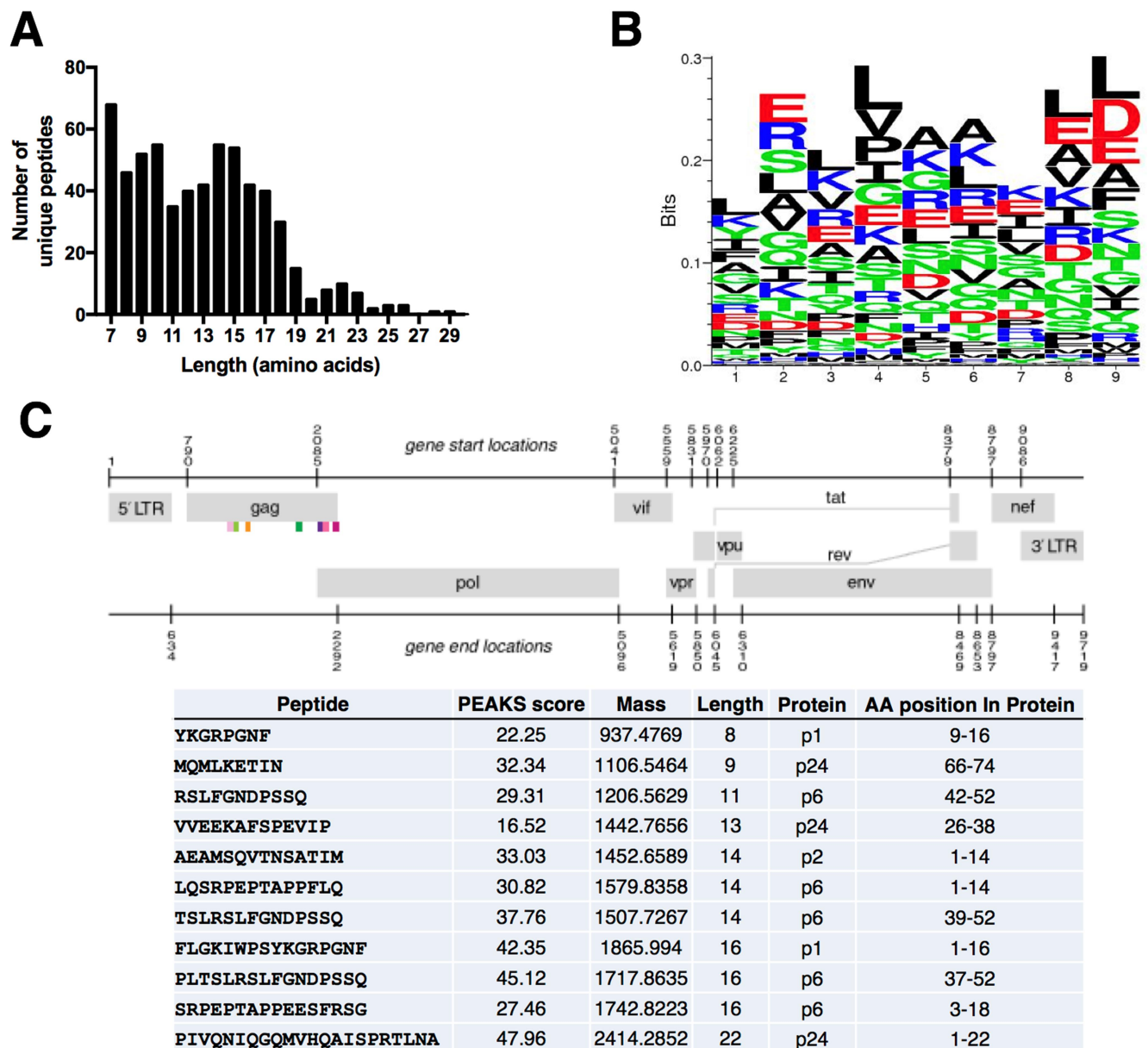
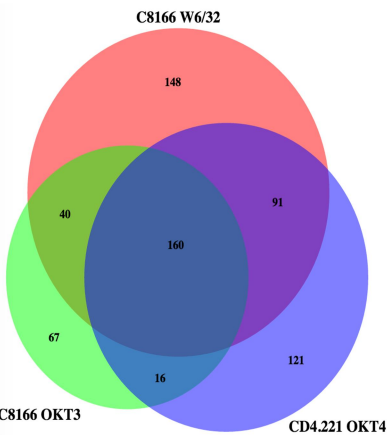


Figure 4.7 *HIV-1 peptides are eluted from CD4 immunoprecipitate of HIV-1-infected CD4.221 cells.* 10^8 CD4.221 cells were infected with HIV-1 IIIB for 3 days by which time they were $>50\%$ p24⁺ as measured by intracellular p24 staining. Cells were lysed and immunoprecipitation was performed using the anti-CD4 antibody OKT4. Resultant peptides were purified by RP-HPLC and sequenced on an Orbitrap Fusion Lumos mass spectrometer. **A.** Size distribution of peptides identified from CD4.221 OKT4 IP (n=611) **B.** Sequence motif of 9-mers identified in CD4.221 OKT4 IP. Polar amino acids are green, basic amino acids are blue, acidic amino acids are red and hydrophobic amino acids are black. **C.** Map of HIV-1 epitopes identified in OKT4 IP. Epitopes are shown below proteins on the HIV-1 genome map as coloured blocks. The table below the schematic indicates the sequence of each peptide, the PEAKS software score, mass of the peptide, peptide length and the source HIV-1 sub-protein in Gag as well as its amino acid position in the protein.

B



C8166 W6/32 IP				C8166 OKT3 IP			
Description	Coverage (%)	#Peptides	#Unique	Description	Coverage (%)	#Peptides	#Unique
MHC class I antigen A*1	32	11	1	MHC class I antigen A*1	14	5	3
MHC class I antigen A*11	28	8	0	MHC class I antigen E	6	2	1
MHC class I antigen A*26	21	7	0	MHC class I antigen B*47	7	2	1
MHC class I antigen A*29	25	8	0	MHC class I antigen B*82	7	2	0
MHC class I antigen A*3	30	9	0	MHC class I antigen Cw*1	7	2	0
MHC class I antigen A*32	25	9	0				
MHC class I antigen A*33	18	5	0				
MHC class I antigen A*43	21	7	0	CD4.221 OKT4 IP			
MHC class I antigen A*69	22	7	0	MHC class I antigen A*3	16	5	0
MHC class I antigen A*74	25	9	0	MHC class I antigen A*32	17	6	0
MHC class I antigen A*80	24	8	0	MHC class I antigen A*33	11	4	0
MHC class I antigen B*13	19	7	0	MHC class I antigen A*34	16	5	0
MHC class I antigen B*14	22	7	0	MHC class I antigen A*69	15	5	0
MHC class I antigen B*18	16	5	0	MHC class I antigen B*14	12	4	0
MHC class I antigen B*27	21	8	0	MHC class I antigen B*18	14	5	1
MHC class I antigen B*38	24	9	0	MHC class I antigen B*38	14	5	0
MHC class I antigen B*39	24	9	0	MHC class I antigen B*57	14	5	0
MHC class I antigen B*40	20	7	0	MHC class I antigen B*78	12	4	0
MHC class I antigen B*44	24	8	0	MHC class I antigen Cw*1	9	3	0
MHC class I antigen B*47	20	7	0	MHC class II DQA1	5	2	0
MHC class I antigen B*51	24	7	0	MHC class II antigen DRB1*15	3	1	1
MHC class I antigen B*52	24	7	0				
MHC class I antigen B*57	23	8	0				
MHC class I antigen B*59	24	9	0				
MHC class I antigen B*73	15	5	0				
MHC class I antigen B*78	24	7	0				
MHC class I antigen B*82	19	7	0				
MHC class I antigen Cw*1	17	5	0				
MHC class I antigen Cw*12	20	6	1				
MHC class I antigen Cw*16	20	6	1				
MHC class I antigen Cw*18	16	5	0				
MHC class I antigen Cw*3	16	4	0				
MHC class I antigen Cw*6	16	5	0				
MHC class I antigen Cw*7	16	5	0				

4.2.3 Novel epitopes derived from the HIV-1 5' leader sequence translated and presented by MHC class I on infected CD4⁺ T cells

In addition to epitopes arising from conventional HIV-1 proteins, CD8⁺ T cells are also known to target epitopes resulting from translation of alternative reading frames of the HIV-1 genome^{174,176,597,598}. Therefore when searching mass spectra for HIV-1 epitopes, a 6 frame translation of the entire HIV-1 IIIB genome was included in order to allow the identification of cryptic epitopes. In experiments where primary CD4⁺ T cells were infected with HIV-1 IIIB, a number of cryptic epitope candidates were detected. Interestingly, 2 such overlapping epitopes detected in one donor were derived from the 5' 'untranslated' region, henceforth called the 5' leader region (Figure 4.9). As the percentage of productively infected cells generally ranged from just 10-25% in a donor-dependent fashion, fewer HIV-1 peptides were detected in a primary CD4⁺ T cells in comparison to the highly infectable C8166 CD4⁺ T cell line. Of the 3383 unique peptides identified (data were acquired on a TripleTOF 5600 mass spectrometer) in primary CD4⁺ T cells from donor 97 (HLA-A*01:01, HLA-A*02:01, HLA-B*07:02, HLA-B*08:01, HLA-C*07:01, HLA-C*07:02), 4 HIV-1 peptides were identified (Figure 4.10A, B). FLGKIWPS and FLGKIWPSY are likely to be HLA-A*02:01-restricted, as NetMHC predicted that FLGKIWPS would bind HLA-A*02:01, and an extended version of the epitopes (FLGKIWPSYK) has been previously described as an HLA-A*02:01-restricted epitope targeted by CD8⁺ T cells during HIV-1 infection^{120,615}. The two overlapping 5' leader-derived peptides (LSLGALWL and LSLGALWLAREPTA) arose from the TAR loop structure of HIV-1 RNA where a one-off Kozak sequence allows alternative ribosomal initiation (unpublished data; Ranjit Warriar, Edward Kreider & George Shaw, University of Pennsylvania, US). The correct assignment of the 5'leader-derived peptides was confirmed by fragmenting a synthetic peptide of the same sequence under the same conditions as the original sample by LC-MS/MS. Both the sample and synthetic standard spectra were doubly-charged and detected at a similar retention time and m/z ratio (Figure 4.10C). Both peptides were assigned relatively confident PEAKS software identification scores (34.98 and 41.30; with scores of 10-15 usually just above the 5% FDR threshold). Moreover, a similar fragmentation pattern was observed whereby the y₈ ion was the most intense followed by y₇ and y₉ ions. These results demonstrate that peptides derived from

alternative reading frames in the so-called ‘untranslated region’ of the HIV-1 genome can be produced and presented naturally on HLA class I of infected cells for potential recognition by CD8⁺ T cells.

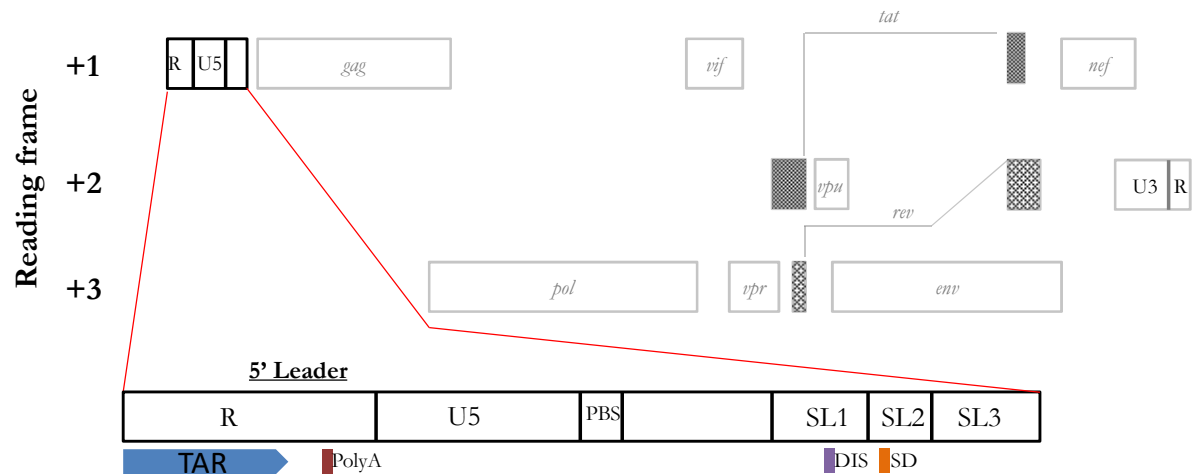


Figure 4.9 *The HIV-1 5' leader region.* In the HIV-1 mRNA transcript is the 5' leader region, which contains the ‘R’ and the ‘U5’ regions of the proviral 5’LTR. Shown in the schematic are important functional sites and domains within the 5’ leader region. TAR; transactivating response element, PBS; primer binding site, DIS; dimer initiation signal, SL1/2/3; stem loop 1/2/3, SD; splice donor site, PolyA; polyadenylation signal site.

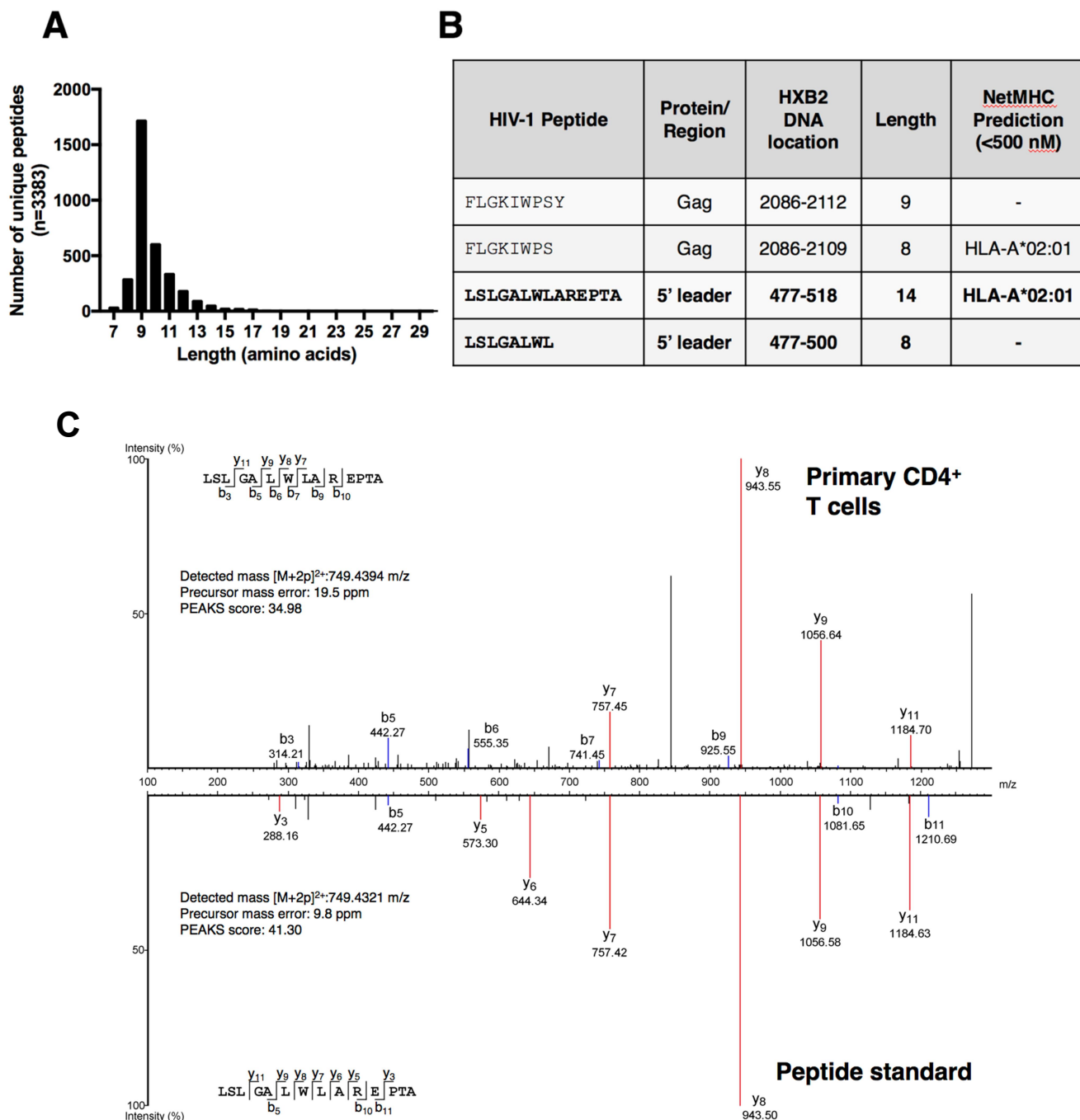


Figure 4.10 Peptides derived from the HIV-1 5'leader sequence are presented with MHC class I in infected CD4⁺ T cells. **A.** Length distribution of HLA class I peptides eluted from 1.2×10^8 donor 97 primary CD4⁺ T cells infected with HIV-1 IIIB. **B.** List of HIV-1 peptides eluted from donor 97 primary CD4⁺ T cells infected with HIV-1 IIIB. Putative HLA restriction was predicted using NetMHC3.4. **C.** Comparison of experimental LSLGALWLAREPTA fragmentation spectra with synthetic peptide standard. For each spectrum, the detected mass over charge ratio $[M+np]^n/n$ (where n is the number of charges and p is the mass of a proton), the PEAKS software peptide score and the measured precursor mass error (in parts per million) are shown. The detected fragment ions in each spectrum are displayed in the peptide sequence where b and y represent N- and C-terminal fragment ions, respectively.

4.2.4 HIV-1 epitopes presented by the non-classical MHC molecule HLA-E

In addition to discovery of epitopes derived from novel regions of the HIV-1 proteome, epitope targets restricted by unconventional HLA class Ib alleles could be beneficial for a vaccine. Since HLA-E is highly conserved across human populations, characterisation of HLA-E-restricted viral epitopes could be exploited in a universal prophylactic approach. Therefore, attempts were made in this study to develop a method to identify HLA-E-restricted HIV-1 peptides by mass spectrometry. Two methods were utilised to achieve this:

- 1) Using HLA-E-specific antibodies to purify peptide-HLA-E complexes from cells containing classical HLA class I alleles
- 2) Isolating HLA-E from cells which only express HLA-E in the absence of functional HLA class Ia alleles

4.2.4.1 Elution of peptides from naturally expressed HLA-E

The pan HLA class I-specific antibody W6/32 recognises HLA-E in addition to HLA-A, -B, and -C. Indeed, the canonical signal peptides which are known to bind with high affinity to HLA-E were detected in most W6/32 IP samples. However, whether other HLA-E-restricted peptides were also detected in these samples was not clear. Gibbs clustering of HLA-bound peptides identified from samples immunoprecipitated with W6/32 did not robustly identify a group of peptides likely to have been bound to HLA-E, but the HLA-E peptide binding motif has not been definitively characterised (and it has been speculated that MHC-E may bind a broader range of peptides than class Ia molecules¹⁵¹) and peptides restricted to HLA class Ia alleles are likely to be more abundant than those bound to the relatively lowly-expressed HLA-E, making the latter more difficult to detect. To focus in on HLA-E peptides, the HLA-C- and E-specific antibody DT9 was used to immunoprecipitate peptide-HLA complexes from the CD4⁺ T cell line PM1 (homozygous for HLA-A*01:01, B*15:03 and C*03:03) which was either HIV-1 IIIB-infected or uninfected. PM1 cells were chosen because 1) they are easily infected by diverse HIV-1 strains and 2) their homozygosity in the HLA-C locus for HLA-C*03:03 would allow more straightforward discrimination between HLA-C*03:03- and HLA-E-bound peptides. 771 and 548 unique peptides were identified in the uninfected and HIV-1 IIIB-infected samples, respectively. The peptide length distribution was

bimodal, as two localised peaks at 9 and 16 amino acids were observed (Figure 4.11A). However, the majority of peptides comprised a typical HLA class I distribution, with 64.9% and 56.6% of all peptides being 8-11 amino acids in length for the uninfected and IIIB-infected samples, respectively. As with a typical W6/32 IP, most peptides sequenced by LC-MS/MS were doubly charged although singly-charged and triply-charged peptides were also common (uninfected PM1 DT9 IP sample shown in Figure 4.11B). Considerable overlap between the uninfected and HIV-1 IIIB-infected samples was also observed (288 shared peptides; Figure 4.11C). To determine whether a HLA-E binding motif could be distinguished from the known HLA-C*03:03 binding motif, 9-mers were grouped by Gibbs Clustering. Two clusters were formed in the uninfected DT9 IP sample, whilst only one cluster was the optimal fit for the HIV-1 IIIB-infected sample (Figure 4.11D). One of the uninfected DT9 clusters (253 peptides) clearly represented HLA-C*03:03 peptides, as a preference for a hydrophobic C-terminal anchor, alanine or serine at position 2 anchor, and a preference for phenylalanine or tyrosine at the position 1 auxiliary anchor was observed (Figure 4.11D, top left panel). Although the MHC motif viewer indicates a preference for proline at the position 2 anchor residue (<http://www.cbs.dtu.dk/biotools/MHCMotifViewer/HLA-c.html#27>), a more comprehensive study based on experimental MHC dissociation assays determined the HLA-C*03:03 binding motif as described above⁶¹⁶. Interestingly, proline was the most common amino acid at position 4 in this cluster; the same position where proline is conserved in HLA class I signal peptide sequences which bind tightly to HLA-E. However, amino acid preferences at other anchor positions in these peptides with proline at position 4 still indicated a HLA-C*03:03 binding motif (data not shown) and could therefore not be assigned as putative HLA-E-restricted peptides. The other cluster of 9-mers in the uninfected sample comprised of 95 peptides representing a mix of HLA-A*01:01 and HLA-B*15:03-restricted peptides, because enrichment of serine/threonine at position 2 as well as aspartate at position and tyrosine at position 9 is reminiscent of the known HLA-A*01:01 binding motif (Figure 4.11D, bottom left panel). Additionally, glutamine enrichment at position 2 and phenylalanine at the C-terminal anchor residue suggests the presence of HLA-B*15:03-bound peptides. When the outlier peptides not grouped into either cluster were examined, no discernible motif or pattern emerged (data not shown). The 1 cluster identified in the IIIB-infected sample seemed to represent all three HLA class Ia alleles, but was again most enriched for HLA-C*03:03 peptides (Figure 4.11D, right panel).

In the HIV-1 IIIB-infected sample, 4 HIV-1 peptides were detected. Three of these were Gag-derived and ranged from 11-19 amino acids in length (Figure 4.11E). Since these extended peptides were amongst those found to be non-specific contaminants in HLA IPs (see section 4.4.2), they were not considered to be HLA-specific peptides here. None of these contaminant peptides were predicted to bind to any of the HLA class Ia alleles or HLA-E by NetMHC4.0. However, one 9-mer (YSTQVDPEL; YL9) originating from the HIV-1 Vif protein was predicted to bind strongly to HLA-C*03:03 (% rank of 0.17) (fragmentation spectra shown in Figure 4.10F). Indeed, the serine at the position 2 anchor and hydrophobic leucine C-terminal anchor concord with the described HLA-C*03:03 motif. To my knowledge, this Vif YL9 epitope has not been described until now, and therefore represents another potential target for HIV-1-specific CD8⁺ T cell responses.

As DT9 IP predominantly isolated HLA-C-restricted peptides, a HLA-E-specific antibody (clone: 4D12) was utilised to exclude HLA class Ia-bound peptides. C8166 cells, either infected with HIV-1 IIIB or uninfected, were lysed then HLA-E was immunoprecipitated with 4D12. Similarly to the DT9 IP, a bimodal distribution was observed whereby 9-mers were the most common length of peptide detected along with a long tail of peptides >14 amino acids (Figure 4.12A). Respectively, 1532 and 1511 peptides were identified in the uninfected and IIIB-infected C8166 IP samples of which 629 were common to both samples (Figure 4.12B). When 9-mers from the uninfected C8166 4D12 IP were clustered, 3 distinct motifs emerged which were representative of the binding motifs for HLA-A*01:01, HLA-B*08:01 and HLA-B*44:02 expressed by the C8166 cell line (Figure 4.12C), indicating that classical HLA class I complexes were contaminating the 4D12 IP sample. Pulldown of classical HLA class I complexes may have resulted from cross-reactivity of the 4D12 antibody for particular HLA class I alleles. To test this hypothesis, the cross-reactivity of 4D12 antibody to a panel of 100 classical HLA alleles on the surface of LABScreen beads was measured and normalised relative to the reactivity of W6/32 antibody. Although 4D12 exhibited cross-reactivity of up to 30% of that observed for W6/32 to particular alleles, the reactivity to HLA-A*01:01, HLA-B*08:01 and HLA-B*44:02 was relatively low (Figure 4.12D), suggesting that antibody cross-reactivity was not behind the observed presence of HLA class Ia complexes in 4D12 IPs. Numerous HIV-1 peptides were also observed in the HIV-1 IIIB-infected C8166 4D12 IP sample (Figure 4.12E). These

peptides were predominantly Gag- or Pol-derived and many were nested sets (data not shown) identified as potential non-specific contaminants as demonstrated in section 4.2.2.

In summary, the use of HLA-E-specific antibodies was not sufficient to elucidate a clear HLA-E binding motif due to contamination with peptide-HLA class Ia complexes. However, HLA-E-restriction of some of the peptides identified here cannot be precluded and could be confirmed through further validation.

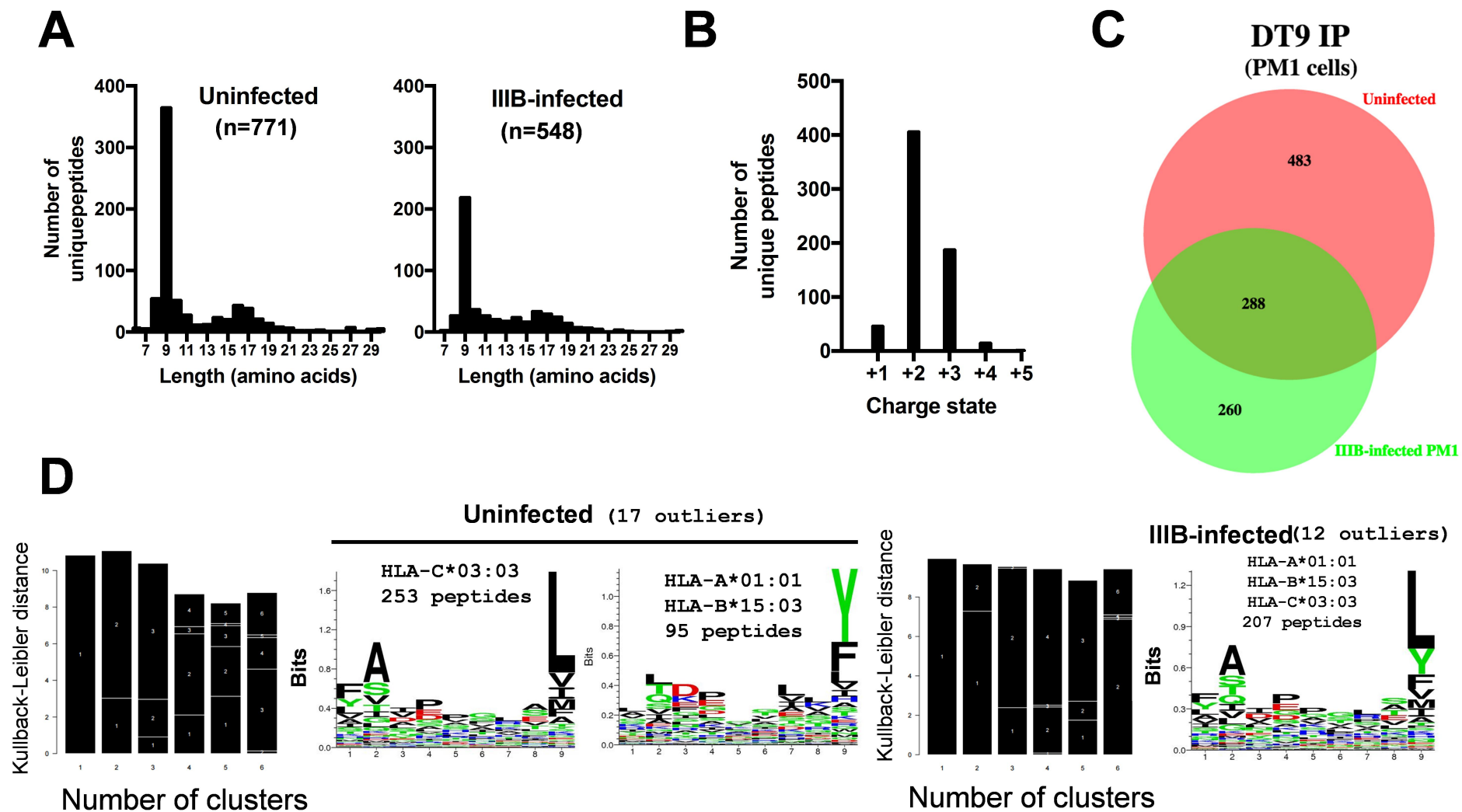


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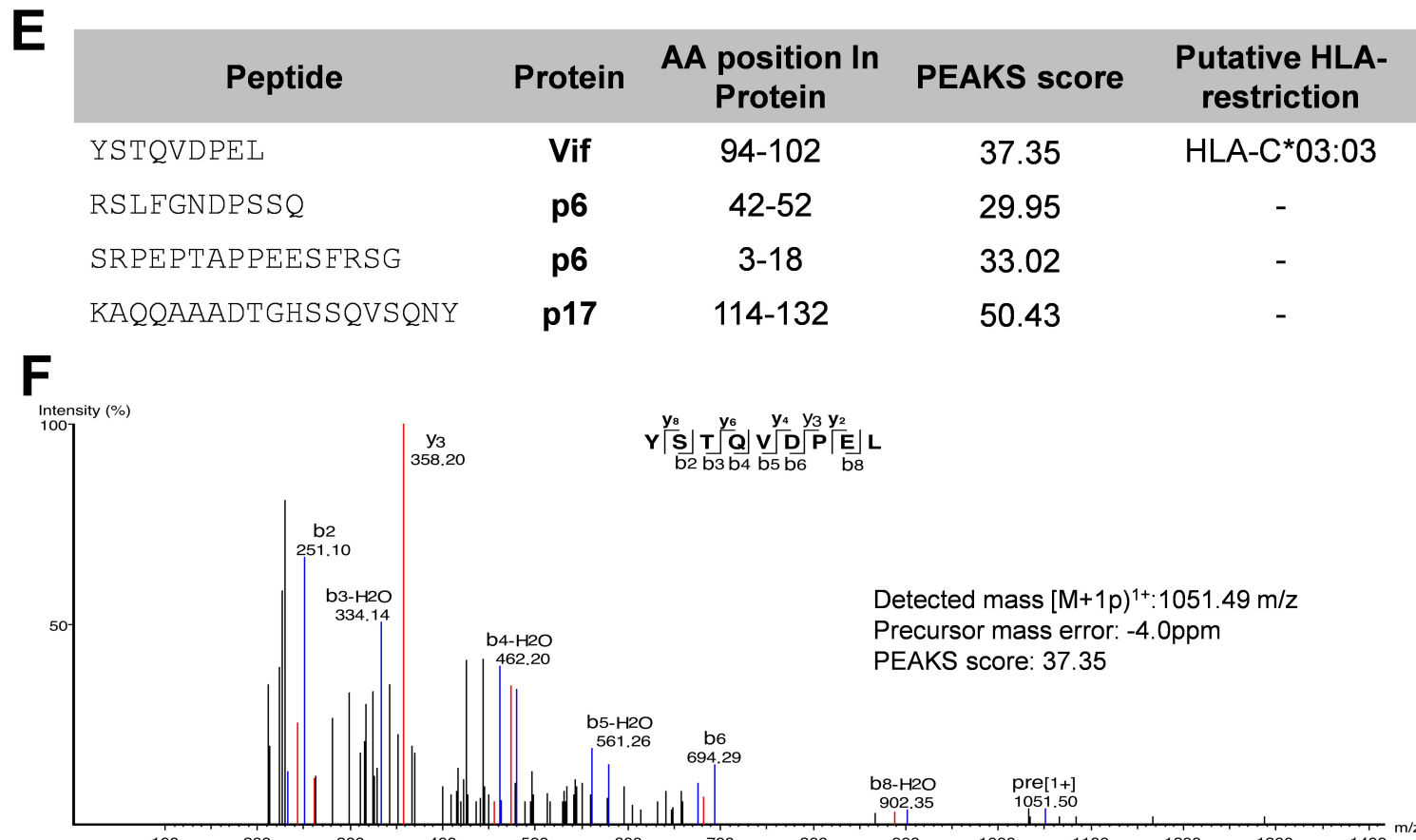


Figure 4.11 Peptide elution from HIV-1-infected PM1 cells using DT9 immunoprecipitation. 4.5×10^7 PM1 cells were infected with HIV-1 IIIB for 4 days until they were $>43\%$ p24⁺ as measured by intracellular p24 staining. Cells were lysed and HLA-C/HLA-E was immunoprecipitated using DT9 antibody-cross-linked Sepharose. Data were acquired on an Orbitrap Fusion Lumos tandem mass spectrometer. **A.** Size distributions of peptides eluted from uninfected (n=771) and HIV-1 IIIB-infected (n=548) PM1 DT9 IP samples. **B.** Charge states of peptides detected in the uninfected PM1 cell DT9 IP sample. **C.** Overlap in peptide IDs between the uninfected and IIIB-infected DT9 IP samples. **D.** 9-mers identified in each IP were grouped into distinct clusters using the Gibbs Clustering algorithm. 2 clusters were formed for the uninfected PM1 sample whereas for the IIIB-infected sample, 1 cluster was the optimal fit. The number of peptides in each cluster is shown below the putative HLA type expressed by PM1 cells for which the binding motif matches with described motifs. Polar amino acids are green, basic amino acids are blue, acidic amino acids are red and hydrophobic amino acids are black. **E.** Table depicting the HIV-1 peptides identified in the HIV-1 IIIB-infected DT9 IP sample. The source HIV-1 protein, location within the protein as well as the PEAKS identification score and putative HLA-restriction are shown. HLA-restriction was predicted using the NetMHC4.0 online server. Peptides with a % rank lower than 2% in relation to a library of 400,000 random peptide sequences were assigned as putative binders to the indicated allele. **F.** Fragmentation spectra of the putative HLA-C*03:03 Vif epitope YSTQVDPEL. For each spectrum, the detected mass over charge ratio $[M+np]^n/n$ (where n is the number of charges and p is mass or proton), the PEAKS software peptide score and the measured precursor mass error (in parts per million) are shown. The detected fragment ions in each spectrum are displayed in the peptide sequence where b and y represent N- and C-terminal fragment ions, respectively.

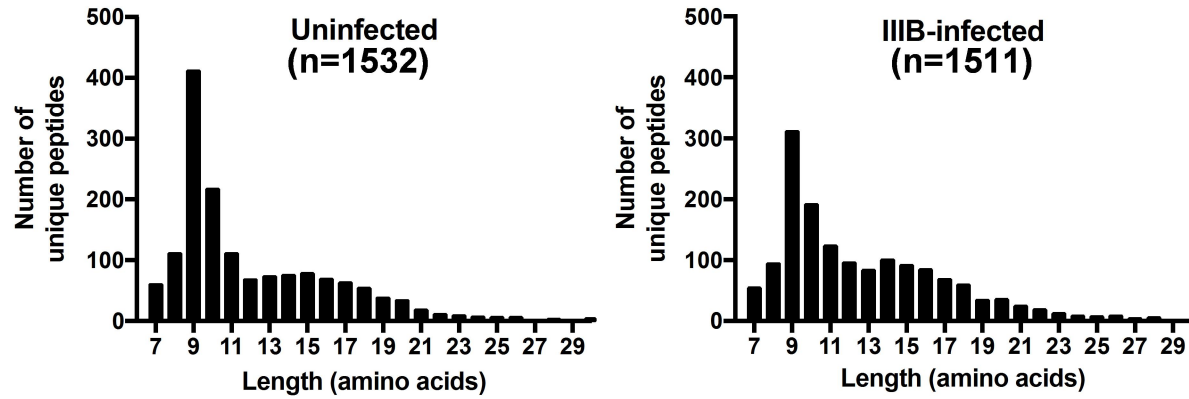
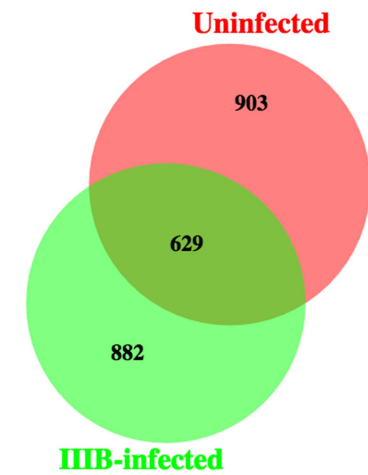
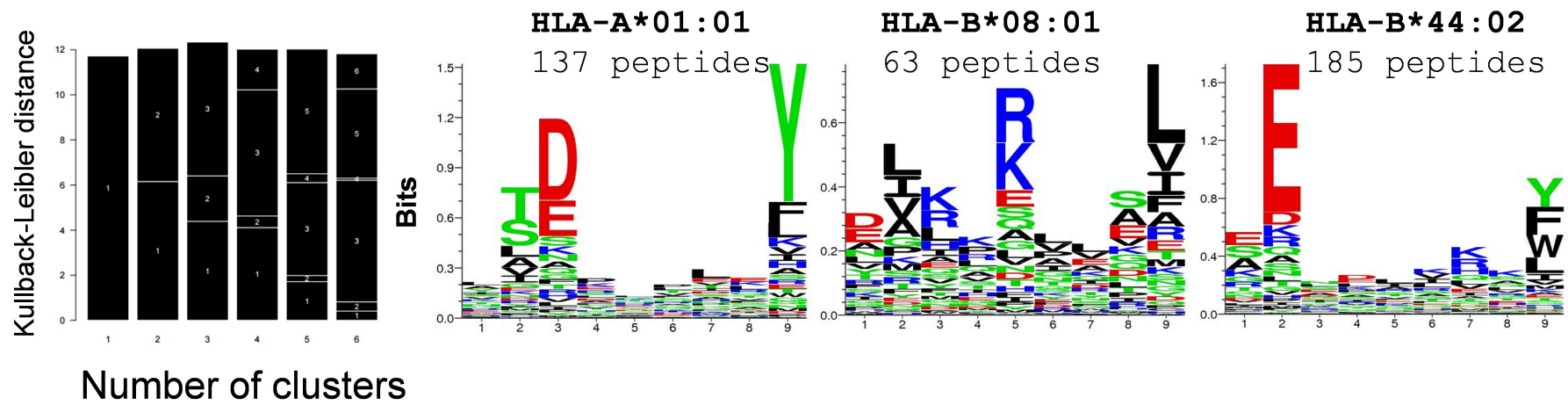
A**B****C**

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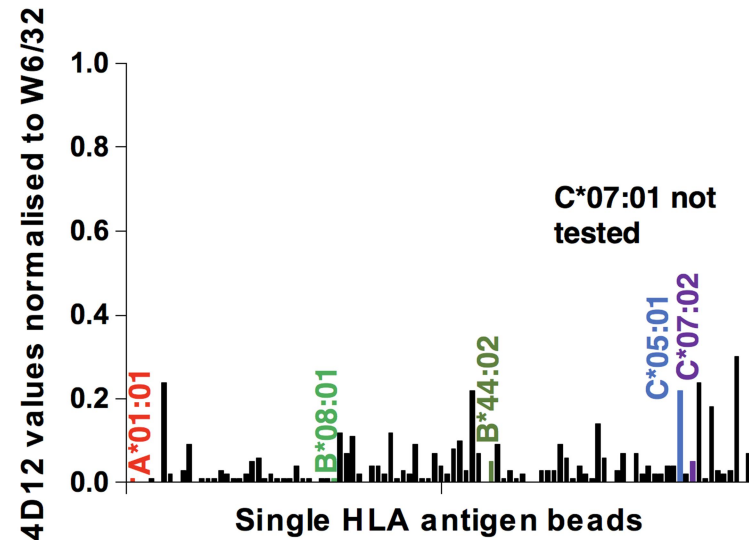
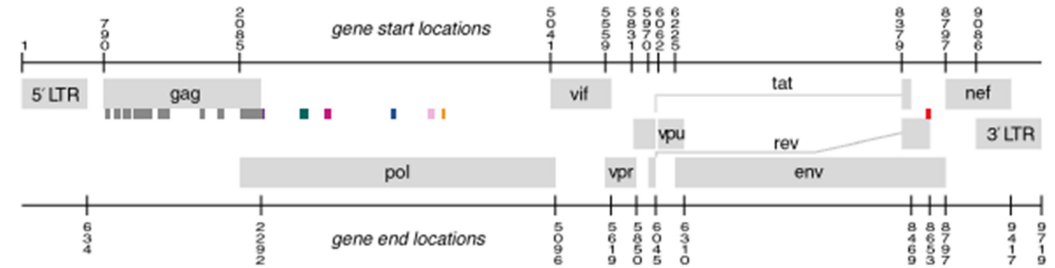
D**E**

Figure 4.12 *Peptide elution from HIV-1-infected C8166 cells using HLA-E-specific 4D12 immunoprecipitation.* 10^8 C8166 cells were infected with HIV-1 IIIB for 3 days until $>50\%$ cells were $p24^+$ or left uninfected then lysed and anti-HLA-E antibody 4D12 was used for immunoprecipitation and peptide elution. Isolated peptides were sequenced on an Orbitrap Fusion Lumos mass spectrometer. **A.** Size distribution of all unique peptides eluted from uninfected (left) or HIV-1 IIIB-infected (right) C8166 4D12 IP. **B.** Overlap in peptide identifications between uninfected and HIV-1 IIIB-infected C8166 4D12 IPs. **C.** All 9-mers identified in the uninfected 4D12 IP were grouped using the online Gibbs clustering server. 3 distinct clusters were formed and assigned to HLA-A*01:01, HLA-B*08:01 or HLA-B*44:02 upon comparison to known peptide binding motifs as detailed in the online MHC motif viewer⁸⁹⁹. 72 peptides were excluded by the clustering algorithm as outliers. In the sequence logos, polar amino acids are green, basic amino acids are blue, acidic amino acids are red and hydrophobic amino acids are black. **D.** 4D12 antibody was tested for cross-reactivity towards a panel of 100 single HLA molecules presented on LABScreen beads. Values shown were normalised by division by the value of W6/32 reactivity with each allele. Bars depicting alleles expressed by C8166 cells are highlighted and annotated with the name of the allele in the same colour. HLA-C*07:01 expressed by C8166 cells is not included in the set of HLA class I LabScreen beads. However, HLA-C*07:02, which differs by only 2 amino acids from HLA-C*07:01 is highlighted instead. **E.** Schematic of the HIV-1 genome showing location of HIV-1 peptides identified in the IIIB-infected C8166 4D12 IP. Each peptide (or overlapping/nested set) is shown as a block underneath/above the epitope's location within the protein. The graphic was produced by the QuickAlign tool in the HIV Molecular Immunology database at Los Alamos National Laboratories (http://www.hiv.lanl.gov/content/sequence/QUICK_ALIGNv2/QuickAlign.html).

4.2.4.2 An unconventional HLA-E binding motif is revealed by elution of peptides from HLA class Ia negative CD4.221 cells

As a negative control in a few experiments, I immunoprecipitated HLA class I from the HLA class Ia negative cell line expressing CD4 (CD4.221) infected with HIV-1 IIIB using W6/32 antibody. Similar to previous experiments using CD4.221 cells in section 4.2.2, relatively few peptides were detected (n=278), but in contrast to previous experiments where no enrichment for 9-mers was observed, a striking number of 9-mers (n=66) were detected after W6/32 IP (Figure 4.13A). Fewer than 20 peptides were seen for any other peptide length studied. The reason for this unexpected enrichment of 9-mers is unclear, but one explanation could be that the cells were passaged for longer in culture than in other experiments. Given that CD4.221 do not express classical HLA class I molecules but do express HLA-E⁶¹⁷, these 9-mers were examined for their potential HLA-E restriction. A sequence logo incorporating all 9-mers demonstrated a previously undescribed preference for proline at position 3 within the 9-mers (Figure 4.13B). Additionally, the canonical leucine anchor at the C-terminal residue was preferred. Interestingly, the most common residues at position 2 were alanine (19/66) and serine (12/66), whereas the canonical position 2 methionine was only present in 3 peptides (2 of which were redundant due to the inability to distinguish between leucine and isoleucine of VMAPRTLIL/VMAPRTLIL). When 9-mers were tested for their ability to bind HLA-E by NetMHC4.0 prediction, 24/66 unique peptides were found to have a % rank lower than 2% relative to a library of 400,000 random peptides, indicating that they were likely to bind to HLA-E (listed in Figure 4.13C). Intriguingly, despite reports suggesting that CD4.221 cells do not express classical HLA class I, the signal sequence of HLA-A*01:01 (VMAPRTLIL; here also denoted as VMAPRTLIL) was detected in the sample. The majority of the remaining predicted binders contained a proline residue at position 3 within the 9-mers. 3 HIV-1 peptides were detected, all of which were Gag-derived including one 9-mer (MQMLKETIN). The other 2 peptides were determined to be extended contaminant peptides as described in section 4.4.2. Thus, these data show that putative HLA-E-restricted epitopes can be identified by taking advantage of cell lines which express HLA-E in the absence of classical HLA class I expression.

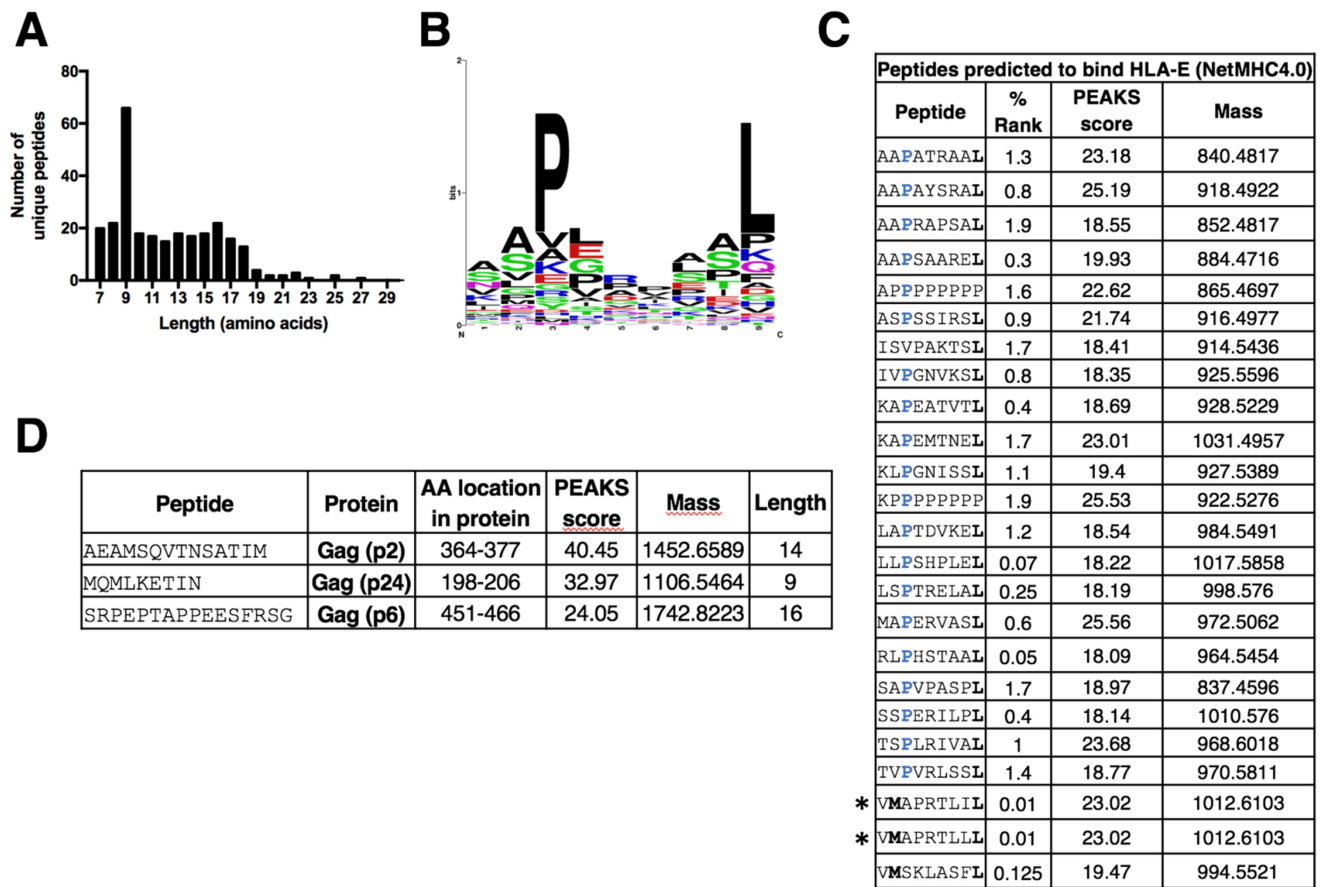


Figure 4.13 Peptide elution from HLA-E expressed by HIV-1-infected CD4.221 cells using W6/32 immunoprecipitation. 10^8 CD4.221 cells were infected with HIV-1 IIIB for 3 days until they were $>50\%$ p24⁺ as measured by intracellular p24 staining. Cells were lysed and HLA-E was immunoprecipitated using W6/32 antibody-cross-linked Sepharose beads. Data were acquired on an Orbitrap Fusion Lumos mass spectrometer. **A.** Size distributions of peptides eluted from W6/32 IP of HIV-1 IIIB-infected CD4.221 cells (n=278). **B.** Sequence logo depicting the 9-mers (n=66) detected in the CD4.221 W6/32 IP sample. Polar amino acids are green, basic amino acids are blue, acidic amino acids are red and hydrophobic amino acids are black. **C.** Table showing all 9-mer peptides predicted to bind to HLA-E (n=24) by the online NetMHC4.0 algorithm. The canonical signal sequence peptides derived from HLA class I are indicated with an asterisk (*). The canonical anchor residues at position 2 (methionine) and the C-terminal position 9 (leucine) are bolded where they are present within the peptide. Where a proline residue was present at position 3 within the peptide, it is bolded and coloured blue. **D.** Table depicting the HIV-1 peptides identified in the HIV-1 IIIB-infected CD4.221 W6/32 IP sample. The source HIV-1 protein, location within the protein as well as the PEAKS identification score, peptide mass length are shown in the table.

4.2.4.3 Stabilisation of HLA-E by peptides on the surface of TAP-deficient T2 cells

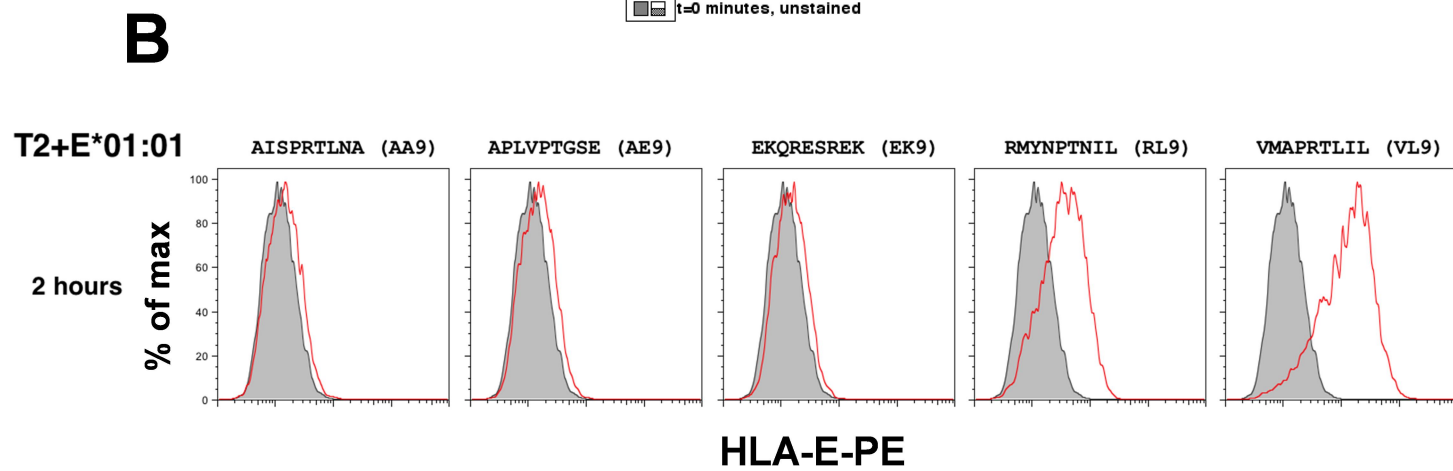
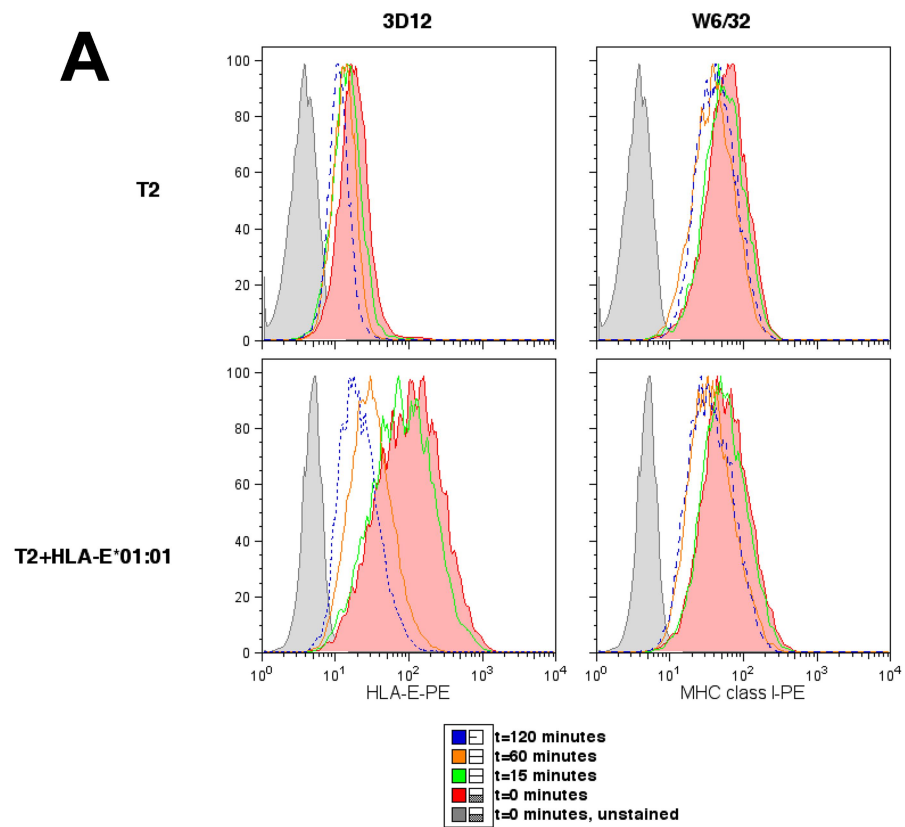
To determine whether HIV-1 peptides identified from 4D12 IP or CD4.221 W6/32 IP eluates were truly bound to HLA-E, an assay to assess stabilisation of HLA-E by synthetic peptides was implemented. The assay used T2 cells overexpressing recombinant HLA-E*01:01 (T2E1) or HLA-E*01:03 (T2E3), the two alleles of HLA-E that are expressed in genetically-diverse populations, which differ by just one arginine to glycine substitution at position 107 within the heavy chain sequence. Under normal conditions at 37°C, the TAP-deficient cells are unable to transport peptides into the endoplasmic reticulum for binding to and stabilisation of MHC molecules. Thus T2 cells display low levels of surface-expressed MHC at 37°C. However, at 26°C, empty MHC molecules (without peptide binding in the groove) become thermodynamically stable. This feature can be exploited to determine whether particular peptides bind to HLA molecules expressed in this cell line, by adding synthetic peptides to cell culture medium of T2 cells at 26°C, warming the cells to 37°C, and measuring cell-surface levels of stabilised MHC⁶¹⁸.

In silico modelling suggested that HLA-E is potentially more stable in the empty conformation relative to classical alleles such as HLA-A*02:01¹⁵¹. However, no experimental evidence for this proposal exists. To examine this hypothesis, parental T2 cells or T2E1 cells were cultured at 26°C overnight then placed at 37°C in the absence of peptides and HLA-E (detected using the HLA-E-specific antibody 3D12) or total HLA class I (detected using W6/32) surface expression was followed up to 2 hours thereafter by flow cytometry. Parental T2 cells initially stained weakly for HLA-E then expression was further reduced as time progressed (Figure 4.14A). Similar results were observed for W6/32 staining, although the fluorescence levels were higher. HLA-E staining was initially higher on T2E1 cells and large decreases in fluorescence intensity were observed by 15, 60 and 120 minutes of culture at 37°C. Similar results were observed for the T2E3 cells (data not shown). Although W6/32 staining seemed to be minimal by 1 hour post-37°C incubation whereas HLA-E staining continued to decline for at least 2 hours, the range of HLA-E staining levels was greater, so it was not possible to draw a firm conclusion from these results as to whether HLA-E remains comparably stable in the empty conformation relative to other HLA molecules.

Recently, rhesus macaques vaccinated with a live RhCMV vector expressing SIV Gag were shown to mount extraordinarily broad CD8⁺ T cell responses, one third of which targeted MHC-E-restricted Gag epitopes containing non-canonical anchor residues¹⁵¹. Given that macaque MHC-E has a very similar sequence to HLA-E⁶¹⁹ and may bind similar sets of peptides, the MHC-E-restricted Gag 9-mers identified by Hansen *et al*, and also the AA9 (AISPRTLNA) HIV-1 Gag peptide, which has previously been reported to bind HLA-E^{620,621} were synthesised and incorporated into the T2 HLA-E stabilisation assay. As expected, high concentrations of the canonical HLA class I leader sequence peptide VL9 stabilised HLA-E at 2 hours post-37°C incubation (Figure 4.14B). The SIV Gag RL9 epitope, which contains the canonical methionine and leucine anchor residues, also noticeably stabilised HLA-E. The AA9 (AISPRTLNA) HIV-1 Gag peptide only showed small shifts in fluorescence. Similarly, the SIV Gag AE9 and EK9 epitopes did not stabilise HLA-E to a great extent. The 8 remaining non-classical SIV Gag epitopes failed to show convincing stabilisation of HLA-E*01:01 at concentrations of 2-200 µm (Figure 4.14C). Similar results were observed for the HLA-E*01:03 allele (data not shown). When small positive shifts in fluorescence were observed, these did not seem to titrate out between the 2 and 200 µm concentrations. On the other hand, peptides previously reported to bind HLA-E from GroEL, Hsp60 and Epstein-Barr virus (EBV) BZLF-1 did prominently stabilise surface HLA-E at the high concentration. Stabilisation with these peptides was markedly reduced at the lower peptide concentration of 2 µm. Therefore, definitive characterisation of the SIV Gag epitopes as HLA-E binders cannot be confirmed with these data.

Multiple HIV-1 peptides were identified in the C8166 4D12 IP (section 4.2.4.1) and also by W6/32 IP from CD4.221 cells. Furthermore, nested sets of the CLIP peptide derived from the invariant chain (Ii/CD74) were also identified in both samples (data not shown). To determine whether any of these peptides could bind to and stabilise HLA-E, synthetic versions were prepared and incorporated into the T2 assay. Even at a high concentration of 100 µm, none of the 9-mers or extended nested sets of HIV-1 Gag peptides could demonstrate any evidence of stabilisation at the 3 hour timepoint (Figure 4.14D). Interestingly however, a 9-mer present within the CLIP peptide (CLIP₁₀₆₋₁₁₄; KMRMATPLL), which contains the canonical anchor residues, stabilised HLA-E similarly to the VL9 positive control.

Altogether, these results suggest that peptides containing canonical anchor residues retain the greatest ability to stabilise surface HLA-E expression on TAP-deficient cells. Furthermore, none of the HIV-1 peptides identified in previous elution experiments could be confirmed in this system. However these data do not preclude the possibility that the HIV-1 peptides bind HLA-E, as many of the SIV Gag peptides that failed to stabilise HLA-E in the T2 assay could be demonstrated to bind to MHC-E using alternative binding assays¹⁵¹.



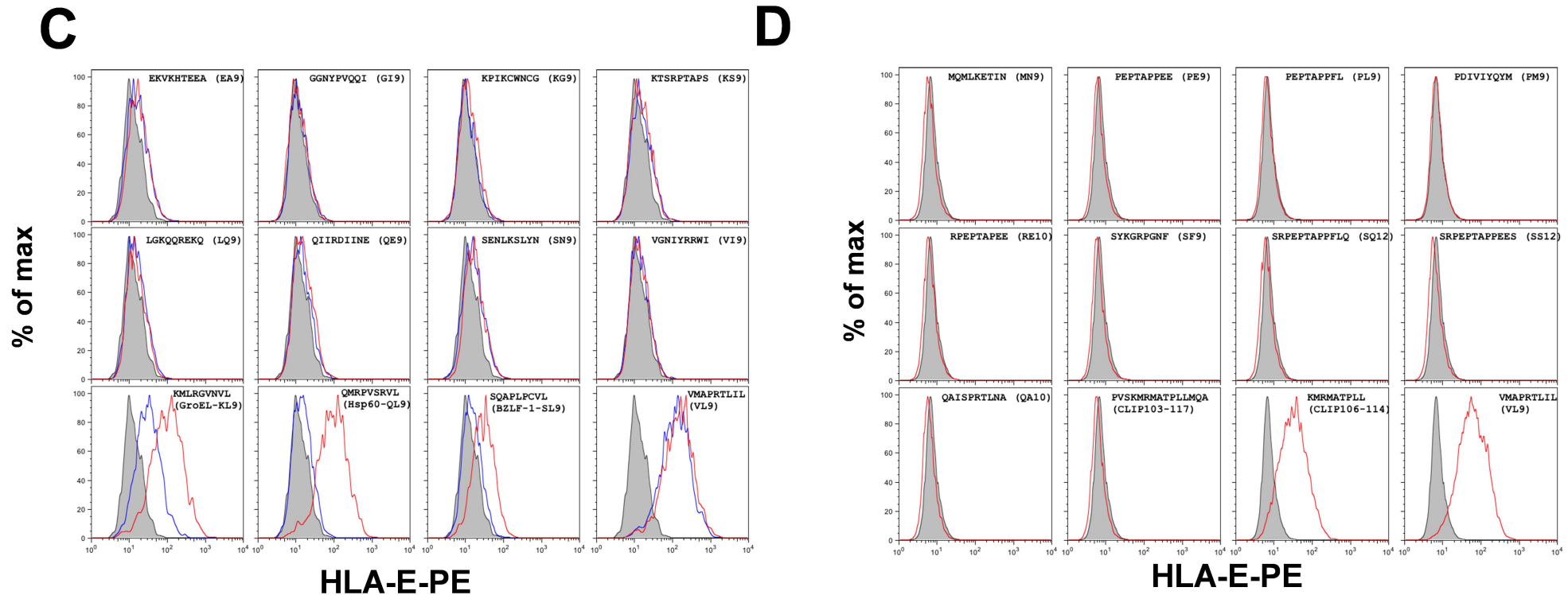


Figure 4.14 *HLA-E stabilisation by synthetic peptides on TAP-deficient T2 cells.* **A.** Parental T2 cells or T2E1 (overexpressing HLA-E*01:01) were cultured overnight at 26°C then placed back into 37°C for 0 (filled red lines), 15 (green line), 60 (orange lines) or 120 (dashed blue lines) minutes. Surface expression of HLA-E and total HLA class I were measured by PE-conjugated 3D12 and W6/32 antibody staining at each timepoint. Filled grey lines indicated the unstained control cells **B.** T2E1 cells were cultured overnight at 26°C then pulsed with the 200 μ M SIV Gag HLA-E restricted epitopes AE9 (APLVPTGSE), EK9 (EKQRESREK) and RL9 (RMYNPTNII), the known HLA-E-restricted HIV-1 epitope AA9 (AISPRTLNA), or the signal sequence VL9 (VMAPRTLII) peptide. Cells were then cultured at 37°C for 2 or 16 hours and HLA-E expression was determined for each condition. Filled grey lines indicate T2E1 cells cultured in equivalent amounts of DMSO without peptide whilst red lines indicate the test peptide. **C.** T2E1 cells were cultured at 26°C overnight then pulsed with 2 (blue line) or 200 μ M (red line) of the indicated peptides for 3 hours at 37°C. Cells were then washed and stained with 3D12 antibody. Filled grey lines indicate T2E1 cells cultured in equivalent amounts of DMSO without peptide whilst blue and red lines indicate the test peptide. **D.** T2E1 cells were cultured at 26°C overnight then pulsed with the indicated peptides at a concentration of 100 μ M for 3 hours, washed and stained with 3D12 antibody. Filled grey lines indicate T2E1 cells cultured in equivalent amounts of DMSO without peptide whilst red lines indicate the test peptide.

4.3 Discussion

In this chapter I established a qualitatively and quantitatively reproducible HLA IP protocol to elucidate the composition of the immunopeptidome of HIV-1-infected CD4⁺ T cells. I determined a set of peptides which elute non-specifically from IPs and highlighted evidence of the presence of non-specific HIV-1 peptide contaminants in HLA IP samples. Also identified were multiple previously-undescribed HLA class I-bound peptides derived from canonical HIV-1 proteins as well as from so-called ‘untranslated regions’ of the HIV-1 genome. Finally, a potentially novel HLA-E binding preference for peptides with proline at position 3 within the peptide was revealed by HLA-E IP from a HLA class Ia-negative cell line.

Methodology and reproducibility

In line with the results of other studies, a greater proportion of peptides eluted from the HLA IP were predicted to bind HLA class I than peptides isolated by MAE (Figure 4.2)^{622,623}. The main advantages of MAE are that peptides identified are eluted from the cell surface (although lysed cells within the overall population would constitute a source of contaminating intracellularly-derived peptides) and that peptides of varying affinities can be identified. On the other hand, HLA IP is expected to purify HLA class I complexes from all compartments of the cell (the exact contribution from each compartment is not well-defined, but up to 90% of IP’d HLA class I complexes may derive from the cell-surface, (personal communication, Anthony Purcell, University of Melbourne, AU)), and is biased towards high affinity ligands on the basis of stringent wash steps and lysis conditions^{623,624}. However, given that high affinity ligands induce the dissociation of tapasin and the PLC from HLA class I complexes²²⁷, most of the peptides isolated by HLA IP would likely be at, or on their way to the cell surface regardless. Furthermore, the HLA IP method is versatile in that different HLA-specific antibodies can be used sequentially to isolate distinct classes or alleles of HLA molecules. Another method which was not explored in this thesis is the isolation of recombinantly-expressed soluble peptide-HLA class I complexes secreted by transfected cells. Although a large amount of HLA class I complexes can be purified, and the repertoire of peptides identified using this technique has been shown to correspond to the peptides identified in membrane IPs of cancer cells⁶²⁵, there are two main drawbacks to this method. Firstly, overexpression of HLA molecules may alter quantitative

and qualitative characteristics of the immunopeptidome⁶²². Secondly, this technique is only practically available for use in cell lines which can be stably transfected with the HLA molecule of interest. Thus HLA IP was the method of choice for this thesis due to its high specificity, versatility and utility in primary cell samples.

The reproducibility of the HLA IP method was assessed in this chapter using biological and technical replicates of peptide samples eluted from C8166 cells. The ~70% and ~80% overlap in peptide identifications between biological and technical replicates (Figure 4.3) is comparable to that observed in other studies¹⁶⁵, and could possibly be further improved by using a more stringent 1% FDR rather than the 5% FDR used here. Taking advantage of the latest state-of-the-art Orbitrap Fusion Lumos mass spectrometer, >10,000 peptides were identified in some samples, making it one of the largest datasets arising from a single replicate to date. Mommen *et al* reported that the use of multiple peptide fragmentation methods such as electron-transfer/higher-energy collision dissociation (EtHCD) could significantly increase the number of HLA class I peptide identifications (>12,000) over HCD fragmentation alone⁶²⁶. In this thesis HCD fragmentation was used, but additional fragmentation methods could be used in future studies to delve deeper into the immunopeptidome. Additional pre-fractionation of peptides by RP-HPLC (e.g. 10 fractions instead of the 2 fractions used here) would also be expected to reduce sample complexity and thereby improve the number of peptide identifications. It is estimated that between 10,000-500,000 HLA class I molecules are expressed on a given cell (dependent on cell type)⁶²⁷. Theoretically, this is the maximum number of unique peptides which could be presented on a cell, however one study demonstrated that just two peptides could occupy half of the MHC molecules during a viral infection³³³, and it may now be possible to identify peptides present in just one copy per cell using targeted approaches (personal communication, Anthony Purcell, University of Melbourne, AU). This suggests that the immunopeptidomic method used here identifies a substantial fraction of the immunopeptidome, but lower abundance peptides are likely to be below the detection limit.

In most datasets studied in this chapter, Gibbs clustering of 9-mers was sufficient to separate distinct peptide groups conforming to HLA-A and HLA-B alleles expressed by the cells used. However, clusters representing HLA-C alleles were often not determined as observed in other mass

spectrometry-based immunopeptidomic studies of HLA class I alleles^{165,628}. *In silico* separation of HLA-A- and HLA-B-binding peptides may be facilitated by distinct and non-overlapping binding motifs in each allotype⁶²⁹. On the other hand, the overlap in binding motifs between HLA-C and HLA-A/B alleles is not known because many HLA-C binding motifs have not been elucidated. One study systematically revealed binding motifs for multiple HLA-C alleles, concluding that most alleles prefer a hydrophobic C-terminal anchor and consequentially present a much more limited repertoire of peptides than HLA-A and HLA-B⁶¹⁶. The lower expression levels¹⁴⁷ and more limited peptide repertoire of HLA-C alleles in addition to potential overlap with HLA-A/B alleles could explain the inability of Gibbs clustering to distinguish HLA-C binding motifs.

Interestingly, differences in the numbers of peptides conforming to motifs for each allele were observed after Gibbs clustering. For example, almost 3 times as many 9-mers clustered in the motif representing HLA-B*44:02 than were clustered in the HLA-A*01:01 and HLA-B*08:01 motifs, despite homozygosity for HLA-A*01:01 in the C8166 cell line (Figure 4.3). This difference could potentially be explained by differences in the size of the peptide repertoire bound by different alleles, as predicted by a computational study¹⁴⁵ and experimentally demonstrated for a limited set of 3 HLA class I alleles¹⁴⁶. However, the differences in number of peptides conforming to each motif in this thesis could be biased by different length preferences for each allele (only 9-mers were clustered here). For example, HLA-A*01:01 seems to have higher propensity to bind longer peptides and, unusually for HLA class I, binds an approximately equal number of 9-mers and 10-mers⁶³⁰. Furthermore, one study showed that alleles such as HLA-B*44:03 prefer to bind peptides of at least 9 amino acids in length, whereas HLA-B*18:01 almost exclusively binds 8-mers and 9-mers⁶³¹. It would be interesting to further characterise the size of the peptide repertoire of different HLA class I alleles. In fact, in collaboration with others in the group, I have recently begun to comparatively evaluate the peptide repertoire presented by HLA class I alleles associated with slow or fast HIV-1 disease progression¹⁰⁸ using CD4.221 cells stably transfected with individual HLA class Ia alleles that are infected with HIV-1 and analysed using an immunopeptidomics approach (unpublished data not included in this thesis).

Non-specific HIV-1/self-peptide background

Particular species of unusually long HIV-1 Gag peptides in HLA class I IP preparations have been identified in 3 separate prior studies that used different methodologies to identify novel CD8⁺ T cell targets in HIV-1-infected cells: HLA IP from whole cells⁵⁸¹, secreted HLA-A*11:01 IP⁵⁸² or MAE from cell surface⁵⁸³. These peptides cannot simply be untrimmed species bound to ER-associated HLA class I molecules, as they were also identified using the acid elution and secreted HLA methodologies. Although some long Gag peptides were recognised by HIV-1⁺ patient PBMC in ELISPOT assays in these studies, whether the IFN γ -producing cells were CD4⁺ or CD8⁺ was not explored. Additionally, no peptide fine mapping was performed to determine whether smaller optimal-length peptides within the long sequence could stimulate the same PBMC (many of the long peptides derive from highly immunogenic regions containing many 9-mer epitopes restricted to diverse HLA alleles; <http://www.hiv.lanl.gov/content/immunology/maps/ctl/Gag.html>). Furthermore, Yaciuk *et al* used an *in vitro* HLA-A*11:01 peptide binding assay to show that many of the long peptides identified in the 3 studies (e.g. AM14; AEAMSQVTNANTSIM, SF13; SRPEPTAPPEESF and PQ16; PLASLRSLFGSDPSSQ) showed minimal or no capacity to replace a fluorescent peptide standard on HLA-A*11:01. Given that the HLA IP method is biased towards peptides with high affinity for HLA class I^{623,624}, it is highly suspicious that such long peptides with low affinity for HLA-A*11:01 would also be present in HLA class I peptide samples from multiple donors of diverse HLA types. Many peptides are thought to bind two or more HLA class I molecules⁶³², but unusually long peptides binding promiscuously across many donor HLA class I types would be unprecedented. A recent study of HLA A*02:01 ligands in *Toxoplasma gondii*-infected cells showed that C-terminally-extended peptides were eluted (the HLA-A*02:01-binding motif was at the N-terminus), and a crystal structure demonstrated that a peptide extended by one lysine at the C-terminus could displace the Tyr84 residue at the end of the binding groove, thus making the groove ‘open-ended’⁵⁹⁶. Although most of the long peptides identified (just a 12-mer and a 16-mer bound with IC50 <500 nm) could not be confirmed to bind HLA-A*02:01 *in vitro* (speculated by the authors to require molecular chaperones), the aforementioned study merits further careful consideration of long HLA class I peptide candidates.

My data clearly show that long HIV-1 Gag peptides co-elute with membrane CD4 IPs prepared from lysates of HLA class Ia-negative CD4.221 cells (Figure 4.7), as well as from membrane CD3 IPs from HLA class Ia-expressing C8166 cells (Figure 4.6). These peptides could be HLA-E-restricted, thus explaining their presence in samples of diverse HLA types (as HLA-E is highly conserved across human populations). However, I could not show any significant HLA-E stabilisation by a subset of these HIV-1 peptides in a T2 HLA-E stabilisation assay (Figure 4.14) and 9-mers are the main peptide length experimentally reported to bind HLA-E⁶⁰⁸ or predicted to bind HLA-E by computational studies¹⁵¹. Alternatively, these peptides could be HLA class II-restricted (HLA class II would be present on activated CD4⁺ T cells and is expressed by CD4.221 cells). Supporting this hypothesis is the known promiscuity of peptides which bind to multiple HLA-DR molecules^{633–635} and the presence of nested sets of the long peptides in these studies, which is a well-described feature of HLA class II-bound peptides^{636,637}. However, in preliminary analyses of HLA-DR-bound peptides (using L243 antibody, data not shown) the nested sets of Gag peptides did not substantially overlap with the long HIV-1 Gag peptides observed in W6/32 IPs. Hence the peptides are unlikely to be HLA-DR-restricted, although it is conceivable that they are HLA-DP or HLA-DQ-restricted. A comprehensive study of each peptide's capacity to support *in vitro* refolding, stabilisation or peptide replacement on various HLA class I and HLA class II molecules would clarify these results.

Lastly, the presence of these longer Gag peptides could be explained by the inherent properties of Gag for membrane association and proteolytic processing. By virtue of its co-translational N-terminal myristoylation, Gag associates with membranes where it forms nascent virions incorporating *pol* gene products such as INT, RT and PR. The maturation process of the virion requires the protease (PR) to cleave the precursor Gag (pr55) sequentially into MA, CA, p2, NC, p1 and p6 fragments (Figure 4.15). The p2 (AEAMSQVTNVATIM) and p1 (FLGKIWPSYKGRPGNF) fragments generated by this process were amongst the long HIV-1 Gag peptides identified in the 3 immunopeptidomic studies of HIV-1-infected cells cited above. Thus it is conceivable that proteolytic products of Gag are associated with membranes, virions or other compartments within a HIV-1-infected cell and are co-purified by W6/32 IPs. The incorporation of HLA class I (and II) into virions⁴⁹⁵ supports this hypothesis, as IP of semi-intact virions or Gag-containing membranous vesicles then acid elution

would liberate Gag fragments from the membrane. Nested sets of these fragments may be a result of differential proteolytic processing or degradation during sample collection. It is possible that other membrane-associated proteins could co-elute with W6/32 IP preparations (unusually long Pol, Rev and self-peptides were also noted in this thesis), but this phenomenon may be most noticeable in the

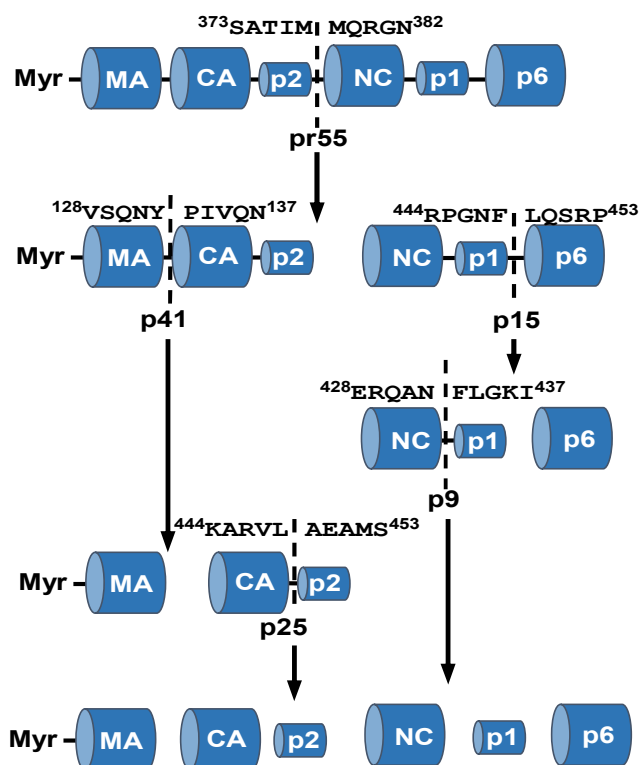


Figure 4.15 *Proteolytic processing of Gag antigen within maturing virions.* Precursors pr55 Gag is processed by the viral protease (PR) enzyme in a sequential fashion to yield MA (matrix), CA (capsid), p2, NC (nucleocapsid), p1 and p6 fragments within the virion. The sequences surrounding sites of proteolysis are shown. Myr = myristoylation site at the N-terminus. Diagram modified from⁷⁷⁹.

case of HIV-1 Gag as it is one of the most abundant proteins produced within infected cells (see Chapter 5). Therefore I conclude that the long HIV-1 Gag peptides are non-specific contaminants found in W6/32 IP preparations and result either from 1) binding to HLA class II molecules which co-purify with HLA class I immunoprecipitate or 2) membrane-associated Gag fragments co-purifying with W6/32 IP preparations. This result is of great significance, as the incorporation of these long HIV-1 Gag peptides into candidate vaccines on the notion that they would potentially elicit CD8⁺ T cell responses in all individuals could be detrimental to the overall immunogenicity of such a vaccine.

Another non-specific contamination issue highlighted by the CD3 membrane IP, was that peptide-HLA class I/II complexes can be co-precipitated in IPs using off-target antibodies. I found that HLA class I heavy chain was present in the immunoprecipitate of both CD3 and CD4 IPs from C8166 and CD4.221 cells (Figure 4.8). Furthermore, 9-mers were the most common peptide length detected in the CD3 IP, and these peptides could be clustered into groups with motifs reminiscent of motif preferences of alleles expressed by the C8166 cell line (Figure 4.5). Thus I speculate that the IP method used in this thesis generates ‘membrane fragments’ which contain other host/viral proteins in addition to the molecule of interest. In the future, alternative detergents, detergent concentrations and extra sonication steps could be explored in order to obtain a purer membrane-associated HLA preparation. This optimisation may have to strike a balance between purity and yield, as high concentrations of detergent may cause HLA class I complex dissociation (personal communication, Anthony Purcell, University of Melbourne, AU). This finding clearly has implications for epitope discovery, as 1) HLA class II molecules could potentially contaminate W6/32 IP preparations (as hypothesised in the previous paragraph) and 2) when purifying particular molecules using allele-specific antibodies, other HLA class I allele complexes could potentially contaminate IP preparations.

HLA-E-restricted HIV-1 epitope discovery

This cross-contamination was evident when I attempted to IP HLA-E using the 4D12 antibody. Although fewer peptides were identified in the 4D12 IP than in the W6/32 IP (the W6/32 IP biological replicate #2 dataset in Figure 4.3 was generated from the same C8166 cells as used in the 4D12 IP), the 9-mers identified were shown to cluster into motifs representative of HLA class Ia alleles expressed by C8166 cells (Figure 4.12). The presence of these peptides was likely not due to cross-reactivity of the 4D12 antibody for HLA class Ia alleles as demonstrated in Figure 4.12. Therefore the most likely scenario is that membrane fragments containing both HLA-E and HLA class Ia were co-IP’d. Most of the 9-mers identified in the 4D12 IP conformed to HLA class Ia motifs but HLA-E-bound peptides were probably also purified because the canonical VL9 (VMA^PRTLIL) peptide was observed in the sample. However, because the binding motif of HLA-E is not well-characterised (indeed, the MHC-E-restricted Gag epitopes identified by Hansen *et al* did not show

evidence of a common motif¹⁵¹), I was not able to clearly distinguish other HLA-E-bound peptides. HLA-E heavy chain was detected using a standard proteomics approach in the CD3 IP for C8166 cells, but not in the W6/32 IP (C8166 cells) or the CD4 IP from CD4.221 cells (Figure 4.8). The lack of detectable tryptic peptides corresponding to HLA-E probably results from its low expression¹⁴⁷, and the low depth proteomic approach used. Indeed, extensive pre-fractionation of peptides can allow a deep proteomic analysis where almost all of the proteins expressed in a given cell can be detected⁶³⁸. Furthermore, the sequence similarity of HLA-E to classical alleles is enough that certain peptides assigned to HLA class Ia alleles could also be derived from HLA-E. Because the expression levels of HLA-E are significantly lower than HLA class Ia molecules, the issue of HLA class Ia co-IP with HLA-E will persist in HLA class Ia-expressing cells unless 1) levels of HLA-E can be boosted, 2) HLA class Ia expression is reduced, 3) I optimise IP methods to reduce co-IP of other membrane proteins or 4) a clear HLA-E binding motif is defined so peptides can be unambiguously assigned as HLA-E-restricted.

The other approach that was taken in this chapter to identify HLA-E-restricted peptides was to IP HLA-E from the HLA class Ia-negative cell line CD4.221. Although initial experiments showed a remarkably clean background (110 peptides or less from $>10^8$ cells; Figure 4.4), another replicate revealed a striking enrichment for 9-mers, indicative of HLA class I-bound peptides (Figure 4.13). It should be noted that just 237 peptides were identified in the latter sample, which is still comparable to the former samples and contrasting with the $>10,000$ peptides identified in HLA class Ia-expressing cells using the same mass spectrometer. The reason for the discrepancy in proportion of 9-mers identified between these samples could be a result of cellular changes happening after extended times in culture. Interestingly, the HLA-A*01:01 signal peptide (VMA¹PTLLL) was identified amongst the enriched 9-mers. Clearly, HLA-E is the only functional HLA class I molecule present in the CD4.221 parental cell line and derivatives thereof (e.g. CD4.221)⁶¹⁷. However, to my knowledge, the MHC gene region has never been sequenced. Therefore it is possible that short fragments or heavily mutated sequences of HLA class Ia molecules are produced in the cell line under certain conditions (e.g. extended cell culture or stress) which could explain the identification of the VL9 peptide in W6/32 IP of CD4.221 cells.

Interestingly, analysis of the remaining 9-mers in W6/32 IP from CD4.221 cells revealed enrichment for prolines at position 3. Furthermore, one third of the eluted 9-mers were predicted to bind HLA-E by NetMHC, likely in part due to the C-terminal leucine found in these peptides. Some investigators have reported that HLA-E can bind a repertoire similar to that of HLA-A*02:01⁶⁰⁸, whilst others described a remarkably diverse set of SIV Gag peptides presented by Mamu-E, which is very similar to HLA-E¹⁵¹. Other studies suggested that unusually long peptides could be presented by HLA-E, but no negative controls were presented in these studies and many peptides originated from common non-specific contaminant proteins^{609,610}. There is a precedent for HLA-E binding peptides containing proline at position 3, as 4/18 of the *Mycobacterium tuberculosis*-derived HLA-E-restricted peptides which have been extensively studied contain a proline at position 3⁶³⁹. In future studies, overexpression of HLA-E within the CD4.221 cell line would be expected to increase the number of peptide identifications in order to provide a more complete snapshot of the HLA-E peptide repertoire. Furthermore, the use of MAE to elute surface-expressed HLA-E-bound peptides from CD4.221 (with appropriate negative controls to rule out HLA class II-bound peptides) would possibly result in more identifications, as the non-canonical MHC-E-bound peptides reported by Hansen *et al* seem to have a relatively low affinity for HLA-E¹⁵¹.

Speculatively, the altered HLA-E peptide binding motif observed in this chapter could result from VL9 peptide loading of nascent HLA-E allowing its transfer to another cellular compartment, in a manner similar to that observed for HLA class Ia alleles associating with CD74 for the process of cross-presentation²⁶⁸. In fact the CLIP peptide (or invariant chain; Ii) derivative of CD74, which typically associates with HLA class II for transfer to endosomes¹⁵³, was found in both W6/32 and 4D12 IPs. Ii has been found associated with various MHC class I molecules over the years^{640–647}, and the CLIP peptide fragment MRMATPLLM has even been eluted from HLA class I molecules^{648,649}. Furthermore, fusion of vaccine antigens to Ii has been reported to significantly enhance CD8⁺ T cell responses to a variety of antigens^{650–653}. I showed that a 9-mer within the CLIP peptide (**KMRMATPLL**), like other peptides which contain the methionine at position 2 and leucine at position 9, could substantially stabilise HLA-E on the surface of TAP-deficient cells (Figure 4.14). However, this short fragment was not specifically detected in HLA IP samples and the full length CLIP variant

did not noticeably stabilise HLA-E in the timeframe studied. It would be interesting to determine whether CD74/Ii co-localises with HLA-E and to study the intracellular trafficking of HLA-E in the absence of CD74.

An alternative explanation for the unusual preference for proline at position 3 is that these peptides may actually be HLA-F or HLA-G-restricted. Although HLA-F is expressed by 721.221 cells⁶⁵⁴, it is an unlikely candidate as HLA-F is predominantly found in an open conformer form and no peptide-bound forms have ever been described^{655,656}. On the other hand, HLA-G is known to be recognised by W6/32 and displays a preference for proline at position 3 and leucine at position 9 within the limited peptide repertoire described to date⁶⁵⁷⁻⁶⁵⁹. The 721.221 cell line was originally described as HLA-G negative⁶⁵⁴ but perhaps extended culture or alteration by transfection with CD4 has induced variable HLA-G expression in CD4.221 cells. In the future it will be imperative to determine levels of HLA-G expression in CD4.221 cells to determine their contribution to the peptides eluted from W6/32 IPs.

Lastly, I attempted to verify the HLA-E restriction of a subset of the HIV-1 peptides identified in the 4D12 IP by incorporating them into the T2 HLA-E stabilisation assay. None of these synthetic peptides were able to significantly stabilise HLA-E (Figure 4.14). Furthermore, all but one (**RMYNPTNILL**) of the recently reported SIV Gag peptides which bind Mamu-E/HLA-E¹⁵¹ also did not noticeably stabilise HLA-E in this assay (whereas all other peptides containing methionine at position 2 and leucine at position 9 showed noticeable stabilisation). It is likely that this flow cytometric readout of HLA-E stabilisation is not sensitive enough to detect small changes in surface expression by low affinity peptides. In contrast, the *in vitro* HLA-E refolding assay and the single chain HLA-E heavy chain/ β 2m/peptide expression assays used by Hansen *et al* were capable of detecting low affinity peptide binding to HLA-E, as they used either a very high concentration of peptide in artificial conditions, or used a flexible linker region in the trimer to keep the peptide in the vicinity of the binding groove. The study by Hansen *et al* also used computational modelling to suggest that HLA-E is more stable than HLA-A*02:01 in their empty states. To test this I compared the downregulation over time of HLA-E and total HLA class I on HLA-E-expressing T2 cells and parental T2 cells, respectively. Although HLA-E may not have been completely removed from the

surface by 2 hours post incubation at 37°C, no firm conclusions could be drawn from this experiment because the dynamic range of downregulation of surface HLA class I on T2 cells was relatively small. Future experiments could examine the surface expression of individual alleles such as HLA-A*02:01 with specific antibodies such as BB7.2 in T2 cells in order to provide a more direct comparison between HLA class Ia and Ib alleles.

Novel HLA class Ia-restricted targets for CD8⁺ T cells

The immunopeptidomics approach in this chapter identified numerous putative novel HLA class Ia-restricted epitopes. For example, the putative Pol VL9 (VSAGIRKIL) epitope has not been experimentally reported previously. One reason these novel epitopes may not have been reported previously is that the sequence of lab-adapted clade B HIV-1 IIIB differs from that of natural HIV-1 clade B strains infecting patients. For example, the HLA-B*44:02-restricted EW9 (EEM**N**LPGRW) epitope seems to be more commonly detected than the variant described in this thesis (EEM**S**LPGRW). Furthermore, CTL responses towards these novel epitopes could be subdominant/low in magnitude (or the epitope may not induce a response at all) explaining why responses to these epitopes were not detected using the most common epitope mapping techniques (e.g. ELISPOT) which are biased by natural CTL immunodominance hierarchies. Identification of these novel epitopes is important as sustained or enhanced responses against typically subdominant epitopes in mice infected with LCMV, MCMV or *Trypanosoma cruzi* parasites has been associated with pathogen clearance⁶⁶⁰⁻⁶⁶². Furthermore, responses towards epitopes associated with relative viral control during HIV-1 infection are often subdominant⁶⁶³. Hence identification of novel HIV-1 epitopes through this immunopeptidomics approach for testing of their immunogenicity is an important step in the development of candidate prophylactic/therapeutic vaccines.

My data also suggest the existence of epitopes derived from the HIV-1 5' leader sequence (also known as the 5'UTR) presented on HLA class I by infected cells (Figure 4.10). CTL targeting of cryptic epitopes derived from ARFs within the coding region of the HIV-1 genome are well-described^{174,176,597,598}, but researchers have typically ignored the flanking 'untranslated' regions. Independently of this thesis, others have recently demonstrated rapid selection for mutants in the 5' leader region of longitudinal viral sequences in the plasma of SIV-infected macaques and HIV-1-

infected individuals during early infection (unpublished data; Edward Kreider & George Shaw, University of Pennsylvania, US). Moreover, ribosomal scanning revealed non-canonical translation initiation sites within the 5'leader sequences of SIV/HIV-1 RNA species (unpublished data; Ranjit Warrier, George Shaw, UPenn, US). Furthermore, infected macaques and HIV-1⁺ patients mount detectable CD8⁺ T cell responses against 5'leader-derived peptides, recognition of which is abrogated by selected escape mutants (unpublished data; Katja Pfafferoth, Katherine Roberts, Persephone Borrow, University of Oxford, UK). Therefore, these 5'leader peptides represent a new form of DRiPs (as they are likely to be degraded quickly following translation) which could be rapidly presented on infected cells. The two 5'leader peptides identified in this chapter (LSLGALWL and LSLGALWLAREPTA) are predicted to bind HLA-A*02:01 because they contain hydrophobic leucine anchor residues. This would imply that the 14-mer peptide is extended at the C-terminus, which has been recently reported for HLA class I ligands⁵⁹⁶. Furthermore, I performed another W6/32 IP of HLA-A*02:01⁺ cells from a different donor which had been infected *in vitro* with HIV-1 IIIB. After analysis of this sample by the data-independent acquisition (DIA) mass spectrometry method 'SWATH', spectral evidence was found for the presence of peptides DLSLGALWL and LSLGALWL (data not shown). The DIA method provides high-depth coverage of peptides within a complex sample because it fragments all peptides within distinct mass windows (SWATHs)⁶⁶⁴. This technique has gained popularity recently and has been used to measure viral protein expression kinetics⁶⁶⁵ as well as for identification of HLA ligands by mass spectrometry⁶⁶⁶. The software to analyse this technically-challenging data is still undergoing development, but it would be interesting to use DIA analysis in the future for analysis of low-abundance HLA class I peptides.

Targeting of these 5'leader-derived epitopes could be beneficial for two reasons. Firstly, the 5'leader sequence is present at the start of every HIV-1 RNA transcript, thus peptides arising from non-canonical 5'leader translation could be some of the first and most abundant epitopes presented by infected cells (although incoming virion proteins could be presented more or equally rapidly). CD8⁺ T cells recognising 5'leader-derived epitopes may thus be able to eliminate target cells very early after they become infected, enabling potent antiviral efficacy. Secondly, the 5'leader sequence is the most conserved region within the HIV-1 genome at the nucleotide level (hiv.lanl.gov) because it

contains important structural and regulatory sites involved in virus replication⁶⁶⁷⁻⁶⁶⁹. As discussed earlier, T cell responses targeting epitopes in functionally-constrained regions of the viral genome are difficult for the virus to escape without high costs to fitness, so are associated with good HIV-1 control⁶⁷⁰.

Peptides derived from other ‘untranslated’ regions within the human genome have been shown to comprise up to 10% of the HLA class I immunopeptidome¹⁷². Preliminary analysis of my W6/32 IP data in the context of a database containing short ORFs (sORFs)⁶⁷¹ revealed numerous potential HLA class I ligands (8-11 amino acids in length) from sORFs within 5’UTRs in the human genome, some of which were only identified in HIV-1-infected samples (data not shown). Speculatively, CTL could potentially recognise infected cells through increased presentation of these self-peptides. It would be interesting to perform transcriptomic/ribosomal scanning analysis in parallel with immunopeptidomic analysis of HIV-1-infected cells in order to generate a cell-specific database of potential sORFs and characterise a set of HIV-1-induced self-peptides then screen for CD8⁺ T cell responses to these peptides in HIV-1⁺ patients.

Also, I have not explored HLA class I peptide post-translational modifications (PTMs) in this thesis. For example, phosphorylated HLA class I ligands (where the phosphate faces away from the groove and can interact with the TCR surface) have been reported⁶⁷²⁻⁶⁷⁴. In early HIV-1 infection, the tryptophan catabolism pathway is enhanced⁶⁷⁵⁻⁶⁷⁷, hence kynurenine (metabolised tryptophan) could form a component of peptides presented by HLA class I, thus altering TCR recognition. In the future it would be interesting to enrich peptides containing various PTMs to determine their contribution to the HIV-1 immunopeptidome.

Nef epitopes are rarely detected on HIV-1-infected CD4⁺ T cells

Lastly, I clearly showed in this chapter that only a minority of epitopes were Nef-derived. In contrast, Gag, Pol and Env-derived epitopes were far more commonly detected on infected CD4⁺ T cells. These data contrast with the description of Nef (along with Gag) as the most CTL epitope-dense proteins within the HIV-1 proteome (hiv.lanl.gov)⁶⁷⁸. This finding could potentially result from the use of lab-adapted HIV-1 IIIB and NL4.3^{582,583}, which are known for high *in vitro* replicative capacity. The differences between lab-adapted and primary HIV-1 isolates which would impact upon

Nef epitope presentation are not clear, but a careful examination of the levels of Nef expression by each isolate would potentially provide useful information.

These data also do not support a hypothesis in which Nef-specific CTL responses are expanded during AHI because HIV-1-infected cells preferentially present Nef epitopes. It is possible that more Nef epitopes may have been detected if the HLA class I had been immunoprecipitated at an earlier timepoint post-infection (as Nef is produced early in the infection lifecycle). However, the cells used in my experiments were not synchronously infected and therefore represent a mixture of cells at various stages of infection. To determine whether Nef epitopes are presented during the early stages of infection lifecycle, I could use magnetic CD44 microbeads to synchronously infect CD4⁺ T cells⁶⁷⁹ and examine peptide presentation over time with immunopeptidomics or using T cell clones specific for different viral proteins. In the general discussion (Chapter 7), I will discuss the implications of this finding in the context of the hypothesis of this thesis and the results of other chapters.

Chapter 5: Characterisation of extracellular vesicles secreted by HIV-1-infected cells

5.1 Introduction

In this thesis I hypothesise that in the primary HIV-1-specific CD8⁺ T cell response towards recognition of epitopes derived from Nef (and also Gag) may be due to preferential cross-presentation of epitopes derived from these proteins by DCs, leading to early priming of Nef-(and Gag)-specific responses. In chapter 4 I identified numerous epitopes derived from Gag, (and Pol and Env) proteins, but just 2-3 Nef-derived epitopes overall were observed presented by HIV-1-infected CD4⁺ T cells, likely due to the abundance of Gag (but not Nef) in infected cells. While infected CD4⁺ T cells constitute one source of antigen for cross-presentation by DCs, DCs could potentially also acquire antigen by capturing EVs or virions released from infected cells. Hence, in this chapter I focused on characterisation of EVs (and virions) secreted by HIV-1-infected cells.

EVs are small membrane-bound particles secreted by all cells. EVs are categorised as either microvesicles or exosomes depending upon their intracellular origin. Microvesicles are relatively large vesicles (0.2-1 μ m in diameter) budded directly from the plasma membrane. Exosomes are generally <200 nm in diameter, bud into multivesicular bodies (MVB) and are released into the extracellular space as MVB fuse with the plasma membrane⁴⁴²(see Figure 1.9 in the introduction). Initially thought to act as a cell's 'waste disposal' mechanism, EVs are now known to perform a wide variety of functions in intercellular signalling and regulation of immunity⁶⁸⁰.

Since the discovery that EVs such as exosomes can directly activate T cells⁶⁸¹ and transport pathogen or cancer antigens between cells²⁷⁸, there has been a great deal of interest from immunologists in dissecting the immunomodulatory properties of EVs⁶⁸⁰. In fact, numerous strategies employing DC-derived immunosuppressive or immunostimulatory EVs have been proposed in the settings of cancer, autoimmunity, transplantation and vaccination⁶⁸⁰. The existence of immunomodulatory EVs seems to be a common theme across species, as even intracellular/extracellular parasites such as Leishmania, malaria, trypanosomes, fungi and helminths release EVs to alter the immune microenvironment⁶⁸².

The protein, RNA, lipid and metabolite cargo enveloped by EV reveals their cellular origin and impacts the downstream effects on recipient cells. Dependent on the cell type, exosomes are enriched

in tetraspanins (e.g. CD9, CD63, CD81), proteins involved in vesicle biogenesis (e.g. Alix and Tsg101), signal transduction proteins, heat shock proteins and immunoregulatory molecules such as MHC class I/II. Non-coding microRNAs (miRNAs) are also transported between cells by exosomes and can repress gene expression in the recipient cell⁶⁸³. Small molecules and metabolites may also be packaged into EVs. Recently, the intracellular second messenger cyclic GMP-AMP (cGAMP) was found to be incorporated into exosomes and HIV-1 virions^{684,685}. cGAMP, which is synthesised by cyclic GMP-AMP synthase (cGAS) upon encounter with cytosolic DNA during viral infections, activates stimulator of interferon genes (STING) resulting in a downstream antiviral response. The packaging of cGAMP into EVs therefore represents a novel mechanism by which danger signals can be communicated between cells. Host protein cargo such as APOBEC3G⁶⁸⁶ and other undefined factors in exosomes from plasma, semen or breast milk have also been shown to inhibit HIV-1 infection⁶⁸⁷.

On the other hand, viruses such as HIV-1 may also appropriate the EV system to enhance infection. In fact, based on the observed biochemical and physical similarities between HIV-1 virions and exosomes, Gould *et al* proposed the ‘Trojan exosome hypothesis’, which states that retroviruses evolved to exploit cellular pathways of intercellular vesicle trafficking⁶⁸⁸. Although this hypothesis has been disputed⁶⁸⁹ because most evidence suggests that HIV-1 virion budding occurs at the plasma membrane in T cells, budding at the plasma membrane or the endosome (particularly in macrophages) both seem to require ESCRT proteins involved in exosome biogenesis^{690–693}. Other viruses also use the EV system to their advantage. HSV-infected cells secrete microvesicles containing envelope glycoproteins^{694–696} which are thought to sensitise cells for enhanced viral replication⁶⁹⁷. EBV-infected cells secrete immunosuppressive exosomes containing viral oncoprotein LMP-1 and galectin-9^{698–700}. Exosomes containing viral proteins are also detected in sera of patients with Hepatitis B Virus (HBV) and Hepatitis C virus (HCV) infection, and are hypothesised to hide HCV particles or act as a distraction for the immune system^{701,702}. Hence, characterisation of the EVs secreted by HIV-1-infected cells in an inflammatory microenvironment could be important in determining mechanisms of viral persistence and pathogenesis.

Transfer of proteins such as the HIV-1 co-receptors CXCR4 and CCR5 by microvesicles has been shown to render recipient cells susceptible to infection^{703,704}. Incorporation of increased levels of CD45, CD86 and HLA class II in microvesicles originating from HIV-1-infected cells appeared in one study to enhance cell death upon exposure⁴⁹⁵. HIV-1-derived TAR RNA, TAR miRNAs and unspliced genomic RNA were also shown to be packaged into exosomes secreted by infected cells^{705–707}. TAR RNA-containing exosomes increased inflammatory cytokine production and susceptibility to infection in HIV-naïve recipient cells. ADAM17 packaged into exosomes was recently identified as a key mediator of cellular activation and increased susceptibility to HIV-1 infection of recipient cells^{708,709}. ADAM17 is a metalloprotease responsible for cleaving the membrane-associated pro-TNF α molecule into its active form^{710,711} and was therefore hypothesised to contribute to the inflammatory environment observed in HIV-1 infection. Indeed, a recent study demonstrated that ADAM17 activity in plasma exosomes correlated with low CD4⁺ T cell counts during chronic HIV-1 infection⁷¹². Interestingly, this study also showed that the HIV-1 Nef protein is abundant alongside ADAM17 within plasma-derived exosomes in both viraemic and non-viraemic chronic infection.

A sharp increase in secretory activity in cells has been observed in cells expressing Nef⁴⁵². Furthermore, Nef itself is packaged into exosomes secreted by HIV-1-infected or *nef*-transfected cells^{450–453}. Nef induces its own secretion in EVs through critical domains located in the N-terminal portion of the protein⁷¹³. A cluster of arginine residues at amino acids 17, 19, 21 and 22, the phosphofurin acidic cluster sequence (PACS; ⁶²EEEE⁶⁵), and in particular the so-called ‘secretion modification region’ (SMR; located at residue positions ⁶⁶VGFPV⁷⁰) were shown to be critical in Nef’s propensity to be secreted. Although alanine substitution in the SMR did not significantly abrogate viral replication in human PBMC, apoptosis and inflammatory cytokine production was dulled in PBMC and humanised mice infected with the mutated SMR strain, indicating the important role of Nef secretion in modulating key aspects of HIV-1-induced disease⁴⁶⁶.

Nef exosomes have been reported to induce apoptosis of CD4⁺ T cells *in vitro* and investigators have therefore speculated on the possibility that Nef exosomes contribute to CD4⁺ depletion *in vivo*^{451,467}. Conversely, exosomal HIV-1 TAR RNA was shown to downregulate apoptosis in recipient cells⁷⁰⁶. Additionally, Nef promoted HIV-1 infection of bystander cells by reducing the levels of CD4 on the

surface of exosomes which can otherwise hinder interaction with target cells by binding enveloped virions⁷¹⁴. Nef expression also substantially altered the miRNA profile of exosomes secreted by Nef-transfected U937 monocytic cells or HIV-1-infected macrophages^{468,715,716}. A burgeoning role for Nef exosomes in HIV-associated neurocognitive impairment has also been suggested, as Nef transfer to neuroblastoma cells subsequently increased amyloid beta secretion⁷¹⁷ and disrupted an *in vitro* model of the blood brain barrier⁷¹⁸. Other consequences of extracellular Nef uptake by cells include broad changes in cell signalling molecules and transcription factors (e.g. NFκb, IRF3, STAT2)^{458,459}, downregulation of CD36⁴⁵⁶, CD4⁴⁵⁵, HLA class I and mannose receptor in macrophages and DC, upregulation of CD1a, HLA-DR and CD80⁴⁶⁰, production of inflammatory cytokines or inhibition of production of IgG, IgA and IgE from B cells⁴⁶³. These vast alterations caused by extracellular Nef in the immune environment therefore seem to have far-reaching consequences for the pathology observed during HIV-1 infection. However, the role that exosomal Nef plays in antigen presentation by DC and CD8⁺ T cell responses remains unexplored.

Although exosomal Nef is present in plasma of HIV-1-infected patients at very high levels of approximately 10 ng/ml^{453,454}, the *in vivo* source of these Nef exosomes has not yet been explored in great detail. Deciphering their cellular origin would garner important information about Nef-induced immunopathogenesis and generate targets for HIV-1 therapy or vaccination. Most studies of Nef exosomes have focused on the main cellular target of HIV-1, CD4⁺ T cells. However, a recent study suggested that Nef exosomes may originate from a myeloid source as evidenced by an enrichment of inflammatory molecules such as IL-12p40, TNFα, RANTES and GM-CSF in plasma exosomes of HIV-1⁺ patients⁷¹².

As the field of EV research continues to grow, more methods of vesicle isolation are being developed. The gold standard method for isolation and characterisation of exosomes is differential ultracentrifugation of diluted body fluids or cell culture supernatants depleted of bovine vesicles⁷¹⁹. Further separation of EVs from non-membranous aggregates is achieved by density gradient ultracentrifugation or immunoprecipitation using antibodies against the vesicular protein of interest. Other methods being developed include microfiltration, microfluidic devices and precipitation

technologies such as ExoQuick⁷²⁰. Although these methods may be useful in some settings, the yield or purity of EV isolated may be reduced for these protocols⁷²¹.

In this chapter I aimed to:

- Validate a differential ultracentrifugation method for isolation of microvesicles and exosomes
- Validate a method to separate exosomes from virions
- Investigate the characteristics of EV present in patient plasma or secreted by HIV-1-infected cells in order to explore the potential *in vivo* source of Nef exosomes.

5.2 Results

5.2.1 Validation of a differential ultracentrifugation method for isolation of exosomes

Although multiple protocols have been described for the isolation of exosomes from supernatants and tissues, differential ultracentrifugation remains the gold standard technique. A differential ultracentrifugation protocol was implemented and validated in order to isolate and characterise vesicles secreted by HIV-1-infected cells. Jurkat (a CD4⁺ T cell line), U937 (a lymphoblast line with monocytic characteristics) and primary CD4⁺ T cells were cultured in exosome-free R10 for 24 hours. Microvesicles and exosomes were isolated by differential ultracentrifugation at 20,000 x g and 100,000 x g, respectively. NTA demonstrated that the 100,000 x g pellet (exosomes) from CD4⁺ T cells, Jurkat and U937 cells respectively consisted of particles with a modal diameter of 126, 112 and 116 nm (Figure 5.1A). In contrast, the microvesicle pellet was comprised of heterogeneous populations of vesicles with diameters up to ~1000 nm in addition. No particles were detected in unconditioned exosome-free R10 or PBS (data not shown). The ratio of numbers of exosomes/microvesicles secreted by Jurkat T cells, primary CD4⁺ T cells and U937 cells, was 10.95, 2.21 and 0.39 respectively, indicating cell type-specific differences in vesicle subtype secretion.

Alix and syntenin-1 are proteins reported to be enriched in exosomes whereas the ER marker calnexin is thought to be excluded from exosomes^{442,722}. EVs were isolated from the MT-2 CD4⁺ T cell line and HEK293T cells (embryonic kidney cells commonly used for lentiviral production) cultured in exosome-free R10 or D10 for 24 hours, respectively. Exosomes from HEK293T cells were enriched in Alix and syntenin-1 protein (Figure 5.1B). Exosomal syntenin-1 was detected at a higher molecular weight than cell-associated syntenin-1. Interestingly, exosomes from MT-2 cells were not significantly enriched in syntenin-1 and Alix protein was barely detectable, possibly indicating cell type-specific differences in exosome composition. As expected, calnexin was most enriched in cell lysates, cell debris and microvesicle samples whereas relatively little calnexin was detected in exosome lysates.

Extracellular vesicles are too small for analysis using standard flow cytometer setups. Therefore EV isolated from the supernatant of PM1 cells (a CD4⁺ T cell line) cultured in exosome-free R10 were

passively adhered to latex beads and EV-bead complexes were stained with antibodies specific for the tetraspanin molecules CD9, CD63 and CD81. A small proportion of the 2000 x g pellet stained weakly positive for CD81, whereas microvesicles and exosomes both stained strongly positive for CD81 (Figure 5.1C). CD63 expression was also observed on the surface of microvesicles and exosomes. In contrast, CD9 expression was not detected on PM1-derived EV.

To determine whether the isolated vesicles were able to interact with DCs, exosomes were isolated from the supernatant of Jurkat cells then stained with the membrane dye, Bodipy-FL maleimide and washed to remove free dye. Stained vesicles were incubated with MDDCs differentiated in the presence of IFN α and GM-CSF (IFN-DC). At 1, 2, 4 or 20 hours of incubation, the fluorescence of IFN-DCs was measured by flow cytometry. When IFN-DCs were incubated with a media control (exosome-free R10 cultured under same conditions without cells was ultracentrifuged then the 'pellet' was stained with Bodipy-FL and washed similarly to exosomes) without exosomes, cells were only weakly positive for Bodipy-FL (Figure 5.1D). IFN-DCs incubated with stained exosomes for 1 hour at 4°C showed intermediate fluorescence, indicating that exosomes were adhered to IFN-DC plasma membrane. When IFN-DCs were incubated with stained exosomes for 1 hour or more at 37°C, fluorescence was increased relative to the 4°C control (and was maximal at 2 hours after exosome addition). Together, these data indicate that a differential ultracentrifugation protocol is able to isolate vesicles with size and protein cargo characteristic of exosomes and which interact with antigen-scavenging cells such as monocyte-derived DC.

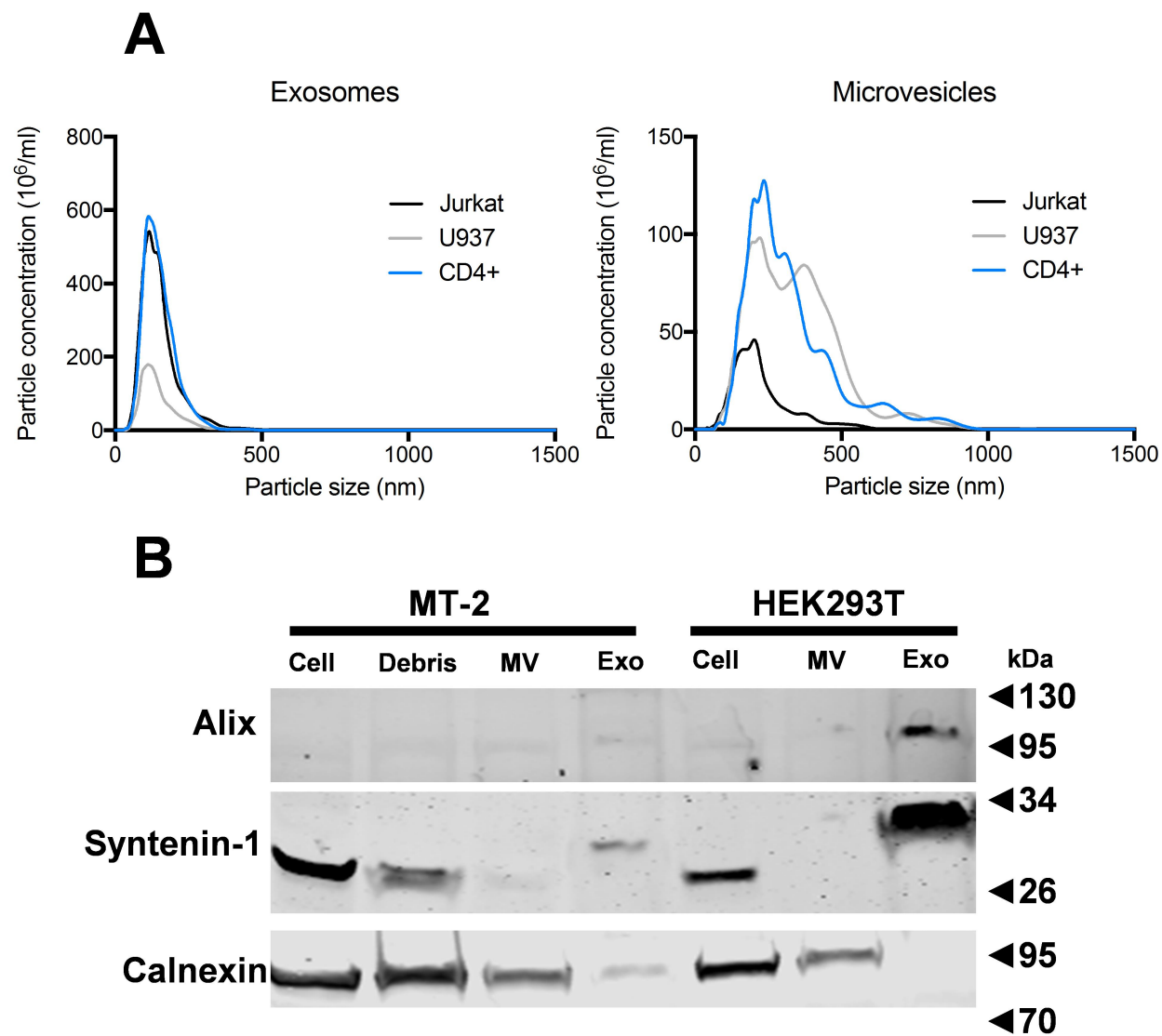


Figure 5.1 continued next page

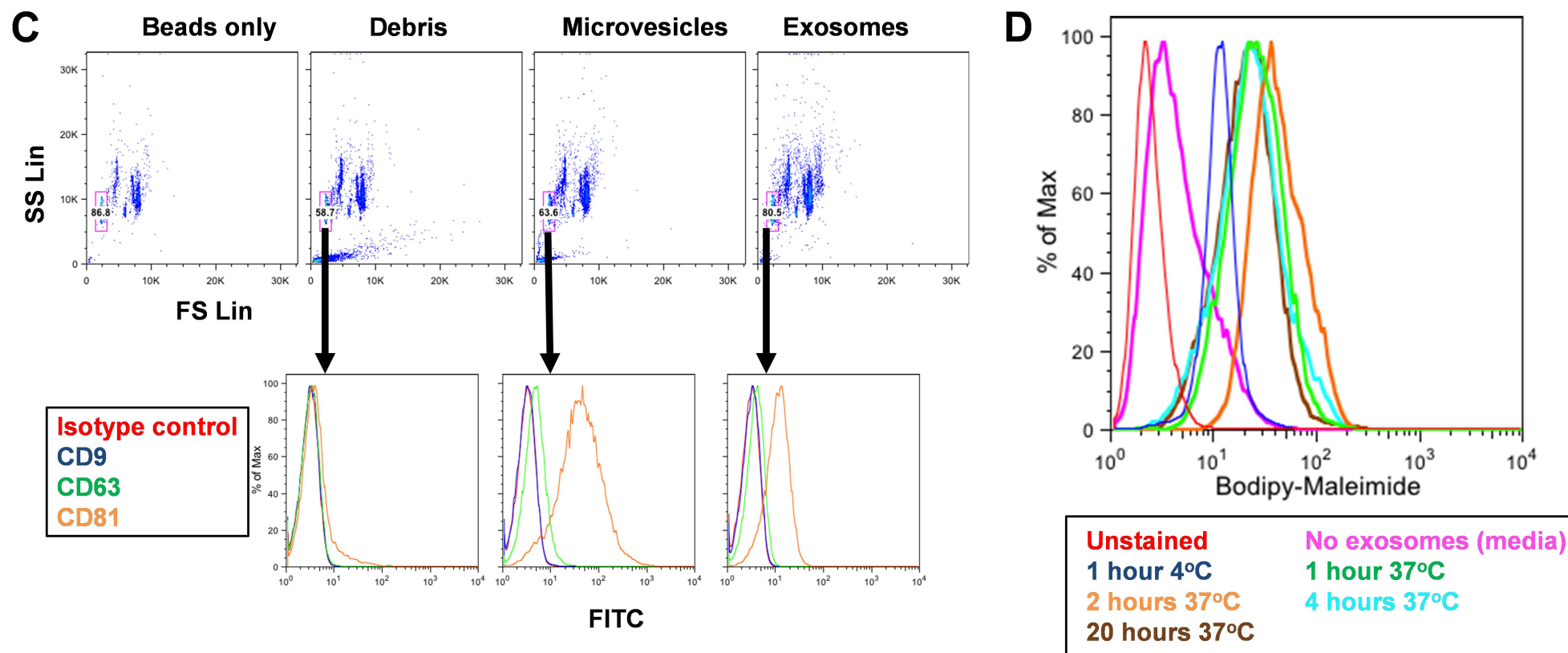


Figure 5.1 Validation of a differential ultracentrifugation protocol to isolate extracellular vesicles. **A.** Jurkat, U937 and primary CD4⁺ T cells were cultured in exosome-free R10 for 24 hours then the supernatant was harvested for EV isolation. Microvesicle or exosome pellets were resuspended in PBS then particle size and concentration was measured by Nanoparticle Tracking Analysis. **B.** MT-2 cells and HEK293T cells (85% confluent) were cultured in exosome-free R10 or D10 for 24 hours, respectively. Cells, debris, microvesicles and exosomes were isolated by differential ultracentrifugation then protein was isolated for immunoblotting. Equal amounts of cell or EV lysates were loaded into each lane (20 µg for MT-2 cells/EV; 10 µg for HEK293T cells/EV) then probed for Alix, Syntenin-1 and Calnexin. Molecular weight markers are shown on the right of each blot. **C.** PM1 cells were cultured in exosome-free R10 for 24 hours then EV were harvested from the supernatant. EV were then passively adhered to latex beads and probed for tetraspanin markers CD9, CD63 and CD81 by flow cytometry using FITC-labelled antibodies. The top row shows the forward scatter/sidescatter profiles for latex beads. Histograms displaying the FITC fluorescence intensity for each sample are shown in the second row. **D.** Jurkat cells were cultured in exosome-free R10 for 5 days then exosomes were isolated by differential ultracentrifugation. Exosomes were stained with 0.2 mM Bodipy-FL Maleimide then washed and resuspended in 125 µl PBS. 20 µl stained exosomes were added to IFN-DCs for the times indicated in the legend then cells were washed and fixed for flow cytometry analysis. IFN-DCs not treated with exosomes are labelled 'Unstained'. Conditioned exosome-free media 100,000 x g pellet was used as a negative control for IFN-DC particle uptake. IFN-DCs pulsed with exosomes for 1 hour at 4°C served as a negative control for particle endocytosis.

5.2.2 Nef is abundant in the plasma of HIV-1⁺ patients

Other groups have reported that ~10 ng/ml vesicle-associated Nef protein is present in the plasma of HIV-1⁺ patients^{453,454}. Interestingly, a more recent study demonstrated that Nef protein remains detectable in purified exosomes from the plasma of patients receiving antiretroviral therapy with undetectable viral loads⁷¹². To confirm these results and gain more insight into the levels of Nef present in plasma at different stages of infection, plasma samples from a cohort of patients who were recruited at the Mortimer Market (MM) centre in London during acute infection and sampled at serial timepoints into early chronic HIV-1 infection were used. Total EVs were isolated from MM patients at various stages of acute and early chronic infection, including timepoints after commencement of antiretroviral therapy (Figure 5.2A). 50 µg protein from each EV sample were then separated by SDS-PAGE and immunoblots were probed for the presence of vesicle markers and viral proteins. In addition, vesicles were isolated from plasma samples from two healthy, HIV-seronegative control volunteers. As positive controls for viral proteins, microvesicles and exosomes/virions were isolated from the supernatant of D10/HuT-78 cells which are chronically infected with a primary replication-competent HIV-1 strain⁷²³.

CD45 (isoforms of which vary between 180-220 kDa in molecular weight) was detected in EVs from all MM plasma samples at similar levels, however, a lower molecular weight band (~70 kDa) was strongly enriched in particular patients (Figure 5.2B). Given that total EV were isolated from plasma, it was unsurprising to see calnexin present in all MM samples. Although Tsg101, a marker thought to be enriched in exosomes, was identified in most MM samples, it was below detectable levels in the healthy control samples. The exosome-enriched marker Alix was not detected in any sample (data not shown). Nef was detected in plasma samples from HIV-1⁺ subjects at a molecular weight of ~35 kDa, whereas Nef secreted by D10/HuT-78 cells appeared to be in a lower molecular weight form. Interestingly, Nef was even present in plasma EVs derived from patients on therapy, one of whom had a viral load of less than 50 RNA copies/ml. In stark contrast, no Gag protein was detected in these treated patients. Small amounts of Gag protein in both p24 and p55 kDa form were observed in untreated HIV-1⁺ patients. On the other hand, large amounts of p24 Gag protein were seen in exosome/virion pellet isolated from D10/HuT-78 cells. Indeed, the quantitated Nef/Gag ratio was >1

for all MM patient plasma samples whereas the ratio was <1 for the D10/HuT-78 cells. In conclusion, it is shown that Nef contained within plasma vesicles is relatively abundant in comparison to Gag during acute, early chronic and chronic treated HIV-1 infection. The source of this vesicle-associated Nef remains unclear. Further work is needed to address whether Nef is similarly abundant in plasma prior to the peak in viraemia in AHI, but if so, this could constitute a source of antigen for induction of CD8⁺ T cell responses to Nef epitopes during primary infection.

A

Timepoint	Stage	VL	CD4	DFOSx
MM49.3	Late acute	1929700	420	35
MM49.7	Early chronic (low CD4)	>500000	200	280
MM49.9	Chronic on therapy	70	580	506
MM60.1	Late acute	55000	820	23
MM60.8	Early chronic	9600	560	233
MM64.12	Chronic on therapy	<50	1020	2 years
MM72.1	Post-peak acute	2100000	510	15
MM72.6	Early chronic	350000	410	200
MM75.2	Post-peak acute	2400000	290	16
NO81	Seronegative control	-	-	-
NO85	Seronegative control	-	-	-

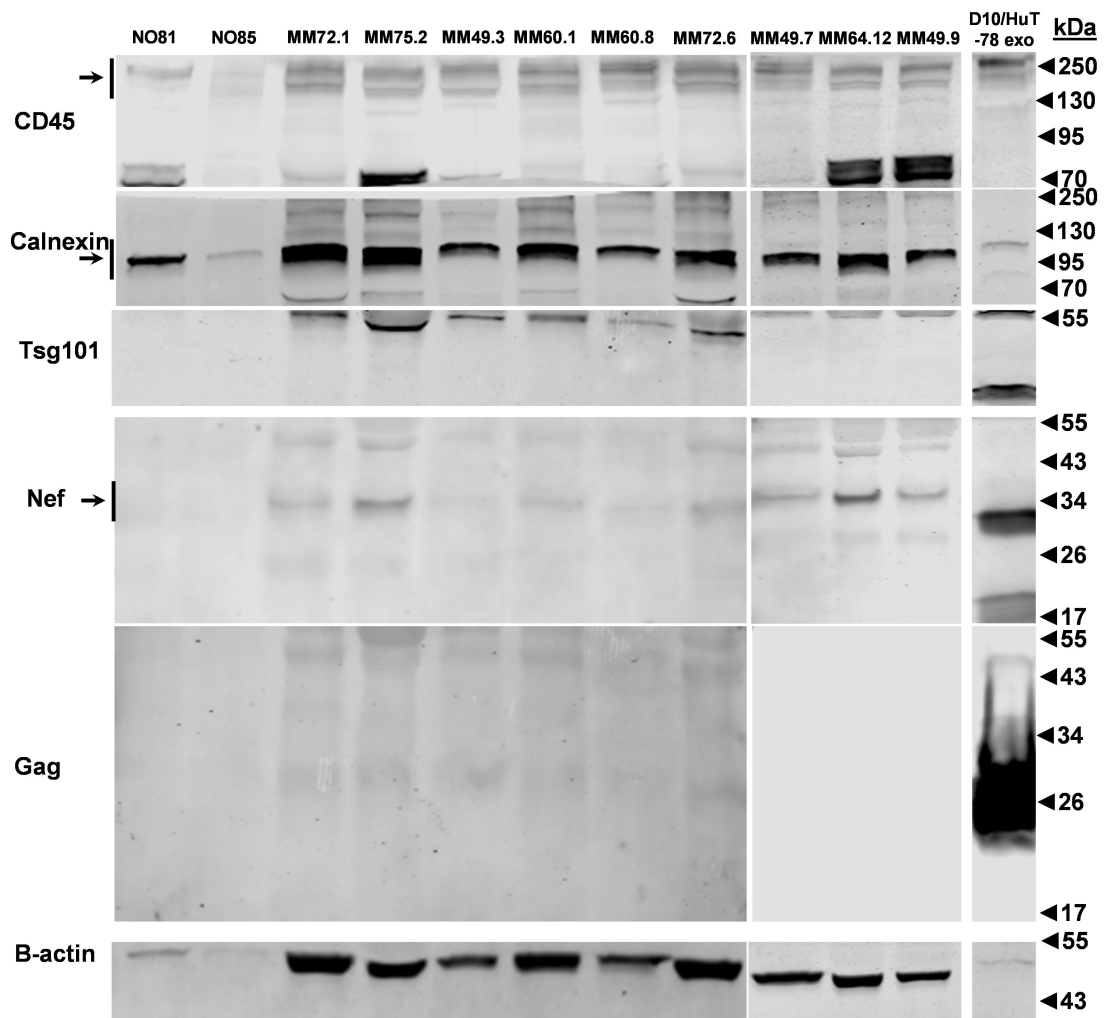
B

Figure 5.2 *Nef* is abundant in the plasma of HIV-1⁺ patients. **A.** Table displaying clinical data for all patient plasma samples used. The patient identifier (e.g. MM49) is followed by the visit number (e.g. MM49.3). Viral load (VL:RNA copies/ml), CD4 count (per mm³) and days following onset of symptoms (DFOSx) are listed for each patient. NO81 and NO85 are HIV-seronegative controls. **B.** Total EVs (microvesicles and exosomes) were isolated from plasma samples then lysed for immunoblot analysis. Proteins were separated via SDS-PAGE and probed for the proteins indicated on the left of each blot. EVs isolated from the supernatant of D10/HuT-78 cells, which are chronically infected with a replication-competent HIV-1 strain, were used as a positive control for Gag and Nef. 50 µg protein was loaded in each lane with the following exceptions due to low protein yield: MM60.8 - 21.5 µg; MM49.7 - 35 µg. Molecular weight markers are listed on the right of each blot and arrows on the left indicate the expected size of bands for CD45, calnexin and Nef.

5.2.3 Gag is the most abundant HIV-1 antigen secreted by infected CD4⁺ T cells

Having confirmed that vesicle-associated Nef is abundant in plasma from HIV-1⁺ patients, I next wanted to determine the cellular origin of these EV. Since CD4⁺ T cells are a major target of HIV-1 infection, and are known to secrete Nef-containing exosomes, the possibility that such cells may constitute a relevant source of Nef *in vivo* was examined.

Given that HIV-1 virions and exosomes share approximately the same diameter, it is not possible to separate the two populations using the gold standard differential ultracentrifugation method and vesicles are a common contaminant of virus preparations^{724,725}. Two previously described methods have been commonly used to separate exosomes and virions; iodixanol density gradient ultracentrifugation⁴⁹² and CD45 immunoaffinity depletion⁷²⁶. The former method relies on the greater speed with which HIV-1 virions move through a 6-18% iodixanol gradient due to their dense Gag core, whilst the latter takes advantage of the observation that the bulky molecule CD45 is not incorporated into HIV-1 virions^{494,495,727}. To separate exosomes from virions and to determine the relative distribution of Nef and Gag within these distinct populations, these methods were implemented.

5.2.3.1 Separation of exosomes and virions using an iodixanol gradient

Previous studies have used AchE activity as a specific marker of exosomes in iodixanol density gradients^{493,728,729}. 6-18% iodixanol gradients were set up in order to determine their ability to resolve AchE⁺ exosomes from the comparably dense p24⁺ virion fractions. HIV-1 strain IIIB stock was first harvested from the supernatant of the infected T cell line C8166, and concentrated through a sucrose cushion then resuspended in RPMI 1640 medium. This stock was overlaid onto an iodixanol density gradient then 1 ml fractions harvested after the 1.5 hour spin. Exosome-free R10 was loaded onto a separate gradient as a negative control. The density of fractions 1-11, as measured by absorbance at 340 nm, increased with the percentage of iodixanol (Figure 5.3A). Next, the AchE activity and the presence of infectious virus (measured by TZM-B1 luciferase assay) were measured in each fraction. As described in prior studies, the majority of the infectious virus was present in fractions with 13.2-18% iodixanol (Figure 5.3B). However, AchE activity was only detected in the media control fractions and not in the HIV-1 IIIB fractions (Figure 5.3B). To rule out a cell line-

dependent effect, primary CD4⁺ T cells were infected with HIV-1 IIIB then on day 3 cells were cultured in exosome-free R10 media for 24 hours. The purified exosome/virion pellet was subjected to iodixanol density gradient ultracentrifugation and each resulting fraction tested for AchE activity, reverse transcriptase (RT) activity and p24 concentration by ELISA. RT activity and p24 concentration both peaked near the bottom of the gradient in the 14.4% iodixanol fraction (Figure 5.3C). Interestingly, significant AchE activity was measured in the CD4⁺ exosome/virion pellet both before and after separation on the gradient (Figure 5.3C). However the majority of this activity was also noted in the exosome-free R10 media control, indicating that soluble proteins present in FBS may be the source of the observed AchE activity.

In related experiments I carried out as part of a collaborative study aiming to determine whether the second messenger cGAMP is packaged into virions and/or exosomes⁶⁸⁴, a similar pattern of AchE activity and p24 concentration was observed. Here, 293T cells were transfected with a plasmid encoding the lab-adapted infectious molecular clone NL4.3 alone or together with plasmids expressing the wildtype cGAS protein or the catalytically-inactive cGAS mutant and exosomes/virions were isolated from the cell supernatant and separated by iodixanol density gradient ultracentrifugation. Again, the majority of AchE activity was measured in low density fractions whereas p24 concentration was highest in the high density fractions (Figure 5.3D). Taken together, these results suggest that extracellular protein complexes containing AchE derived from FCS proteins present in exosome-free media may constitute the bulk of the observed AchE activity in the low density fractions of 6-18% iodixanol density gradients. On the basis of these results, AchE activity was disregarded as a marker of exosomes in iodixanol density gradients used for this study.

Instead, exosome and viral protein markers present in density gradient fractions were examined by the more direct method of immunoblotting. Exosomes/virions were isolated from HIV-1 IIIB-infected PM1 cells (CD4⁺ T cell line; derivative of HuT-78) by differential ultracentrifugation then loaded onto an iodixanol density gradient. Protein from resulting fractions was precipitated, separated via SDS-PAGE and probed for exosomal/viral markers via immunoblotting. Multiple isoforms of CD45 were detected in all fractions, although the highest signal was observed towards the high density fractions (Figure 5.4). Notably, the exosomal markers Alix and Tsg101 were both

detected in the high density fractions, but at low to undetectable levels in lower-density fractions. A lower molecular weight isoform of Alix was also observed in addition to the expected 95 kDa protein. The exosome-enriched marker Syntenin-1 was not reliably detected in any fractions (data not shown). Similarly to Alix and Tsg101, HIV-1 Nef protein was only detected in high density fractions. HIV-1 Gag was observed in all fractions as the cleaved p24 form present in mature virions. However, the majority of the detectable Gag was present in the higher density fractions in both precursor uncleaved p55 and intermediate isoforms. It is possible that some exosomes were being separated on the iodixanol gradient, but that the quantity of exosomes present in the lower-density fractions from IIIB-infected PM1 cells was too low to enable detection of Alix and Tsg101 by immunoblotting. Any small pieces of cell debris or vesicular aggregates present in the preparations would likely have co-purified with virions in the higher-density fractions. High-sensitivity cGAMP analysis performed by Dr Bridgeman on the fractions I had separated from the supernatant of plasmid-transfected 293T cells in the cGAMP experiment described above indicated the presence in fractions towards the middle of the gradient of particles containing lower levels of cGAMP than those detected in virions present in the higher-density fractions⁶⁸⁴, consistent with the presence of exosomes here. However, because of the difficulties in verifying whether Nef-containing exosomes could be separated from p24-containing virions in supernatants of infected CD4⁺ T cell lines, I decided to test the other described method for separating exosomes from virions; CD45 depletion.

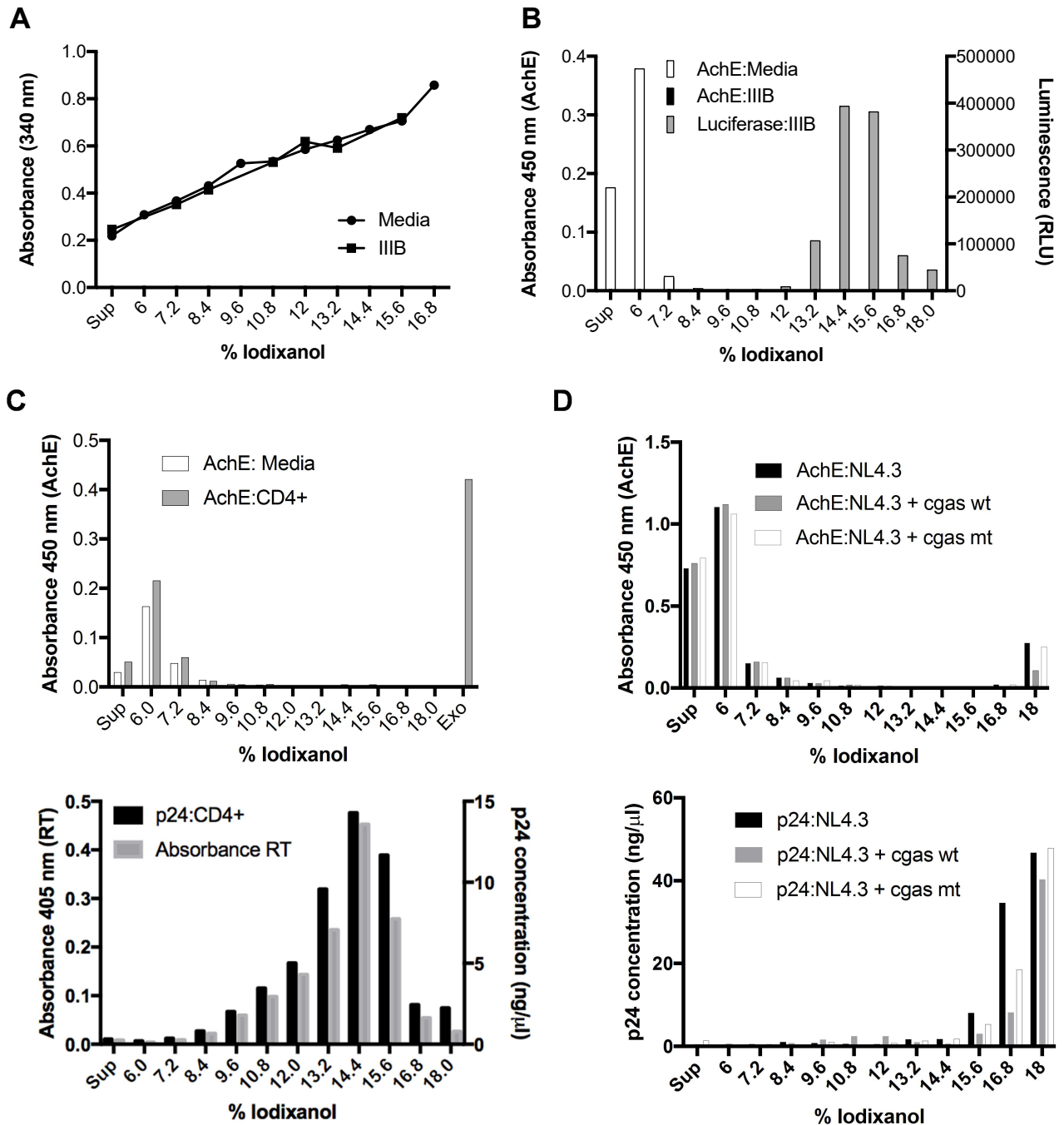


Figure 5.3 Substantial acetylcholinesterase activity associated with foetal bovine serum. **A.** HIV-1 IIIB stock derived from infected C8166 cells was first pelleted on a sucrose cushion and resuspended in RPMI. 1 ml of HIV-1 IIIB or exosome-free R10 ('Media') was loaded onto a 6-18 iodixanol gradient and spun at 200,000 x g for 1.5 hours. The density of collected fractions was tested by absorbance at 340 nm. 'Sup' represents the top fraction. **B.** Samples were prepared as described in 'A' then tested for acetylcholinesterase activity and for replication-competent virus using the TZM-B1 luciferase assay. **C.** Primary CD4⁺ T cells were infected with HIV-1 IIIB for 3 days then washed and cultured in exosome-free R10 for 24 hours. Exosomes/virions were isolated from supernatant by differential ultracentrifugation then loaded onto a 6-18% iodixanol gradient. Resulting fractions were assayed for AchE activity, RT activity and p24 concentration. 'Exo' indicates the pre-fractionated exosome/virion pellet. **D.** 293T cells were transfected with the HIV-1 infectious molecular clone NL4.3 only, NL4.3 and wildtype 'wt' cGAS, or NL4.3 and the catalytically-inactive mutant 'mt' cGAS. Exosomes/virions were isolated then loaded onto a 6-18 iodixanol gradient. Resulting fractions were assayed for AchE activity and p24 concentration. AchE activity and RT activity are expressed in arbitrary absorbance units.

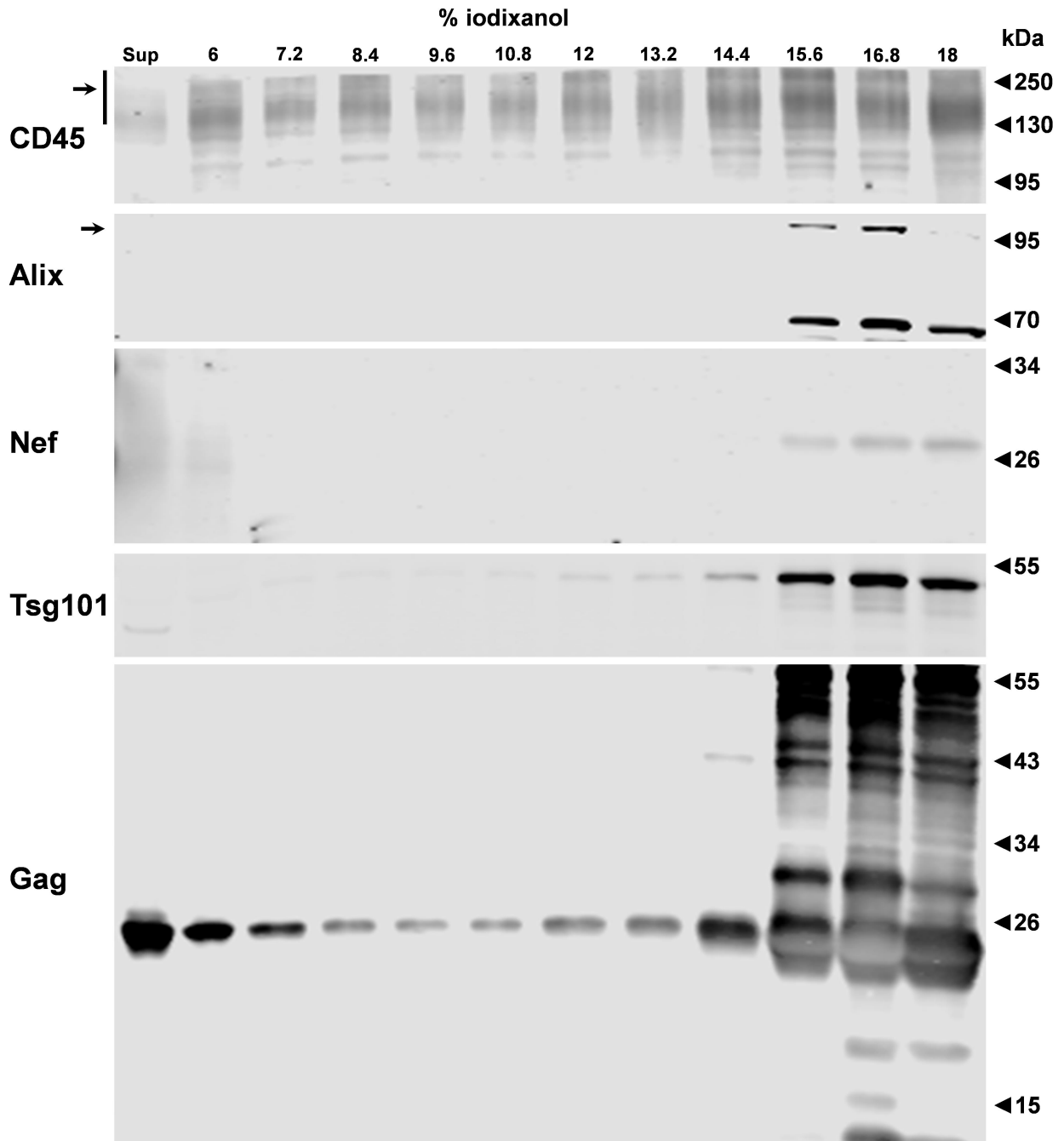


Figure 5.4 *Iodixanol gradients do not appear to separate Nef exosomes from virions secreted by the CD4⁺ T cell line PM1.* The CD4⁺ T cell line PM1 was infected with HIV-1 IIIB for 3 days then cultured in exosome-free R10 for 24 hours. Cells, large cell debris and microvesicles were removed by differential ultracentrifugation then the 100,000 x g pellet was overlayed onto a 6-18% iodixanol density gradient and spun at 200,000 x g for 1.5 hours. Proteins were extracted from resulting fractions by TCA precipitation then separated by SDS-PAGE. Blots were probed for the proteins indicated on the left with specific antibodies. Molecular weight markers are indicated on the right of each blot. ‘Sup’ indicates the top fractions where the 100,000 x g pellet was originally overlayed, whilst the 18% iodixanol fraction represents the densest fraction at the bottom of the tube.

5.2.3.2 Separation of exosomes and virions using CD45 depletion: Nef is predominantly contained within CD45⁺ exosomes

Nguyen and Hildreth first showed that the bulky CD45 molecule is selectively excluded from Jurkat-derived HIV-1_{RF} virions⁴⁹⁴. Subsequently, these results were extended to HIV-1 virions derived from infected PBMC, T cells, B cells and macrophages, all of which express high surface levels of CD45 that is not incorporated into HIV-1 virions⁴⁹⁵. The same group exploited this observation by developing a CD45 immunoaffinity depletion protocol to separate HIV-1 virions from contaminating exosomes⁷²⁶. I aimed to use this CD45 depletion protocol to confirm, as others have previously shown, that Nef is enriched in CD45⁺ exosomes secreted by HIV-1-infected cells⁴⁵³.

Firstly, I examined the viral stock derived from the supernatant of HIV-1 IIIB-infected C8166 cells (a CD4⁺ T cell line). Viral stock was first purified through a sucrose cushion then separated using an optimised CD45 depletion protocol and assessed by immunoblotting. As expected, CD45 was only detected in the starting material and bead-bound eluate (Figure 5.5A). Interestingly, the exosome-enriched marker Alix was detected in both the starting material and the flowthrough (CD45⁻) off the column but not the eluate, whilst Tsg101 was detected in all samples. Calnexin isoforms were only observed in pre-separated viral stock and the CD45⁺ material. Gag p24 protein was observed in the starting material, the flowthrough and the CD45⁺ fraction. Strikingly however, Nef was significantly enriched in only the CD45⁺ material. Following on from this result, Nef enrichment in CD45⁺ vesicles secreted from infected cells was confirmed in a different CD4⁺ T cell line whilst comparing to uninfected cells. HuT-78 cells were infected with HIV-1 IIIB for 3 days (>50% p24⁺ by intracellular staining; data not shown) then cultured in exosome-free R10 for 24 hours. HuT-78 cells and microvesicles pelleted at 300 x g and 20,000 x g respectively were lysed then exosomes/virions pelleted at 100,000 x g were separated by CD45 depletion and lysed for immunoblots. Again, CD45 was detected in cell lysates, microvesicles and eluates but was under-represented in the flowthrough fraction (Figure 5.5B). Similarly, calnexin was abundant in cell and microvesicle lysates, but was under-represented in CD45 depletion flowthrough and eluate. However, multiple isoforms of calnexin were more abundant in HIV-1-infected samples, possibly as a result of release of apoptotic vesicles, alteration of calnexin expression or non-specific recognition of infection-induced proteins.

Relative to cell and microvesicle lysates, Alix and Tsg101 were enriched in CD45 depletion flowthrough and eluate fractions. HIV-1 Gag p24 was abundantly enriched in microvesicles, flowthrough and eluate samples relative to cell lysates. On the other hand, Nef was predominantly enriched in only the CD45⁺ eluate sample, although small amounts were detected in cells, microvesicles and CD45⁻ flowthrough. From these results it was therefore concluded that Nef secreted by infected CD4⁺ T cells is predominantly found in association with CD45⁺ exosome-like vesicles.

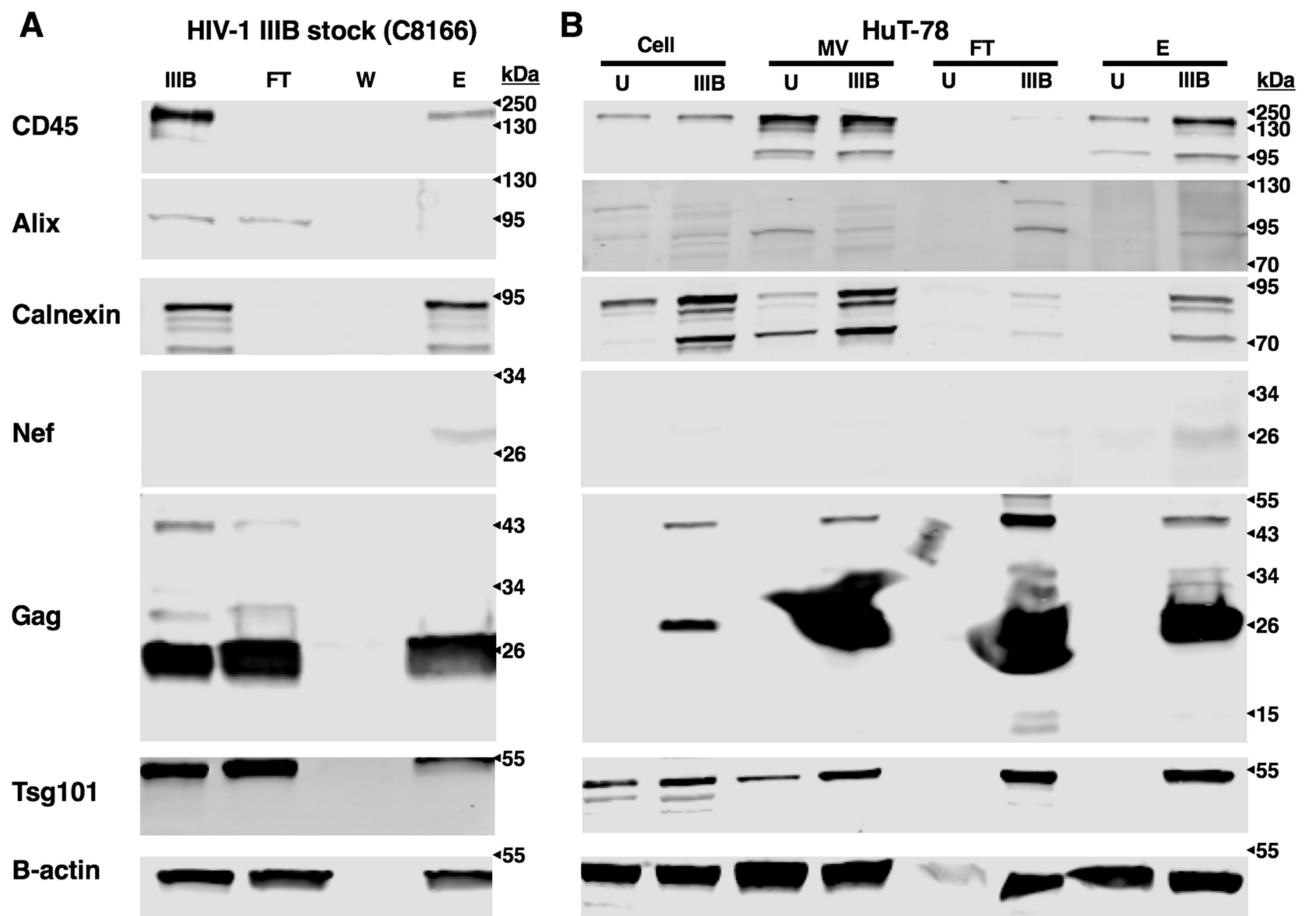


Figure 5.5 *Nef* is predominantly contained within $CD45^{+}$ exosomes released into HIV-1-infected cell supernatants. **A.** HIV-1 IIIIB stock derived from the supernatant of infected C8166 cells was separated into $CD45^{+}$ and $CD45^{-}$ fractions using $CD45$ microbeads. ‘IIIIB’ indicates the unseparated stock; ‘FT’ represents the flowthrough fraction off the column; ‘W’ is the additional wash fraction and ‘E’ is the eluate fraction bound to the beads. 10 μ g of protein for each sample (with the exception of ‘W’, where little protein was isolated) was separated by SDS-PAGE and probed with antibodies specific for the proteins indicated on the left. Molecular weight markers are shown to the right of each blot. **B.** The $CD4^{+}$ T cell line HuT-78 was infected with HIV-1 IIIIB at a MOI of 0.01 then cultured for 24 hours in exosome-free R10. Cell lysates, microvesicles ‘MV’ and exosome pellets were isolated. Exosomes were separated from virions using $CD45$ microbeads. 20 μ g of protein for each sample (with the exception of uninfected FT and uninfected E where 2.5 μ g was loaded due to very low protein yield) was separated by SDS-PAGE and probed with antibodies specific for the proteins indicated on the left. Molecular weight markers are shown to the right of each blot. ‘U’ indicates uninfected HuT-78 cells or vesicles whilst ‘IIIIB’ denotes HIV-1 IIIIB-infected samples. ‘FT’ and ‘E’ represent the flowthrough and eluate fractions, respectively.

5.2.3.3 **Gag is the most abundant antigen in vesicles secreted by infected CD4⁺ T cells and cell lines**

Experiments thus far have shown that Nef is predominantly associated with CD45⁺ vesicles and is found at relatively high levels compared to Gag in the plasma of HIV-1⁺ patients. However, in contrast to this, although the relative levels of Gag and Nef proteins cannot be accurately inferred from the immunoblots shown in Figures 5.4 and 5.5, Gag protein seemed to be more abundant than Nef in CD45⁺ vesicles secreted by infected CD4⁺ T cells, as well as being present at high levels in virions and microvesicles. Furthermore, in Chapter 4 it was demonstrated that Gag-derived epitopes substantially outnumber Nef-derived epitopes in the repertoire of HLA class I-bound peptides detected by mass spectrometry in infected CD4⁺ T cells and T cell lines. Given that antigen abundance and turnover is often predictive for likelihood of being presented on MHC class I¹⁶⁵, abundant production and secretion of Gag-containing vesicles/virions by infected CD4⁺ T cells may explain this result. To gain more accurate insight into the relative amounts of viral proteins in cells and vesicles, these were determined using mass spectrometry.

Cells and supernatant cell debris, microvesicles and exosome/virion pellets were isolated from HIV-1 IIIB-infected Jurkat cells and primary CD4⁺ T cells. Protein was extracted from these cells and vesicle populations then subjected to LC-MS/MS analysis and the relative abundance of each protein was calculated using Intensity-Based Absolute Quantitation (iBAQ) in MaxQuant software. No HIV-1 proteins/peptides were detected in the uninfected control samples (data not shown). HIV-1 Gag, Pol, Env and Nef proteins were detected in microvesicle pellets of both primary CD4⁺ T and Jurkat cells (Figure 5.6). Interestingly however, no HIV-1 proteins were detected in primary CD4⁺ T cells and only Gag and Env were detected in the Jurkat cell lysate, which was likely due to Pol and Nef-derived peptides being present in amounts below the limit of detection. Gag was the most abundant HIV-1 protein in microvesicles and exosome/virion pellets secreted by both cell types. The next most abundant HIV-1 protein in vesicular samples was Pol, followed by Env and Nef. These data concur with previous immunoblotting results where Gag was abundant in cells and vesicles secreted by infected CD4⁺ T cell lines whereas Nef was barely detectable in unseparated vesicle pellets.

	CD4 ⁺ Cell		CD4 ⁺ 2000 x g		CD4 ⁺ MV		CD4 ⁺ Exosomes	
Protein	#peps	iBAQ	#peps	iBAQ	#peps	iBAQ	#peps	iBAQ
Gag	0	0	6	1169900	30	414320000	27	37672000
Env	0	0	0	0	2	599050	0	0
Nef	0	0	0	0	2	868530	0	0
Pol	0	0	0	0	12	2624100	1	126800

	Jurkat Cell		Jurkat 2000 x g		Jurkat MV		Jurkat Exosomes	
Protein	#peps	iBAQ	#peps	iBAQ	#peps	iBAQ	#peps	iBAQ
Gag	11	5478100	18	4802100	40	459430000	53	408110000
Env	1	44898	0	0	8	1322400	9	1119300
Nef	0	0	0	0	2	395610	1	167700
Pol	0	0	1	35217	38	6642700	49	11184000

Figure 5.6 *Gag* is the most abundant antigen produced and secreted by HIV-1 IIB-infected CD4⁺ T cells. Primary CD4⁺ T cells from one donor or the CD4⁺ T cell line Jurkat were infected with HIV-1 IIB for 4 days or left uninfected as a negative control. On day 4, cells, cell debris, microvesicles (MV) and exosomes/virions were harvested by differential ultracentrifugation. Cells or vesicles were lysed in RIPA buffer then protein was extracted and digested with trypsin for LC-MS/MS analysis on a QExactive instrument. Raw spectra files were analysed in MaxQuant software and intensity based absolute quantitation (iBAQ) values were used to compare relative amounts of different HIV-1 proteins present within each sample. **A.** Table showing the number of identified peptides in each sample associated with each viral protein alongside the calculated iBAQ value.

5.2.4 Characterisation of exosomes secreted by HIV-1-infected macrophages

Multiple lines of evidence thus far indicated that CD4⁺ T cells release large amounts of Gag-containing MVs and virions/exosomes, hence although Nef is enriched in exosomes, much more Gag is released overall. Therefore it seemed unlikely that Nef-containing exosomes secreted by HIV-1-infected CD4⁺ T cells are the major source of Nef in the plasma of HIV-1⁺ patients. Macrophages are known to be infected by HIV-1 *in vivo* and are now thought to be a significant cellular reservoir of HIV-1 in chronic infection⁷³⁰. Macrophages are long-lived, accumulate in tissues throughout HIV-1 infection⁷³¹ and are thought to accumulate unintegrated HIV-1 DNA which can produce detectable levels of Nef protein^{732,733}. *In vitro* infection experiments have indicated that ‘macrophage-like’ THP-1 cells and primary MDM secrete relatively large amounts of Nef when cultured for extended periods of time⁴⁵³. Additionally, recent evidence hinted that Nef-containing exosomes from HIV-1⁺ patient plasma are derived from a myeloid cell source⁷¹². Therefore the ratio of Nef and Gag protein production in long-lived MDM infected *in vitro* was examined.

MDM were infected with the lab-adapted CCR5-tropic recombinant virus NL4.3 BaL *env* (which has the NL4.3 backbone but the *env* gene from the CCR5-tropic HIV-1 BaL strain) then cells, cell debris, microvesicles and exosomes secreted from days 4-7 post-infection were collected. At day 7 post-infection, >70% of macrophages were live and p24⁺ (data not shown). The small amounts of vesicular protein that could be isolated from the supernatant of cultures were fractionated by differential ultracentrifugation and examined by immunoblotting for the presence of viral proteins Nef and Gag as well as exosome markers. CD45 was detected in both uninfected and HIV-1-infected macrophage cells and secreted vesicles (Figure 5.7). As expected, the ER marker calnexin was enriched in cells but barely detectable in the exosome/virion pellets. The exosome marker Tsg101 was expressed at higher levels in uninfected than in infected macrophages. However Tsg101 was only detectable in the extracellular vesicles secreted from HIV-1-infected macrophages, in line with its role in HIV-1 virion biogenesis⁷³⁴⁻⁷³⁶. Nef and Gag were detected in HIV-1-infected macrophages and in debris, microvesicles and exosomes. When the Nef/Gag ratio was quantitated in each sample, the results suggested that relatively more Gag was secreted by macrophages than Nef at the timepoint studied, although the ratio was closer to 1:1 in cells and debris (Figure 5.7B).

Since MDM appear to go through a productive phase of HIV-1 infection from approximately 4-14 days post-infection which subsequently subsides⁷³⁷, the change in the ratio of Nef/Gag secretion in exosomes over time after the productive phase of macrophage HIV-1 infection was examined. MDM were infected with NL4.3 BaL *env* and cultured longitudinally for 42 days. Exosomes/virions were isolated weekly for 6 weeks then lysed and probed for Nef, Gag, CD45, calnexin and β -actin by immunoblotting. At day 7, live and infected macrophages were $\sim 24\%$ p24⁺ as determined by intracellular p24 staining (data not shown). Similarly to the previous experiment, both Nef and Gag were detected in infected macrophages at day 7 (Figure 5.8A). As expected, CD45 was detected in all samples and calnexin was below detectable levels for all exosome/virion samples. Gag was enriched in exosome/virion pellets at all timepoints tested and seemed most abundant by days 14-21 post-infection. Nef was barely detectable at the day 7 timepoint but was present in greater amounts from day 14 onwards. When the Nef/Gag ratio was quantitated in each EV sample, the ratio was highest at day 42 post-infection (Figure 5.8B), therefore indicating that relatively less Gag is released as compared to Nef by macrophages infected for extended periods of time in comparison to macrophages recently infected with HIV-1 (i.e. <7 days). Although this experiment was performed with only 1 donor, these preliminary data provide an indication that macrophages may constitute a physiological source of cell-associated or EV-associated Nef antigen *in vivo*.

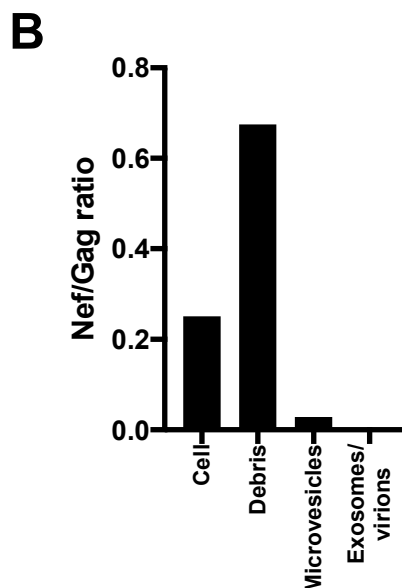
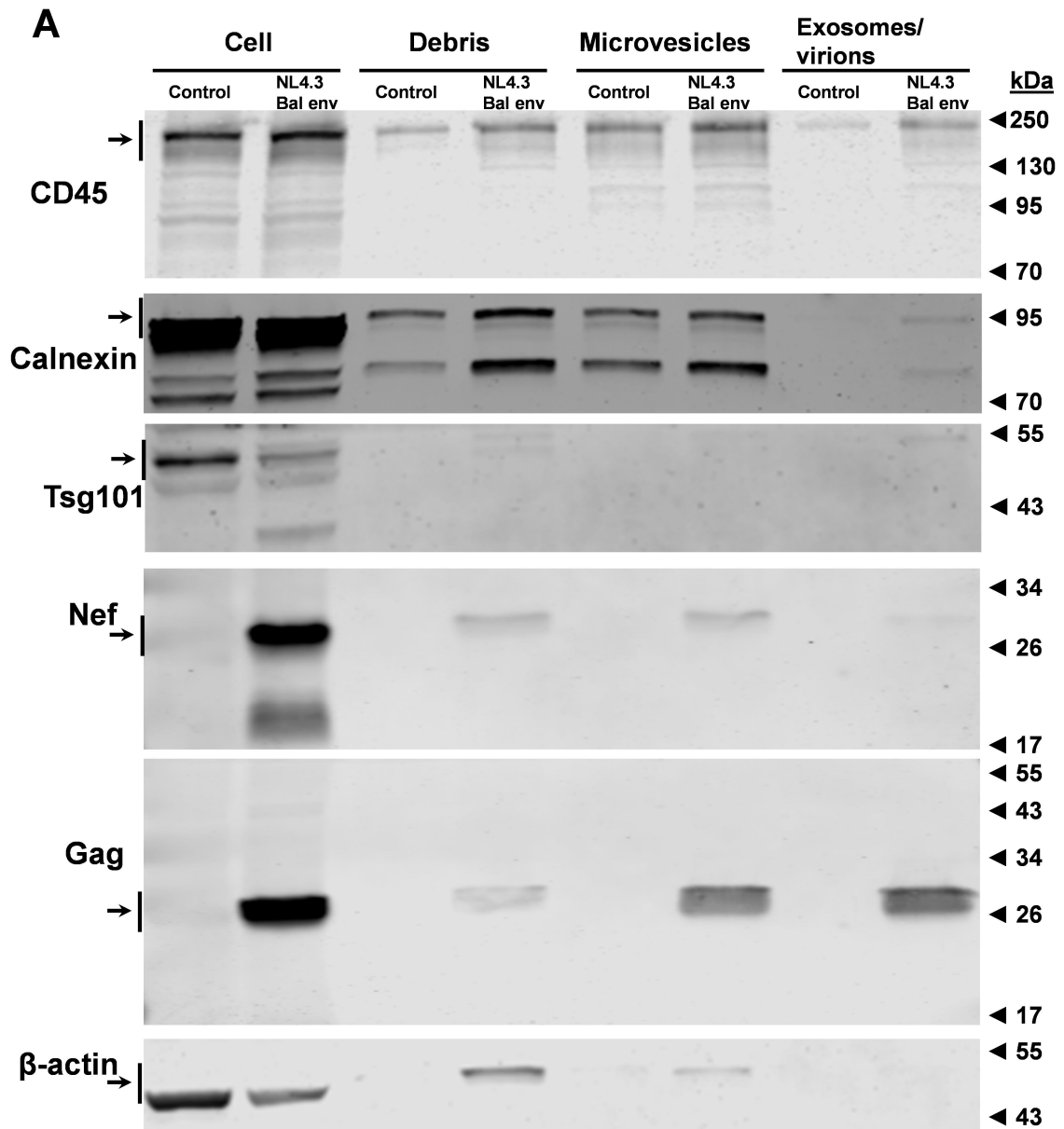


Figure 5.7 *Characterisation of vesicles secreted by HIV-1-infected macrophages.* **A.** Monocytes were isolated from a HIV-1 seronegative donor then differentiated into macrophages and infected with NL4.3 Bal env (or left uninfected as a negative control). On day 4 post-infection, macrophages were washed once in PBS then cultured in exosome-free R10 for 3 days. Cells, cell debris (pelleted at 2000 x g), microvesicles (20,000 x g) and exosomes/virions (100,000 x g) were then isolated through differential ultracentrifugation and lysed for analysis of the indicated markers via immunoblotting. 50 µg of cell lysates were loaded. Note that uneven protein amounts were loaded across wells due to low yield of vesicle protein isolated from supernatant. Control debris, 3.15 µg; NL4.3 Bal env debris, 5.78 µg; Control microvesicles, 3.5 µg; NL4.3 Bal env microvesicles, 4.88 µg; Control exosomes/virions, 1.48 µg; NL4.3 Bal env exosomes/virions, 1.26 µg. **B.** Nef and Gag bands were quantitated on Li-Cor software. The ratio of Gag/Nef quantitative values is shown on the y-axis for NL4.3 Bal env-infected macrophage cells, debris, microvesicles and exosome/virion pellets.

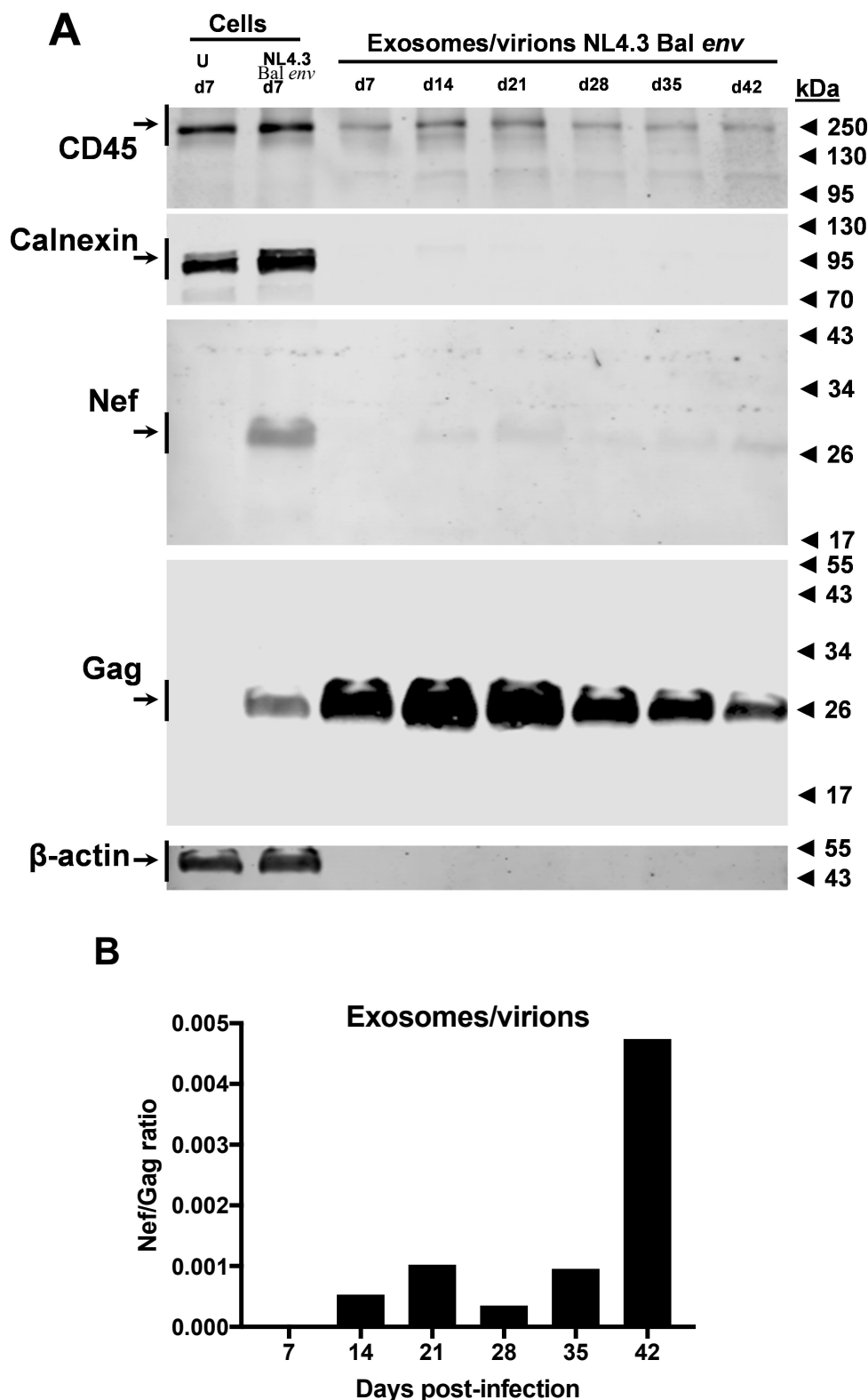


Figure 5.8 Longitudinal characterisation of vesicles secreted by HIV-1-infected macrophages. **A.** Monocytes were isolated from a HIV-1 seronegative donor then differentiated into macrophages and infected with NL4.3 Bal env (or left uninfected as a negative control). On day 4 post-infection, macrophages were washed once in PBS then cultured in exosome-free R10 for 3 days. Fresh exosome-free R10 was replaced every 3-4 days for until 42 days post-infection. Cells, cell debris (pelleted at 2000 x g), microvesicles (20,000 x g) and exosomes/virions (100,000 x g) were isolated weekly (i.e. on day 7, 14, 21, 28, 35 and 42) through differential ultracentrifugation and lysed for analysis of the indicated markers via immunoblotting. 10 µg of cell lysates were loaded. Note that uneven protein amounts were loaded across wells containing exosome/virion lysates due to low yield of vesicle protein isolated from supernatant. Day 7, 1.9 µg; Day 14, 2.7 µg; Day 21, 2.5 µg; Day 28, 2.3 µg; Day 35, 1.3 µg; Day 42, 1.5 µg. U = uninfected, NL4.3 Bal env = infected. **B.** Nef and Gag bands were quantitated on Li-Cor software. The ratio of Nef/Gag quantitative values is shown on the y-axis for NL4.3 Bal env-infected macrophage exosomes/virion pellets on the day post-infection indicated on the x-axis.

5.3 Discussion

In this chapter I validated a differential ultracentrifugation protocol which allowed the efficient isolation of cells, MV and exosomes in cell culture supernatants and plasma samples. This method was used to show that relatively more Nef than Gag is found in EV present in the plasma of HIV-1⁺ patients during acute and early chronic infection. In contrast, infected CD4⁺ T cells were found to release large amounts of EV/virion-associated Gag (and relatively little Nef) after *in vitro* infection with HIV-1 IIIB. The small amount of Nef detected was predominantly associated with CD45⁺ EV. Lastly, preliminary data suggested that HIV-1-infected macrophages may produce increased amounts of Nef relative to Gag protein after extended periods of infection.

Validation of differential ultracentrifugation protocol to isolate EVs

Exosomes are commonly reported to be 30-100 nm in diameter with a cup-shaped morphology in studies using electron microscopy for vesicle characterisation⁴⁴². However, exosomes derived from cell culture supernatants are generally between 90-160 nm in diameter when measured by NTA, a difference which is thought to result from shrinkage artefacts following vesicle fixation for electron microscopy (although some NTA instruments may overestimate diameter due to low resolution of EV <90 nm)⁴⁴⁴. My NTA data are consistent with this size range, as the modal diameter of 100,000 x g pellets (exosomes) isolated for 3 different cell lines was between 112-126 nm (Figure 5.1). Importantly, a media control (exosome-free R10 cultured without cells) contained a negligible number of detectable particles as measured by NTA, indicating that the EV detected in cell culture supernatants were secreted by cells and not derived from FBS. The presence of FBS-derived exosomes (which contain bovine RNA and protein) can have significant functional consequences, as cells treated with FBS-containing medium not depleted of EV behave differently to cells treated with exosome-depleted medium⁷³⁸. On the other hand, cells cultured in exosome-depleted media show a reduced rate of proliferation and survival relative to media containing FBS EV⁷³⁹. This reduced survival might partially explain the vastly different exosome/microvesicle ratios found in Jurkat, U937 and primary CD4⁺ T cells, as activated or apoptotic cells tend to release larger vesicles^{740,741}. Thus it is conceivable that differential sensitivity of each cell type to exosome-free R10 could result in differential release of larger apoptotic microvesicles in the 24 hour period studied here.

My data also suggest there are cell-specific differences in EV protein cargo. For example, HEK293T-derived exosomes were enriched in syntaxin-1 as reported previously⁷⁴², but syntaxin-1 was not enriched in MT-2-derived exosomes despite detectable expression in MT-2 cell lysates (Figure 5.1). Such differences could result from altered exosome biogenesis in each cell type (e.g. T cells tend to bud exosome-like vesicles from the plasma membrane⁶⁹¹). Another potential explanation is that MT-2 cells are also chronically infected with a retrovirus, HTLV-1, and therefore secreted virions which co-purify with exosomes⁷⁴³ may harbour low levels of syntaxin-1. The low levels of calnexin observed in exosome pellets for both cells supports the conclusion that exosomes were isolated through this differential ultracentrifugation protocol, as ER-retained proteins such as calnexin and calreticulin are generally not secreted⁷⁴⁴. Although proteomic composition profiles may characterise particular subgroups of EV, no single marker exists which is unique to EVs⁷⁴⁵. Tetraspanins such as CD9, CD63 and CD81 are commonly enriched in exosomes from different cell types⁷⁴⁶. I did not probe for the presence of tetraspanins by immunoblotting here, because this class of proteins requires native or non-reducing gels for adequate detection of glycosylated forms. Therefore I used a previously-described passive bead adsorption method⁷⁴⁷ to detect CD9, CD63 and CD81 on the surface of PM1 cell-derived EVs isolated by differential ultracentrifugation. CD81 and CD63 were enriched in microvesicles and exosomes relative to cell debris which pellets at 2000 x g, but CD9 was not detected (Figure 5.1). CD9 is not detected on Jurkat T cell-derived exosomes⁷⁴⁸, so a lack of exosomal CD9 might be a feature of particular T cell lines such as Jurkat and PM1 cells.

Altogether, the NTA, immunoblotting and flow cytometry data suggest that exosomes are isolated by the ultracentrifugation method employed in this chapter. It is possible that some exosomes are also pelleted by the 20,000 x g spin step (which is faster than the 10,000 x g step employed by many studies⁷⁴⁹) and therefore co-purify with larger microvesicles. Absolute separation of EV by size is hampered by EV aggregation, varying EV densities and the observation that small EV with less distance to travel (i.e. near the bottom of the tube) will pellet with larger vesicles at low speed⁷⁵⁰. Co-sedimentation of exosomes and microvesicles likely occurred in the 20,000 x g pellets in this chapter, as suggested by the multimodal microvesicle distribution shown in Figure 5.1. However, this co-purification of exosomes and microvesicles in the 20,000 x g spin is acceptable in this thesis because

it means that the exosome population pelleted at 100,000 x g is likely to be highly pure (devoid of larger microvesicles). In the future I could use electron microscopy and an increased number of specific antibodies to further characterise EVs secreted by HIV-1-infected cells. The size characteristics of EVs secreted by HIV-1-infected cells could be analysed.

Lastly, I showed that exosomes isolated by this differential ultracentrifugation protocol interact with DCs. A small amount of uptake of the membrane dye was observed in the negative control (no exosomes), likely as a result of uptake of free- or protein complex-associated dye by IFN-DCs. IFN-DC cultured at 4°C for one hour with dyed exosomes stained positive for fluorescent dye, indicating that the exosomes adhered to the membrane (endocytosis is abrogated at low temperature). On the other hand, it remains unclear whether IFN-DC cultured at 37°C had endocytosed the exosomes or simply adhered more exosomes on their cell surface. Future experiments could address the routes of exosome uptake (e.g. fusion, phagocytosis, receptor-mediated endocytosis⁷⁵¹) by utilising alternative dyes such as pHrodo, which fluoresces at the low pH found in phagosomes and has been used in previous studies to study exosome uptake⁷⁵². To distinguish EV endocytosis from fusion with cellular membranes, the self-quenching octadecyl rhodamine B dye could be utilised (dye will fluoresce once membrane fusion occurs)⁷⁵³. Furthermore, the receptors which mediate uptake of exosomes (by DCs in particular) during HIV-1 infection are not well understood. It would be interesting to use blocking antibodies against receptors such as CD169 which could mediate exosome uptake to explore this. In addition to its role in HIV-1 trans-infection⁴⁰, CD169 (sialoadhesin) has been reported to mediate exosome capture in lymphoid tissues⁷⁵⁴ and is expressed by specialised macrophages in the subcapsular sinus of lymph nodes which are known to be important for CD8⁺ T cell priming and induction of tolerance against dead cell-associated or apoptotic antigen through antigen transfer to lymph node-resident DCs or cross-presentation to CD8⁺ T cells^{755–759}.

Methods to separate exosomes from virions

In this chapter I evaluated two methods designed to separate exosomes and HIV-1 virions secreted by infected CD4⁺ T cells. My data show that the first method, iodixanol density gradient ultracentrifugation, was unable to efficiently separate exosomes containing Nef from virions, although more sensitive readouts suggested that some exosomes may have separated from virions⁶⁸⁴.

Isolation of exosomes by ultracentrifugation is known to cause exosome aggregation⁷¹⁹. Furthermore, activation or death of HIV-1-infected CD4⁺ T cells may result in the release of DNA into the extracellular environment, which could potentially cause aggregation of vesicles⁷⁶⁰. Supporting this are my own proteomic data (not shown) and reports demonstrating that EVs released by apoptotic cells contain histones in association with chromatin fragments which pellet at the bottom of density gradients^{493,761}. Such clumps of exosomes/virions may have moved through the density gradient at a greater rate than single particles, resulting in the bulk of exosome-associated protein co-purifying with virions. Furthermore, the greater ability to separate exosomes from virions in work I performed for the collaborative study of cGAMP may be partially explained by the low levels of cell death which occur in transfected 293T cells. Similarly, there may be cell type-specific differences in exosome composition which affect the rate at which exosomes move through the density gradient. Various groups have reported successful separation of exosomes from virions derived from plasma of HIV-1⁺ individuals^{728,762}, infected PBMC⁴⁵¹, primary CD4⁺ T cells^{706,708,763}, U937 cells^{707,729}, MDDCs⁷⁶⁴ and HEK293T cells⁴⁹³, although the identification of exosomes was sometimes based on AchE activity alone, which, as discussed below, can be misleading. Given that electron microscopy and immunoblotting for exosome markers confirmed exosome isolation in many of these studies, it is likely that the particularities of my method resulted in inefficient separation. Perhaps extra vortexing steps or reduction in cellular apoptosis would facilitate more monodisperse populations of EVs in ultracentrifugation pellets.

Many investigators have used AchE activity as a surrogate marker for exosomes, based originally on findings that reticulocyte exosomes contained AchE activity⁷⁶⁵. In many cases, AchE may be erroneously assumed to associate with vesicles⁷⁶⁶, because AchE is present in both membrane-associated and (predominantly) soluble enzyme forms^{767,768}. In my hands, the majority of AchE activity observed near the top of the gradient was already present in exosome-free media (Figure 5.3), indicating that this enzymatic activity was caused by soluble bovine AchE. In many studies, it is unclear whether such media controls were used, although some studies confirmed the presence of AchE in exosome lysates by immunoblotting⁴⁵¹. My results concur with other studies where the majority of Nef was located in higher-density fractions despite AchE activity in lower-density

fractions^{451,769}. Thus it seems that AchE may variably associate with vesicles depending on the experimental system or cellular model used. In fact, Luo *et al* found Nef associated with lower-density AchE⁺ fractions in EV secreted by 293T cells but not by Jurkat cells. They also proposed distinct subsets of AchE⁺CD81^{low}Tsg101^{low} and AchE⁺CD81^{high}Tsg101^{high} exosomes (although the former subset's detergent insolubility may indicate soluble protein rather than EVs). In that sense, I cannot absolutely rule out AchE activity as a marker of a subset of EVs, because a small amount of AchE activity observed in lower-density fractions of gradients separating EVs secreted by primary CD4⁺ T cells could not be explained by soluble bovine AchE alone. In the future I could perform AchE-specific immunoblotting and electron microscopy to confirm the presence of EVs in these lower-density fractions.

The second method commonly used to separate exosomes and virions is CD45 depletion, which is based on the knowledge that CD45 is excluded from virions^{494,495,726,770}. My data showed that CD45 depletion could separate Nef-containing exosomes from HIV-1-infected C8166 cells or HuT-78 cells, as Nef was predominantly observed in CD45⁺ eluates and not in the CD45 negative flowthrough fractions (Figure 5.5), a finding that is consistent with other studies⁴⁵³. In line with their role in virion/exosome biogenesis⁷⁷¹, the exosome markers Alix and Tsg101 were found in both CD45⁺ and CD45⁻ flowthrough of HuT-78 EVs. However, the lack of detectable Alix protein in the CD45⁺ eluate of C8166-secreted EVs is likely due to the presence of large amounts of large microvesicles (which are not enriched in Alix). Similarly, the calnexin observed in the CD45⁺ fraction is probably located within (possibly apoptotic) microvesicles, as observed for HuT-78-secreted microvesicles here and in numerous studies⁷⁷²⁻⁷⁷⁴. Interestingly, greater amounts and alternative forms of calnexin were relatively enriched in EVs secreted by HIV-1-infected HuT-78 cells compared to uninfected cells. Again, apoptosis caused by activation and abortive infection could potentially result in release of calnexin within microvesicles and smaller exosome-like vesicles.

Other groups have used a similar immunodepletion protocol to separate exosomes from virions. For example, anti-AchE was used by Cantin *et al* to isolate exosomes⁴⁹³, and Lee *et al* utilised anti- $\alpha v \beta 3$ to identify Nef-containing exosomes in the plasma of HIV-1-infected individuals⁷¹². It would be

interesting to separate EV populations secreted by HIV-1-infected macrophages or other myeloid cells by immunodepletion in order to compare their proteomic composition to those found in plasma.

Relative amounts of Nef and Gag in plasma EV and secreted by HIV-1-infected CD4⁺ T cells and macrophages

The data presented in this chapter support the findings of other studies, which showed that vesicle-associated Nef is present in the plasma of HIV-1⁺ patients at about 10 ng/ml^{453,454}. In contrast, Gag p24 protein concentration in plasma has been estimated at much lower levels of ~5 pg/ml⁷⁷⁵. In accordance with this, my data demonstrated that Gag was barely detectable in plasma EVs of most subjects who were at varying stages of disease, whereas Nef was detectable at all stages (Figure 5.2). In fact, the quantitated ratio of Nef/Gag was >1 for all subjects, supporting the notion that Nef is the predominant viral antigen in the plasma of HIV-1⁺ patients. Interestingly, Nef was detected in two patients on antiretroviral therapy, one with a viral load of <50 copies/ml. Detectable Nef in plasma of non-viraemic patients has been reported previously^{453,712}, and has important implications for HIV-1-associated disease because of the central role Nef plays in immunopathogenesis³⁶⁶. It would be interesting to determine the abundance of Nef in plasma EVs before the peak of viraemia when the first wave of CD8⁺ T cell responses is presumably primed by DCs.

As I did not separate plasma microvesicles from exosomes (and exosomes from virions) due to limited sample availability, I could not determine whether Nef was packaged in just one or both of these EV subtypes, although the authors of a recent study specifically purified plasma exosomes by density gradient ultracentrifugation and showed that Nef is abundant within these exosomes⁷¹². Furthermore, no statement can be made here about whether Nef is associated with the surface or the lumen of EVs, although one study used subtilisin treatment to cleave proteins unprotected by vesicular membrane to show that the majority of Nef was contained within the lumen⁴⁵⁰.

Proteins purified from plasma EVs of healthy seronegative donors showed a considerably different profile to those of plasma from HIV-1⁺ MM cohort patients. For example, the amounts of CD45, calnexin and β -actin were lower in plasma EVs of healthy controls than HIV-1⁺ plasma EVs. It is unlikely that this is a result of alterations caused by HIV-1-infection alone, as such differences were not observed by Lee *et al.* Alternatively, different sample processing could explain these differences,

as MM plasma samples were processed in the presence of EDTA anticoagulant, whereas blood from seronegative donors was drawn into heparinised tubes. Furthermore, specific bands for the proteins measured here were not detected in EVs isolated from plasma of a donor sampled at a timepoint prior to peak viraemia (data not shown). This plasma was processed in the presence of 4% citrate. One study suggested that citrate and EDTA anticoagulants decreased the microvesicle yield from plasma by inducing microvesicle adhesion to platelets⁵⁴⁴. Therefore it is conceivable that use of different anticoagulants at the time of blood collection could affect downstream EV characteristics or EV yield in plasma.

The molecular weight of Nef detected in EVs secreted by the chronically HIV-1-infected D10/HuT-78 cells was noticeably smaller than Nef detected in patient plasma EVs. Conceivably, the Nef from patient virus isolates was different in sequence and therefore a different molecular weight relative to the virus isolate within D10/HuT-78 cells. Alternatively, variable post-translational modification could explain the size difference, although Nef is not glycosylated under normal circumstances^{776,777}. Otherwise, the difference in size could simply be an artefact of the different amount of liquid loaded into each lane of the gel (less was loaded into D10/HuT-78 due to a higher concentration of protein in the lysate).

Gag p24 protein was extremely abundantly expressed within D10-/HuT-78 cells in contrast to plasma EVs. I observed a similar pattern of viral protein expression (Gag>Nef) by immunoblotting of EVs secreted by the CD4⁺ T cell lines C8166, HuT-78 and PM1 cells infected with HIV-1 IIIB. This pattern is not unique to artificial T cell lines, as a proteomic analysis of primary CD4⁺ T cells (and Jurkat T cells) also suggested that Gag was by far the most abundant protein. This expression pattern concurs with the known stoichiometry of abundant Gag protein, at least in HIV-1 virions where Gag comprises 50% of the virion mass^{778,779}.

The copious amount of Gag in HIV-1-infected CD4⁺ T cells provides a plausible explanation for the abundance of Gag-derived epitopes (and scarcity of Nef-derived epitopes) identified by immuno-peptidomics in chapter 4. However, there is a clear disconnect between the low Nef/Gag ratio in EVs secreted by infected CD4⁺ T cells and the high Nef/Gag ratio in plasma EVs of HIV-1⁺ individuals. Seeking to explain this disconnect, I examined EV secreted by HIV-1-infected

macrophages and found that expression of Nef and Gag was more balanced in this cell type. These data are supported by another study showing THP-1 macrophage-like cells secreted far more extracellular Nef than Gag, whereas Jurkat cells produced relatively large amounts of virion-associated Gag⁴⁵³. The potential mechanisms underlying this differential expression are not well understood, but some studies have reported that unintegrated HIV-1 genomes in circular ‘1-LTR’ or ‘2-LTR’ episomes can stably persist for unusually long times in non-dividing mature macrophages^{732,780}. Furthermore, transcription from these unintegrated episomes has repeatedly been shown to be biased towards expression of early genes Tat, Rev and Nef, although Nef is the only protein unequivocally shown to be translated from these transcripts^{733,781,782}. I also showed in one experiment that the Nef/Gag ratio in EV secreted by HIV-1-infected macrophages may increase over time in long-term cultures. Supporting this result, a study by Brown *et al* used a Nef-GFP bicistronic reporter virus to monitor the relative proportions of Nef-GFP⁺ and p24⁺ macrophages over 78 days. Interestingly, they observed a notable increase in a Nef-GFP^{hi}p24^{lo} population over the culture period which was suggested to represent a subset of macrophages harbouring unintegrated HIV-1 DNA⁷³⁷. Future experiments could examine timepoints past 42 days, or incorporate integrase inhibitors and integrase-defective viruses into macrophage infections to examine any alterations in viral protein expression. Whether treatment of macrophages with maturation agents or cytokines produced during AHI influences the kinetics of HIV-1 protein expression could also be explored. In addition, virus-like particles containing Vpx could be utilised in parallel to HIV-1 infection to examine EV secretion during HIV-2 infection. Modelling HIV-2 infection in the context of HIV-1 infection would be interesting because Nef is rarely targeted by CTL during HIV-2 infection^{359,360} and the mechanisms underlying this phenomenon are completely unknown. The highly productive phase of macrophage infection by HIV-2 is early, transient (similar to HIV-1-infected CD4⁺ T cells) and differs significantly from the kinetics of HIV-1 infection⁷⁸³. Speculatively, different kinetics of viral protein expression and cell death could account for potential differences in viral protein cargo within EVs from HIV-1 or HIV-2-infected macrophages, and therefore differential cross-priming in each infection.

Other cell types such as fibrocytes are infected by HIV-1 *in vitro*⁷⁸⁴. Fibrocytes are monocyte-derived cells exhibiting features of both macrophages and fibroblasts which have been shown to be capable of priming CD8⁺ T cells under some circumstances^{785,786}. Hashimoto *et al* directly compared macrophage and fibrocyte infection *in vitro* to suggest that fibrocytes display a much lower level of virion production which persists for longer periods of time than macrophages. Determining Nef expression and secretion in vesicles over time in fibrocytes would also be of interest.

Determining the *in vivo* cellular source of the abundant Nef-containing exosomes is of importance, as these exosomes represent a key mediator of immunopathogenesis during HIV-1 infection and the cells producing them constitute a target for prophylactic or therapeutic intervention. My data hint at the possibility of long-lived macrophages being this Nef-producing population. Long-lived macrophages are known to accumulate in gut mucosa and other local tissues and to sustain viral replication in the absence of T cells during HIV/SIV infection^{731,787–790}, hence if a proportion of these cells secreted Nef it could explain the abundance of Nef exosomes.

In summary, the data in this chapter indicate that:

- A differential ultracentrifugation protocol efficiently separates EVs (microvesicles and exosomes) from cells and cell debris in cell culture supernatants
- Nef is predominantly contained within CD45⁺ EV secreted by HIV-1-infected CD4⁺ T cells and not virions
- Gag is by far the most abundant antigen produced by HIV-1-infected CD4⁺ T cells; but relatively more Nef than Gag is present in plasma EVs of HIV-1⁺ patients during early, chronic and treated infection
- Macrophages potentially represent a source of abundant EV-associated Nef antigen

Chapter 6: The cross-presented HIV-1 immunopeptidome of DCs

6.1 Introduction

The first wave of CD8⁺ T cell responses during AHI is biased towards recognition of Nef epitopes. This thesis attempts to test the hypothesis that DCs preferentially cross-present Nef epitopes to naïve CD8⁺ T cells during AHI. Cross-presentation, the process by which bystander APC are able to endocytose exogenous antigen and present peptides on MHC class I, is believed to be important for immunity to viral pathogens which do not naturally infect DCs. Although the relative importance of direct- and cross-presentation for immunity against particular pathogens is still debated, examples of non-infected DCs cross-priming CTL *in vivo* have been documented. For example, lymph node-resident CD8α⁺ DCs in mice seem to play an important role in activating CTL targeting HSV-1 infections of distal skin sites by receiving antigen from migratory CD103⁺ DCs^{791–793}. Other herpesviruses such as CMV encode numerous proteins to evade MHC class I recognition by CTL and therefore infected DCs are unable to prime naïve CD8⁺ T cells⁷⁹⁴. Instead, cross-presentation seems to be the dominant mode of CD8⁺ T cell priming during CMV infection *in vivo*^{795,796}. During pulmonary influenza infections of mice, multiple subsets of DCs have been shown to cross-present antigen for CD8⁺ T cell cross-priming in temporally distinct phases^{797–800}. Lastly, in Batf3-deficient mice, which do not develop the cross-presenting CD8⁺ and CD103⁺ DC subsets, activation of Sendai virus- or West Nile virus-specific CD8⁺ T cell immunity is impaired^{801,802}.

Conversely, the relative importance of cross-presentation in development of immunity to HIV-1 is not resolved. DCs can be infected with HIV-1, as they express CD4 and chemokine co-receptors, but infection is generally not productive, in large part due to restriction factors such as SAMHD1 which inhibits reverse transcription of the HIV-1 genome^{803,804}. Cross-presentation may therefore be important for priming of naïve CD4⁺ and CD8⁺ T cells during AHI. A number of studies showed that DCs pulsed with live or aldrithiol-inactivated HIV-1 virions are able to induce expansion or activation of HIV-1-specific T cells, and this cross-presentation relied upon virion-receptor interactions and membrane fusion^{533,805–808}. Aldrithiol-inactivated SIV administered on the tonsils was shown to partially protect macaques against repeated viral challenge⁸⁰⁹, and *in vitro* cross-presentation of inactivated SIV by DCs was shown to activate CTL⁸¹⁰. Furthermore, therapeutic

vaccination of SIV-infected macaques or HIV-1-infected individuals with DCs pulsed with inactivated SIV/HIV-1 particles was shown to reduce viral load^{811,812}. Targeting of HIV-1 protein to particular receptors has also been investigated as a potential HIV-1 vaccine candidate. For example, targeting of HIV Gag protein to DEC-205 was shown to enhance cross-presentation by DCs in comparison to DC-SIGN-targeted antigen²³⁹. The maturation state of the cross-presenting DCs also plays a role, as LPS maturation during virion capture enhanced the ability of DCs to activate T cells whereas prior LPS maturation was detrimental⁸¹³.

DC maturation state also affects cross-presentation of antigen derived from live and apoptotic HIV-1-infected cells by DCs. In one study, immature DCs loaded with necrotic HIV-1-infected cells were superior to matched matured DCs in terms of CD8⁺ T cell activation⁵³⁶. Furthermore, therapeutic vaccination with autologous DCs loaded with autologous HIV-1-infected apoptotic CD4⁺ T cells modestly reduced plasma viral load in untreated individuals⁸¹⁴. The source of antigen for DC cross-presentation is not limited to apoptotic or necrotic cells, as IL-4-DCs also cross-present HIV-1 antigen efficiently from live cells⁸¹⁵.

An important question for vaccinologists is whether DC cross-presented HIV-1 epitopes are also directly-presented by HIV-1 infected cells. In murine CMV infection, where cross-presentation is the main mechanism of CD8⁺ T cell priming, cross-presented epitopes differ from directly-presented epitopes and therefore cross-primed CTL do not recognise infected cells⁷⁹⁶. Interestingly, 'cross-presentation' of incoming virion-derived protein by newly-infected CD4⁺ T cells can be recognised by CTL in the first few hours of infection^{124,125,816}, although CD4⁺ T cells are not capable of priming naïve CD8⁺ T cells. Differential antigen processing between cell types has been documented. Dinter *et al* used *in vitro* peptide degradation assays to demonstrate that CD4⁺ T cells, DCs, macrophages and monocytes have differential antigen-processing activities which contribute to significantly altered levels of recognition by CTL clones^{559,560}. These antigen degradation patterns were also shown to correlate with the production of immunodominant epitopes⁵⁶¹. The same group also recently showed that protease inhibitors prescribed to HIV⁺ patients can differentially modulate antigen processing activities in CD4⁺ T cells and myeloid cells⁸¹⁷. However, the overall similarity between the DC and CD4⁺ T cell immunopeptidomes has not been explored to date. Knowledge of

immuno-peptidomic overlap between cross-presenting cells and infected cells would facilitate rational design of CD8⁺ T cell vaccines, enabling induction of maximally-effective CTL.

Another mechanism that DCs can use to activate CD8⁺ T cells *in vivo* is 'cross-dressing', i.e. the acquisition of fully formed peptide-MHC complexes from cells or vesicles²⁷³. This alternative method of antigen presentation could be advantageous because it would encourage priming of CD8⁺ T cells specific for epitopes directly-presented on HIV-1-infected cells. DC cross-dressing in mouse models of viral infection has been demonstrated to activate both naïve and memory CD8⁺ T cells *in vivo*^{272,818}. CD8⁺ T cell priming by cross-dressed DCs has also been demonstrated in the context of cancer immunity^{280,819}. However, whether DCs can be cross-dressed by peptide-HLA class I complexes derived from HIV-1-infected cells and their functional relevance has not yet been studied.

Over the years, the nature of cross-presented proteins has been a matter of debate. Although both mechanisms are not mutually exclusive, two main models have been postulated to explain the sources of cross-presented antigen. In the first model it was proposed that peptides which had been degraded in the donor cell were bound and chaperoned by heat shock proteins such as gp96 then taken up by receptor-mediated endocytosis into antigen-presenting cells for cross-presentation^{820–823}. However, the role of gp96 in cross-presentation is contested, as knockout of gp96 in donor cells did not reduce their ability to cross-prime CD8⁺ T cells *in vivo*⁸²⁴. In fact, it is now generally accepted that intact protein is the main source of antigen for cross-presentation, although exceptions exist where unique extended peptides or protein fragments can escape degradation and be cross-presented^{825–828}. Evidence for a role for intact protein in cross-presentation came from studies where the cross-priming activity localised in the cellular compartments containing intact antigen rather than with heat shock proteins or peptide degradation products⁸²⁹. Secondly, particular long-lived and stable proteins seem to be cross-presented more efficiently than unstable products^{829–833}. Furthermore, proteasome activity in the donor cell does not seem to be required for cross-presentation⁸³⁴. Lastly, when immunogenic peptides are inserted into protein signal sequences which are cleaved during biogenesis, they are poorly cross-presented⁸³⁵. However, although whole protein seems to be the dominant source of antigen for cross-presentation, a role for heat shock proteins in chaperoning such proteins cannot be excluded and may be important in certain circumstances^{836,837}.

The subcellular location of antigen is also thought to be important in determining cross-priming of CTL responses, although most of the data is based solely on various ovalbumin (OVA) constructs. For example, Shen *et al* showed that a membrane-associated OVA had a significantly longer half-life and stimulated greater T cell activity than cytosolic OVA⁸²⁹. Similarly, Henning *et al* used OVA constructs expressed by adenoviral vectors to show that the subcellular location was important for the immunogenicity of OVA⁸³⁸. Moreover, one group suggested that vaccinia virus avoids cross-presentation by sequestering protein in ‘virus factories’ within cells during late infection⁸³⁹. Similarly, the lack of cross-presentation of LCMV glycoprotein (GP) could be overcome by expression of an ER-retained GP in infected cells which resulted in significantly larger CTL responses⁸⁴⁰. Lastly, one study found that cross-presentation is most efficient when antigen is produced in large amounts and is also associated with apoptosis^{841,842}. Furthermore, chemotherapy-induced tumour cell apoptosis was shown to increase the availability of nuclear antigen for cross-presentation in another study⁸⁴³. Although these studies have greatly contributed to the collective knowledge of cross-presentation, a limited number of antigens were investigated overall. Therefore, an exploration of the global repertoire of peptides/proteins cross-presented by DCs would provide useful information for vaccine development and understanding of the mechanisms of cross-presentation.

In this thesis I hypothesised that the expansion of Nef-specific CD8⁺ T cell responses during AHI may result from preferential cross-presentation of Nef epitopes by DCs. As highlighted above, little is known about the repertoire of HIV-1 peptides cross-presented by DC endocytosing various forms of antigen. To explore this hypothesis, I implemented the immunopeptidomics approach introduced in Chapter 4 to define the DC cross-presented HIV-1 immunopeptidome.

In this chapter I aimed to:

- Develop an *in vitro* cross-presentation assay to examine the repertoire of HIV-1 peptides cross-presented by DC pulsed with infected cellular antigen or EV
- Comparatively evaluate the immunopeptidomes of *in vitro* monocyte-derived DC and autologous CD4⁺ T cells
- Assess the repertoire of proteins which are sampled by the DC cross-presentation pathway

6.2 Results

6.2.1 Development of a method to investigate the dendritic cell cross-presented immuno-peptidome

Previously, cross-presentation has predominantly been studied in the context of a limited number of model antigens containing well-described epitopes such as SIINFEKL from ovalbumin using T cell clones as a readout²³⁵. However, no studies have so far used a proteomics approach to discern the repertoire of epitopes cross-presented or directly-presented by DCs on HLA class I in the setting of viral infections. To identify cross-presented HIV-1 epitopes, a cross-presentation assay was developed whereby HIV-1 epitopes were naturally processed and presented by monocyte-derived DCs endocytosing antigen from HIV-1-infected cells or EV. The HLA immunoprecipitation and LC-MS/MS approach described in Chapter 4 was used to determine the identity of HIV-1 epitopes cross-presented by DCs.

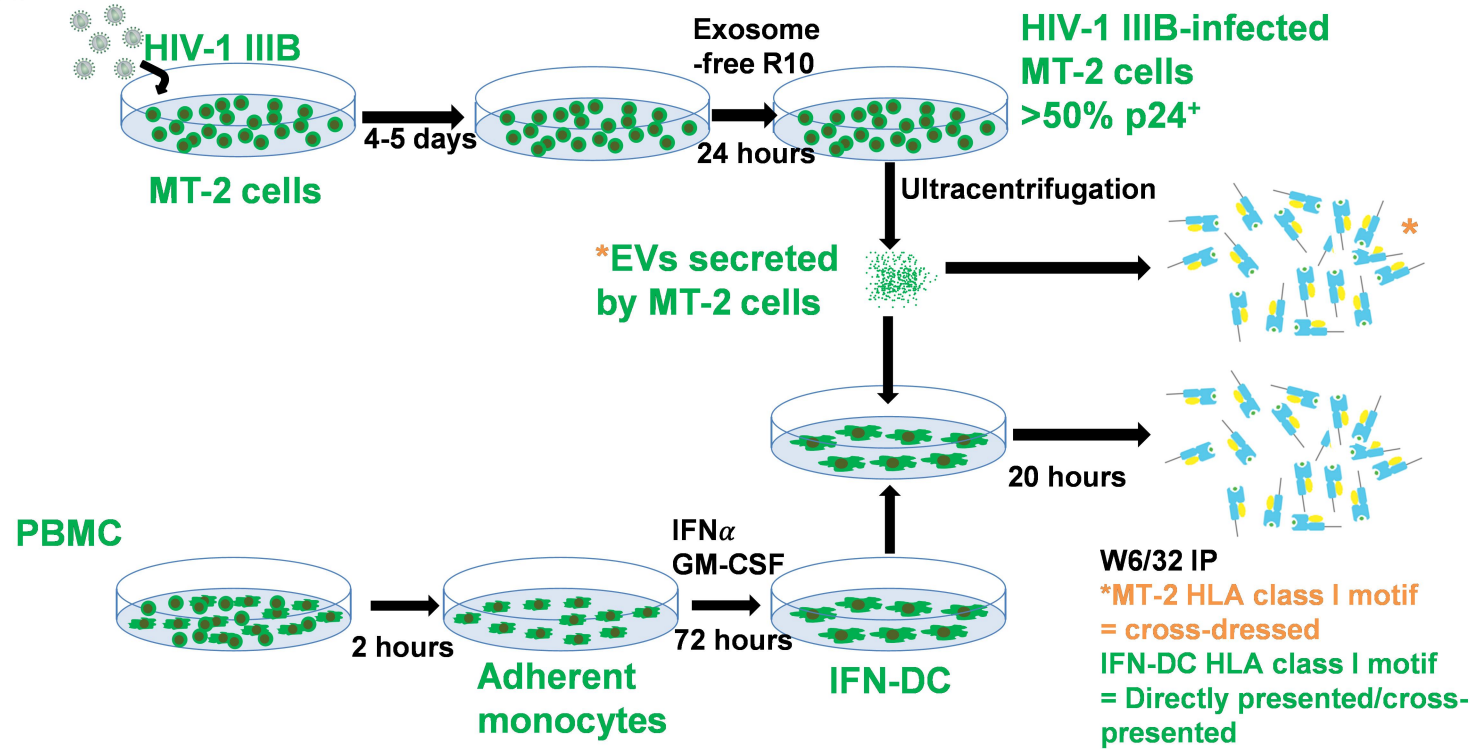
IL-4-DCs, which are differentiated by culturing monocytes in the presence of IL-4 and GM-CSF, are commonly used for *in vitro* assays of DC function. However, as IL-4 cytokine levels are not elevated in plasma during AHI⁴³, the *in vivo* equivalents of *in vitro* IL-4-DCs are unlikely to be present in great numbers within tissues. On the other hand, IFN α is strongly elevated during AHI and remains high during subsequent untreated chronic HIV-1 infection⁴³. Therefore, IFN-DCs which are differentiated with IFN α and GM-CSF, were the antigen-presenting cell of choice due to their reported superior cross-presentation ability, relative resistance to HIV-1 infection and physiological relevance⁵¹²⁻⁵¹⁵. Reasoning that IFN-DCs are unlikely to become productively infected with HIV-1 in less than 24 hours after exposure to HIV-1-infected antigen, co-cultures of IFN-DCs and HIV-1-infected cells were incubated overnight to allow sufficient time for IFN-DCs to endocytose, process and present HIV-1 antigen on HLA class I.

A key component of the working hypothesis of this thesis was that DCs uptake and process EVs present during AHI as a source of antigen for cross-presentation. EVs are abundantly secreted during AHI⁴⁵ and can be a rich source of peptide-HLA complexes for cross-dressing or viral antigen for cross-presentation by DCs²⁷⁷. To gauge the contribution of EVs secreted by HIV-1-infected cells to cross-dressing or cross-presentation by DCs, the repertoire of HIV-1 peptides cross-presented by

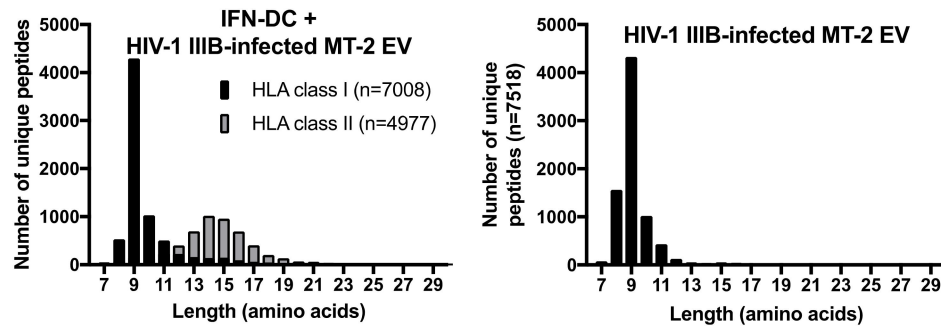
DCs interacting with EVs derived from HIV-1-infected cells was examined. The CD4⁺ T cell line MT-2 was infected with the lab-adapted X4-tropic HIV-1 strain IIIB until >50% of cells were live and p24⁺ as determined by intracellular p24 staining (data not shown). Total EVs (both microvesicles and exosomes/virions) secreted by HIV-1 IIIB-infected or matched uninfected cells were subsequently isolated and added to IFN-DCs overnight then HLA IP was performed sequentially with W6/32 and L243 antibodies for HLA class I and HLA-DR, respectively. Because EVs such as exosomes are known to present peptide-MHC class I complexes capable of activating T cells, HLA class I was also immunoprecipitated from isolated MT-2-derived EVs in order to determine the contribution of ‘cross-dressing’ to the IFN-DC immunopeptidome (Figure 6.1A). 7008 unique peptides were identified in the W6/32 IP from IFN-DCs pulsed with EVs secreted by HIV-1 IIIB-infected MT-2 cells (Figure 6.1B). From the same cells, 4977 peptides with a modal length of 14 amino acids were identified in the L243 IP, in accordance with the known peptide length preferences of HLA class II. 7158 peptides were observed in the W6/32 IP from MT-2 EVs alone. Although 9-mers were the most common length of peptide identified in both W6/32 IPs, a lower proportion of 8-mers were observed in the IFN-DC sample than for MT-2 EVs, likely reflecting a difference in peptide length preferences for the HLA expressed by each cell type. Despite this divergence in length distribution, 1039 peptides were common to both samples (Figure 6.1C). When 9-mers from the IFN-DC sample were clustered (using the online Gibbs Clustering server) into groups with shared sequence motifs, 3 distinct clusters emerged which represented the known binding preference for HLA-A*01:01/HLA-B*15:01, HLA-A*02:01 and HLA-B*40:01 expressed by the IFN-DC donor (Figure 6.1D, top row). Divergent clusters indicative of HLA-C-bound peptides were not observed. Similarly, 3 individual sequence motifs emerged from clustering of 9-mers from the HIV-1 IIIB-infected MT-2 EVs (Figure 6.1D, bottom row). These motifs were comparable to the known binding motifs for HLA-A*24:02, HLA-B*40:02 and HLA-B*51:01, which are expressed by MT-2 cells. Examination of 9-mers identified in both samples revealed binding motifs representative of HLA-A*24:02, HLA-B*51:01 and HLA-B*40:01/HLA-B*40:02, indicating that peptide-HLA complexes derived from MT-2 EVs had been transferred to IFN-DC through cross-dressing (Figure 6.1E). Shared HIV-1 peptides were also isolated in IFN-DC and MT-2 EV samples, including the known HLA-A*24:01-restricted gp160 epitope RYLKDQQLL, and Gag p2 fragments

FLGKWIPS/FLGKIWPSY (Table 6.1). Additionally, a 17-mer cryptic peptide from a negative sense translation of the HIV-1 genome and 10-mer from Pol (LTQQIRKSLY; LY10) were identified in the IFN-DC sample. Although, LY10 is predicted to bind either HLA-A*01:01 or HLA-B*15:01 expressed by the IFN-DC donor, both LY10 and the cryptic 17-mer were assigned relatively low confidence identification scores by PEAKS, and would therefore require further validation by synthetic peptides.

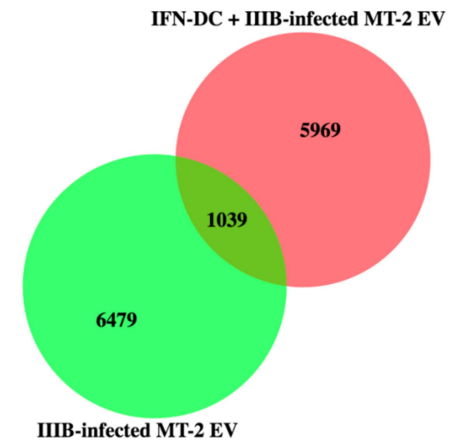
A



B



C



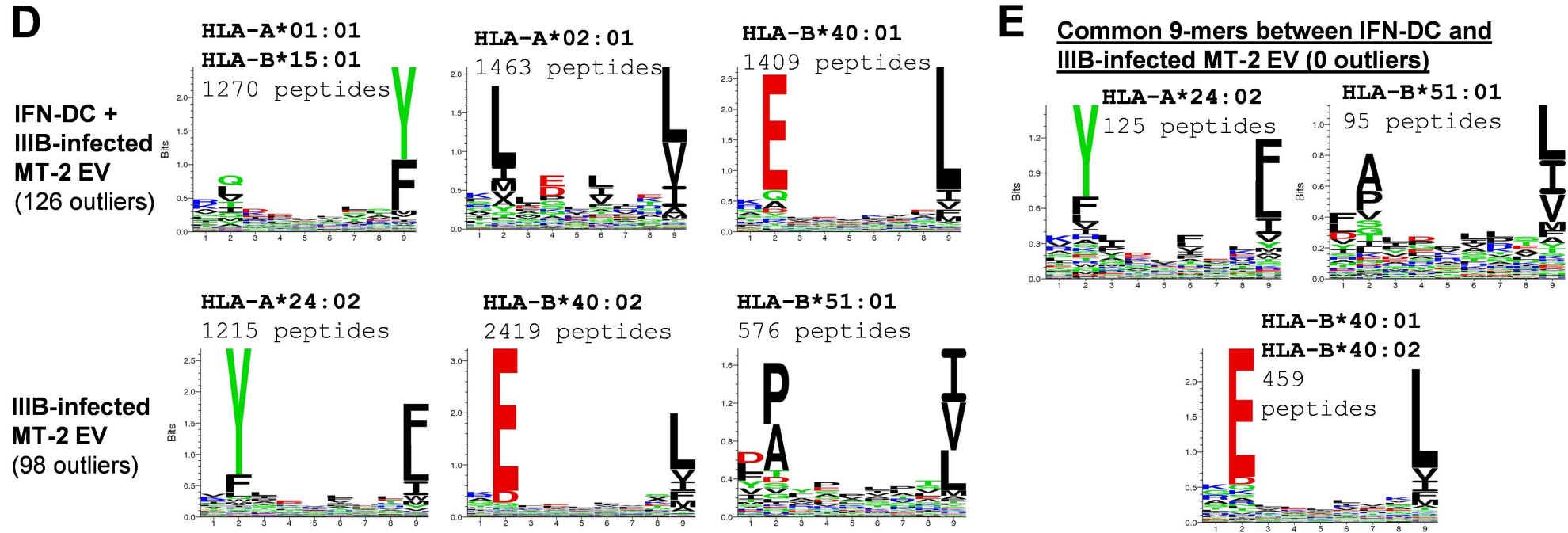


Figure 6.1 *IFN-DCs cross-present and are cross-dressed with HIV-1 epitopes from EVs secreted by HIV-1 IIIB-infected MT-2 cells.* **A.** 1.9×10^8 IFN-DCs from donor 222 (HLA: A*01:01, A*02:01, B*15:01, B*40:01, C*03:03, C*07:02) were co-cultured with total EVs isolated from the supernatant of 2×10^8 uninfected or HIV-1 IIIB-infected MT-2 cells (HLA: A*24:02, B*40:02, B*51:01, C*03:03, C*14:02) for 20 hours. IFN-DC were then lysed and MHC class I peptides were immunoprecipitated with W6/32-linked Sepharose beads. HLA-DR was also immunoprecipitated from IFN-DC flowthrough lysate using the L243 antibody. HLA class I peptides were also eluted directly from total EVs isolated from 2×10^8 uninfected or HIV-1 IIIB-infected MT-2 cells using W6/32-linked Sepharose. Peptides were analysed by LC-MS/MS on a TripleTOF 5600 mass spectrometer. Peptides which were previously identified in CD4.221 cells alone by W6/32 IP or HIV-1 peptides identified by OKT4 IP (see Chapter 4) were excluded from further analyses (exclusion list shown in Appendix 9.3, Tables 9.9, 9.10). **B.** Size distribution of peptides eluted from IFN-DCs pulsed with EVs from HIV-1 IIIB-infected MT-2 cells and of peptides eluted directly from IIIB-infected MT-2 EVs. **C.** Overlap in peptide identifications between IFN-DCs pulsed with EVs from IIIB-infected MT-2 cells and the EVs. **D.** 9-mers identified in either uninfected or IIIB-infected co-cultures were grouped using the online Gibbs Clustering server. The optimal fit (Kullbach-Leibler Distance; KLD) made 3 distinct clusters for which the sequence logos are shown for each sample. Sequence logos were assigned to one of the HLA types expressed by the IFN-DC donor by comparison to known motifs in the literature and MHC Motif Viewer. **E.** 9-mers shared between IFN-DCs pulsed with MT-2 EVs and MT-2 EVs alone were clustered using the Gibbs Clustering server as in D.

IFN-DC (HLA: A*01:01, A*02:01, B*15:01, B*40:01, C*03:03, C*07:02) + MT-2 EV (HLA:A*24:02, B*40:02, B*51:01, C*03:03, C*07:02)							
LGPRDEMLGGCQTSTLT	17	Cryptic (- sense)	-	14.72	1777.8339	-	-
FLGKIWPS	8	Gag	433-440	21.08	946.5276	-	HLA-A*02:01
FLGKIWPSY	9	Gag	433-441	52.74	1109.5909	HLA-A*01:01/HLA-B*15:01	-
LTQQIRKLSY*	10	Pol	523-532	14.55	1248.719	HLA-A*01:01/HLA-B*15:01	-
RYLKDQQLL	9	gp160	585-593	22.5	1175.6663	HLA-A*24:02/HLA-C*07:02/HLA-C*14:02	HLA-A*24:02
MT-2 EV (HLA:A*24:02, B*40:02, B*51:01, C*03:03, C*07:02)							
FLGKIWP	7	Gag	433-439	27.78	859.4956	-	-
EPTAPPFL	8	Gag	454-461	20.74	870.4487	HLA-B*51:01	-
FLGKIWPS	8	Gag	433-440	17.81	946.5276	-	HLA-A*02:01
LYNTVATL	8	Gag	78-85	22.11	893.4858	-	HLA-A*24:02/HLA-C*14:02
TERQANFL	8	Gag	427-434	19.05	977.493	HLA-B*40:02	HLA-B*40:02
YESPHPRI	8	Vif	44-51	22.36	997.4981	-	-
AFHHVAREL	9	Nef	190-198	18.94	1078.5671	HLA-C*14:02	HLA-A*24:02
FLGKIWPSY	9	Gag	433-441	36.87	1109.5909	-	-
KELYPLTSL	9	Gag	481-489	33.11	1062.5961	HLA-B*40:02	HLA-B*40:02
PEPTAPPFL	9	Gag	453-461	12.95	967.5015	-	-
YSTQVDPFL	9	Vif	94-102	26.7	1050.4869	HLA-C*03:03/HLA-C*14:02	-
AEWDRVHPVH	10	Gag	210-219	13.98	1244.605	HLA-B*40:02	-
IEIKDTKEAL	10	Gag	92-101	14.86	1158.6495	HLA-B*40:02	HLA-B*40:02
PSYKGRPGNF	10	Gag	439-448	28.64	1121.5618	HLA-A*24:02	-
AEWDRVHPVHA	11	Gag	210-220	22.71	1315.6421	-	-
GENTSLHPVSL	12	Nef	159-170	49.5	1265.6615	HLA-B*40:02	-
TDIQTKEK	8	Pol	921-928	28.18	946.4971	-	HLA-B*40:02
ADRQGTVSF	9	Pol	46-54	16.2	979.4723	-	-
KEYAFFYKL	9	gp160	171-179	33.28	1207.6277	HLA-B*40:02	-
RYLKDQQLL	9	gp160	585-593	25.75	1175.6663	HLA-A*24:02/HLA-C*14:02	HLA-A*24:02
YELHPDKWTVPPIVL	15	Pol	387-401	25.23	1836.9774	HLA-B*40:02	-
YQYNVLPQGKGGSPA	15	Pol	299-313	26.81	1706.8416	-	-

On the other hand, 22 HIV-1 peptides were identified as presented by HLA class I on EVs secreted by HIV-1-infected MT-2 cells. 15/22 peptides have been reported or were predicted to bind to one of the alleles expressed by MT-2 cells (8 for HLA-B*40:02, 4 for HLA-A*24:02). For example, the Gag epitopes TERQANFL and KELYPLTSL have both been described previously¹²⁰, whilst gp160-derived KEYAFFYKL represents a new target for HLA-B*40:02-restricted CD8⁺ T cells. In summary, these data indicate that IFN-DCs may be cross-dressed by peptide-HLA class I complexes present in EVs secreted by HIV-1-infected cells. Hence in this particular experimental setup I was unable to confidently separate the process of cross-presentation from cross-dressing.

In vivo, DCs are likely to interact directly with HIV-1-infected cells as well as with vesicular products such as exosomes. IFN-DCs from donor 293 (HLA: A*01:01, A*03:01, B*35:01, B*40:02, C*02:02, C*04:01) were co-cultured overnight with autologous CD4⁺ T cells infected with HIV-1 IIIB then the two cell populations were separated using CD3 microbeads (based on the assumption that only CD4⁺ T cells would express CD3). Although more larger and more granular cells were observed in the CD3⁻ flowthrough, a substantial population (17.4% of events) of small cells with lower expression of HLA-DR and CD11c remained (Figure 6.2A). Similarly, large and granular cells with high HLA-DR and CD11c expression (16% of events) were observed in the CD3⁺ eluate fraction. A greater number of peptides were eluted from the W6/32 IP of the CD3⁻ than from the CD3⁺ cell fraction (CD3⁻ n=2199; CD3⁺ n=783) (Figure 6.2B). Equally, in accordance with the elevated expression of HLA class II on IFN-DCs, greater than 10 times the number of unique HLA-DR-restricted peptides were identified in the CD3⁻ than in the CD3⁺ sample (CD3⁻ n=5040, CD3⁺ n=415). As expected, the majority of HLA class I peptides were between 8-11 amino acids in length, whilst the peptides eluted from L243 IP were predominantly between 13-17 amino acids in length. Furthermore, 54% (n=423) of the HLA class I peptides identified in the CD3⁺ fraction were also observed in the CD3⁻ sample (Figure 6.2C). 9-mers identified in the W6/32 IP were clustered and 4 distinct motifs were clustered for the CD3⁻ fraction, whilst just 2 motifs were identified for the CD3⁺ fraction (Figure 6.2D). These clusters corresponded to the known HLA class I alleles expressed by donor 293. Unfortunately, no HIV-1 epitopes were identified in either sample tested, probably because the CD4⁺ T cells from this donor were relatively poorly infected with HIV-1 IIIB. These

data indicated that the separation of IFN-DC from CD4⁺ T cells was incomplete (or that exchange of peptide HLA complexes through cross-dressing had occurred) and therefore that peptides identified in each fraction may have been presented on HLA class I by either cell population.

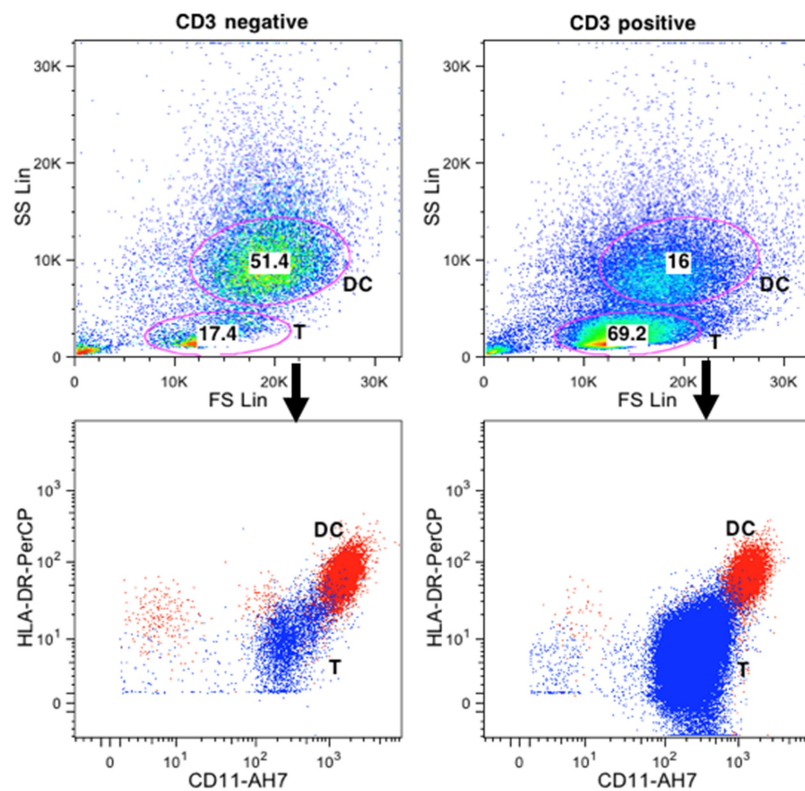
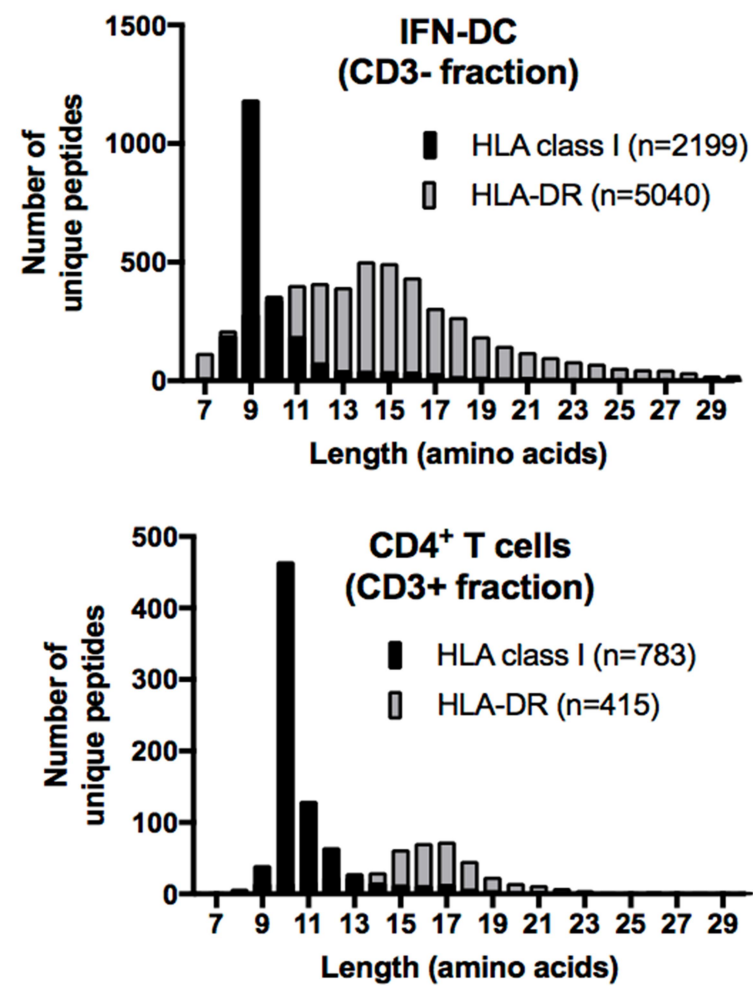
A**B**

Figure 6.2 continued on next page

Figure 6.2 (continued)

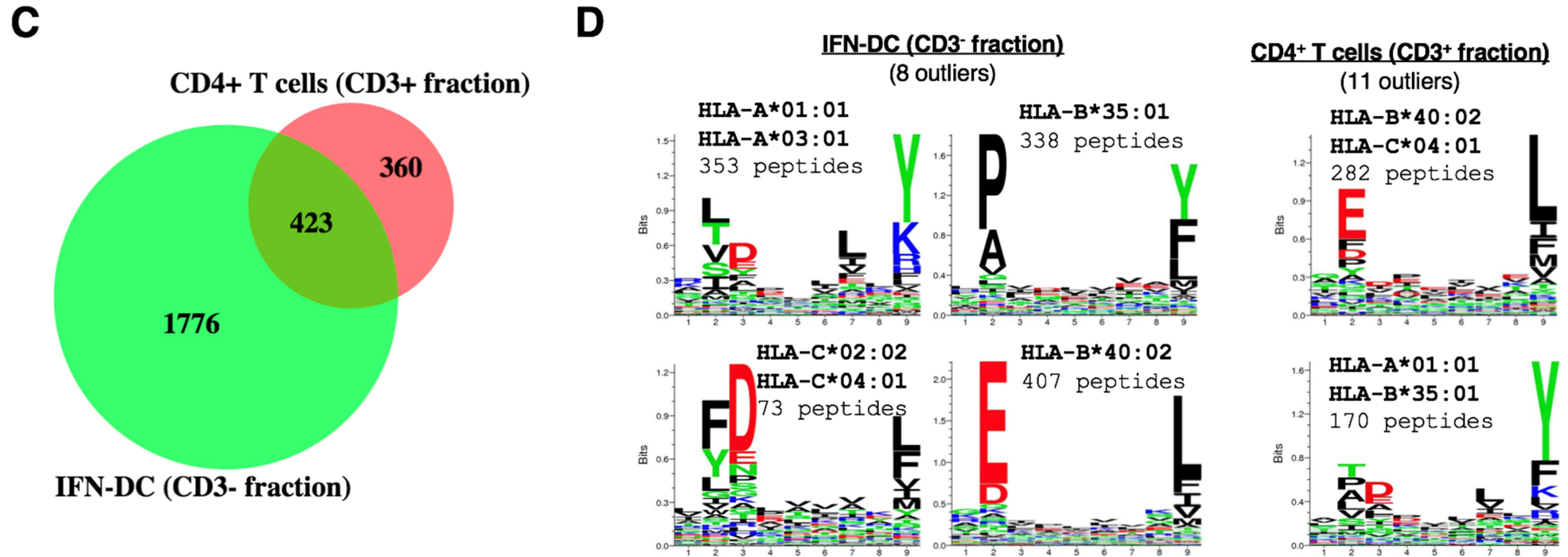


Figure 6.2 *Elution of peptides from IFN-DCs co-cultured with primary CD4⁺ T cells.* 8.4×10^7 IFN-DCs from donor 200 (HLA: A*01:01, A*03:01, B*35:01, B*40:02, C*02:02, C*04:01) were co-cultured with 8.4×10^7 autologous uninfected or HIV-1 IIIB-infected primary CD4⁺ T cells at a ratio of 1:1 for 20 hours. Co-cultures were then separated using CD3 microbeads into CD3⁻ (3×10^7 cells) and CD3⁺ (5.4×10^7 cells) fractions. Cells were then lysed and HLA class I was immunoprecipitated with W6/32-linked Sepharose beads and bound peptides eluted. HLA-DR-bound peptides were also subsequently eluted from L243 IP. Data were acquired by LC-MS/MS on a TripleTOF 5600 mass spectrometer. **A.** Flow cytometry analysis of CD3⁻ and CD3⁺ fractions. Based on forward scatter and side scatter characteristics a large, granular DC gate and a smaller T cell gate were set as shown. The expression of HLA-DR and CD11c on each population is shown in the bottom row. **B.** Size distribution of HLA class I (black) and HLA-DR (grey) peptides eluted from CD3⁻ and CD3⁺ cell fractions. **C.** Overlap in peptide identifications between CD3⁻ (presumed IFN-DCs) and CD3⁺ (presumed CD4⁺ T) cells. **D.** 9-mers identified in either CD3⁻ or CD3⁺ fractions were grouped using the online Gibbs Clustering server. The optimal fit (Kullback-Leibler Distance; KLD) made 4 distinct clusters in the CD3⁻ fraction and 2 distinct clusters in the CD3⁺ fraction for which the sequence logos are shown. Sequence logos were assigned to one of the HLA class I types expressed by the IFN-DC/CD4⁺ T cell donor by comparison to known motifs in the literature and MHC Motif Viewer.

The low efficiency of the separation may have resulted from the acquisition of CD3 molecules by IFN-DCs through membrane transfer from CD4⁺ T cells. Hypothesising that the reverse process whereby IFN-DCs transfer membrane fragments to CD4⁺ T cells may happen less frequently, a strategy for positive selection of IFN-DCs was devised. Because *in vitro*-generated IFN-DCs upregulate BDCA4 (CD304) expression⁸⁴⁴, BDCA4 microbeads were utilised to enable separation of IFN-DCs from CD4⁺ T cells after overnight co-culture. In this experiment, the CD4⁺ T cell line PM1 was co-cultured with IFN-DCs for two reasons; 1) PM1 cells are easily infected with HIV-1 IIIB (>50% live p24⁺ cells) and 2) the different set of HLA class I alleles expressed by PM1 cells and donor IFN-DCs would potentially allow greater discrimination between peptides presented by each cell type.

Approximately 2×10^8 and 5×10^7 cells were isolated in the BDCA4⁻ and BDCA4⁺ populations after co-culture, respectively. A greater number of unique HLA class I peptides were eluted from the BDCA4⁻ population (n=6559) than from the BDCA4⁺ presumptive IFN-DC-enriched population sample (n=3091) (Figure 6.3A). Similarly, more unique HLA-DR-associated peptides were identified in the BDCA4⁻ sample (n=6498) than in the BDCA4⁺ cells (n=4089). This may have been because the BDCA4⁻ fraction contained more cells, and/or because this fraction included both PM1 cells and IFN-DC that had not been enriched from them by positive selection. Strikingly, of the 3091 peptides observed in the W6/32 IP of the BDCA4⁺ cell population, >75% (n=2345) were also seen in the BDCA4⁻ population (Figure 6.3B), supporting the notion that IFN-DC and PM1 cells were not fully separated. When 9-mers from each sample were grouped using the online Gibbs Clustering server, 3 motifs emerged which were representative of the HLA-A*01:01, HLA-B*44:05 and HLA-A24:02/HLA-B*15:01 (Figure 6.3C). These three motifs were conspicuously similar comparatively between the BDCA4⁻ and BDCA4⁺ samples. Of the 14 HIV-1-derived peptides identified in the BDCA4⁻ negative sample, 6 were also observed in the BDCA4⁺ sample (Table 6.2). Two cryptic peptides in a negative sense translation of the HIV-1 genome were seen, but the identification scores were relatively low so these peptides would require further validation. The majority of the HIV-1 peptides derived from Gag, Pol and gp160 proteins, although one Tat epitope (ITKALGISY) reportedly restricted by HLA-B*15:01⁸⁴⁵ was present in the BDCA4⁻ sample. In fact, 9/15 HIV-1

peptides observed were predicted to bind to one of the alleles expressed by either the donor IFN-DCs or PM1 cells. In conclusion, these data indicate that separation of IFN-DCs from HIV-1-infected PM1 cells using BDCA4 microbeads was unsuccessful and that cross-presented HIV-1 epitopes cannot be determined using this method.

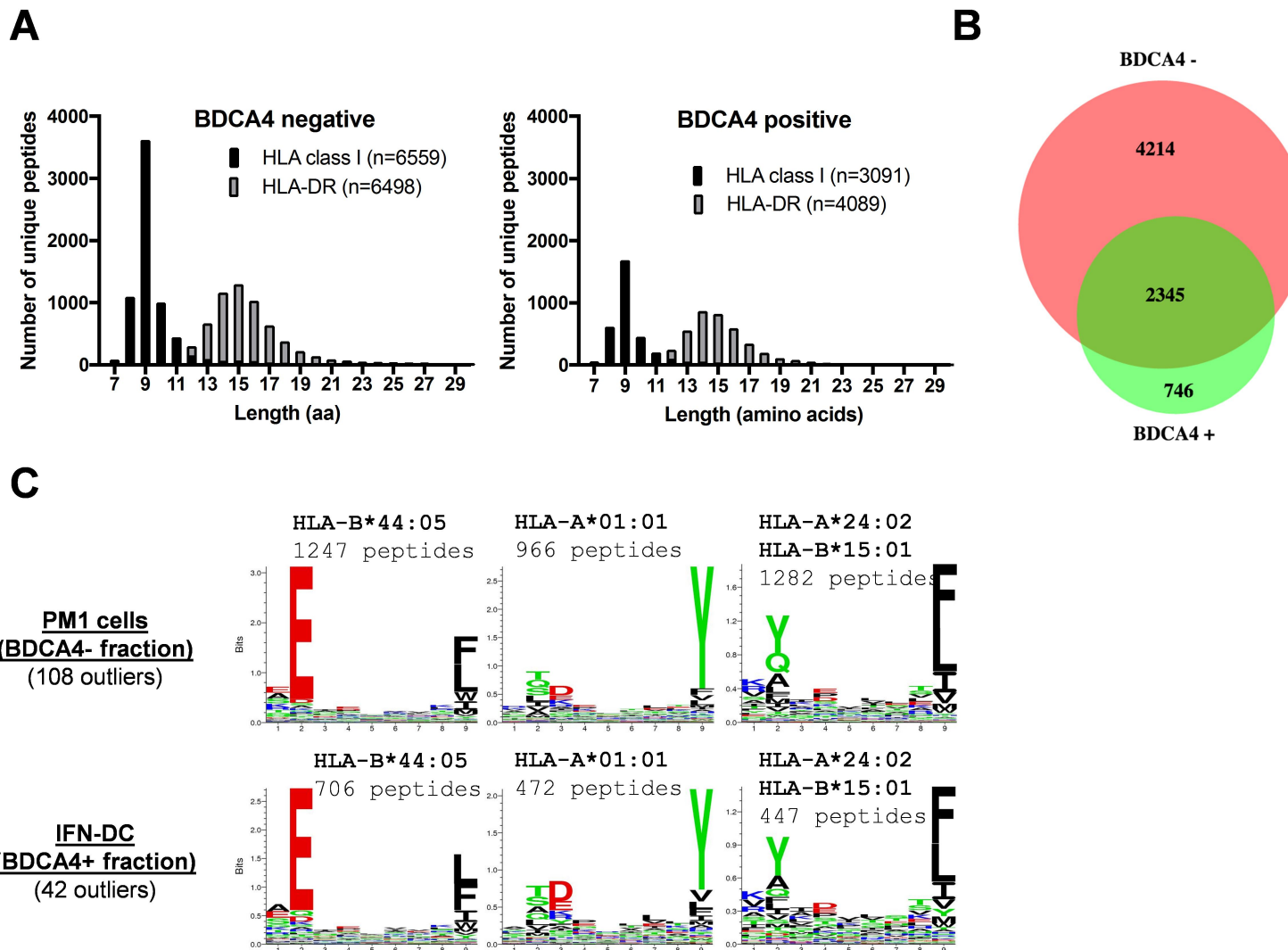


Figure 6.3 Elution of peptides from IFN-DCs co-cultured with HIV-1 IIIB-infected PM1 cells. IFN-DCs from donor 279 (HLA: A*01:01, A*24:02, B*44:05, B*52:01, C*02:02, C*12:02) were co-cultured with HIV-1 IIIB-infected PM1 cells (HLA:A*01:01, B*15:01, C*03:03 homozygous) for 20 hours then separated using BDCA4 microbeads into BDCA4⁻ and BDCA4⁺ cell populations. Cells were then lysed for W6/32 IP followed by L243 IP to elute HLA class I and HLA-DR peptides, respectively. Data were acquired by LC-MS/MS on an Orbitrap QExactive mass spectrometer. **A.** Size distribution of peptides eluted from BDCA4⁻ or BDCA4⁺ cell populations. **B.** Overlap in HLA class I peptide identifications between BDCA4⁻ and BDCA4⁺ cell populations. **C.** 9-mers identified in either BDCA4⁻ or BDCA4⁺ populations were grouped using the online Gibbs Clustering server. The optimal fit (Kullback-Leibler Distance; KLD) made 3 distinct clusters for which the sequence logos are shown for each sample. Sequence logos were assigned to one of the HLA types expressed by the IFN-DC donor or PM1 cells by comparison to known motifs in the literature and MHC Motif Viewer.

BDCA4 negative							
VSFVTLGKGGF	11	Cryptic (- sense)	-	19.26	1110.6073	-	-
QYCRRFHQY	9	Cryptic (- sense)	-	15.85	1299.5931	HLA-A*24:02	-
ITKALGISY	9	Tat	39-47	28.63	964.5593	HLA-A*01:01/HLA-B*15:01	HLA-B*15:01
IQKQGQGQW	8	Pol	484-492	21.16	851.5116	-	-
IQKQGQGQWTY	11	Pol	484-494	47.24	1071.5461	HLA-B*15:01	-
KMIGGIGGF	9	Pol	101-109	37.26	878.4684	HLA-B*15:01/HLA-A*24:02	HLA-B*15:01
LKNSAVSLLN	10	gp160	807-816	12.12	1053.5243	-	-
LPPVVAKE	8	Pol	743-750	34.25	1193.5757	-	-
LQVWGRDNNSPSEAG	15	Pol	31-45	33.61	1045.5808	-	-
RQGYSPLSF	9	gp160	709-717	36.2	1057.6132	HLA-B*15:01/HLA-A*24:02/HLA-B*52:01	-
VQKEYAFFY	9	gp160	169-177	14.38	1335.6571	HLA-A*01:01/HLA-B*15:01	-
YTSLIHSLI	9	gp160	638-646	9.68	1628.7543	HLA-A*01:01/HLA-A*24:02/HLA-B*52:01/HLA-C*02:02/HLA-C*03:03/HLA-C*12:02	-
SYKGRPGNF	9	Gag	440-448	53.28	1109.5909	HLA-A*24:02	-
FLGKIWPSY	9	Gag	433-441	54.25	1024.509	HLA-A*01:01/HLA-B*15:01	-
BDCA4 positive							
VSFVTLGKGGF	11	Cryptic (- sense)	-	16.28	1110.6073	-	-
FLGKIWPSY	9	Gag	433-441	39.71	1109.5909	HLA-A*01:01/HLA-B*15:01	-
SYKGRPGNF	9	Gag	440-448	14.18	1024.509	HLA-A*24:02	-
LPPVVAKE	8	Pol	743-750	32.74	851.5116	-	-
LQVWGRDNNSPSEA	14	Pol	31-44	22.59	1053.5243	-	-
LQVWGRDNNSPSEAG	15	Pol	31-45	15.2	1571.7328	-	-
RQGYSPLSF	9	gp160	709-717	20.55	1628.7543	HLA-B*15:01	-

Co-culture of IFN-DCs with a HIV-1-infected cell line which does not express any HLA class I would not require separation of the two cell types, as any peptides eluted by W6/32 IP would elute from HLA expressed by IFN-DCs. In Chapter 4, it was shown that relatively few peptides (possibly of HLA-E origin) were eluted after W6/32 IP from the 721.221 lymphoblastoid cell line expressing CD4 (CD4.221). Given that this cell line is highly susceptible to infection with HIV-1 IIIB and only express low levels HLA-E, CD4.221 cells were incorporated into the cross-presentation assay. To determine whether cross-presented HIV-1 epitopes can be identified using this system, HIV-1 IIIB-infected CD4.221 or matched uninfected CD4.221 cells were co-cultured with IFN-DCs from donor 293 (HLA: A*02:01 homozygous, B*27:05, B*51:01, C*02:02, C*14:02) overnight then all cells were lysed together for isolation of HLA class I peptides. Non-specific contaminants or potential HLA-E-bound peptides as identified in W6/32 IPs of CD4.221 cells alone (see Chapter 4) were removed from the data. Similarly, extended nested sets of HIV-1 Gag peptides identified as non-specific contaminants by OKT4 IP were also removed from the data (list of non-specific contaminant peptides shown in Appendix 9.3, Tables 9.9, 9.10). For the IFN-DCs co-cultured with uninfected and HIV-1 IIIB-infected CD4.221, 7165 and 8016 unique peptides were identified, respectively (Figure 6.4A). 88.2 and 86.5% of these peptides were of the canonical length (8-11 amino acids) for HLA class I ligands in the uninfected and HIV-1 IIIB-infected co-cultures, respectively. Unsurprisingly, given IFN-DCs from the same donor were used in each sample, ~65% of the peptides observed in the uninfected co-culture were also identified in the HIV-1 IIIB-infected co-culture (Figure 6.4B). When 9-mers for each sample were clustered, 4 distinct motifs emerged (Figure 6.4C). These peptide groups were representative of the binding motifs for HLA-A*02:01, HLA-B*27:05, HLA-B*51:01 and HLA-C*02:02/HLA-C*14:02 expressed by the IFN-DC donor. Interestingly, more peptides conformed to the binding motif for HLA-A*02:01 (n=1887 uninfected; n=2083 IIIB-infected) and HLA-B*27:05 (n=1139 uninfected; n=1340 IIIB-infected) than for HLA-B*51:01 (n=745 uninfected; n=776 IIIB-infected) or the HLA-C alleles (n=469 uninfected; n=538 IIIB-infected). Given that the motifs detected within the co-culture immunopeptidome match with the HLA class I type of the IFN-DC donor, these data suggest that any HIV-1 peptides identified would arise solely (or almost exclusively) from IFN-DC-expressed HLA molecules. Indeed, 9 HIV-1 peptides (excluding non-specific contaminants) originating from Gag, Pol, gp160 and Rev were detected in

the HIV-1 IIIB-infected sample only (Table 6.3). Amongst these peptides were the well-studied immunodominant HLA-B*27:05-restricted KK10 epitope (KRWIILGLNK) and the HLA-B*51:01-restricted LI9 (LPPVVAKEI). Thus these data confirm that a system employing IFN-DCs co-cultured with CD4.221 cells can allow the identification of cross-presented epitopes.

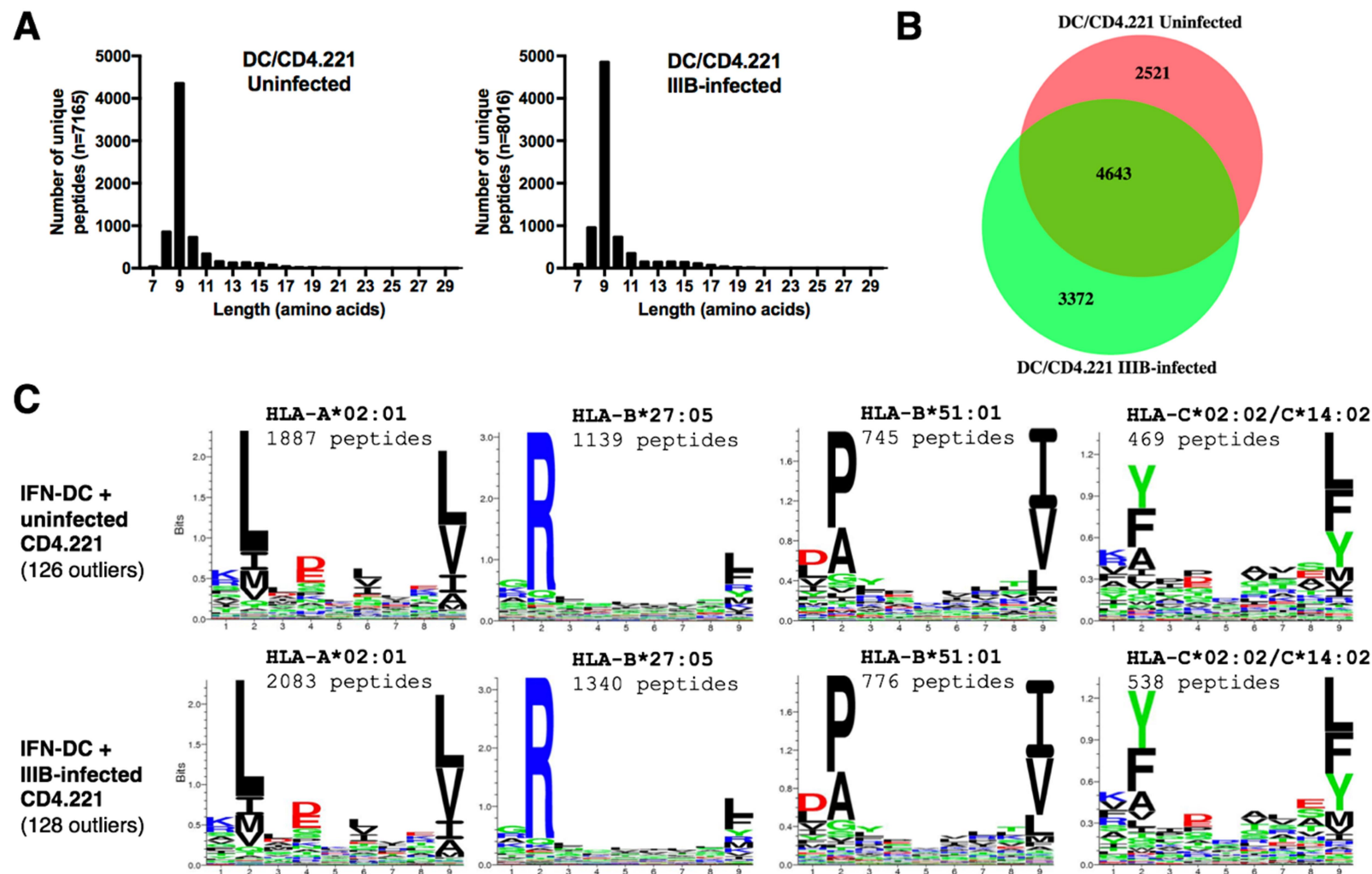


Figure 6.4 *IFN-DC co-culture with CD4.221 cells.* 1.4×10^8 and 2×10^8 IFN-DCs from donor 293 (HLA: A*02:01, B*27:05, B*51:01, C*02:02, C*14:02) were co-cultured with uninfected or HIV-1 IIIB-infected CD4.221 cells at a ratio of 2:1 for 20 hours, respectively. Cells were then lysed and MHC class I peptides were immunoprecipitated with W6/32-linked Sepharose beads. Data were acquired by LC-MS/MS on a Fusion Lumos instrument. Peptides which were previously identified in CD4.221 cells alone by W6/32 IP or HIV-1 peptides identified by OKT4 IP (see Chapter 4) were excluded from further analyses (exclusion list shown in Appendix 9.3, Table 9.10, 9.11). **A.** Size distribution of HLA class I peptides eluted from IFN-DC/CD4.221 co-cultures. **B.** Overlap in peptide identifications between IFN-DCs co-cultured with uninfected and HIV-1 IIIB-infected CD4.221 cells. **C.** 9-mers identified in either uninfected or IIIB-infected co-cultures were grouped using the online Gibbs Clustering server. The optimal fit (Kullback-Leibler Distance; KLD) made 4 distinct clusters for which the sequence logos are shown for each sample. Sequence logos were assigned to one of the HLA types expressed by the IFN-DC donor by comparison to known motifs in the literature and MHC Motif Viewer.

Table 6.3. HIV-1 peptides cross-presented by IFN-DC co-cultured with HIV-1 IIB-infected CD4.221 cells

Peptide	Polyprotein	AA position in Polyprotein	Length	PEAKS score	Mass	Putative HLA restriction
VESPTVLESGTKE	Rev	104-116	13	37.03	1374.6877	-
GIKQLQARILAVE	gp160	572-584	13	35.92	1437.8667	-
GQETAYFLK	Pol	809-818	10	12.98	1168.6128	-
IPEWEFVNTPPLVK	Pol	566-579	14	60.71	1667.8922	HLA-B*51:01
LPPVVAKEI	Pol	743-751	9	25.09	964.5957	HLA-B*51:01
MRQAHCNI	gp160	326-333	8	11.9	971.4429	-
FLGKIWPS	Gag	433-440	8	38.11	946.5276	HLA-A*02:01
FLGKIWPSY	Gag	433-441	9	50.65	1109.5909	HLA-A*02:01
KRWIILGLNK	Gag	263-272	10	33.78	1239.7815	HLA-B*27:05
MLKETINEEA	Gag	200-209	10	22.35	1176.5696	-

A possible caveat to the previous experiment where HIV-1 epitopes were presented by IFN-DCs co-cultured with infected CD4.221 cells was the possibility that IFN-DCs can become productively infected with HIV-1 IIIB. Although it was unlikely that IFN-DCs produced nascent viral proteins within 20 hours of exposure to infected cells, the effects of the antiretroviral nucleoside analog reverse transcriptase inhibitor AZT on IFN-DC antigen uptake were tested. IFN-DCs cultured in the presence of 10 μ M AZT showed no difference in uptake of dextran-FITC over the course of 1 hour in either donor tested (Figure 6.5). Moreover, no differences in cell viability after culture were seen in the presence or absence of AZT (data not shown). Thus the use of AZT was optimised for incorporation into the *in vitro* cross-presentation system in order to inhibit *de novo* infection of IFN-DCs.

Antigen processing activities are known to vary depending on the cell type studied^{559,560}. These disparities are thought to account for quantitative differences in direct and cross-presentation of particular model antigens. However, whether these cell-specific divergences in antigen processing activities translate to physiologically relevant changes across the whole immunopeptidome has not been explored. Furthermore, the characteristics of proteins sampled by the cross-presentation pathway have not been defined in the literature. To explore these questions, the cross-presentation assay method was modified in two ways (method shown in Figure 6.6A):

- Primary CD4⁺ T cells and IFN-DCs were isolated from the same donor in order to directly compare the immunopeptidomes of each cell type.
- CD4.221 cells were cultured and infected with HIV-1 IIIB in the presence of stable heavy isotopes of leucine, lysine and arginine. Full incorporation of these stable isotopes into the proteome of CD4.221 cells would allow demarcation of epitopes derived from CD4.221 proteins (heavy; cross-presented by IFN-DCs) or from IFN-DC endogenous proteins (light; direct presentation). Leucine, lysine and arginine were chosen because >97% of peptides eluted from most donors will contain at least one of these amino acids.

A possible shortcoming of this cross-presentation assay method is that heavy isotope-labelled CD4.221 cells placed back into standard ‘light’ isotope amino acid-containing media will immediately start to produce nascent ‘light’ proteins. To halt nascent protein translation, CD4.221

cells were cultured for 4 hours in standard R10 media containing the irreversible protein synthesis inhibitor emetine (or the reversible inhibitor cycloheximide (CHX) or without drugs as negative controls) then washed thoroughly. Subsequently, CD4.221 cells were transferred into media containing heavy leucine, lysine and arginine for 16 hours to determine whether new proteins were made following treatment with emetine. Total protein was then extracted from cells for proteomic analysis and the percentage of detected peptides containing heavy isotopic amino acids was calculated. As expected, treatment with CHX showed only a minimal effect on protein translation as 11.1% and 12.6% of detected peptides contained heavy isotopes in the uninfected CHX-treated and no drug controls, respectively (Figure 6.6B). In contrast, just 0.3% of detected peptides contained heavy leucine, lysine or arginine in the emetine-treated uninfected sample. Interestingly, just 0.8% of peptides in the HIV-1 IIIB-infected CD4.221 cells not treated with any drug contained heavy amino acids. This result could reflect the high amount of cell death which occurs by day 3 of infection with HIV-1 IIIB (data not shown). The percentage of heavy-labelled peptides dropped to 0.3% for the HIV-1 IIIB-infected CD4.221 cells treated with emetine. This residual protein translation occurring in emetine-treated cells is thought to partially result from emetine-resistant mitoribosomal translation⁸⁴⁶. Overall however, these data show that emetine irreversibly inhibits the majority of protein translation in CD4.221 cells and will therefore considerably reduce the proportion of cross-presented peptides falsely assigned in the cross-presentation system as originating from proteins endogenously-expressed by IFN-DC.

Although experiments were performed to assess the suitability of AZT and emetine for use in inhibiting infection of IFN-DCs and halting protein translation in CD4.221 cells, the experiments described in the rest of this chapter, which aimed to determine the viability of the cross-presentation assay incorporating amino acids containing stable isotopes, did not employ these compounds, as they were carried out before the use of AZT and emetine had been explored. However these compounds can be used in future work.

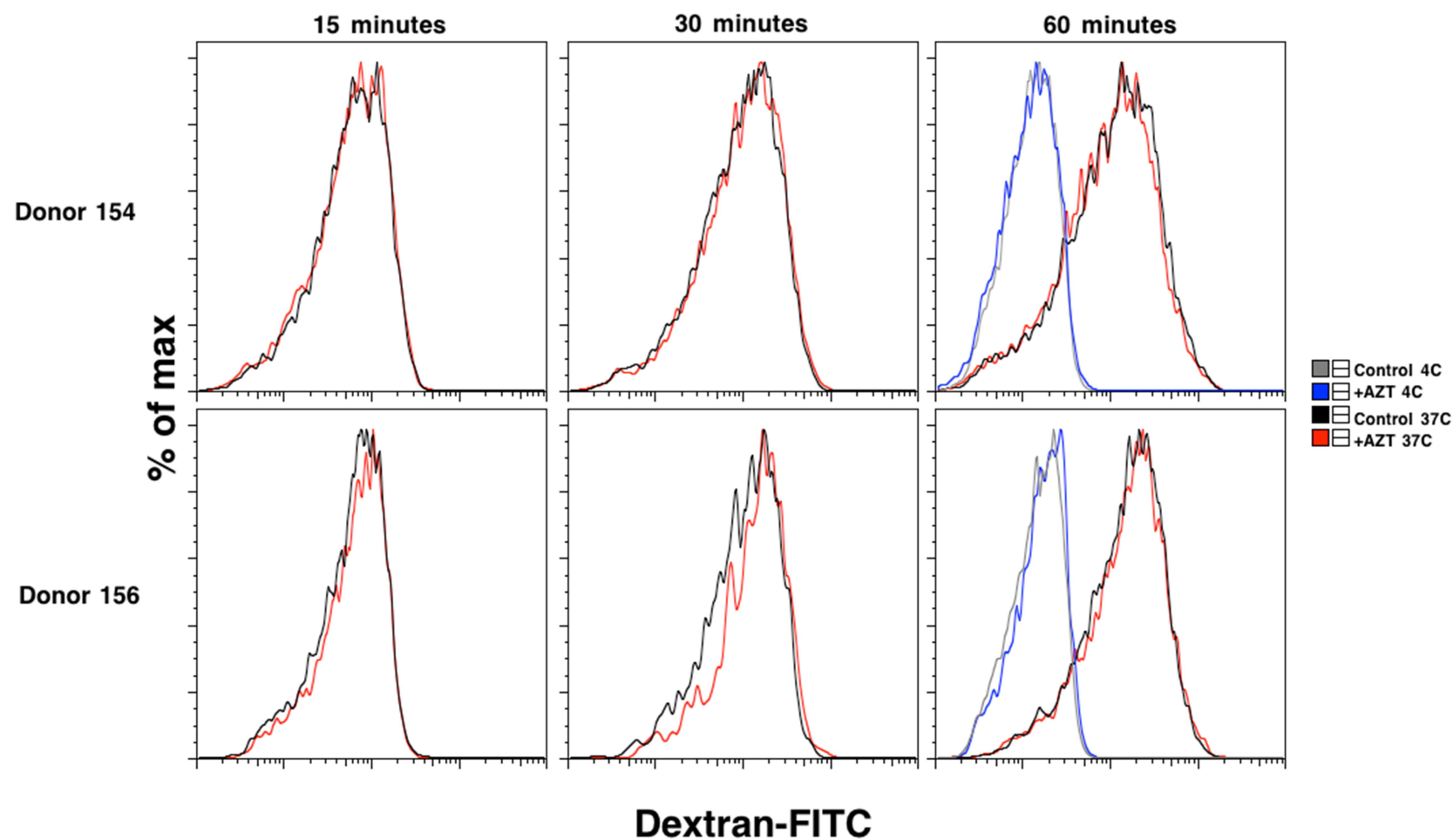


Figure 6.5 Effects of AZT on IFN-DC dextran-FITC endocytosis A. IFN-DC were pre-incubated with 10 μ M AZT for 30 minutes then 1 mg/ml dextran-FITC was added to the cultures for the indicated times. Dextran-FITC uptake by IFN-DCs was measured by flow cytometry. IFN-DCs cultured at 4°C for 60 minutes were included as a negative control.

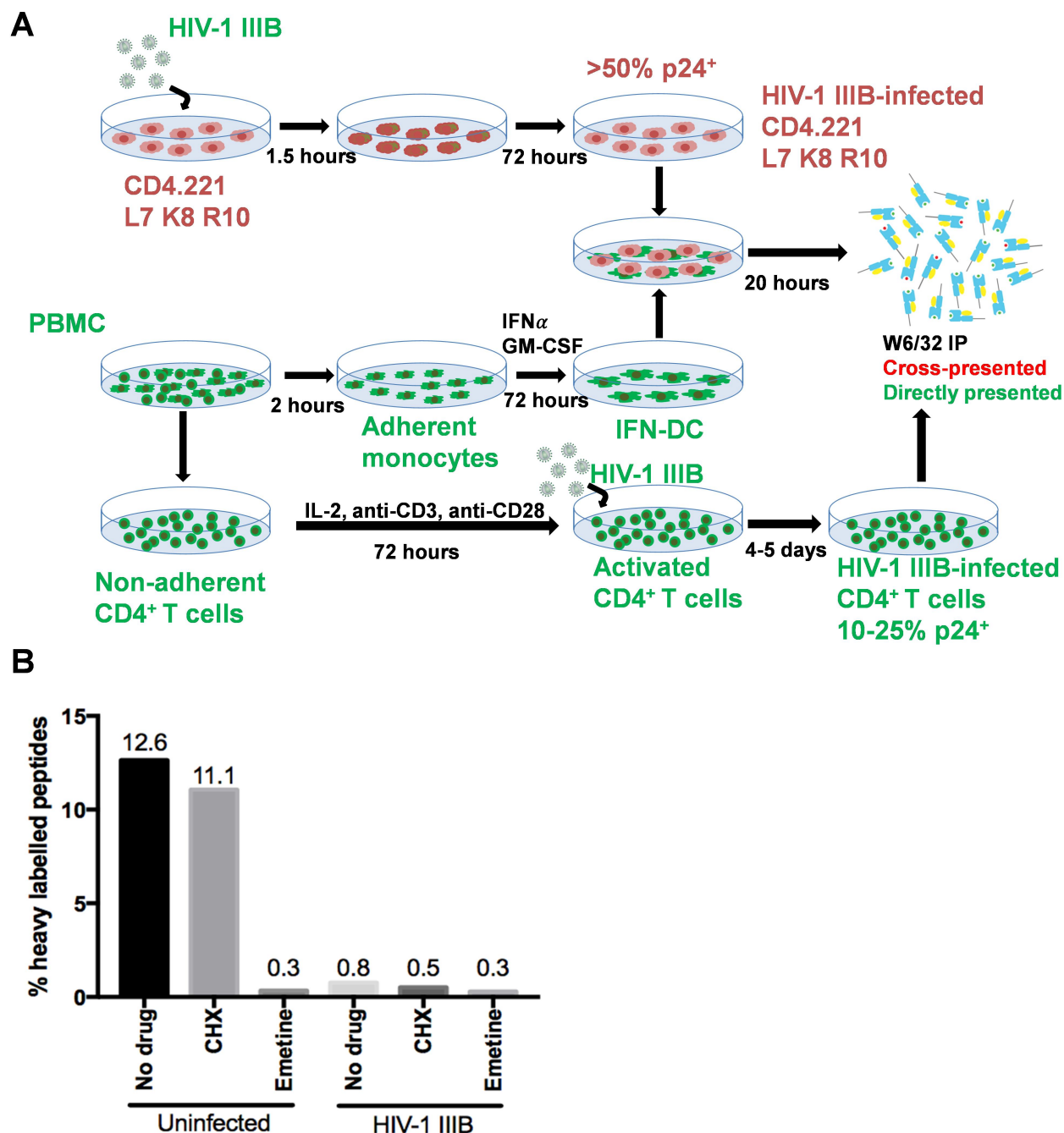


Figure 6.6 *Optimisation of IFN-DC cross-presentation assay.* **A.** Schematic of the IFN-DC cross-presentation assay where antigen from CD4.221 cells is cross-presented, and the cross-presented immunopeptidome compared to that directly-presented on infected CD4⁺ T cells derived from the same donor as the IFN-DCs. HLA class I negative CD4.221 cells were infected with HIV-1 IIIB for 3 days until >50% p24⁺ as determined by intracellular p24 staining. In parallel, IFN-DCs were differentiated from adherent monocytes of a leukocyte cone donor. The non-adherent cells were harvested and primary CD4⁺ T cells were isolated, activated and infected with HIV-1 IIIB separately until 10-25% p24⁺. On day 3 post-infection of CD4.221 cells, IFN-DC were co-cultured with uninfected or IIIB-infected CD4.221 at a ratio of 1:1-2 for 20 hours. Subsequently, HLA class I (which can only be IFN-DC-expressed) was isolated by W6/32 IP. In some experiments, HLA-DR (which is expressed by both IFN-DCs and CD4.221 cells) was also isolated sequentially after HLA class I with the L243 antibody. In some experiments, CD4.221 cells were cultured in SILAC R10 containing heavy leucine, lysine and arginine (L7 K8 R10, labelled cells shown in red) in order to allow discrimination between cross-presented and directly-presented HLA class I peptides on IFN-DCs. **B.** CD4.221 cells were left uninfected or infected with HIV-1 IIIB for 3 days until cells were >50% p24⁺ as determined by intracellular p24 staining. CD4.221 cells were then pulsed with 100 µg/ml cycloheximide (CHX) or emetine for 4 hours then drug was washed out. Cells were then cultured in SILAC R10 (containing heavy leucine, lysine and arginine) for 16 hours then protein was extracted from cell lysates and digested with trypsin. Data were acquired by LC-MS/MS on a QExactive-HF instrument. Shown is the percentage of detected peptides which contained a heavy version of leucine, lysine or arginine in each sample.

6.2.2 Comparison of the immunopeptidomes presented by DCs and CD4⁺ T cells

Investigators have reported a differential efficiency with which DC subsets or other types of APC present particular epitopes as measured using T cells or TCR-like antibodies which recognise specific peptide-MHC complexes^{559,561,847}. However to my knowledge, an immunopeptidomics approach has not yet been utilised to comparatively evaluate the repertoire of peptides presented by different primary cell types from the same individual.

To explore this, IFN-DCs and CD4⁺ T cells were isolated from the same donor, incubated with uninfected/HIV-1-infected SILAC-labelled CD4.221 cells or directly infected with HIV-1, respectively as shown in Figure 6.6A, and their immunopeptidomes were analysed. Approximately 4.25×10^7 IFN-DCs (co-cultured with CD4.221 cells) and 2×10^8 CD4⁺ T cells were harvested for the peptide isolation in either uninfected or HIV-1 IIIB-infected cultures. Peptides bound to HLA class I were isolated by W6/32 IP and analysed. More peptides were identified from CD4⁺ T cell cultures (uninfected n=10835, IIIB-infected n=6747) than from IFN-DCs (uninfected CD4.221 co-culture n=3394, IIIB-infected CD4.221 co-culture n=5470), likely as a result of the greater number of CD4⁺ T cells isolated (Figure 6.7A). The peptides isolated from all samples showed a similar size distribution, with 9-mers being the most common peptide length identified, followed by 8-mers then 10-mers. HLA-DR-bound peptides were also isolated from IFN-DCs by L243 IP from flowthrough lysate. 2499 and 3556 HLA-DR-associated peptides were detected in the uninfected and HIV-1 IIIB-infected IFN-DC co-cultures, respectively. Clustering of 9-mers identified in W6/32 IP'd samples from uninfected CD4⁺ T cells and uninfected IFN-DC co-cultures into groups displaying similar motifs revealed 4 distinct clusters which were representative of the binding motifs for HLA-A*01:01, HLA-B*08:01, HLA-B*57:01 and HLA-C*06:02/HLA-C*07:02 expressed by the donor cells used (Figure 6.7B). Very similar results were obtained when 9-mers from HIV-1 IIIB-infected CD4⁺ T cells or IFN-DC co-cultures were clustered (data not shown). Interestingly, the number of peptides conforming to the HLA-B*57:01 motif was generally greater than for other alleles (e.g. 1795 peptides in HLA-B*57:01 cluster, 1230 in HLA-B*08:01 cluster and 738 in HLA-A*02:01 cluster for uninfected CD4⁺ T cells). Overall, the binding motifs arising from CD4⁺ T cells or IFN-DCs appeared to be identical. Nine HIV-1-derived peptides were identified as presented by infected CD4⁺

T cells (Table 6.4). Amongst these epitopes were the well-described B*08:01-restricted Nef FL8 (FLKEKGGL), HLA-B*57:01-restricted Gag SW9 (STLQEQIGW) and gp160 KW11 (KAYDTEVHNVW) epitopes. Additionally, a putative novel gp160 epitope restricted by HLA-B*08:01 was identified (EL9; ELKNSAVSL). Although one cryptic epitope was identified, this peptide would have to be validated by extra experiments because the sequence was not predicted to bind any of the HLA alleles expressed by the donor, and the identification score assigned by PEAKS software was relatively low. One epitope was found to be presented on CD4⁺ T cells as well as cross-presented by IFN-DCs. This gp160-derived RW10 (RVKEKYQHLW) epitope is predicted to bind both HLA-B*08:01 (RVKEKYQHL) and HLA-B*57:01 (RVKEKYQHLW)^{848,849}. Just one of the two lysines (K) in the peptide detected in the IFN-DC sample contained heavy isotopes and also the leucine (L) was not heavy-labelled. This indicates that the RW10 cross-presented peptide originated from gp160 protein newly synthesised in CD4.221 cells which incorporated residual stores of heavy amino acids and unlabelled amino acids present in the media.

I also attempted to determine whether the immunopeptidomes of CD4⁺ T cells and IFN-DCs are quantitatively similar. Due to limitations on the number of cells available, biological replicates of W6/32 IPs from cells of the same donor could not be performed. Therefore, a full quantitative statistical analysis of peptides which are over-presented in one cell type relative to the other was not possible. Nonetheless, a considerable overlap in peptide identifications was observed between CD4⁺ T cells and IFN-DCs; 71.2% (n=2415) and 58.4% (n=3195) of peptides in uninfected and IIIB-infected IFN-DC co-cultures were also identified in matched CD4⁺ T cell eluates (Figure 6.7C). Bearing in mind that I showed in Chapter 4 that ~70% of peptides were identified in both biological replicates of a single cell line, the large overlap between IFN-DC and CD4⁺ T cell immunopeptidomes indicated considerable similarity in the types of peptides presented by either cell type. To determine whether peptides common to both samples were presented in a similar abundance hierarchy, the measured peptide intensities in each sample were plotted against each other. A clear positive correlation between the peptide abundance in IFN-DCs and in CD4⁺ T cells was observed (uninfected, $r=5272$ $p<0.0001$; IIIB-infected, $r=0.6132$ $p<0.0001$) (Figure 6.7D). Therefore a peptide relatively abundantly-presented by CD4⁺ T cells is also likely to be abundantly presented by

IFN-DCs from the same donor. In summary, these data indicate that the immunopeptidomes of IFN-DCs and CD4⁺ T cells are qualitatively and quantitatively similar.

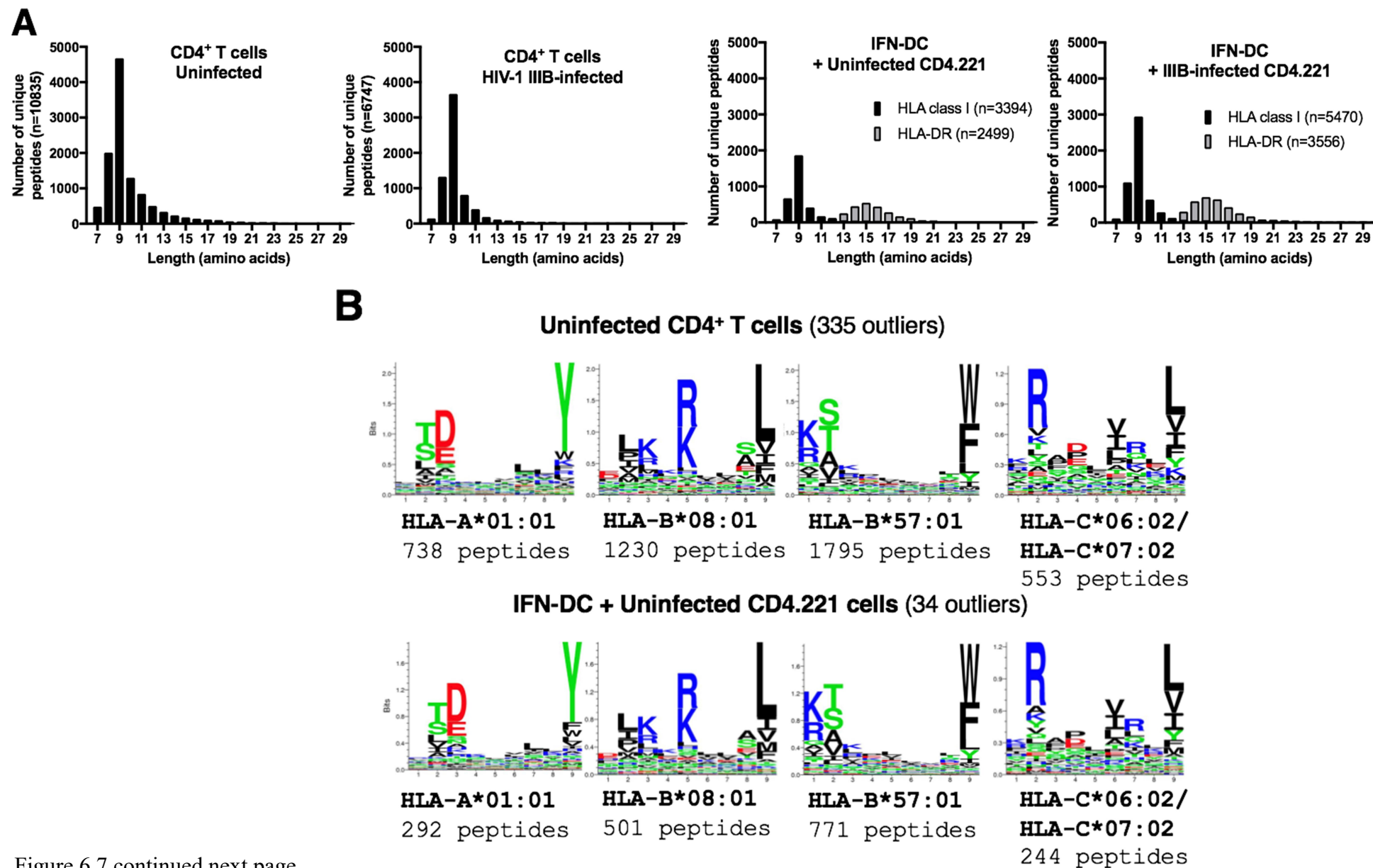


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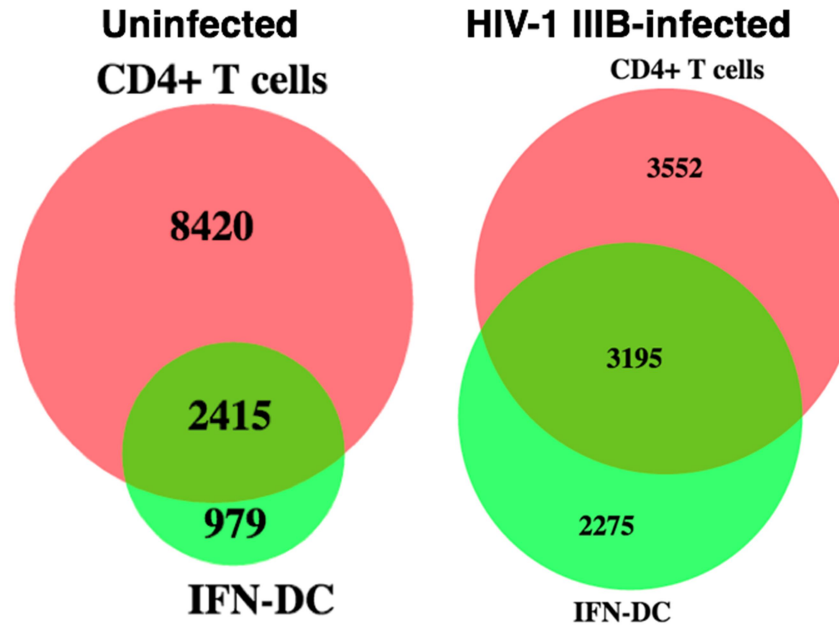
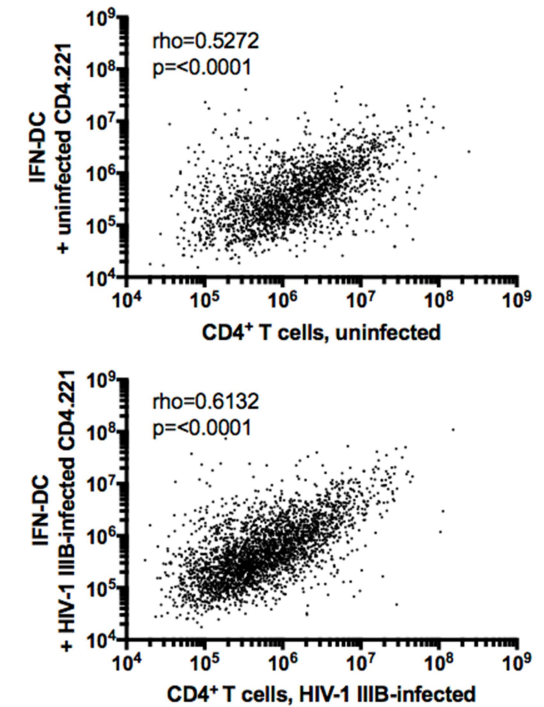
**D**

Figure 6.7 IFN-DCs and CD4⁺ T cells present qualitatively and quantitatively similar immunopeptidomes. IFN-DCs and CD4⁺ T cells from donor 231 (HLA:A*01:01, B*08:01, B*57:01, C*06:02, C*07:02) were differentiated and activated as shown in Figure 6.6A. CD4.221 cells were cultured in SILAC R10 (containing heavy leucine, lysine and arginine) for 10 days until completely labelled. CD4.221 cells were then either left uninfected or infected with HIV-1 IIIB at a MOI of 0.1 for 3 days until >50% cells were p24⁺ as determined by intracellular p24 staining (data not shown). Then, IFN-DCs were co-cultured with either uninfected or HIV-1 IIIB-infected CD4.221 cells overnight. Primary CD4⁺ T cells were either left uninfected or infected with HIV-1 IIIB for 5 days until ~12.5% p24⁺ as determined by intracellular p24 staining (data not shown). HLA class I peptides were isolated from IFN-DCs or CD4⁺ T cell samples using W6/32 IP. HLA-DR-bound peptides were also isolated from IFN-DC by L243 IP. LC-MS/MS data were acquired on a Fusion Lumos mass spectrometer. **A.** Size distribution of peptides eluted from primary CD4⁺ T cells or IFN-DC/CD4.221 co-cultures. HLA class I peptides are shown in black whilst HLA-DR peptides are shown in grey. **B.** 9-mers identified in uninfected CD4⁺ T cells (top row) or the uninfected DC/CD4.221 co-culture (bottom row) were grouped using the online Gibbs Clustering server. The optimal fit (Kullback-Leibler Distance; KLD) made 4 distinct clusters for which the sequence logos are shown for each sample. Sequence logos were assigned to one of the HLA types expressed by the CD4⁺ T cell/IFN-DC donor by comparison to known motifs in the literature and MHC Motif Viewer. **C.** Overlap in peptide identifications between CD4⁺ T cells and IFN-DCs co-cultured with uninfected and HIV-1 IIIB-infected CD4.221 cells. **D.** Shared peptides between the IFN-DC and CD4⁺ T cell W6/32 IP samples (uninfected n=2122, HIV-1 IIIB-infected n=2915) with assigned peptide intensity values by PEAKS software were plotted against each other. Spearman's rank correlation and the associated p value are shown.

Table 6.4. HIV-1 epitopes identified in autologous IFN-DC and primary CD4⁺ T cells

HIV-1 Peptide	Length	Polyprotein	AA position in Polyprotein	PEAKS score	Mass	Putative HLA restriction
<u>CD4⁺ T cells</u>						
SYLIPGLSIF	10	Cryptic (- sense)	-	11.59	1108.6168	-
FLKEKGGL	8	Nef	90-97	13.11	890.5225	HLA-B*08:01
FLGKIWPSY	9	Gag	433-441	46.03	1109.5909	HLA-A*01:01
STLQEQIGW	9	Gag	241-249	24.61	1060.5189	HLA-B*57:01
VCFITKAL	8	Tat	36-43	10.62	893.5045	-
ELKNSAVSL	9	gp160	806-814	16.11	959.5287	HLA-B*08:01
RVKEYQHLW*	10	gp160	2-11	47.7	1385.7567	HLA-B*08:01 HLA-B*57:01
KAYDTEVHNVW	11	gp160	59-69	59.81	1360.6411	HLA-B*57:01
YVDGAANRETKLGKAGY	17	Pol	596-612	24.35	1811.9165	HLA-A*01:01
HIV-1 Peptide	Length	Polyprotein	AA position in Polyprotein	PEAKS score	Mass	Putative HLA restriction
<u>IFN-DC</u>						
PVAAPRLLPCAL	12	Cryptic (- sense)	-	10.22	1219.7111	-
RVKEK (+8.01) YQHLW*	10	gp160	2-11	24.66	1393.771	HLA-B*08:01 HLA-B*57:01

The peptide identified in both CD4⁺ T cells and cross-presented by IFN-DC is bolded and indicated with an asterisk (*). Peptides containing heavy isotopes of leucine, lysine or arginine are listed with the mass shift in parentheses

6.2.3 Analysis of peptides and proteins cross-presented by IFN-DCs

Despite the rapid increase in sensitivity of current mass spectrometers and the parallel increase in the numbers of studies reporting large immunopeptidome datasets, no analyses exist which detail the repertoire of peptides or proteins cross-presented by professional APCs. In the experiment described in section 6.2.2, IFN-DCs were cultured with heavy amino acid isotope-labelled CD4.221 cells, which enabled HLA class I-bound peptides containing heavy isotopes (cross-presented) or those without heavy isotopes (assumed to be directly-presented) to be distinguished. Importantly, peptides which were shown to elute non-specifically from CD4.221 cells were excluded from analyses (see Chapter 4).

Of the 3394 and 5470 peptides identified in the W6/32 IP of IFN-DCs co-cultured with uninfected or HIV-1 IIIB-infected CD4.221 cells, 292 and 244 peptides contained heavy leucine, lysine or arginine, respectively (Figure 6.8A). The majority of these peptide species were between the lengths of 8-11 amino acids, although some labelled peptides were up to 30 amino acids in length. Interestingly, the measured intensities of heavy cross-presented epitopes were significantly lower than those of the unlabelled peptides in the same sample (IFN-DCs with uninfected CD4.221 co-culture, $p=0.0002$; IFN-DCs with HIV-1 IIIB-infected CD4.221 co-culture, $p<0.0001$) (Figure 6.8B). This result indicates that peptides cross-presented by IFN-DCs are likely to be lower in abundance than other presented peptides derived from endogenously synthesised proteins. Interestingly, 8-11-mers identified in IFN-DC W6/32 IP samples showed allelic-restriction differences when comparing heavy and light peptides (Table 6.5). A greater proportion of peptides in the 'light' (directly-presented) subset were predicted to bind to HLA-B*08:01 than were predicted for the same allele in the 'heavy' (cross-presented) peptide subset. On the other hand, a greater proportion (46.3%) of the heavy peptides were predicted to bind to HLA-B*57:01 than to the same allele in the light peptide subset (39.5%) of the IFN-DC uninfected co-culture. However, no similar difference was observed for the HIV-1 IIIB-infected CD4.221 IFN-DC co-culture, raising the possibility that the differences seen in the uninfected CD4.221/IFN-DC co-cultures may have occurred by chance (as the total number of heavy peptides identified was relatively low, and peptide-HLA binding prediction algorithms are not wholly accurate).

To examine the contribution of a protein's abundance to its likelihood of being cross-presented, I analysed the relative abundance of proteins expressed by the CD4.221 line or the EVs secreted by CD4.221 cells. CD4.221 cells were infected with HIV-1 IIIB (or left uninfected) for 3 days then proteins were extracted from cell or EV lysates, digested with trypsin and analysed by LC-MS/MS. Proteins detected in uninfected or HIV-1 IIIB-infected CD4.221 cells or EVs were then assigned iBAQ values by MaxQuant software. Plotted in Figure 6.8C are the iBAQ values in either CD4.221 cells or EVs of any cross-presented protein for which a heavy-labelled peptide was detected in the IFN-DC immunopeptidome. Similarly, the iBAQ values in cells or EVs of any protein which were not cross-presented are shown. Proteins for which no expression data was available were excluded from the analysis. Proteins cross-presented by IFN-DCs co-cultured with HIV-1-infected CD4.221 cells were found to be significantly more abundant in CD4.221 cells ($p < 0.0001$) or EVs ($p < 0.0001$) than proteins which were not cross-presented by IFN-DCs (Figure 6.8C). Equally, proteins cross-presented by IFN-DCs co-cultured with uninfected CD4.221 cells were significantly more abundant in CD4.221 cells ($p < 0.0001$) or EVs ($p = 0.001$) than proteins not cross-presented by IFN-DCs. These data suggest that protein abundance may be an important factor in determining whether epitopes derived from it are cross-presented or not.

Protein length has been shown to correlate positively with the number of HLA class I peptides presented by cells¹⁶⁵. However, the effect of protein length on its probability of peptides being cross-presented is not known. The proteins from which peptides were cross-presented were found to be significantly longer than those from which peptides were only directly-presented on IFN-DCs co-cultured with uninfected CD4.221 cells ($p = 0.007$) (Figure 6.8D). However the difference in length between proteins from which peptides were cross-presented and directly-presented by IFN-DCs co-cultured with HIV-1-infected CD4.221 cells did not reach statistical significance ($p = 0.0651$).

Protein degradation has also been reported to positively correlate with presentation on HLA class I¹⁶⁵. The turnover rates of cross-presented and directly-presented proteins were extracted from a previous study⁸⁵⁰. No difference in the turnover rate was found between cross-presented and directly-presented proteins ($p = 0.881$ uninfected co-culture; $p = 0.1392$ HIV-1-infected co-culture) (Figure 6.8E).

Hypothesising that the subcellular location of a protein may influence its likelihood of entering the cross-presentation pathway, I extracted the subcellular locations of proteins catalogued in Ingenuity Pathway Analysis (IPA) software. In general, a greater percentage of cross-presented peptides originated from membrane proteins than was the case for directly-presented peptides (uninfected co-culture, 18.06% 'heavy' and 9.81% 'light'; HIV-1 IIIB-infected co-culture, 15.74% 'heavy' and 10.02% 'light') (Figure 6.8F). In summary, these data indicate that cross-presented peptides may differ from directly-presented peptides in the following respects:

- Cross-presented peptides were significantly lower in abundance than peptides directly-presented by IFN-DCs
- Cross-presented proteins were significantly more abundant in the donor CD4.221 cells or donor-secreted EVs than proteins which were not cross-presented
- Cross-presented proteins may be longer than directly-presented proteins
- Plasma membrane proteins may be sampled more by the cross-presentation pathway than the direct-presentation pathway.

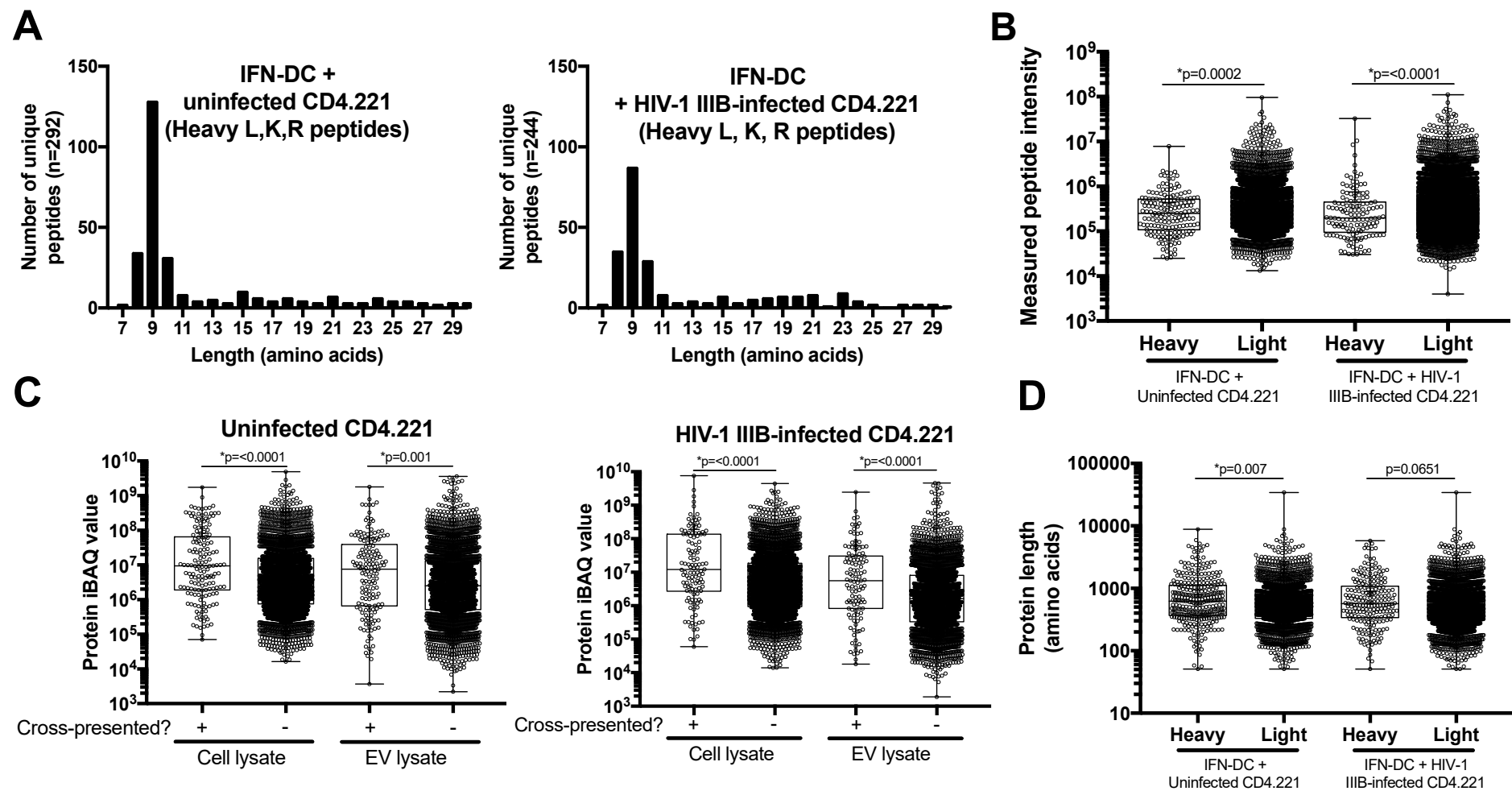


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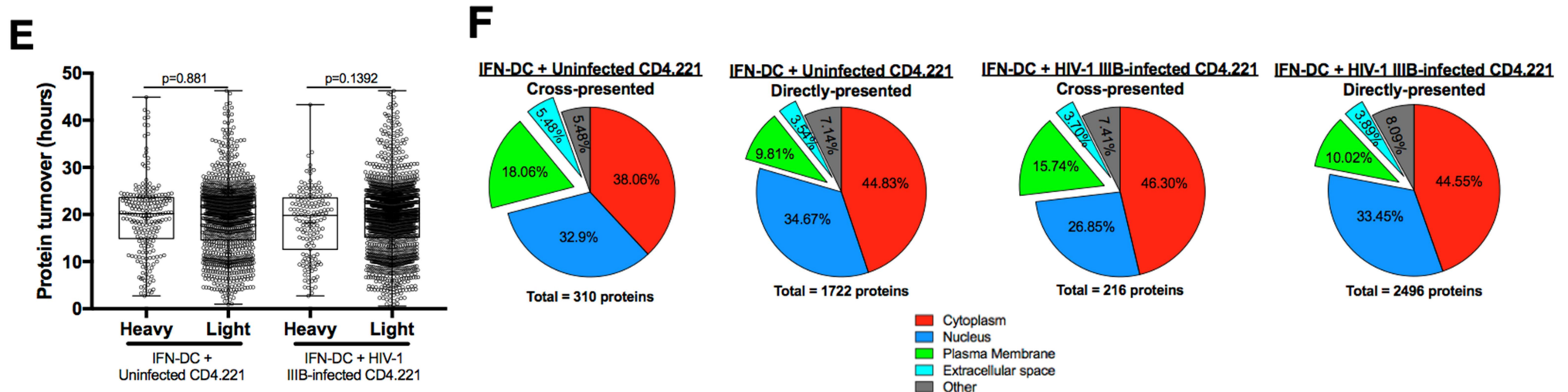


Figure 6.8 Analysis of peptides/proteins cross-presented by IFN-DCs. IFN-DCs from donor 231 (HLA:A*01:01, B*08:01, B*57:01, C*06:02, C*07:02) were differentiated and activated as shown in Figure 6.6A. CD4.221 cells were cultured in SILAC R10 (containing heavy leucine, lysine and arginine) for 10 days until completely labelled. CD4.221 cells were then either left uninfected or infected with HIV-1 IIIB at a MOI of 0.1 for 3 days until >50% cells were p24⁺ as determined by intracellular p24 staining (data not shown). Then IFN-DCs were co-cultured with either uninfected or HIV-1 IIIB-infected CD4.221 cells overnight. HLA class I peptides were isolated from IFN-DC/CD4.221 co-cultures using W6/32 IP. LC-MS/MS data were acquired on an Orbitrap Fusion Lumos mass spectrometer. Separately, CD4.221 cells (uninfected or HIV-1 IIIB-infected) were lysed then protein was extracted and digested with trypsin for proteomic analysis on a QExactive HF instrument. Raw data were analysed in MaxQuant software and iBAQ values were extracted. **A.** Size distribution of ‘heavy’ HLA class I peptides eluted from either uninfected or HIV-1 IIIB-infected IFN-DC/CD4.221 co-cultures are shown. A non-parametric Mann-Whitney test was used to assess the statistical significance of differences between heavy and light peptide intensities within each sample. **B.** The measured peptide intensities of ‘heavy’ or ‘light’ peptides for the uninfected or HIV-1 IIIB-infected co-cultures are shown. A non-parametric Mann-Whitney test was used to assess the statistical significance of differences between heavy and light peptide intensities within each sample. **C.** The iBAQ values (in CD4.221 cells) of proteins from which any HLA class I heavy peptide was detected (cross-presented by IFN-DCs) or not detected (not cross-presented by IFN-DCs) are plotted. Proteins for which a HLA class I peptide was detected but for which no expression data is available were excluded from the analysis. Mann-Whitney tests were performed to assess the statistical significance of differences in iBAQ values between proteins cross-presented and not cross-presented. **D.** The length (in amino acids) of proteins which were cross-presented (heavy) or only directly-presented (light) is plotted. Mann-Whitney tests were performed to assess the statistical significance of differences in each sample. **E.** The whole protein turnover (in hours) of each protein cross-presented (heavy) or directly-presented (light) in each sample (as determined by ⁸⁵⁰) is shown. **F.** The subcellular location of proteins identified as cross-presented or directly-presented was determined by Ingenuity Pathway Analysis software. Shown in each pie chart is the percentage of proteins classified as located in the indicated subcellular compartment.

The percentage (%) of 8-11-mers predicted (NetMHC4.0) to bind to the indicated alleles is shown					
	HLA-A*01:01	HLA-B*08:01	HLA-B*57:01	HLA-C*06:02	HLA-C*07:02
Uninfected 'heavy' (n=201)	15.4	19.4	46.3	12.4	19.4
Uninfected 'light' (n=2837)	15.5	30.5	39.5	16.1	16.8
HIV-1-IIIB-infected 'heavy' (n=159)	9.4	23.2	38.4	11.9	10.1
HIV-1-IIIB-infected 'light' (n=4737)	16.2	28.3	39.4	16.1	16.7

6.3 Discussion

In this chapter I incorporated an immunopeptidomics workflow into an optimised *in vitro* DC cross-presentation assay in order to identify and differentiate between directly-presented epitopes and cross-presented epitopes derived from exogenous EVs or cellular antigen. EVs derived from MT-2 cells were found to be a source of pre-formed peptide-HLA class I complexes for DC cross-dressing as well as a source of HIV-1 protein antigen for cross-presentation. I was unable to efficiently separate co-cultures of IFN-DCs from primary CD4⁺ T cells or PM1 cells using immunodepletion approaches, so I incorporated the CD4.221 cell line into co-culture experiments to identify cross-presented HIV-1 epitopes derived from infected CD4.221 cells. A SILAC labelling approach also allowed preliminary evaluation of the properties of cross-presented proteins.

Contributions of EV antigen to cross-dressing and cross-presentation by IFN-DCs

By isolating HLA class I from EVs secreted by HIV-1-infected MT-2 cells (Figure 6.1), I showed that a broad range (>7500) of peptides are presented by HLA class I molecules loaded into EVs. To my knowledge, this is the first study to analyse the immunopeptidome of EVs by a mass spectrometry approach. The EV immunopeptidome could be grouped into clusters representative of the binding motifs for alleles expressed by MT-2 cells. Furthermore, the EV immunopeptidome largely overlapped (~70%) with the immunopeptidome of their MT-2 cellular origin in terms of peptide identity (uninfected cells/EV, data not shown). Interestingly, most 9-mers eluted from MT-2 EV were sorted into the HLA-B*40:02 cluster, whilst the fewest peptides were sorted into the HLA-B*51:01 cluster. A comparison to the proportion of 9-mers in allele-specific clusters from MT-2 cells would be of interest, as I speculate that different HLA class I alleles may enter the EV secretion pathway more efficiently than other alleles. Because exosomes and microvesicles were not separated here, the relative contribution of each EV subtype to the EV immunopeptidome cannot be ascertained. It would be interesting to determine whether there are any differences between the immunopeptidomes of microvesicles, exosomes and virions secreted from HIV-1-infected cells.

EVs have long been known to contain peptide-HLA complexes for presentation to T cells⁴⁴⁵, although it seems that only exosomes derived from mature DCs can efficiently directly stimulate T cells^{446,851,852}, probably due to the incorporation of co-stimulatory molecules into exosomes. In fact,

exosomes secreted by tumour cells generally seem to require uptake by DCs in order to stimulate T cell immunity^{278,447}. In this setting, it is not entirely clear how antigen transferred by exosomes is utilised by DCs for T cell stimulation. There are at least 4 possibilities; 1) exosomes bound to DC membrane stimulate T cells through exosomal peptide-HLA class I complexes with co-stimulation provided by DCs, 2) exosomal peptide-HLA class I complex is recycled onto DC plasma membrane for T cell stimulation (cross-dressing), 3) exosomes are degraded within DCs and peptides are re-loaded onto endogenous HLA class I molecules or 4) protein antigen within exosomes is cross-presented on endogenous HLA class I complexes. Pre-formed exosomal peptide-HLA class I complexes do seem to be functional, as peptide-loaded HLA-A*02:01⁺ exosomes could induce stimulation of clones or prime T cells specific for peptide-HLA-A*02:01 complexes when presented by HLA-A*02:01⁻ DCs^{853,854}. However, another study suggested that pre-formed exosomal peptide-HLA class I complexes were not required for OVA-specific T cell responses when OVA protein was loaded into exosomes, because exosomes from MHC^{-/-} mice stimulated T cell immunity at the same magnitude as MHC^{+/+} exosomes⁸⁵⁵. Thus it seems that both cross-dressing and cross-presentation of whole antigen may contribute to immunity under different circumstances. Although the immunogenicity of EV-derived antigen was not tested in this thesis, my data support a role for both cross-dressing and cross-presentation of EV antigen by DCs. Greater than 1000 peptides were identified in both MT-2-derived EVs and DCs treated with MT-2 EV, many of which contained a binding motif for HLA-B*51:01 and HLA-A*24:02 alleles expressed by MT-2 cells (Figure 6.1). Conservatively, when peptides which could bind either IFN-DC expressed HLA-B*40:01 or MT-2 expressed HLA-B*40:02 were excluded, at least 300 different peptide-HLA class I complexes would have cross-dressed IFN-DCs. Comparing that to the 292 or 244 ‘heavy’ peptides cross-presented by IFN-DC co-cultured with uninfected or HIV-1-infected CD4.221 cells respectively (Figure 6.7), it seems that both processes of cross-dressing and cross-presentation occurred in the system used in this chapter. It would be interesting to directly compare the relative contribution of EV cross-dressing and cross-presentation to the DC immunopeptidome. This could be achieved by heavy isotope labelling a cell line (e.g. MT-2) then isolating EV and pulsing HLA-mismatched (in order to differentiate the HLA class I complex origin) IFN-DCs with these EVs. Directly-presented peptides (‘light’) could

then be differentiated from cross-presented peptides ('heavy' with IFN-DC HLA class I binding motif) and cross-dressed peptides ('heavy' with MT-2 HLA class I binding motif).

The data presented here also suggest that EV-derived HIV-1 peptide-HLA class I complexes were cross-dressed on IFN-DC. For example, the HLA-A*24:02-restricted Env-RL9 (RYLKDQQLL) was presented by IFN-DC and MT-2 EVs (Table 6.1). On the other hand, the Pol-LY10 (LTQQIRKLSY) is likely to be HLA-A*01:01- or HLA-B*15:01-restricted, was not identified in MT-2 EV, and therefore was probably cross-presented. Thus both processes could contribute to induction of HIV-1-specific T cell immunity. Whether the cross-dressing observed here occurs by adherence of EVs to IFN-DC membrane or by EV endocytosis then recycling of peptide-HLA class I complexes is not obvious, although it is clear that DCs can internalise exosomes into endocytic compartments for antigen processing⁸⁵⁶. This could be addressed in future studies by microscopy examination of stained exosomes interacting with DCs over time. Given the demonstration of cross-priming or memory cell stimulation by cross-dressed DCs *in vivo* by multiple studies^{272,280,857,858}, it is possible that cross-dressing plays a significant role in priming CTL responses during AHI. However, many of the aforementioned studies suggested that, rather than exosomal transfer, cell-cell contact was required for efficient cross-dressing. Furthermore, it is not clear whether the quantities of MT-2 EVs used in my experiments are representative of physiological conditions *in vivo*.

Optimisation of whole cell/IFN-DC co-culture cross-presentation assay

Using immunodepletion methods, I could not reliably separate IFN-DC from primary CD4⁺ T cells or PM1 cells after co-culture and therefore could not determine the cellular origin of HIV-1 peptides identified. Trogocytosis (i.e. 'nibbling'; intercellular transfer of cellular membrane) could be partially responsible for this inefficient separation. Originally described in T cells nibbling membrane at immunological synapses^{275,859}, bi-directional transfer of DC-CD4⁺ T cell membrane may have occurred to result in CD3⁺ DCs which would be retained in the CD3⁺ eluate. Furthermore, PM1 cells may have acquired BDCA4 expressed by IFN-DC through trogocytosis and leading to their retention in the BDCA4⁺ eluate. Engulfment of whole cells by DCs would also raise uncertainty about whether efficient separation had been achieved or whether cross-dressing or cross-presentation had occurred (as engulfed cells containing peptide-HLA class I complexes could be recycled). The difficulties in

separating IFN-DC from PM1 cells are reflected in the similar binding motifs identified in the BDCA4⁻ and BDCA4⁺ populations isolated and the fact that ~75% of peptides identified in the BDCA4⁻ population were also identified in the BDCA4⁺ cells (Figure 6.3). This experiment yielded numerous HIV-1 epitopes, some of them previously undescribed (Table 6.2). However, robust conclusions about whether they were directly-presented or cross-presented cannot be made. It may be possible to achieve separation of the two populations by fluorescence activated cell sorting (FACS).

Given the difficulties in ensuring separation of an acceptably pure population of IFN-DCs after co-culture with HIV-1-infected CD4⁺ T cells, I utilised the HLA class I negative CD4.221 cells as a solution. In Chapter 4 I demonstrated that relatively few peptides (<300) were eluted from W6/32 IPs of CD4.221 cells. By excluding any of these previously identified peptides, I could ensure that peptide-HLA class I complexes identified in W6/32 IPs of IFN-DC/CD4.221 co-cultures originated from IFN-DC (and not from CD4.221-expressed HLA-E/G). However, it was not possible to compare cross-presentation to HLA class II presentation in this system, because HLA class II is expressed by CD4.221 cells⁶¹⁷. Greater than 7000 peptides were eluted from W6/32 IPs of these co-cultures, 9-mers of which were clustered into each of the HLA class I alleles expressed by the IFN-DC donor, thus confirming the ability of this approach to specifically elute ligands from IFN-DC-expressed HLA class I in CD4.221 co-cultures. 10 HIV-1-derived peptides were identified, excluding non-specific Gag contaminant peptides (Table 6.3). Although previous studies have analysed HLA class II presentation of exogenous proteins by mass spectrometry^{860–863}, the data presented here constitute the first example of analysis of naturally processed and cross-presented HLA class I epitopes by immuno-peptidomics to my knowledge. Many of the cross-presented HIV-1 peptides identified have not been described previously, were longer than usual (up to 14 amino acids in length), and would therefore need validation in additional HLA peptide binding assays to confirm their specificity. However, the well-described immunodominant HLA-B*27:05-restricted Gag KK10 (KRWIIILGLNK) and HLA-B*51:01-restricted LI9 (LPPVVAKEI) epitopes were also identified with high confidence. Given that immunodominance has been associated with efficient presentation by both DCs and target cells in viral infection models in mice^{328,864,865}, the identification of these immunodominant HIV-1 epitopes may support this association, although the relative abundance of

individual immunodominant epitopes detected (or undetected subdominant epitopes) cross-presented by IFN-DCs cannot easily be inferred from the data generated here. Differential antigen processing of immunodominant and subdominant HIV-1 epitopes could be an important factor in determining immunodominance hierarchies, as was suggested by one study of HLA-B*57:01-restricted epitopes⁵⁶¹. Comparative quantitative immunopeptidomic analysis of cross-presentation by IFN-DCs from more donors expressing HLA types with well-described immunodominance hierarchies would provide more information about the relative cross-presentation of immunodominant/subdominant HIV-1 epitopes.

One caveat to these results is that no AZT was incorporated into these assays and therefore IFN-DC may have become infected and produced HIV-1 proteins endogenously. However, this is unlikely because the HIV-1 lifecycle is typically longer than the 20 hour incubation used here⁸ and DCs are generally not productively infected with HIV-1⁴⁷⁶. Future studies could utilise AZT to inhibit HIV-1 infection of DCs, as my data showed no effect of AZT on IFN-DC cell death or phenotype. Alternatively, HIV-1 mRNA could be transferred to IFN-DCs via exosomes or phagocytosis enabling HIV-1 proteins to be produced endogenously within IFN-DCs. In fact miRNAs and mRNAs (although they tend to be unusually short (<700 nt)) have been demonstrated to be transferred intercellularly by exosomes^{866,867}. One study even showed that unspliced but not spliced HIV-1 transcripts associated with exosomes⁷⁰⁷. Therefore it is possible that Gag or Pol epitopes could be translated endogenously by DCs from unspliced genomic HIV-1 transcripts transferred by exosomes. Regulatory/accessory HIV-1 proteins could potentially be translated within DCs from singly- or multiply-spliced HIV-1 transcripts internalised by phagocytosis of infected CD4⁺ T cells. However, the amount of HIV-1 genomic mRNA transferred by exosomes was reported to be <10% of that transferred by virions⁷⁰⁷, and direct translation of viral proteins from incoming virion-encapsidated genomic mRNAs has not been demonstrated. Furthermore, the amount of transferred mRNAs (in the absence of cDNA) required for production of sufficient viral proteins and epitopes to be detected by mass spectrometry is not clear, but it may potentially require at least a few thousand copies as are observed in productively infected cells⁸⁶⁸. For the aforementioned reasons, the heavy isotope

labelling of HIV-1-infected CD4.221 is the best solution to unequivocally identify cross-presented 'heavy' HIV-1 peptides.

Similarity between CD4⁺ T cell and IFN-DC HLA class I immunopeptidomes

No discernible differences in the types of peptides presented by CD4⁺ T cells and IFN-DCs were observed, as 9-mers clustered into very similar binding motifs specific for the alleles expressed by the donor. Furthermore, a large overlap in peptide identifications showed that the peptides presented originated from similar proteins (Figure 6.6). HLA class I binding motifs generally seem to be rigid in the presence of TAP, tapasin and other peptide-editing mechanisms which optimise the presentation of high affinity ligands. Hence an altered binding motif for 9-mers or peptide length would only be expected if one cell type was deficient for components of antigen processing or peptide loading machinery^{608,869}. The large overlap in peptide identifications likely reflects presentation of epitopes from proteins constitutively expressed by both cell types. Likewise, given that protein abundance is predictive of HLA class I presentation¹⁶⁵, the positive correlation between the measured abundance of shared peptides also likely mirrors the constitutive expression of proteins required for basic cellular function in both cell types. Moreover, the HLA-B*57:01-restricted Env-RW10 (RVKEKYQHLW) epitope was identified in both CD4⁺ T cells and cross-presented by IFN-DCs (Table 6.4). The lack of detection of the other 7 CD4⁺ T cell-presented HIV-1 epitopes derived from conventional proteins in IFN-DCs could be due to the relatively lower efficiency of cross-presentation, increased sample complexity due to heavy isotope labelling, or differential antigen processing which limits presentation of these other HIV-1 epitopes. Indeed, evidence obtained from *in vitro* proteasomal digests and T cell stimulation assays suggests that significant differences in the generation of individual HIV-1 epitopes exist between cell types^{559–561,870}. Although it was not possible here due to limitations in cell numbers, if biological replicates were performed for each donor this would enable more precise determination of the extent of immunopeptidome overlap and any quantitative differences between DCs and CD4⁺ T cells. In this respect, I would expect there to be subsets of peptides uniquely presented by one cell type or the other, due to differential protein expression. I would also expect there to be subsets of peptides presented by both cell types which are present in different quantities due to the aforementioned altered antigen processing mechanisms.

Quantitative targeted mass spectrometry techniques with increased sensitivity could be utilised to comparatively evaluate the abundance of particular epitopes on each cell type. Elucidating the similarities and differences between immunopeptidomes of priming DCs and target CD4⁺ T cells is important for vaccine development, as selection of conserved epitopes presented by both cell types for incorporation into a vaccine would be desirable.

Properties of peptides and proteins cross-presented by IFN-DC

What little is known about the nature of cross-presented proteins is based on study of a few model antigens. In this chapter I provided the first immunopeptidomic study of cross-presentation to date, which allowed me to analyse the properties of peptides and proteins cross-presented by IFN-DCs. One issue which became apparent during analysis was that many of the peptides containing heavy isotopes were not fully labelled; that is, some of the leucines, lysines and arginines within the peptide contained the 'light' isotope (see Table 6.4; RW10 epitope as example). Cells contain an intracellular store of amino acids, but this store is quickly replaced by extracellular amino acids provided in cell culture media. It is unlikely that IFN-DCs reused heavy isotope labelled amino acids for endogenous protein synthesis in high enough amounts for any single partially labelled heavy peptide to be detected, as excess extracellular 'light' amino acids would quickly dilute the relatively small intracellular stores of heavy isotopes. Given that I had confirmed complete labelling of the CD4.221 cell line proteome with heavy isotopes (data not shown), the cross-presented semi-labelled heavy peptides are likely due to ongoing translation occurring in CD4.221 when placed into 'light' isotope-containing medium. As most 'heavy' peptides detected were incompletely labelled, this could potentially indicate that cross-presentation favoured recently translated proteins here. Speculatively, there could also be transfer of actively-translating ribosomes between the cells, as has been observed from Schwann cells to axons⁸⁷¹. If such a mechanism existed in this setting, it would be unlikely to occur through exosomes, as ribosomal RNA is rarely detected in exosomes⁸⁶⁷. However, this observation could also be an artefact of the 20 hour timepoint when IFN-DCs were lysed. Perhaps at an earlier timepoint the predominant proteins cross-presented would be completely labelled with heavy isotopes. In future experiments, the irreversible protein synthesis inhibitor emetine could be

incorporated into the assay to block translation of nascent proteins in CD4.221 cells and therefore ensure cross-presented peptides are fully labelled with heavy isotopes.

The measured abundance of peptides cross-presented by IFN-DC was significantly lower than the abundance of directly-presented peptides (Figure 6.7). Intuitively this makes sense, as exogenous proteins must be internalised in sufficient quantities and avoid excessive degradation in lysosomes to enter the cross-presentation pathway, whereas endogenous proteins do not need to meet these prerequisites. In fact, a study using targeted mass spectrometry analysis of the chicken OVA H-2K^b-restricted SIINFEKL peptide demonstrated that DCs presented approximately 10 times less exogenous OVA-derived SIINFEKL peptide *in vivo* than EG-7 cells which endogenously express OVA and directly present SIINFEKL⁸⁷². Thus cross-presented peptides may generally be less abundantly presented than directly-presented peptides, as supported by this study and my results.

Just one study has directly examined the effect of protein abundance on cross-presentation, concluding that only highly abundant proteins can be cross-presented in sufficient quantities for naïve T cell priming⁸⁴¹. This study was based purely on the model antigen OVA, so it is unclear whether this result is generalizable to all cross-presented proteins. By calculating iBAQ values for proteins expressed in CD4.221 cells or EV, I showed that cross-presented proteins were significantly more abundant than proteins which were only directly-presented. This difference was observed for proteins in both HIV-1-infected CD4.221 cells and EVs secreted by HIV-1-infected CD4.221s. However, whether the cross-presented proteins originated from EVs or were cell-associated is unknown. A caveat in this analysis is that many proteins did not have expression data available due to their abundance being below the detection limit of the low-depth proteomic approach used. A deep proteomic analysis achieved by extensive pre-fractionation of peptides would allow the identification of up to ~20,000 proteins present in the CD4.221 cell line.

Uninfected CD4.221-derived proteins cross-presented by IFN-DCs were significantly longer than endogenous proteins directly-presented within the IFN-DCs. This result may simply arise from the previously observed correlation between number of observed HLA class I ligands and protein length¹⁶⁵. As shown by Bassani-Sternberg *et al*, normalising for protein length by computing the theoretical number of HLA class I ligands within these cross-presented or directly-presented proteins

to calculate a HLA class I ‘sampling density’ could remove bias based on this correlation. Bassani-Sternberg *et al* also showed that a subset of proteins with fast turnover rates were more likely to be directly-presented. In the IFN-DC immunopeptidome, I found no significant difference in the turnover rates of proteins which were cross-presented or directly-presented. As these turnover rates were calculated in another study using different cells⁸⁵⁰, they may not reflect the turnover rates in the cell types used here. Furthermore, the turnover rates were not calculated for many proteins for which peptides are presented by HLA class I in my data, creating a potential bias. The lack of difference observed in these data contrasts with the multiple studies suggesting that long-lived, stable proteins are a rich source of antigen for cross-presentation^{829–833}.

The subcellular location of proteins seems to be important for their propensity to be cross-presented, as membrane-forms of OVA or ER-retained LCMV GP are cross-presented more efficiently than their cytosolic/soluble versions^{829,838,840,873}. However, whether the cross-presentation characteristics of these 2 model antigens can be generalised to the whole proteome is not clear. In support of these studies, Figure 6.7E suggests that membrane-associated proteins may be more enriched in the cross-presentation pathway relative to proteins found in other cellular compartments. This enrichment may parallel the enriched membrane protein presentation by HLA class II molecules⁶³⁴, as endocytosis of membranous vesicles or trogocytosis is followed by degradation in the endolysosomal pathway. Hence this membrane protein enrichment could result from enhanced membrane protein acquisition from exogenous sources, decreased stability of non-membranous proteins or increased membrane protein presentation by the vacuolar cross-presentation pathway where membrane proteins are predominantly degraded within the cell. Interestingly, the proportion of proteins classified as extracellular was increased in the cross-presented group relative to directly-presented proteins in uninfected IFN-DC/CD4.221 co-cultures, but not in HIV-1-infected co-cultures. Because DCs efficiently acquire antigen from apoptotic cells for cross-presentation^{841,842}, perhaps the increased levels of apoptosis expected to occur in HIV-1-infected cells would result in increased uptake of cellular antigen for cross-presentation and therefore a lower proportion of the cross-presented proteins would arise from extracellular sources.

For all of these analyses, peptides of any length were included as there is some evidence that longer peptides (i.e. >14 amino acids) can bind HLA class I (discussed in chapter 4). However, it is possible that HLA class II-bound or non-specifically-eluted peptides were included in these analyses, and could therefore alter results. Although these extended peptides constitute a minority of those identified in W6/32 IPs, it would be of interest to determine whether exclusion of these peptides alters the associations detailed in this chapter.

Lastly, analysis of 8-11-mers revealed differences in the proportion of peptides predicted to bind divergent HLA class I alleles. For example, predicted HLA-B*08:01 ligands constituted a lower proportion of the ‘heavy’ cross-presented peptides than in the directly-presented subset (Table 6.5). Speculatively, individual HLA class I alleles may have differential access to the vacuolar cross-presentation pathway. CD74/Ii has been implicated as an important mediator in the cross-presentation pathway by chaperoning HLA class I alleles from the ER into the endolysosomal system for peptide loading²⁶⁸. Furthermore, Ii expression has been shown to increase surface expression of HLA class I in an allele-dependent manner^{642,643}. The identification of CD74-derived peptides in many W6/32 IPs here supports a role for CD74 in the HLA class I presentation pathway (data not shown), although this could result from HLA class II contamination. Thus one hypothesis for the allelic differences observed in cross-presented or directly-presented ligands here, is that some alleles have lower affinity (e.g. HLA-B*08:01) for Ii (or some other undetermined chaperone) and therefore do not enter the vacuolar cross-presentation pathway as frequently as other alleles. The demonstration that TAP-independent endolysosomal peptide loading is allele-dependent supports this hypothesis⁸⁷⁴.

In the system used here, cross-presentation could potentially be enhanced by culturing cells in serum-free medium, as scavenging receptors would then not be saturated by proteins present within FBS or human serum.

Relatively few Nef epitopes cross-presented

In the experiments shown in this chapter, just 3 unique Nef epitopes were identified on infected CD4⁺ T cells or EVs secreted by MT-2 cells and 0 *bona fide* cross-presented Nef epitopes were identified. Obviously, these data do not support a hypothesis in which DCs preferentially cross-

present Nef epitopes from HIV-1-infected CD4⁺ T cells or EVs secreted by infected CD4⁺ T cells. This could partially be a result of the timing of Nef and Gag expression in the HIV-1-infected CD4⁺ T cells used here. Perhaps pulsing DCs with EVs or whole cells during the early stages of the infection lifecycle (e.g. ~12-24 hours post-infection) would result in enhanced Nef epitope presentation. However, the infection method used in this thesis is not synchronous, and the population of infected cells is likely to represent a mix of cells at various stages of the infection lifecycle. Alternatively, other cell types (e.g. macrophages, discussed in Chapter 5) infected with HIV-1 may produce more Nef protein, and DCs are therefore more likely to preferentially present Nef epitopes from these different cellular sources *in vivo*. In Chapter 7, I will place these results in the context of the overarching research questions and hypothesis for this thesis.

Chapter 7: General discussion

This thesis aimed to address the role of DCs in determining the Nef-biased CTL immunodominance hierarchy during AHI and the delay before expansion of the second wave of CTL responses with broader epitope specificity. To explain the delayed expansion of additional CTL responses, I hypothesised that shortly after priming of CTL responses to a small number of the most rapidly and/or abundantly presented epitopes, DC function is impaired by soluble or particulate factors released into tissue fluids/plasma during AHI. As for the bias towards targeting of HIV-1 Nef by CTL during AHI, I hypothesised that abundant Nef-containing exosomes secreted by HIV-1-infected cells were captured by DCs and Nef epitopes were preferentially cross-presented by DCs. Here I will discuss the experimental evidence provided by this thesis in the context of these hypotheses.

Impairment of CD8⁺ T cell priming during AHI

The mechanisms behind the delay between the expansion of the first and second waves of HIV-1-specific CD8⁺ T cells responses during AHI are unknown. Given the crucial role of mDCs in priming CD8⁺ T cells²⁸², impairment of mDC function shortly after responses are primed to a limited number of epitopes could hypothetically explain this delayed CD8⁺ T cell expansion. Naïve CD8⁺ T cells are thought to require just one contact with antigen presented by mDCs in order to divide and expand⁸⁷⁵. Thus CD8⁺ T cell responses induced before mDC impairment during AHI would expand but responses of additional specificities would not be primed. Numerous reports have documented the impaired function of mDCs during chronic infection and in particular, reduced IL-12 secretion^{501,520,876–878}. However, very little is known about mDC function during AHI. The number of circulating mDCs (and also pDCs) drops rapidly as viraemia begins to increase during AHI and never fully recovers⁴⁷. This reduction is likely due to DC activation and migration to lymph nodes, where different studies in SIV-infected macaques have reported that mDCs undergo apoptosis⁸⁷⁹ or accumulate in a putatively dysfunctional state with reduced co-stimulatory molecule expression⁸⁸⁰.

In Chapter 3, I used *in vitro* priming assays to examine some potential mechanisms which could affect CD8⁺ T cell priming during AHI. No consistent effect of plasma sampled from patients at timepoints during the early viral expansion phase of AHI on DC-mediated CD8⁺ T cell priming was

observed. In fact, plasma samples from certain timepoints seemed to increase IL-12 production by IL-4-DCs, in contrast to what was observed in a previous study⁴⁶. As discussed in Chapter 3, the need to treat plasma donor samples with heparin or heat-inactivate them may have resulted in a loss of inhibitory activity. The observed stimulatory effect of certain plasma samples may have resulted from these samples being from timepoints around the peak of viraemia, as Frleta *et al* observed the inhibitory effect only in very early AHI (before the peak of viraemia). The increased IL-12 production observed by the slightly later timepoint plasma-treated DCs in response to TLR stimulation is consistent with the increased *ex vivo* TLR responsiveness observed in peripheral blood mDCs sampled at timepoints around and just after the peak of viraemia during AHI⁴⁷.

Due to the issues associated with plasma donor samples described above, the effect of individual factors present in plasma during AHI on CD8⁺ T cell priming by DCs was examined. Because IFN α is one of the earliest cytokines to peak during the AHI cytokine storm⁴³, I examined its effect on CD8⁺ T cell priming. IFN α -treated IL-4-DCs showed reduced co-stimulatory molecule expression and in some donors, CD8⁺ T cells primed by IFN α -treated IL-4-DCs showed reduced cytokine secretion and antigen-specific expansion. Although no significant effect was observed, the results in Chapter 3 provide a basis for further research into the effects of IFN α on CD8⁺ T cell priming. Thus IFN α could hypothetically be one of the factors which may inhibit CD8⁺ T cell priming during AHI. Importantly, this effect could be temporal in nature, as IFN α generally promotes priming and memory formation when acting directly on CD8⁺ T cells at and just after initial activation^{308,309}, whereas other studies have shown that type I IFNs enhance subsequent apoptosis whilst reducing IL-12 production by mDCs⁵⁰⁹⁻⁵¹¹. Therefore a speculative interpretation of these preliminary data could be that the first wave of responses primed by DCs during AHI benefit from the early peak in IFN α , whilst later responses are impaired due to DC apoptosis and reduced function.

Although these data do not preclude the possibility that the ability of DCs to prime naïve CD8⁺ T cells during AHI is impaired, this thesis did not provide conclusive evidence to affirm the working hypothesis. Therefore it is possible that other DC-independent factors play a more substantial role in determining the delay in expansions of additional CTL responses during AHI. For example, CD8⁺ T cells could be inhibited directly by the cytokine storm following priming, as was suggested recently

by one study examining the expansion of CD8⁺ T cells with reduced function¹³⁶. In fact, CD8⁺ T cells in mice have been found to display markers of exhaustion just hours after infection with LCMV⁸⁸¹. Perhaps a similar inhibition of CD8⁺ T cell function could occur in AHI, a feature which might separate LTNPs from progressors¹²⁹.

Preferential CTL targeting of Nef epitopes during AHI

The specific CTL immunodominance hierarchy observed during AHI has been hypothesised to result from interplay of various factors including kinetics of HIV-1 protein expression in infected cells, the HLA class I genotype of the infected individual and the sequence of the T/F virus³⁵². In the introductory Chapter 1 I laid out reasons to believe that the early kinetics of Nef expression within infected cells or a pre-infection Nef-biased naïve or memory TCR repertoire are unlikely to explain the high proportion of Nef-targeted CTL responses during AHI. Instead, the hypothesis that DCs preferentially present Nef epitopes during AHI was tested in this thesis.

Evidently, CD4⁺ T cells predominantly present epitopes derived from Gag, Pol and Env as demonstrated by sequencing of naturally processed HLA class I ligands by mass spectrometry in Chapter 4 and Chapter 6. Indeed, only 4 Nef-derived peptides were identified in HIV-1-infected CD4⁺ T cells whereas >30 Gag-derived peptides were identified. In accordance with this result, Gag was shown to be by far the most abundant viral protein expressed within infected CD4⁺ T cells, followed by Pol and Env (Chapter 5). The HIV-1 immunopeptidome could potentially change over time depending on viral protein expression kinetics (e.g. early Nef expression, late Gag expression), however cells in culture and *in vivo* are infected asynchronously and are therefore a mixture of cells at varying stage of the infection lifecycle. Nevertheless, a more thorough exploration of synchronous early epitope kinetics could be achieved by synchronous HIV-1 infection of CD4⁺ T cells *in vitro* using CD44 magnetic beads⁶⁷⁹. On the whole however, these data do not support a role for direct presentation of Nef epitopes by infected CD4⁺ T cells in preferential expansion or survival of Nef-specific CTL during AHI. Rather, Nef-bias at the initial priming event seems more likely in view of these data.

Given that DCs are thought to be the crucial cell for CD8⁺ T cell priming *in vivo*²⁸², and HIV-1 does not productively infect DCs⁴⁷⁶, cross-presentation must be important during AHI. This links to my

hypothesis that Nef protein is preferentially cross-presented by DCs in order to prime Nef-specific naïve CD8⁺ T cells during AHI. However, an *in vitro* cross-presentation assay designed to reveal the repertoire of HIV-1 epitopes cross-presented by MDDCs internalising antigen from infected CD4⁺ T cells did not show enrichment for Nef-derived epitopes (Chapter 6). In fact, no Nef epitopes were confirmed to be cross-presented by MDDCs, whilst Gag, Pol, Env and Rev cross-presented epitopes were identified. The absence of detectable Nef epitopes and predominance of Gag epitopes was obvious in experiments where MDDCs were cross-presenting EV antigen or interacting with HIV-1-infected cells. Reflecting this result, relative quantitation of Nef and Gag protein by mass spectrometry and immunoblotting demonstrated that Gag is by far the most abundant protein expressed within CD4⁺ T cells or secreted in EVs/virions (Chapter 5). Therefore, although I didn't explore whether Nef-containing EVs are preferentially internalised by DCs over virions, the results described here make this possibility unlikely. However, only one type of MDDC was used for these experiments, and this *in vitro* system may not reflect the DC subset priming naïve CD8⁺ T cells *in vivo*. It is conceivable that one of the many migratory or lymph-node resident DC subsets present *in vivo* are the drivers of naïve CD8⁺ T cell priming during AHI, and that such a subset may selectively internalise particular forms of antigen through receptor-mediated endocytosis²⁴⁹. For example, BDCA3⁺XCR1⁺ DCs loaded with apoptotic HIV-1-infected CD4⁺ T cells were shown to stimulate HIV-1-specific CD8⁺ T cell clones more efficiently than other DC subsets²⁵⁰. This enhanced cross-presentation could be due to differential antigen retention, degradation or translocation from phagosomes in DCs subsets. However, receptor targeting obviously plays a role in enhancing cross-presentation, as demonstrated by studies showing enhanced cross-presentation by multiple DC subsets following targeting of antigen to DEC-205 in transgenic DEC-205 mice or human DCs^{254,882}. Definition of the receptors which might mediate selective uptake of diverse forms of soluble, particulate, apoptotic or live cellular HIV-1 antigen by cross-priming DC subsets would provide insight into potential mechanisms of CTL immunodominance during AHI.

Besides selective antigen uptake, differences in relative viral protein availability could affect the repertoire of cross-presented HIV-1 peptides. Proteomic evaluation revealed that Gag protein was undoubtedly the most abundant protein produced by HIV-1-infected CD4.221 cells (Chapter 6 and

data not shown). Likewise, primary CD4⁺ T cells and CD4⁺ T cell lines such as Jurkat cells, PM1 cells and HuT-78 cells were shown to express abundant Gag protein and relatively small amounts of Nef (Chapter 5). Presumably, HIV-1-infected MT-2 cells display a similar pattern of viral protein expression in accordance with the 12 Gag peptides identified as presented by HLA class I on EVs secreted by MT-2 cells (Chapter 6). In stark contrast, Nef seems to be the most abundant HIV-1 protein present in the plasma EVs of HIV-1-infected individuals, as suggested by results in Chapter 5 and other studies^{453,454}. This disconnect between the HIV-1 protein expression patterns of infected CD4⁺ T cells and the plasma proteome suggests that CD4⁺ T cells may not be a central source of HIV-1 antigen for cross-priming during AHI.

It has long been known that macrophages are infected with HIV-1 *in vivo*. In fact, infected tissue macrophages persist throughout all stages of infection including during antiretroviral therapy^{787,883}. Macrophages are long-lived cells which accumulate over time in lymphoid tissues during HIV/SIV infection^{731,884}. In Chapter 5, I suggested that macrophages may be a relevant source of Nef exosomes for cross-presentation. Immunoblotting results further indicated that the ratio of Nef/Gag production within EVs increases over time. These results are supported by other studies in which cellular Nef production increases over time after *in vitro* THP-1/MDM HIV-1 infection^{453,737}. Also, an abundance of unintegrated DNA accumulating in macrophages has been noted^{732,780,885}, and preferential transcription of multiply-spliced transcripts produces Nef protein^{733,782}. In this thesis, the X4-tropic HIV-1 IIIB was used for CD4⁺ T cell infections whilst the R5-tropic HIV-1 NL4.3 Bal *env* strain was utilised for macrophage infections. Future work could address whether the differences in viral production reflect the HIV-1 strain used or the cell type infected. Furthermore, future experiments could also address the composition of Nef exosomes secreted by differentially-matured macrophages over time. Lastly, to determine whether Nef epitopes are preferentially cross-presented by DCs internalising macrophage EVs, EVs secreted by HIV-1-infected macrophages could be incorporated into the MDDC cross-presentation assay described in Chapter 6.

If macrophages are indeed major producers of Nef antigen *in vivo*, could it be that Nef-specific CTL primed during AHI play a significant role in control of macrophage infection? Some studies have shown that CTL clones have a much greater ability to suppress HIV-1 replication in macrophages

than CD4⁺ T cells⁸⁸⁶, and that CTL from ECs can inhibit viral replication in alveolar macrophages^{887,888}. However, SIV-infected macrophages seem to be resistant to killing in a Nef-independent manner^{889,890}. Interestingly, *ex vivo* cytotoxic Gag- and Nef-specific CD4⁺ T cells have been shown to inhibit viral replication in SIV-infected macrophages but not infected CD4⁺ T cells⁸⁹¹. Furthermore, Zheng *et al* showed that the specific lysis of HIV-1-infected macrophages by HIV-1 Nef-specific cytotoxic CD4⁺ T cells was much greater than that observed for lysis of infected CD4⁺ T cells⁸⁹². Thus one implication of the potentially greater Nef expression in macrophages is their increased targeting by Nef-specific CTL. To this end, an exploration of the immunopeptidome of HIV-1-infected macrophages would be of interest (i.e. do they present many Nef epitopes?).

Linked in with this Nef expression by macrophages is a speculative alternative hypothesis about generation of Nef-specific CD8⁺ T cells during AHI. Macrophages expressing abundant Nef could also directly present Nef epitopes in lymph nodes to prime CD8⁺ T cells, as has been shown for CD169⁺ macrophages inducing adenoviral or anti-tumour immunity^{755,756}. CD169 is a receptor for exosomes⁷⁵⁴, thus CD169⁺ macrophages or CD169⁺ DCs in the lymph node could potentially capture Nef-containing exosomes and cross-present Nef epitopes to naïve CD8⁺ T cells.

Identification of macrophages as a potential source of Nef *in vivo* also has implications for treatment strategies and immunopathogenesis of HIV-1 infection. Nef is also well known to alter macrophage mesenchymal migration as a possible mechanism of viral spread into tissues⁸⁹³. Furthermore, Nef is thought to play a role in many macrophage-mediated sequelae of HIV-1 infection such as lymphoma, metabolic disorders, and neurocognitive disorders⁸⁹⁴. It should be noted that some analyses of animal models suggest that other unidentified Nef-expressing cells cause organ disease⁸⁹⁵. Targeting such Nef-expressing cells with therapeutic vaccination strategies or pharmaceuticals could potentially alleviate some of the detrimental effects of extracellular Nef on HIV-1-associated disease.

Taken together, the data presented in this thesis suggest that Nef is not preferentially cross-presented by DCs internalising antigen from HIV-1-infected CD4⁺ T cells. Instead, I identified HIV-1-infected macrophages as a potential source of the abundant Nef antigen observed in the plasma of HIV-1-infected individuals. On the basis of these data, I speculatively hypothesise that lymph-node resident CD169⁺ macrophages or lymph-node resident DCs capture Nef-containing exosomes secreted by

infected macrophages from lymph then predominantly cross-present (or are cross-dressed) Nef epitopes to rapidly prime naïve CD8⁺ T cells during AHI.

Conclusion

This thesis provides a number of novel contributions to the field of DC antigen presentation and T cell priming. Firstly, preliminary analyses identified IFN α as a potential mediator of DC dysfunction during subsequent CD8⁺ T cell priming. Multiple novel HLA class I HIV-1 epitopes were identified by an immunopeptidomics approach, including peptides derived from the so-called ‘untranslated’ region of the HIV-1 genome which could potentially be incorporated into innovative vaccination strategies. I also determined that many of the unusual extended Gag peptides identified in previous studies are in fact non-specifically eluted from W6/32 IPs, meaning that these peptides should not be pursued for vaccination purposes. HIV-1-infected macrophages were identified as a potential source of the Nef-containing EVs found in the plasma of HIV-1⁺ individuals. Furthermore, an *in vitro* assay capable of identifying cross-presented and cross-dressed HIV-1/self-peptides was developed. This assay provided novel information about the attributes of cross-presented proteins, and also indicated that HIV-1-infected CD4⁺ T cells are not a source of Nef antigen for preferential Nef epitope presentation. The information provided about the understanding of DC control of CD8⁺ T cell immunodominance and expansion during AHI could potentially be exploited in future development of novel prophylactic and therapeutic strategies against HIV-1.

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9.0 Appendix

9.1 Primer sequences

The primers used for PCR amplification of HIV-1 cDNA are shown in Table 9.1. The primers used for sequencing the HIV-1 IIIB genome are listed in Table 9.2.

Table 9.1. PCR Primer sequences	
Primer name	Sequence (5' → 3')
R3B3R	ACTACTTGAAGCACTCAAGGCAAGCTTTATTG
R3B6R	TGAAGCACTCAAGGCAAGCTTTATTGAGGC
U5B1F	CCTTGAGTGCTTCAAGTAGTGTGTGCCCGTCTGT
B3F1	ACAGCAGTACAAATGGCAGTATT
B3F3	TGGAAAGGTGAAGGGGCAGTAGTAATAC
B5R1	CTTGCCACACAATCATCACCTGCCAT
B5R2	CAATCATCACCTGCCATCTGTTTTCCATA
U5B4F	AGTAGTGTGTGCCCGTCTGTTGTGTGACTC

Table 9.2. Sequencing primer sequences			
Primer name	Sequence (5' → 3')	Primer name	Sequence (5' → 3')
SEQ-A-1	GGATAGAGGTAAAAGACACCAA	SEQ-A-R1	AATTTTTCCAGCTCCCTGCTTGCCCA
SEQ-A-2	AAGTGACATAGCAGGAAGTACTAG	SEQ-A-R2	CTGATAATGCTGAAAACATGGGTAT
SEQ-A-3	CTAGGAAAAAGGGCTGTTGGAAATG	SEQ-A-R3	GACAGGGCTATACATTCTTACTAT
SEQ-A-4	AAAATGATAGGGGAATTGGAGGTTT	SEQ-A-R4	CCAAATTTTCCCTAAAAAATTAGCCT
SEQ-A-5	CAGTACTAGATGTGGGGGATGCATA	SEQ-A-R5	TTACTGGTACAGTTTCAATAGGAC
SEQ-A-6	ACAGCTGGACTGTCAATGATATACA	SEQ-A-R6	GGAATATTGCTGGTGATCCTTTCCA
SEQ-A-7	CCACAGAAAGCATAGTAATATGGGG	SEQ-A-R7	ATCCCTGGATAAATCTGACTTGCCCA
SEQ-A-8	CACACAAAGGATTGGAGGAAATGA	SEQ-A-R8	CCCTATTAGCTGCCCCATCTACATA
SEQ-A-9	AAAATTAGCAGGAAGATGGCCAGT	SEQ-A-R9	TTAAAATCACTAGCCATTGCTCTCC
SEQ-A-10	CGGGTTTATTACAGGGACAGCAGAGA	SEQ-A-R10	CTCCTTGACTTTGGGGATTGTAGGG
SEQ-A-11	GTCAGGGAGTCTCCATAGAATGGA	SEQ-A-R11	ATCATCACCTGCCATCTGTTTTCCAT
SEQ-A-12	ATTGGGTGCCAACATAGCAGAATA	SEQ-A-R12	AGAGATCCTACCTTGTTATGTCTCT
SEQ-A-13	GAGAAAGAGCAGAAGACAGTGG	SEQ-A-R13	CTTCCTGCCATAGGAGATGCCTAAG
SEQ-A-14	TATGGGACCAAAGCCTAAAGCCATGTG	SEQ-A-R14	ACCATGTTATTTTTCCACATGTTAAA
SEQ-A-15	CAGCACAGTACAATGTACACATGGAA	SEQ-A-R15	CTGCCATTTAACAGCAGTTGAGTTGA
SEQ-A-16	TTTAATTGTGGAGGAGAATTTTTCTA	SEQ-A-R16	ATGGGAGGGGCATACATTGCT
SEQ-A-17	AGCAGCAGGAAGCACTATGGGCGC	SEQ-A-R17	CCTGGAGCTGTTTAATGCCCCAGAC
SEQ-A-18	CATATCAAATTGGCTGTGGTATAT	SEQ-A-R18	GGTGAGTATCCCTGCCTAACTCTAT
SEQ-A-19	GGAACCTGTGCCTCTTCAGCTACC	SEQ-A-R19	ACTTTTTGACCACTTGCCACCCAT
SEQ-A-20	CCACAGGTACCTTTAAGACCAATGAC	SEQ-A-R20	TCAACTGGTACTAGCTTGAAGCACCA
SEQ-A-21	GGGACTTTCCACTGGGGAAGTTTCC	5'R (LTR)	GGTCTCTCTGGTTAGACCAGAT
		Gag (LTR)	TCCAGCTCCCTGCTTGCCCATACTA

9.2 Peptide pool sequences

Tables 9.3, 9.4, 9.5 and 9.6 list the pools of 18-mers overlapping by 10 amino acids spanning the HIV-1 clade B consensus sequence. Table 9.7 lists the pool of Flu, EBV and CMV (FEC) epitopes

used as negative control peptides in *in vitro* priming assay ELISPOT readout assays. Table 9.8 lists the peptides used for antigen-specific stimulation of naïve CD8⁺ T cells by peptide-pulsed MDDCs in the *in vitro* priming assay.

Table 9.3. <i>Env</i> peptide pools					
Peptide ID:	Sequence		Peptide ID:	Sequence	
000195	HNWATHACVPTDPNPQE	ENV-1	000567	IGPGRAFYTTGEIIGDIR	ENV-3
000200	EDIISLWDQSLKPCVKLT		000568	TTGEIIGDIRQAHCNISR	
000201	QSLKPCVKLTPLCVTLNC		000569	IRQAHCNISRKWNNTLK	
000242	RPGGGDMRDNRSELYKY		000570	SRAKWNNTLKQIVKKLRE	
000257	DQQLLGIWGCSGKLICTT		000571	LKQIVKKLREQFGNKTIV	
000267	LWNWFDITNWLWYIKIFI		000572	REQFGNKTIVFNQSSGGD	
000271	VLSIVNRVRQGYSPLSFQ		000573	IVFNQSSGGDPEIVMHSF	
000529	MRVKGIRKNYQHLRWGT		000574	GDPEIVMHSFNCGGEFFY	
000530	NYQHLRWGTMLLGMLMI		000575	SFNCGGEFFYCNTTQLFN	
000531	GTMLLGMLMICSAAEKLW		000576	FYCNTTQLFNSTWNGTWN	
000532	MICSAAEKLWVTVYGVVP		000577	FNSTWNGTWNTEGNITL	
000533	LWVTVYGVVPVWKEATTT		000578	WNGTWNTEGNITLPCRI	
000534	VPVWKEATTLFCASDAK		000579	WNTEGNITLPCRIKQII	
000536	AKAYDTEVHNWATHACV		000580	EGNITLPCRIKQIINMWQ	
000537	CVPTDPNPQEVVLENVTE		000581	RIKQIINMWQEVGKAMYA	
000538	QEVVLENVTFNFMWKNN		000582	WQEVGKAMYAPPIRGQIR	
000539	TENFNMWKNNMVEQMHEH		000583	YAPPIRGQIRCSSNITGL	
000540	NNMVEQMHEDIISLWDQS		000585	GLLLTRDGGNNETEIFRP	
000541	LTPLCVTLNCTDLMNATN		000586	GNNETEIFRPGGDMRDN	
000542	LCVTLNCTDLMNATNTNT		000587	DNWRSELYKYKVVKIEPL	
000543	NCTDLMNATNTNTTIIYR		000588	KYKVVKIEPLGVAPTKAK	
000544	TNTNTTIIYRWRGEIKNC		000589	PLGVAPTKAKRRVVQREK	
000545	NTTIIYRWRGEIKNCSFN	ENV-2	000590	AKRRVVQREKRAVGIGAM	ENV-4
000546	YRWRGEIKNCSFNITTSI		000591	EKRAVGIGAMFLGFLGAA	
000547	NCSFNITTSIRDKVQKEY		000592	AMFLGFLGAAGSTMGAAS	
000548	SIRDKVQKEYALFYKLDV		000593	AAGSTMGAASMTLTVQAR	
000549	EYALFYKLDVVPIDNDNT		000594	ASMTLTVQARQLLSGIVQ	
000550	DVVPIDNDNTSYRLISCNT		000595	ARQLLSGIVQQQNNLLRA	
000551	NTSYRLISCNTSVITQAC		000596	VQQQNNLLRAIEAQQHLL	
000552	TSYRLISCNTSVITQACP		000597	RAIEAQQHLLQLTVWGIK	
000553	NTSVITQACPKVSFEPIP		000598	LLQLTVWGIKQLQARVLA	
000554	CPKVSFEPIPIHYCAPAG		000599	IKQLQARVLAVERYLKDQ	
000555	IPIHYCAPAGFAILKCND		000600	LAVERYLKDQQLLGIWGC	
000556	AGFAILKCNDKKFNGTGP		000601	GCSGKLICTTAVPWNASW	
000557	NDKKFNGTGPCNTVSTVQ		000602	TTAVPWNASWSNKSLEI	
000558	GPCTNVSTVQCTHGIRPV		000603	SWSNKSLEIWDNMTWME	

000559	VQCTHGIRPVVSTQLLLN		000604	EIWDNMTWMEWEREIDNY	
000560	PVVSTQLLLNGLSLAEEEV		000605	MEWEREIDNYTSLIYTLI	
000561	LNGSLAEEEVVIRSENFT		000607	LIEESQNQQEKNEQELLE	
000562	EVVIRSENFTDNAKTIIIV		000608	QEKNEQELLELDKWASLW	
000563	FTDNAKTIIIVQLNESVEI		000609	LELDKWASLWNWFDITNW	
000564	IVQLNESVEINCTRPNNN		000610	NWLWYIKIFIMIVGGLVG	
000565	EINCTRPNNNTRKSIHIG		000611	FIMIVGGLVGLRIVFAVL	
000566	NNTRKSIHIGPGRAFYTT		000612	VGLRIVFAVLSIVNRVRQ	
000615	RGPDPRPEGIEEEGGERDR	ENV-5	000613	RQGYSPLSFQTRLPPAPRG	
000616	IEEEGGERDRDRSGRLVD		000614	FQTRLPPAPRGPDPRPEGIE	
000617	DRDRSGRLVDGFLALIWD				
000618	VDGFLALIWDRLSLCLF				
000619	WDDLRLSLCLFSYHRLRDL				
000620	LFSYHRLRDLILLIVTRIV				
000621	DLLLIVTRIVELLGR				
000622	IVELLGRRGWE				
000623	RGWEVLKYWWNL				
000624	WEVLKYWWNLLQYWSQEL				
000625	NLLQYWSQELKNSAVSLL				
000626	ELKNSAVSLLNATAIAVA				
000627	LLNATAIAVAEGTDRVIE				
000628	VAEGTDRVIEVVQRACRA				
000629	IEVVQRACRAILHIPRI				
000630	RAILHIPRRIRQGLERAL				
000631	RIRQGLERALL				
002480	TTLFCASDAKAYDTE				
002481	ASDAKAYDTEVHNW				
002482	IRCSSNITGLLLTRD				
002483	NITGLLLTRDGGNNE				
002484	IDNYTSLIYTLIEES				
002485	SLIYTLIEESQNQQE				

Table 9.4. Gag pools					
Peptide ID	Sequence		Peptide ID	Sequence	
000017	YPIVQNLQGQMVHQAI SP	GAG-1	000421	WMTETLLVQNANPDCKTI	GAG-3
000018	GQMVHQAI SPRTLNAWVK		000422	QNANPDCKTILKALGPAA	
000023	TPQDLNTMLNTVGGHQAA		000423	TILKALGPAAATLEEMMTA	
000026	TINEEAAEWDRLHPVHAG		000424	AATLEEMMTACQGVGGPG	
000027	WDRLHPVHAGPIAPGQMR		000425	TACQGVGGPGHKARVLAE	
000028	AGPIAPGQMRPRGSDIA		000426	PGHKARVLAEAMSQVTNS	
000029	MREPRGSDIAGTTSTLQE		000427	GHKARVLAEAMSQVTNSA	
000051	CRAPRKKGCKGKEGHQ		000428	AEAMSQVTNSATIMMQRG	
000052	CWKCGKEGHQMKDCTERQ		000429	EAMSQVTNSATIMMQRGN	
000053	HQMKDCTERQANFLGKIW		000430	SATIMMQRGNFRNQKTV	
000054	RQANFLGKIWPSHKGRPG		000431	GNFRNQKTVKCFNCGKE	
000387	MGARASVLSGGELDRWEK		000432	TVKCFNCGKEGHI AKNCR	
000388	SGGELDRWEKIRLRPGGK		000433	KEGHI AKNCRAPRKGCW	

000389	EKIRLRPGGKKKYKLKHI		000434	IWPSHKGRPGNFLQSRPE	
000390	GKKKYKLKHIVWASRELE		000435	PGNFLQSRPEPTAPPEES	
000391	HIVWASRELERFAVNPGL		000436	PEPTAPPEESFRFGEETT	
000392	LERFAVNPGLLETSEGCR		000437	PTAPPEESFRFGEETTP	
000393	GLLETSEGCRQILGQLQP		000438	ESFRFGEETTPSQKQEP	
000394	CRQILGQLQPSLQTGSEE		000439	FGEETTPSQKQEPIDKE	
000395	QPSLQTGSEELRSLYNTV		000440	TTPSQKQEPIDKELYPLA	
000396	EELRSLYNTVATLYCVHQ		000441	SQKQEPIDKELYPLASLR	
000397	TVATLYCVHQRIEVKDTK		000442	KELYPLASLRSLFGNDPS	
000398	HQRIEVKDTKEALEKIEE		000443	LRSFLGNDPSSQ	
000399	TKEALEKIEEEQNKSKKK				
000400	EEEQNKSKKKAQQAADT				
000401	KKAQQAADTGNSSQVSQ				
000402	QQAADTGNSSQVSQNYNYP				
000403	DTGNSSQVSQNYPIVQNL				
000404	NSSQVSQNYPIVQNLQGG				
000405	SPRTLNAWVKVVEEKAFS				
000406	VKVVEEKAFSPEVIMFMS				
000407	FSPEVIMFMSALSEGATP				
000408	FSALSEGATPQDLNTMLN				
000409	LNTVGGHQAAQMLKETI				
000410	AAMQMLKETINEEAAEWD				
000411	IAGTTSTLQEQIGWMTNN				
000412	QEQIGWMTNNPPIPVGEI				
000413	NNPPIPVGEIYKRWIILG				
000414	EIYKRWIILGLNKIVRMY				
000415	LGLNKIVRMYSPTSILDI				
000416	MYSPTSILDIRQGPKEPF				
000417	DIRQGPKEPFRDYVDRFY				
000418	PFRDYVDRFYKTLRAEQA				
000419	FYKTLRAEQASQEVKNWM				
000420	OASQEVKNWMTETLLVON				

Table 9.5. <i>Nef</i> /Accessory protein peptide pools					
Peptide ID	Sequence		Peptide ID	Sequence	
000302	EVGFVVRPQVPLRPMTYK	NEFACC-1	000674	EWILSTYLGRPAEPVPLQ	NEFACC-3
000308	WVYHTQGYFPDWQNYTPG		000675	GRPAEPVPLQLPPLERLT	
000318	HMARELHPEYKDC		000676	LQLPPLERLTLDNCNEDCG	
000366	MEQAPEDQGPQREPYNEW		000677	LTLDNCNEDCGTSGTQGVG	
000367	GPQREPYNEWTLLELEEL		000678	CGTSGTQGVGSPQILVES	
000373	VEAIIIRILQQLFIHFRI		000679	VGSPQILVESPAVLESQT	
000374	QQLFIHFIRIGCQHSRIG		000680	ESPAVLESQTKE	
000632	MGGKWSKRSVVGWPTVRE		000681	MENRWQVMIVWQVDRMRI	
000633	SVVGWPTVVRERMRAEPA		000682	IVWQVDRMRIRTWKSIVK	
000634	RERMRAEPAADGVGAVS		000683	RIRTWKSLVKHHMYISRK	
000635	PAADGVGAVSRDLEKHGA		000684	VKHHMYISRKAKGWFYRH	
000636	VSRDLEKHGAITSSNTAA		000685	RKAKGWFYRHHYESTHPR	
000637	GAITSSNTAANNADCAWL		000686	RHHYESTHPRISSEVHIP	
000638	AANNADCAWLEAQEEEEV		000687	PRISSEVHIPLGDARLVI	
000639	WLEAQEEEEVGFVVRPQV		000688	IPLGDARLVITTYWGLHT	
000641	QVPLRPMTYKGALDLSHF		000689	VITTYWGLHTGERDWHLG	
000642	YKGALDLSHFLEKKGGL		000690	HTGERDWHLGQGVSIWR	
000643	HFLKEKGGLEGLIYSQKR		000691	LGQGVSIWRKKRYSTQV	
000644	LEGLIYSQKRQDILDWV		000692	WRKKRYSTQVDPDLADQL	
000645	KRQDILDWVYHTQGYFP		000693	QVDPDLADQLIHLIYFDC	
000646	FPDWQNYTPGPGIRYPLT		000694	QLIHLIYFDCFSESAIRN	
000647	PGPGIRYPLTFGWCFKLV		000695	DCFSESAIRNAILGHIVS	
000648	LTFGWCFKLVPVEPEKVE		000696	RNAILGHIVSPRCEYQAG	
000649	LVPVEPEKVEEANEGENN		000697	VSPRCEYQAGHNKVGSLQ	
000650	VEEANEGENNSLLHPMSL	NEFACC-2	000698	AGHNKVGSLQYLALALAI	
000651	NNSLLHPMSLHGMDPPER		000699	LQYLALALAITPKKIKPP	
000652	SLHGMDPPERVLVWKFD		000700	LITPKKIKPPLPSVTKL	
000653	EREVLVWKFDRLAFHMM		000701	PPLPSVTKLTEDRWNKPKQ	
000654	FDSRLAFHMMARELHPEY		000702	LTEDRWNKPKQTKGHRGS	
000655	MEPVDPRLEPWKHPGSQP		000703	PQTKGHRGSHMTMNGH	
000656	EPWKHPGSQPKTACTNCY		000704	EWTLLELEELKNEAVRHF	

000657	QPKTACTNCYCKKCCFHC		000705	ELKNEAVRHFPRIWLHGL	
000658	CYCKKCCFHCQVCFITKG		000706	HFPRIWLHGLGQHIYETY	
000659	HCQVCFITKGLGISYGRK		000707	GLGQHIYETYGDTWAGVE	
000660	KGLGISYGRKKRRQRRRA		000708	TYGDTWAGVEAIRILQQ	
000661	RKKRRQRRRAPQDSQTHQ		000709	RIGCQHSRIGITRQRRAR	
000662	RAPQDSQTHQVSLSKQPA		000710	IGITRQRRARNGASRS	
000663	HQVSLSKQPASQPRGDPT		000713	IIAIVVWSIVFIEYRKIL	
000664	PASQPRGDPTGPKESKKK		000714	IVFIEYRKILRQRKIDRL	
000665	PTGPKESKKKVERETETD		000715	ILRQRKIDRLIDRIRERA	
000666	KKVERETETDTPVD		000716	RLIDRIRERAEDSGNESE	
000667	MAGRSGDSDEELLKTVRL		000717	RAEDSGNESEGQDELSA	
000668	DEELLKTVRLIKFLYQSN		000718	SEGDQEELSALVEMGHHA	
000669	RLIKFLYQSNPPPSPEGT		000719	SALVEMGHAPWDVDDL	
000670	SNPPPSPEGTRQARRNR		002486	MQSLQILAIVALV	
000671	GTRQARRNRNRWRERQR		002487	LQILAIVALVVAII	
000672	RRRRWRERQRQIRSISEW		002488	LAIVALVVAIIAIV	
000673	QRQIRSISEWILSTYLGR		002489	LVVAIIAIVVWSIV	

Table 9.6. <i>Pol pools</i>					
Peptide ID	Sequence		Peptide ID	Sequence	
000075	GIGGFIKVRQYDQILIEI		000454	DTGADDTVLEEMNLPGRW	
000081	LNFPISPIETVPVKLKPG		000455	LEEMNLPGRWKPKMIGGI	
000082	ETVPVKLKPGMDGPKVKQ		000456	RWKPKMIGGIGGFIKVRQ	
000083	PGMDGPKVKQWPLTEEKI		000457	RQYDQILIEICGHKAIGT	
000088	NPYNTPVFAIKKKDSTKW		000458	EICGHKAIGTVLVGPTPV	
000089	AIKKKDSTKWRKLVDFRE		000459	GTVLVGPTPVNIIGRNLL	
000090	KWRKLVDFRELNRKTQDF		000460	PVNIIGRNLLTQIGCTLN	
000091	RELNRKTQDFWEVQLGIP		000461	LLTQIGCTLNFPISPIET	
000092	DFWEVQLGIPHPAGLKKK		000462	KQWPLTEEKIKALVEICT	
000093	IHPAGLKKKKSVTVLDV		000463	KIKALVEICTEMEKEGKI	
000094	KKKSVTVLDVGDAYFSVP		000464	CTEMEKEGKISKIGPENP	
000097	KYTAFTIPSINNETPGIR		000465	KISKIGPENPYNTPVFAI	
000098	SINNETPGIRYQYNVLPQ	POL-1	000466	DVGDAYFSVPLDKDFRKY	POL-3
000099	IRYQYNVLPQGWKSPAI		000467	VPLDKDFRKYTAFTIPSI	
000100	PQGWKSPAIFQSSMTKI		000468	AIFQSSMTKILEPFRKQN	
000104	QYMDDLTVGSDLEIGQHR		000469	KILEPFRKQNPDIVIYQY	
000108	FTTPDKKHQKEPPFLWMG		000470	QNPDIVIYQYMDDLTVGS	
000109	QKEPPFLWMGYELHPDKW		000471	GSDLEIGQHRTKIEELRQ	
000112	LPEKDSWTVNDIQLVGK		000472	HRTKIEELRQHLLRWGFT	
000113	VNDIQKLVGKLNWASQIY		000473	RQHLLRWGFTTPDKKHQK	
000118	PLTEEALELAENREILK		000474	MGYELHPDKWTVQPIVLP	
000119	ELAENREILKEPVHGVYY		000475	KWTVQPIVLPEDKSWTVN	
000120	LKEPVHGVYYDPSKDLIA		000476	GKLNWASQIYAGIKVKQL	
000132	WQATWIPEWEFVNTPLV		000477	IYAGIKVKQLCKLLRGTK	
000133	WEFVNTPLVKLWYQLEK		000478	QLCKLLRGTKALTEVIPL	
000143	NIVTDSQYALGIIQAQPD		000479	TKALTEVIPLTEEALEL	
000151	KVLFLDGDIDKAEHEHEKY		000480	YYDPSKDLIAEIQKQGQG	
000152	DKAQEEHEKYHSNWRAMA		000481	IAEIQKQGQGQWTYQIYQ	
000158	VDCSPGIWQLDCTHLEK		000482	QGQWTYQIYQEPFKNLKT	
000159	QLDCTHLEKIIILVAVHV		000483	YQEPFKNLKTGKYARMRG	
000160	GKIIILVAVHVASGYIEAE		000484	KTGKYARMRGHAHTNDVKQ	
000161	HVASGYIEAEVIPAETGQ		000485	RGHAHTNDVKQLTEAVQKI	
000169	PYNPQSQGVVESMNKELK		000486	KQLTEAVQKIATESIVIW	
000170	VVESMNKELKKIIGQVRD		000487	KIATESIVIWGKTPKFKL	
000171	LKKIIGQVRDQAEHLKTA	POL-2	000488	IWGKTPKFKLPIQKETWE	POL-4
000172	RDQAEHLKTAVQMAVFIH		000489	KLPIQKETWEAWNTEYWQ	
000173	TAVQMAVFIHNFKRKGGI		000491	LVKLWYQLEKEPIVGAET	
000174	IHNFKRKGGIGGYSAGER		000492	EKEPIVGAETFYVDGAAN	
000181	PAKLLWKGEAVVIQDNS		000493	ETFYVDGAANRETKLGKA	
000182	EGAVVIQDNSDIKVVPRR		000494	ANRETKLGKAGYVTDGR	
000444	FFREDLAFPGKAREFSS		000495	KAGYVTDGRQKVVSLTD	
000445	PQKAREFSSEQTRANSP		000496	GRQKVVSLTDTTNQKTEL	
000446	SSEQTRANSPTRRELQVW		000497	TDTTNQKTELQAIHLALQ	
000447	SPTRRELQVWGRDNNSLS		000498	ELQAIHLALQDSGLEVNI	
000448	VWGRDNNSLSEAGADRQG		000499	LQDSGLEVNIVTDSQYAL	
000449	LSEAGADRQGTVSFSFPQ		000500	ALGIIQAQPDKSESELVS	

000450	QGTVSFSFPQITLWQRPL		000501	PKSESELVSQIIEQLIK	
000451	PQITLWQRPLVTIKIGGQ		000502	VSQIIEQLIKKEKVYLAW	
000452	PLVTIKIGGQLKEALLDT		000503	IKKEKVYLAWVPAHKGIG	
000453	GQLKEALLDTGADDTVLE		000504	AWVPAHKGIGGNEQVDKL	
000505	IGGNEQVDKLVSAGIRKV	POL-5			
000506	KLVSAGIRKVLFLDGIDK				
000507	KYHSNWRAMASDFNLPPV				
000508	MASDFNLPPVVAKEIVAS				
000509	PVVAKEIVASCDKCQLKG				
000510	ASCDKCQLKGEAMHGQVD				
000511	KGEAMHGQVDCSPGIWQL				
000512	AEVI PAETGQETAYFLLK				
000513	GQETAYFLLKLKLAGRWPVK				
000514	LKLAGRWPVKTIHTDNGS				
000515	VKTIHTDNGSNFTSTTVK				
000516	GSNFTSTTVKAACWWAGI				
000517	VKAACWWAGIKQEFGIPY				
000518	GIKQEFGIPYNPQSQGVV				
000519	GIGGYSAGERIVDIIATD				
000520	ERIVDIIATDIQTKELQK				
000521	TDIQTKEKQKQITKIQNF				
000522	QKQITKIQNFRVYYRDSR				
000523	NFRVYYRDSRDPLWKGPA				
000524	SRDPLWKGPAKLLWKGEG				
000525	NSDIKVPRRKAKIIRDY				
000526	RRKAKIIRDYGKQMAGDD				
000527	DYGKQMAGDDCVASRQDE				
000528	DDCVASRQDED				
002478	WEAWWTEYWQATWIP				
002479	TEYWQATWIPWEEFV				

Table 9.7. List of FEC peptides used		
Peptide Sequence	HLA-restriction	Pathogen/Protein
GILGFVFTL	A*02:01	Flu/Matrix 1 (58-66)
FMYSDFHFI	A*02:01	Flu/PA (46-54)
SIIPSGPLK	A*11:01	Flu/Matrix 1 (13-21)
RVLSFIKGTK	A*11:01	Flu/NP (342-351)
KTGGPIYKR	A*68:01	Flu/NP (91-99)
LPFDKTTVM	B*07:02	Flu/NP (418-426)
ELRSRYWAI	B*08:01	Flu/NP (380-388)
SRYWAIATR	B*27:05	Flu/NP (383-391)
ASCMGLIY	B*35:01	Flu/Matrix 1 (25-32)
CLGGLLTMV	A*02:01	EBV/LMP2 (426-434)
GLCTLVAML	A*02:01	EBV/BMLF1 (259-267)
AVFDRKSDAK	A*11:01	EBV/EBNA4 (399-408)
DYCNVLNKEF	A*24:02	EBV/BRLF1 (28-37)
RAKFKQLL	B*08:01	EBV/BZLF1 (190-197)
FLRGRAYGL	B*08:01	EBV/EBNA3A (325-333)
QAKWRLQTL	B*07:02	EBV/EBNA3A (379-387)
RRIYDLIEL	B*27:05	EBV/EBNA3C (258-266)
YPLHEQHGM	B*35:01	EBV/EBNA3A (458-466)
NLVPMTATV	A*02:01	HCMV/pp65 (495-503)
TPRVTGGGAM	B*07:02	HCMV/pp65 (417-426)
SDEEEAIVAYTL	B*18:01	HCMV/IE1 (378-389)
IPSINVHHY	B*35:01	HCMV/pp65 (123-131)
VSDGGPNLY	A*01:01	Flu/PB1 (591-599)
CTELKLSDY	A*01:01	Flu/NP (44-52)
ILRGSAVHK	A*03:01	Flu/NP (265-273)
RVRAYTYSK	A*03:01	EBV/BRLF1 (148-156)
RLRAEAQVK	A*03:01	EBV/EBNA3A (603-611)

IVTDFSVIK	A*11:01	EBV/EBNA3B (416-424)
ATIGTAMYK	A*11:01	EBV/BRLF1 (134-143)
RPPIFIRRL	B*07:02	EBV/EBNA3A (379-387)
EENLLDFVRF	B*44:02	EBV/EBNA3C (281-290)
EFFWDANDIY	B*44:02	HCMV/pp65 (512-521)

Table 9.8 *List of peptides incorporated into in vitro priming assay for naïve CD8⁺ T cell stimulation*

Peptide name	Peptide sequence	HLA-restriction	Origin
Melan-A ₂₆₋₃₅ (A27L)	ELAGIGILITV	A*02:01	MART1/Melan-A
Melan-A ₁₆₋₄₀ (A27L)	GHGHSYTTAEELAGIGILTVILGVL	A*02:01	MART1/Melan-A
PGT ₁₇₈₋₁₈₇	LLAGIGTVPI	A*02:01	Prostaglandin transporter
HCV-NS3 ₁₀₇₃₋₁₀₈₁	CINGVCWTV	A*02:01	Hepatitis C virus non-structural protein 3
A2-SL9	SLYNTVATL	A*02:01	HIV-1 Gag
A2-PL10	PLTFGWCYKL	A*02:01	HIV-1 Nef
A2-VL10	VLEWRFDSSL	A*02:01	HIV-1 Nef
A3-KK9	KIRLRPGGK	A*03:01	HIV-1 Gag
A3-RK9	RLRPGGKKK	A*03:01	HIV-1 Gag
A3-RY10	RLRPGGKKKY	A*03:01	HIV-1 Gag
B7-SV9	SPRTLNAWV	B*07:02	HIV-1 Gag
B7-TL9	TPQDLNML	B*07:02	HIV-1 Gag
B7-GL9	GPGHKARVL	B*07:02	HIV-1 Gag

9.3 Peptides identified by immunopeptidomics

Table 9.9 shows the identity of HIV-1 peptides identified in the HLA-E-specific 4D12 IP from C8166 cells. Table 9.10 shows the list of non-specifically eluted HIV-1 peptides excluded from analyses of HLA class I IPs. Table 9.11 shows the list of self-peptides excluded from

Table 9.9. *Exclusion list of HIV-1 IIB Gag peptides non-specifically eluted from HLA class I IPs*

MQMLKETIN	PLTSLRSLFGND
AEAMSQVTSATIM	PLTSLRSLFGNDP
SRPEPTAPPEESFRSG	VETTTTPQKQEPIDKELY
YKGRPGNF	SRPEPTAPPEESFRSGVETTTTP
RSLFGNDPSSQ	VETTTTPQKQEPIDKELYPLTSLR
VVEEKAFSPEVIP	SRPEPTAPPEESFRSGVETTTTPQKQEPIDKELY
LQSRPEPTAPPFLQ	MQRGNFR
TSLRSLFGNDPSSQ	SLRSLFGND
FLGKIWPYSYKGRPGNF	RFAVNPGLE
PLTSLRSLFGNDPSSQ	SQVTSATIM
PIVQNIQQGMVHQAIAPRTLNA	LQSRPEPTAPP
RSLFGND	TGHSSQVSQNY
KQEPIDKELY	SRPEPTAPPFLQ
LSEGATPQDLNT	SLRSLFGNDPSSQ
SKKKAQQAAADTGHSSQVSQNY	AADTGHSSQVSQNY

Table 9.10. Exclusion list of peptides eluted non-specifically from HLA class I negative CD4.221 cells

AAPATRAAL	LEKGLDGA	SGGGGGGGLSGGGSIRSS	GGPGGVGGL
AAPAYSRA	LFDLSSLSGRAGFT	SGKKLEDGPKFLK	GGYVAYSKAATVTGK
AAPEGKRSL	LGALLTGSL	SGKSTTGHLIYK	GHQQLYWSHPR
AAPLRLIAA	LLPSHPLEL	SGPGPAAAL	GLAVKELK
AAPRAPSA	LPASVHALTGVDLSKIPL	SKAEWSDSAQGSSESHSL	GPRSYTIAVASLGKGVA
AAPSAAREL	LPKPPKPVSKMRMATPLLMQAL	SKGKIYPVG	GQKDSYVGDE
ADDFRIK	LPKPPKPVSKMRMATPLLMQALP	SKGKIYPVGY	GRDLNRSFPDQFSTGEPP
ADDFRLK	LPNGRVYYVDHNTK	SNRVVDLMAHMASKE	GSGGGSYSGSGGGGGHGSY
AEPNYHSLPSARTDEQ	LPPQATFQVLRNGASVG	SPAKPKAVKPKAAKPKAAKPKAAKPKAAKAKKAAKAAK	GSHSLKYF
AGPLRKSSL	LPSYEEALSLPSKTPE	SPEDEYELLMPHRIS	GSHSLRY
AKDIGFIKLD	LQIDNARLAADDF	SPEDEYELLMPHRISSH	GSHSLRYF
AKDVKFGADARAL	LQKEQNYSDDL	SPEDEYELLMPHRISSHF	KKSVQYDDVPEYK
AKTDQAQKAEGAGDAK	LSDYNIQKESTLHL	SPNIVIALAGNKADL	KLPGNISSL
ALEEQLOQIR	LSFPTTKTYFPHF	SPNIVIALAGNKADLA	KLKSVWRDQHF
APGLIATSGVGNL	LSPTRELAL	SPPPPPPPP	KLVPPQQLAH
APGNLSLPIP	LTVGNNTKL	SPRRKLISVDSRSVS	KLVSLSAQNLVD
APPPPPPPP	MALMASLDAEKAQGQK	SPTAYGVIAAAAVLS	KMRMATPLLMQAL
APPPYSSISAESAAYF	MAPERVASL	SRSGGGGGGGLSGGGSIR	KNLDFSVIVANKAAFH
APVRHLIATQLLSN	MATAASSSSLEKS	SRSGGGGGGGLSGGGSIRSS	KNLDFSVIVANKAAAFHK
APSRNGMVLKPHFHKD	MATYKEKKASV	SRSGGGGGGGLSGGGSIRSSY	LAPTDVKEL
AQTAAATAPRIKKF	MFGGPGTASRP	SSHKTFRIKRF	KELRDHADSNIIV
AQYEDIAQK	MFGGPGTASRPS	SSKGSLGGGF	KENAGEDPGLA
AQYEEIAQR	MFGGPGTASRPFSS	SSLLEKGLD	HLHNMPPSAL
ASPSSIRSL	NAAPGVDL	SSLLEKGLDGA	HLPETKFSEL
ATKHYEMLANRTAANG	NAPLVHATL	SSLLEKGLDGAKK	HPPPPPPPP
AVGGLGKLGKDA	NAPLVNVTL	SSLLEKGLDGAKKA	HSPGAVSSL
DAGAGIALNDH	NARLAADDF	SSPERILPL	HTGSWIGLRNLDLKE
DARSYTAAVANRAKGGG	NKQKPITPETAEKLARD	SSPLANQSL	HTGSWIGLRNLDLKEGF
DAVEDLESVVK	NNRSLDL	SSSISSEFKD	HTGSWIGLRNLDLKEGFI
DFLETHFLDEEVKLIK	NPGGYVAYSKAATVTGK	SSSISSEFKDPKIS	IADSFQVIKNSPLSE
DHNTKTTTWEDPRLK	NPGGYVAYSKAATVTGKL	SSSKGSLGGGF	IAPTGHSL
DIKIRLENE	NPKDVLVGADSVRAIT	SSSKGSLGGGFSSG	LDDVAALHGPVVR
DPTLDHHWHLWKKTYGKQ	NPNTVILIGNKADLE	SSSKGSLGGGFSSGGFSSG	LDDVAALHGPVVRQL
DPTWESLDRQLPA	NSDTHSSTF	STGDVNVE	LEGEESRMSGE
DRQHTLKHFEHVR	NSNQIKILGNQGSFL	STKKSQYDDVPEYK	KPVLTLIN
DSFKLQTKEFQVLKSLG	NVEIDPEIQ	SVEADIN	KQKPITPETAEKLAR
DTGSYRAQISTKTSKAK	NVELDPEIQ	SYGGGEEASGSGGGYGGGSGKSSHS	KQKPITPETAEKLARD
DTGSYRAQISTKTSAKL	NVKVDPEIQ	TAAATAPRIKKF	KSIQFHWKNSNQIK
EAQKGFQDVE	PGLARQAPKPRKQ	TAQVILNHPGQISAGYA	KSLTNDWEDHLAVKHF
EAQKGFQDVEAQ	PGVTVKDVNQQEFVRA	TDKTLVLLMGKEGVHGG	KSVQYDDVPEYK
EAQKGFQDVEAQQA	PGVTVKDVNQQEFVRAL	TEELAYLK	YSPEGHVL
EDPGLARQAPKPRKQ	PHPPPPPPP	TGKLVLSLAQNLVD	LEKEIET
EEALILDNKY	PKGKKAKGKKVAPAPAVVKKQEAKKVVNPLF	TGSWIGLRNLDLK	LEKELET
EEANADLEVK	PKGKKAKGKKVAPAPAVVKKQEAKKVVNPLFEKRPK	TGSWIGLRNLDLKE	LEKGLDG
EEANNDLENK	PKPPKPVSKMRMATPLLMQALP	TGSWIGLRNLDLKEGF	GVNYFLDVELGRT
EEANNDLENKIQ	PPAENSSAPEAQGGAE	TIIDTKGVITAL	HAGGLPQ
EEQLQQIR	PPAPPPPPP	TLSDYNIQKESTLHL	HEASAAQKENAGEDPGLARQAPKPRKQ
EERQFFEIQSKEAKER	PPHPPPPPP	TNAENEFVTIK	GTRIEVSGQKIK
EESNYELEGK	PPSPPPPPP	TSPLRIVAL	GVNYFLDVELGR
EESNYELEGKIK	PPSPPPPPP	TTDNANI	IDAPGHRDF
EETIEKMQE	PRKIEEIKD	TTDNANIL	IEKELET
EEVHDLERKY	PSPPPPPPP	TPVRLSSL	KPPKPVSKMRMATPLLMQAL
EFGRFASFEAQGALANI	QKENAGEDPGLA	VAGVNYFLDVELGR	KPPKPVSKMRMATPLLMQAL
EKGLDGAKK	QKPIPETAEKLAR	VAPGKDLTK	KPPKPVSKMRMATPLLMQALP
ELWWQAERASSSK	RLAADDF	VAPVASAL	KPPKPVSKMRMATPLLMQALPM
ELWWQAERASSSKS	RLKYENE	VAPVTHVSV	KPPPPPPPP
EPGEPEFKYIGNMHGNE	RLKYENEVA	VDDTQFVRFDSDAASPR	KPSKPKTNMKHMAGAAAAGAVV
EPGEPEFKYIGNMHGNEV	RLPHSTAAL	VEDLESVGKA	KTTIRLMNSQLVTTE
EPNYHSLPSARTDEQ	RNFERNKAIKVI	VGLGGTKSIS	KVWRDQHFVKIQ
ESVGKGAHV	RNVSTGDVNVE	VMAPRTLIL	LANYVHSGKLKLG

ESVGKGAVHDV	RQGVADADINGL	VMAPRTLLL	YENEVALRQ
ESVGKGAVHDVKD	RQYAKDIGFIKLD	VMIPVPAG	YFAERVTSL
ESVGKGAVHDVKDV	RVEYHFLSPYVSPK	VMSKLASFL	YKEQLAKL
ESVGKGAVHDVKDVL	SAPGLIATGSGVGNL	VNLGGSKISIS	YKLVPQQLAH
ESVGKGAVHDVKDVLDS	SAPVGV TAL	VNVEINVAPGKD	YLNENPLRAL
ETDLLDIRSEY	SAPVPASPL	VNVEINVAPGKDLTKTLND	YPSKTQPLQHHNLL
EVFKYLAKAYASNHP	SEAPSFT	VNVVPTFGKKKGNANS	YQSKYEEL
FEAKKLV	SFEDAKEAL	VPKTFVNITPAEVG	YRSGGGFSSGSAG
FGGPGTASRPS	SGGGGGGGLSGGSIR	VPLVPQH	KAPEATVTL
FNEKSKEL	IEKFEKEAAEMGKG	VS KYIQEQNEKIYAPQ	KAPEMTNEL
FRQYAKD	IEKFEKEAAEMGKGS	VSPVVRVAV	KAPEPLSSL
FRQYAKDIGFIKLD	IEKFEKEAAEMGGSF	VVRHQLLKT	KAVGGLGKLGKDAVED
FTRILKHGAKDKDD	IFVKTLTGKTIT	WISKQEY	KEITALAPSTMKIK
GAVHDVKD	IGFIKLD	YAPKGSSL	KEKAELKQSLPPLAVKELK
GAVHDVKDVLDS	INVAPGKDL	YAPSGNFV	GGGGGGGLSGGSIR
GDAKTDQAQKAEGAGDAK	IPVQTISRAAAELFGN	YAPSGNYV	GGGGGGGLSGGSIRSS
GEDFSRTFNDHKQ	IQRTPKIQVY	YEDIAQK	GGGGGGGLSGGSIRSSY
GEDFSRTFNDHKQK	ISPTGHSL	YEDIAQKS	GGGGGGGLSGGSIR
GELWWQAERASSSKSW	ISVPAKTSL	YENEVALR	GGSSSSGGHKSSSSSGSVGESS SKGP
GEPLSYTRFSLARQVDG	ITPTGHSL	IVPGNVKSL	GGLGKLGKDA
GGPGGFGPG			

10.0 List of Publications

Bridgeman, A., Maelfait, J., Davenne, T., Partridge, T., Peng, Y., Mayer, A., ... Rehwinkel, J. (2015). Viruses transfer the antiviral second messenger cGAMP between cells. *Science*, 349(6253), 1228-1232.

Ternette, N., Yang, H., Partridge, T., Llano, A., Cedeño, S., Fischer, R., ... Hanke, T. (2016). Defining the HLA class I-associated viral antigen repertoire from HIV-1-infected human cells. *European Journal of Immunology*, 46(1), 60-9.